# Protein Kinase Activities in Ripening Mango Fruit Tissue:

# Classification, Purification and Biochemical Characterisation

by

**Lorinda Frylinck**

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Promotor : **Prof. I.A. Dubery**

For:

Wouter and Imke

# Contents

# Summary

This study consists of two parts namely:

- Phosphoproteins in ripening mango fruit tissue: Effect of γ-irradiation and various effectors on protein phosphorylation during the climacteric rise, climacteric peak and post-climacteric stages of ripening.
- Protein kinase (EC 2.7.10 and EC 2.7.11) activities in ripening mango fruit tissue: Classification, purification and characterisation.

Soluble proteins were isolated from 1 kGy γ-irradiated and control mangoes at three different stages of ripening; i.e. climacteric rise, climacteric peak and post-climacteric stage. Phosphorylation of proteins by endogenous kinases was performed in the presence of [$\gamma$-$^{32}$P]ATP. The effects of EGTA, endogenous calcium, exogenous calcium and calmodulin on phosphotransferase activity were determined.

Phosphorylation of endogenous proteins in cytosolic fractions prepared from the six mango fruit test groups as specified above, was analysed by means of SDS-PAGE and autoradiographic techniques. The changes of phosphoprotein patterns, along with the natural ripening process, and as result of γ-irradiation in the absence and presence of calcium and calmodulin, were analysed. From the results it was clear that endogenous calcium and calcium-dependent protein kinases activity play a major role during control of the natural ripening process and that γ-irradiation resulted in the activation, or synthesis, or release from the membrane of certain protein kinase activities that influenced the natural ripening process.

The phosphorylation of endogenous membrane proteins, isolated from the six mango test groups as specified above, was also analysed by SDS-PAGE and autoradiography techniques. In contrast to the soluble protein fraction the membrane protein phosphorylation decreased during senescence and as a result of γ-irradiation. It could be due to selective degradation of protein kinases and substrates or, inhibition of enzyme activities by unfavourable micro-environment conditions.

A highly hydrophobic (membrane associated) $Ca^{2+}$-stimulated dual-specificity protein kinase (PK-I) has been purified from ripening mango fruit using dephosphorylated casein as exogenous substrate. The purification procedure involves 30-70% ammonium sulphate precipitation and sequential column chromatography on DEAE-Sephacel, Cibacron blue-Sepharose, and Sephadex G75. PK-I was purified ca. 40-fold with an overall yield of <1%. The final specific activity in the presence of 1 mM $Ca^{2+}$ was consistently 1-5 nmol/min/mg. Analysis of the most highly purified preparations by SDS-polyacrylamide gel electrophoresis and agar isoelectric focusing revealed single Coomassie-stained bands of molecular weight 30 kDa and pI 5.1 respectively. This molecular weight was consistent with results obtained from filtration gel chromatography on Sephadex G200, indicating that the enzyme exists as a monomer. The pH and temperature optima of the enzyme under standard reaction conditions were 6.5 and 25 °C respectively. PK-I efficiently phosphorylated casein and phosvitin, but did not phosphorylate histone II-S, histone III-S, protamine sulphate and BSA. PK-I was dependent on millimolar $Mg^{2+}$ which can not be substituted with $Mn^{2+}$. PK-I activity was stimulated by, but was not dependent on $Ca^{2+}$. Calmodulin and calmodulin inhibitors (chlorpromazine and calmidazolium) did not affect PK-I activity. Heparin and cAMP inhibited PK-I activity. Possible endogenous phospho-Tyr-protein substrates, as well as phospho-Tyr autophosphorylation were revealed by Anti-P-Tyr antibody screening. Evidence pointed to PK-I being a dual-specificity protein kinase because it also phosphorylated casein on Ser or Thr residues.

Another hydrophobic (membrane associated) calcium-stimulated protein kinase (PK-II) was purified from mango fruit during the climacteric peak ripening stage. PK-II was purified over 1000-fold by

ammonium sulphate precipitation, DEAE-Sephacel, Cibacron blue Sepharose, Toyopearl-HW55, and Sephadex G75 column chromatography, after which it was more than 90% pure with a recovery of 1-5%. The purified PK-II had a specific activity of ca. 30 nmol/min/mg protein in the presence of 0.1 mM calcium, with ATP as phosphoryl donor. PK-II had a pH optimum around 9.6 and resolved in three Coomassie stained bands with pI's of 6.95, 7.5 and 9.9 by means of agar isoelectric focusing, representing the different enzyme species. These results and SDS-PAGE results suggested that the enzyme consists of an $\alpha\beta$ structure that can aggregate to an inactive $(\alpha\beta)_2$ structure with molecular weight of 34.7 kDa. The $\alpha$ and $\beta$ subunits have molecular weights of 24 kDa and < 21.5 kDa. PK-II used casein and phosvitin as effective artificial substrates and histone III-S was less effective, but it had no affinity for histone II-S and protamine sulphate. $Mn^{2+}$ could not substitute the $Mg^{2+}$ needed for PK-II activity. $Ca^{2+}$ stimulated the PK-II activity, but its activity was not dependent thereupon. Although calmodulin had an insignificant stimulating effect on PK-II at low $Ca^{2+}$ concentrations, it was insensitive to calmidazolium and chlorpromazine that strongly antagonise $Ca^{2+}$-calmodulin regulated protein kinases. PK-II's activity was extensively inhibited by heparin, but cAMP had no effect on PK-II activity. Autoradiographic studies revealed a few endogenous protein substrates as well as PK-II's ability to autophosphorylate. These proteins were either phosphorylated on Ser or Thr residues. The characteristics above are typical of a casein kinase 2 type enzyme. Anti-P-Tyr antibody studies suggested that PK-I might phosphorylate PK-II on a Tyr residue indicating an activity control mechanism.

A calcium-dependent protein kinase (PK-III), activated by the direct binding of $Ca^{2+}$, and not requiring calmodulin for activity, was purified from extracts of ripening mango fruit tissue. Purification was achieved by chromatography on DEAE-Sephacel, Phenyl-Sepharose, Cibacron blue Sepharose, and Sephadex G75. The standard purification procedure allowed recovery of 1-5% of the total available kinase activity, and yielded approximately 40-460 µg of purified PK-III from 600g of starting material. The final specific activity in the presence of 1 mM $Ca^{2+}$ was consistently 1-9 nmol/min/mg. The purified enzyme (molecular weight = 50 kDa) resolved into two related protein bands of 50 and 43.7 kDa on 10% SDS gels. PK-III displayed a broad pH optimum (pH 6.6 to 9.5) and the optimum reaction temperature with histone III-S as substrate was 35 °C. Enzyme activity was activated 100-fold by micromolar amounts of free calcium and was dependent upon millimolar $Mg^{2+}$ or $Mn^{2+}$ concentrations. Calmodulin (1 µM) had no effect on PK-III activity but the calmodulin-antagonists, calmidazolium and chlorpromazine, inhibited PK-III in a dose-dependent manner over a range of 0 to 100 µM. The results implicate a regulatory domain that is similar to calmodulin. PK-III phosphorylated lysine rich histone III-S and to a lower extent dephosphorylated casein but did not phosphorylate histone II-S, phosvitin, or protamine sulphate. Phosphorylation of histone III-S was inhibited by <0.3 M NaCl. The enzyme phosphorylated casein and histone III-S as well as autophosphorylated on either serine or threonine but not tyrosine. Some endogenous substrates and the ability to autophosphorylate, were revealed by autoradiographic studies.

The kinetic reaction mechanisms of PK-I, PK-II and PK-III were studied by using casein as kinase substrate. Initial velocity and product inhibition studies with ADP as product inhibitor best fit an ordered Bi Bi kinetic mechanism with the $Mg^{2+}$-ATP complex binding first to the enzyme followed by binding of the protein substrate. The $K_m$ATP's of PK-I, PK-II and PK-III were 20, 14 and 17 µM, respectively and their $K_m$Casein's were 0.2, 0.8, and 1.0 mg/ml respectively. The $K_i$ADP of PK-I and PK-II were 9 and 3.2 µM, respectively.

# Opsomming

Hierdie studie bestaan uit twee dele, naamlik:

- Fosfoproteïene in rypwordende vrugweefsel: Effek van gamma-straling en verskeie effektore op proteïenfosforilasie tydens die klimakteriese styging, klimakteriese piek en na-klimakteriese stadia van rypwording.
- Proteïenkinase (EC 2.7.10 en EC 2.7.11) aktiwiteite in rypwordende vrugweefsel: Klassifikasie, suiwering en karakterisering.

Oplosbare proteïene is vanaf 1 kGy γ-bestaalde en kontrole mangoes in drie verskillende stadia van rypwording, naamlik klimakteriese styging, klimakteriese piek en na-klimakteriese stadium, geïsoleer. Die proteïene is in die teenwoordigheid van [$\gamma$-$^{32}$P]ATP deur endogene proteïenkinases gefosforileer. Die effek van EGTA, endogene kalsium, eksogene kalsium en kalmodulien op proteïenkinase aktiwiteit is bepaal.

Die fosforilasie van endogene proteïene in die sitosoliese fraksies wat van die ses mangovrug toetsgroepe, soos bo gespesifiseer, berei is, is m.b.v. SDS-PAGE en outoradiografiese tegnieke, bestudeer. Die veranderinge van die fosfoproteïenpatrone met gelang van rypwording en a.g.v. γ-bestraling in die teenwoordigheid en afwesigheid van kalsium en kalmodulien is geanaliseer. Dit was duidelik dat endogene kalsium en kalsium-afhanklike proteïenkinases 'n belanglike rol tydens die beheer van die natuurlike rypwordingsproses speel. γ-Bestraling het gelei tot die aktivering, of sintese, of vrystelling van spesifieke proteïenkinase aktiwiteite wat die natuurlike rypwordingsproses beïnvloed het.

Die fosforilasie van die endogene membraanproteïene, berei vanuit die ses mangovrug toetsgroepe soos gespesifiseer, is weereens m.b.v. SDS-PAGE en outoradiografiese tegnieke, geanaliseer. In teenstelling met die oplosbare fraksie, het die fosforilering van sekere proteïene tydens die rypwording en verouderingsproses en a.g.v. γ-bestraling afgeneem. Dit kan die gevolg wees van selektiewe afbraak van proteïenkinases en substrate of, inhibisie van ensiemaktiwiteite a.g.v. ongunstige mikro-omgewings-kondisies.

'n Hidrofobiese (membraan geassosieerde) $Ca^{2+}$-gestimuleerde proteïenkinase (PK-I) met moontlike dubbel-spesifieke aktiwiteite (fosforileer beide Ser/Thr en Tyr) is vanuit mangovrugweefsel in die klimakteriese piekstadium, m.b.v. gedefosforileerde kaseïen as eksogene substraat, geïsoleer. Die suiweringsprosedure het die volgende stappe ingesluit; 30-70% ammoniumsulfaatpresipitasie en drie opeenvolgende kolomchromatografie stappe m.b.v. DEAE-Sephacel, Cibacron-blou-Sepharose en Sephadex G75. PK-I is ongeveer 40 keer, met 'n opbrengs van < 1%, gesuiwer. In die teenwoordigheid van 0.01 mM $Ca^{2+}$, het die gesuiwerde PK-II 'n spesifieke aktiwiteit van 1-5 nmol/min/mg proteïen gehad. Analise van die hoogs gesuiwerde bereidings m.b.v. SDS-poliakrielamied jelelektroforese en agar isoelektrofokusering, toon 'n enkele Coomassie-gekleurde band met 'n molekulêre gewig van 30 kDa en 'n pI van 5.1. Hierdie molekulêre gewig stem ooreen met die resultate verkry vanaf uitsluitings-chromatografie m.b.v. Sephadex G200, wat daarop dui dat PK-I 'n monomeer moet wees. Onder standaard reaksiekondisies is die pH en temperatuur optima onderskeidelik 6.5 en 25 °C. PK-I fosforileer kaseïen en fosvitien effektief, maar fosforileer nie histoon III-S, histoon II-S, protamien-sulfaat en BSA nie. $Mg^{2+}$ en nie $Mn^{2+}$ nie, is noodsaaklik vir PK-I aktiwiteit. $Ca^{2+}$ stimuleer PK-I aktiwiteit, maar PK-I is nie daarvan afhanklik nie. Kalmodulien het nie 'n noemenswaardige effek op PK-I gehad nie en die kalmodulien inhibeerders, chlorpromasien en calmidasolium, het nie die ensiem se aktiwiteit geïnhibeer nie. Heparien en cAMP het die PK-I aktiwiteit geïnhibeer. Moontlike endogene fosfo-Tyr-proteïen-substrate, sowel as fosfo-Tyr outofosforilasie is deur anti-P-Tyr teenliggaamstudies blootgelê. Bewyse dui daarop dat PK-I 'n sogenaamde "dual specificity" proteïenkinase is, omdat dit ook eksogene substrate soos kaseïen op Ser en Thr residue fosforileer.

Nog 'n hidrofobiese (membraan geassosieerde) $Ca^{2+}$-gestimuleerde proteïenkinase met kaseïenkinase 2 tipe eienskappe (PK-II) is vanuit mangovrugweefsel in die klimakteriese piekstadium, m.b.v. gedefosforileerde kaseïen as eksogene substraat, geïsoleer. Die suiweringsprosedure het die volgende stappe ingesluit; 30-70% ammoniumsulfaatpresipitasie en vier opeenvolgende kolomchromatografie stappe m.b.v. DEAE-Sephacel, Cibacron-blou-Sepharose, Toyopearl HW-55 en Sephadex G75. PK-II was ongeveer 600 keer, met 'n opbrengs van 1-5%, gesuiwer. In die teenwoordigheid van 0.1 mM $Ca^{2+}$, het die gesuiwerde PK-II 'n spesifieke aktiwiteit van ongeveer 30 nmol/min/mg proteïen gehad. Die gesuiwerde PK-II het in drie Coomassie gekleurde bande, met pI waardes van 6.95, 7.5 en 9.9 elk m.b.v. agar isoelektrofokusering geskei. Analise van die gesuiwerde bereidings m.b.v. 10% SDS-PAGE dui daarop dat die aktiewe ensiem uit 'n $\alpha\beta$ struktuur bestaan (molekulêre gewig = 34.7 kDa) wat kan aggregeer om 'n onaktiewe $(\alpha\beta)_2$ struktuur te vorm. Die $\alpha$ en $\beta$ subeenhede het molekulêre gewigte van 24 en <21.5 kDa. Onder standaard reaksietoestande is die pH en temperatuur optima onderskeidelik 9.6 en 35 °C. PK-II fosforileer kaseïen en fosvitien effektief en tot 'n mindere mate histoon III-S, maar nie histoon II-S, protamiensulfaat en BSA nie. $Mg^{2+}$ en $Mn^{2+}$ is effektiewe divalente metaalione noodsaaklik vir PK-II aktiwiteit. $Ca^{2+}$ stimuleer PK-II aktiwiteit, maar dit is nie noodsaaklik vir ensiem aktiwiteit nie. Kalmodulien (1 µM) het geen effek of PK-II gehad nie en die kalmodulien inhibeerders, chlorpromasien en calmidasolium, het nie die ensiem se aktiwiteit geïnhibeer nie. Heparien inhibeer PK-II aktiwiteit aansienlik, maar cAMP het geen effek op PK-II aktiwiteit nie. Outoradiografiese studies het 'n paar endogene proteïensubstrate, asook die outofosforileringsvermoë van PK-II, ontbloot. Bogenoemde eienskappe is tipiese kaseïenkinase 2 kenmerke. Anti-Tyr-P-teenliggaampiestudies dui daarop dat PK-I 'n Tyr-eenheid van PK-II fosforileer, wat op 'n aktiwiteitsbeheermeganisme dui.

'n Kalsium-afhanklike proteïenkinase (PK-III), geaktiveer deur direkte $Ca^{2+}$-binding sonder die byvoeging van kalmodulien, is vanuit mangovrugweefsel in die klimakteriese piekstadium, m.b.v. gedefosforileerde kaseïen as eksogene substraat, geïsoleer. Die suiweringsprosedure het die volgende stappe ingesluit; 30-70% ammoniumsulfaatpresipitasie en vier opeenvolgende kolomchromatografie stappe m.b.v. DEAE-Sephacel, Feniel-Sepharose, Cibacron-blou-Sepharose en Sephadex G75. PK-III was ongeveer 70 keer, met 'n opbrengs van 1-5%, gesuiwer. In die teenwoordigheid van 1 mM $Ca^{2+}$, het die gesuiwerde PK-III 'n spesifieke aktiwiteit van 1-9 nmol/min/mg proteïen gehad. In 10% SDS-PAGE het die gesuiwerde ensiem (molekulêre gewig = 50 kDa) in twee verwante proteien bande van 50 en 43.7 kDa elk, geskei. PK-III het onder standaard reaksiekondisies 'n bree pH optimum van 6.6 tot 9.5 getoon en 'n optimale temperatuur van 35 °C. PK-III aktiwiteit word tot 100 keer verhoog in die teenwoordigheid van mikromolare hoeveelhede $Ca^{2+}$ en is afhanklik van millimolare hoeveelhede $Mg^{2+}$ en $Mn^{2+}$. Kalmodulien (1 µM) het geen effek op PK-III aktiwiteit gehad nie, maar die kalmodulien-antagoniste, calmidasolium en chlorpromasien, toon dosis-afhanklike inhibisie t.o.v. PK-III. Die resultate dui op 'n katalitiese streek wat soortgelyk aan kalmodulien is. PK-III fosforileer lisien ryke histoon III-S en tot 'n mindere mate kaseïen, maar fosforileer nie histoon II-S, fosvitien of protamiensulfaat nie. Histoon-III-S fosforilasie word deur <0.3 M NaCl geïnhibeer. Sommige endogene substrate en die vermoë van PK-III om te outofosforileer, is deur autoradiografiese studies blootgelê.

Die kinetiese reaksiemeganismes van PK-I, PK-II en PK-III is bestudeer m.b.v. kaseïen as eksogene substraat. Beginsnelheidstudies en produkinhibisiestudies met ADP as produkinhibeerder pas by 'n geordende Bi-Bi meganisme waar $Mg^{2+}$ATP eerste aan die ensiem bind, gevolg deur die binding van die proteïen-substraat. Die berekende $K_m$ATP waardes van PK-I, PK-II en PK-III is respektiewelik 20, 14 en 17 µM en hul $K_m$Kaseïen waardes is respektiewelik 0.2, 0.8, and 1.0 mg/ml. Die berekende $K_i$ADP waardes vir PK-I en PK-II is respektiewelik 9 en 3.2 µM.

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To the Lord, for strength.

# Symbols and Abbreviations

| | | |
|---|---|---|
| $A_{280\ nm}$ | - | absorption at 280 nm |
| α-AB | - | α-aminobuteric acid |
| ATP | - | adenosine-5'-triphosphate |
| | | |
| BCIP | - | 5-bromo-4-chloro-3-indolyl phosphate |
| BIS-TRIS | - | bis-(2-hydroxyethyl)imino-tris(hydroxymethyl)methane |
| β-ME | - | β-mercaptoethanol |
| BSA | - | bovine serum albumin |
| | | |
| ca. | - | approximately |
| CDPK | - | calcium-dependent protein kinase |
| CHES | - | 2-[N-cyclohexylamino]ethanesulphonic acid |
| CK-2 | - | casein kinase 2 |
| cm | - | centimetre |
| CPK | - | cyclin-dependent protein kinase |
| cpm | - | counts per minute |
| CREB | - | cyclic AMP responsive element binding protein |
| cAkinase | - | cyclic AMP dependent protein kinase |
| | | |
| Da | - | dalton |
| DEAE | - | diethylaminoethyl |
| DIECA | - | diethyldithiocarbamate |
| DMSO | - | dimethylsulphoxide |
| dpm | - | disintegrations per minute |
| DTT | - | dithiothreitol |
| | | |
| E* | - | activation energy |
| E | - | enzyme |
| EGTA | - | ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid |
| EDTA | - | ethylenediaminetetraacetic acid |
| EGF | - | epidermal growth factor |
| EGFR | - | epidermal growth factor receptor |
| | | |
| g | - | gram |
| ΔG* | - | Gibbs free energy of formation of transition state of catalytic reaction |
| GA | - | gibberellic acid |

| | | |
|---|---|---|
| GSK-3 | - | glycogen synthase kinase |
| Gy | - | Gray - dosage unit equal to the absorption of 1 Joule per kilogram of radiated product |
| γ | - | gamma |
| h | - | hour |
| *h* | - | Planck's constant |
| ΔH* | - | enthalpy of formation of transition state of catalytic reaction |
| HEPES | - | N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid) |
| IEF | - | isoelectric focusing |
| INF | - | interferon |
| k | - | kilo |
| °K | - | degrees Kelvin |
| $K_i$ | - | inhibition constant |
| $K_m$ | - | Michaelis constant |
| M | - | molar |
| MAPK | - | microtubule-associated protein kinase |
| ml | - | millilitre |
| mM | - | millimolar |
| $M_r$ | - | molecular weight |
| m/v | - | mass per volume |
| MeOH | - | methanol |
| N | - | Avogadro's number |
| NBT | - | nitro blue tetrazolium |
| ND | - | not determined |
| nm | - | nanometer |
| PAGE | - | polyacrylamide gel electrophoresis |
| pI | - | isoelectric point |
| PIC | - | phosphoinosidase C |
| PMSF | - | phenylmethanesulphonyl fluoride |
| PS | - | Phosphatidyl serine |
| PVDF | - | polyvinyl-difluoride |
| R | - | ideal gas constant = 1.987 kcal x $mol^{-1}$ x $degree^{-1}$ |

| | | |
|---|---|---|
| $R_f$ | - | relative electrophoretic mobility |
| RLK | - | receptor-like kinase |
| | | |
| s | - | second |
| $\Delta S^*$ | - | entropy of formation of the transition state |
| SDS | - | sodium dodecyl sulphate |
| Ser | - | serine |
| SRK | - | self-incompatibility (S-) locus receptor kinases |
| | | |
| T | - | temperature (0 °C = 273 °K) |
| TCA | - | trichloroacetic acid |
| Thr | - | threonine |
| TMK | - | thylakoid membrane kinase |
| TRIS | - | tris(hydroxymethyl)amino methane |
| Tyr | - | tyrosine |
| | | |
| µg | - | microgram |
| µl | - | microlitre |
| µM | - | micromolar |
| UV | - | ultraviolet |
| | | |
| Ve | - | elution volume |
| V = Vmax | - | maximal reaction rate |
| v | - | initial velocity |
| vSRC | - | viral sarcoma oncogene = protein kinase |
| | | |
| w/v | - | weight per volume |
| | | |
| > | - | higher than |
| < | - | lower than |
| ~ | - | approximately |

# CHAPTER 1

# Aim of Study

The early ripening and decay of fruit and vegetable products is a substantial problem in the food industry. Low doses of gamma-irradiation (0.2 - 1 kGy) changes the normal biochemical processes in the fruit cell, resulting in the delay of some physiological processes, e.g. budding, ripening and senescence (Surendranathan & Nair 1980, Dubery et al. 1984, Frylinck et al. 1987). This finding opened a new area in the modern technology to obtain longer shelf lives. Questions have arisen: How are these physiological processes controlled? How does gamma-irradiation effect these natural processes? Does gamma-irradiation change the nutritive value of the food product? Does gamma-irradiation cause the formation of harmful substances?

It is necessary that scientists try to find answers to these questions so that the consumers can decide whether to accept or discard this way of food preservation on the basis of scientific evidence. Gamma-irradiation treatment causes a stress condition in the fruit that, depending on the dose, may lead to browning of the tissue or necrotic decay (Frylinck et al. 1987) as well as distortion of some biochemical processes so that senescence is delayed (Dubery et al. 1984).

The biochemical, and more precise regulatory events that lead to the maturation, ripening and senescence of fruit is largely unclear. The additional effect of gamma-irradiation on these processes is very complex as it could disturb so many aspects of the total picture.

Fruit tissue is exceptional material for the study of physiological and biochemical processes that involve differentiation of structure and function that accompanies development, ripening and senescence (Biale 1964). Previous studies in this field were conducted on mangoes (Dubery et al. 1984, Frylinck et al. 1987), as they are important South African export products. Mangoes were therefore again used as experimental material.

Reversible protein phosphorylation, a reversible post-translational modification of proteins (enzymes), has long been known to regulate enzyme activities (Krebs & Beavo 1979), and has more recently been shown to regulate proteins involved in many fundamental cellular processes (Hunter & Cooper 1985, Edelman et al. 1987, Hanks et al. 1988, Ullrich & Schlessinger 1990). Furthermore, protein kinases and phosphatases are often themselves regulated by phosphorylation, either in direct response to cellular signals or via signal transduction pathways (Ullrich & Schlessinger 1990, Gilman 1987, Kemp & Pearson 1991).

In plants, protein phosphorylation may regulate unique processes, such as photosynthesis, photomorphogenesis and gravitropism, besides pathways common to eukaryotes. Much of what is known about protein phosphorylation in plants comes from biochemical studies, and only very recently have genes involved in protein phosphorylation been isolated and characterised at the molecular level (Ma 1993). The involvement of protein phosphorylation in the regulation of fruit ripening is expected as there is substantial proof that calcium and calmodulin regulated protein phosphorylation regulate biochemical events inside the plant (fruit tissue) cell in response to external stimuli (Poovaiah & Reddy 1987, Ma 1993). Molecular genetic and physiological analyses (Kieber et al. 1993) have already identified a kinase in the ethylene (ripening hormone) response pathway of *Arabidopsis*.

The aim of this study was to obtain more information on the regulation of fruit ripening and senescence by means of phosphorylation and dephosphorylation and to obtain further insight into the effect of gamma-irradiation on the regulatory systems operative during ripening and senescence. This study consists of two parts namely:

- Phosphoproteins in ripening mango fruit tissue: Effect of gamma-irradiation and various effectors on protein phosphorylation during the climacteric rise, climacteric peak and post-climacteric stages of ripening.

Here the aim was to study the effect of gamma-radiation on the *in vitro* phosphorylation of soluble and membrane proteins in relation to ripening and senescence of mango fruit. The role of various natural effectors like calcium, calmodulin, cAMP and phospholipids, was also of concern.

- Protein kinase activities in ripening mango fruit tissue: Classification, purification and characterisation.

Here the aim was to purify protein kinases from Kent mango fruit tissue in the climacteric peak stage of ripening to such an extent that biochemical studies could be performed on them. Biochemical studies are crucial in determining the mechanisms by which these protein kinases function.

# CHAPTER 2

# Literature Survey

# List of Figures and Tables

## Figures



## Tables

# CHAPTER 2

# Literature Survey

## 2.1 Introduction

The study field covering this project is very wide and the information available is voluminous. An attempt is being made to summarise the most important information necessary to understand and conduct projects in this field.

Firstly, a general discussion of protein kinases and protein phosphorylation is given. Emphasis is laid on the classification and properties of protein kinases. Protein tyrosine phosphorylation and protein phosphatases are also discussed.

Secondly, the signal perception and transduction in plants will be discussed against the background of the knowledge obtained from animal cells. Evidence for the role of calcium as second messenger, and the phosphoinositide system as means for signal transduction in plants is presented, after which the enzymes, substrates and regulators known in plants are mentioned.

Lastly, the effect of external signals on protein kinase activity is discussed.



## 2.2 Protein kinases and protein phosphorylation

Since the discovery of cAMP by Sutherland in the 1950s and the subsequent identification of cAMP-dependent protein kinase as the primary intracellular receptor for cAMP, it has been clear that protein phosphorylation plays a major regulatory role in eukaryotic cells (Krebs & Beavo 1979).

The initial studies focused in particular on glycogen metabolism and attempted to define the specific molecular events that correlated hormone-mediated activation of adenylate cyclase with enhanced glycogenolysis. The primary event following the elevation of intracellular cAMP is the activation of cAMP-dependent protein kinase. A series of phosphorylation events were identified subsequently that correlated specific phosphorylations with functional changes in several enzymes including glycogen synthase, glycogen phosphorylase and phosphorylase kinase (Krebs & Beavo 1979, Cohen 1983). Although many enzymes and proteins now are known to be regulated by phosphorylation, the regulation of glycogen metabolism remains as one of the best understood examples where protein phosphorylations at multiple targets combine to give a single concerted response in a given tissue. The role of phosphorylation in regulating glucose metabolism in the liver, also includes inhibition of phosphatases, inhibition of glycolysis, and the activation of gluconeogenesis as co-ordinated events which further facilitate the release of glucose. The catalytic subunit of cAMP-dependent protein kinase directly mediates the phosphorylation of at least five different proteins in these combined pathways (Krebs & Beavo 1979).

Reversible phosphorylation is now recognised to be one of the most widely used mechanisms for signal transduction in eukaryotic cells (Krebs 1993). Many critical proteins and enzymes are regulated by the reversible addition of a phosphate, and the family of enzymes catalysing this reaction is large and diverse (Hanks et al. 1988). The diversity of the kinase family extends from the large growth factor receptors that span the plasma membrane to cell cycle-control protein kinases. There are two major

classes of kinase: those showing specificity for Ser/Thr and those that transfer phosphate to Tyr (Krebs & Beavo 1979). The essential nature of these molecular switches is obvious from the many oncogenic protein kinases known to disrupt normal signalling pathways. Several hundred protein kinases can be classified according to their mode of regulation or substrate specificity (Hunter 1991) (Table 2.2.1).

## 2.2.1 Classification of Protein Kinases

Among the large family of Ser/Thr kinases (E.C. 2.7.10) (Hunter 1991) ,

- some are dependent on the second messengers and
- others on specific components of the system that they are called upon to regulate. Such is the case of the heme-regulated kinase that blocks the initiation of globin synthesis when heme or iron becomes limiting, or the double-stranded RNA-dependent kinase that is induced by interferon in cells under viral attack.
- There are also the so-called "independent kinases" such as the casein kinases for which no precise mode of control has been elucidated as yet.

Next come the mixed-function kinases that can phosphorylate their substrates on both tyrosine or serine/threonine, or that can be regulated by tyrosine and serine/threonine phosphorylation. This is the case for the enzymes that Krebs (Seger et al. 1992, Lindberg et al. 1992) had described: microtubule-associated protein (MAP) kinase, the MAP kinase kinase, perhaps *raf*, or the cell cycle kinases such as p34$^{cdc2}$ (Fischer 1993). They stand guard at crucial crossroads of signal transduction. They form the link between a signalling system that originates at the membrane level and relies on tyrosine phosphorylation, and the more widespread serine/threonine phosphorylation reactions that occur downstream. Their dual control and specificity would ensure that no accidental initiation of important cellular events occurs at inappropriate times (Fischer 1993).

The large class of tyrosine kinases (E.C.2.7.11) (Hunter 1991) will be discussed later.



Table 2.2.1 Classification of protein kinases (PKs).

**I. Second messenger-dependent Ser/Thr PKs**
- A. cyclic nucleotides: cAMP, cGMP PKs
- B. $Ca^{2+}$/calmodulin (CaM)-dependent kinase: phosphorylase kinase, Myosin Light Chain Kinase, CaM kinase II
- C. diacylglycerol (DAG)/$Ca^{2+}$-protein kinase (PKC)

**II. Second messenger-independent Ser/Thr PKs**
- A. heme-, ds RNA-, (INF)-dependent eIF2 PKs
- B. CK-I, CK-II, GSK-3, S6 kinases

**III. Dual specificity, (Ser Thr and Tyr) PKs**
MAPK, MAPKK
*Weel*

**IV. Protein tyrosine kinases (PTKs)**
- A. cellular or viral (oncogenic) PTKs
- B. receptor-linked PTKs

The less common histidine kinases (E.C. 2.7.12) (Hunter 1991) such as those involved in bacterial chemotaxis (Bourret et al. 1991) and the double-headed kinase/phosphatase that regulates bacterial isocitrate dehydrogenase (La Porte 1985), are not listed.

## 2.2.2 Properties of protein kinases

All the protein kinases have homologous catalytic domains but vary greatly in the structure of their regulatory segments. They have consensus sequences, such as the motifs that are involved in the binding of ATP, by which they can be identified by searching the database (Hanks et al. 1988). Most are regulated by segments that block their activity, often by virtue of the fact that they contain pseudo-substrate motifs that interact with and shield, their catalytic sites. These auto-inhibitory domains can exist on separate subunits as in the cAMP-dependent protein kinase (cAMP PK) first characterised by Krebs (Walsh et al. 1968), or within the same peptide chain, as in the cGMP PK, where the two segments have become fused in the course of evolution. Initially, for the cAMP PK, the reaction seemed simple enough: the enzyme exists as an inactive complex between catalytic and regulatory subunits: cAMP induces a change in conformation in the regulatory subunits, resulting in the dissociation of the enzyme and the liberation of active catalytic subunits. It soon became apparent, however, that the inactive complex had a more substantial purpose, namely, to prevent the translocation of the free catalytic subunits to other compartments of the cell, particularly the nucleus (Castagna et al. 1975).

The regulation of protein kinases is even more sophisticated: the regulatory subunits themselves contain structural determinants that allow them to recognise and bind with high affinity to anchoring proteins distributed at specific locations within the cell (Leiser et al. 1986, Bergman et al. 1989, Carr et al. 1992). More than two dozen of these have been identified; they are particularly abundant in the brain and the thyroid. Conceivably, some of these could co-localise with particular cAMP-generating receptors. This would confer a certain degree of selectivity to the hormonal response, by targeting the kinase toward a given set of substrates (Carr et al. 1992) (Figure 2.2.1).



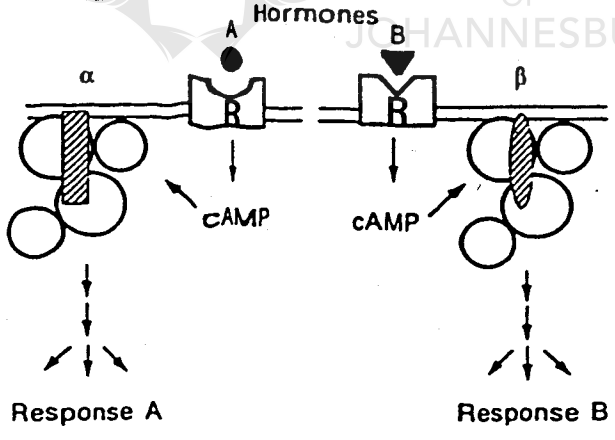



Figure 2.2.1 Hypothetical selectivity in hormonal response by juxtaposition of cAMP-dependent protein kinase anchoring proteins with specific adenylate cyclase receptors (Fischer 1993).

Enzyme translocation may also play an important role in the regulation of protein kinase C first described by Nishizuka et al. (Takai et al. 1979, Nishizuka 1992). Depending on which subspecies of PKC is involved, the enzyme contains up to three regulatory domains responsible for the binding of $Ca^{2+}$, diacylglycerol, and phospholipids. Binding of these allosteric effectors can promote the translocation of the enzyme to the plasma membrane where it could bind to specific anchoring proteins (Mochly-Rosen et al. 1991). This could determine which particular signal pathway would become affected.

Enzyme translocation might also be one of the functions of the cyclins, those regulatory subunits that are transiently expressed during various phases of the cell cycle. They associate strongly with the cell cycle-dependent kinases and operate particularly at the G1/S and G2/M transitions (Nurse 1990, Norbury & Nurse 1992). In *Saccharomyces cerevisiae,* for instance, between four and five dozen cyclins have been identified (Norbury & Nurse 1992). While their multiplicity, would provide the cell with the redundancy it needs to protect itself from accidental failures, it would seem unlikely that their sole purpose would be to modulate the activity of the kinases. Some of these complexes must become operative at other set points along the cell cycle. More importantly, perhaps, they could be essential to target the enzymes toward those elements that become operative during the profound cytoskeletal reorganisations that accompany cell division (Fischer 1993).

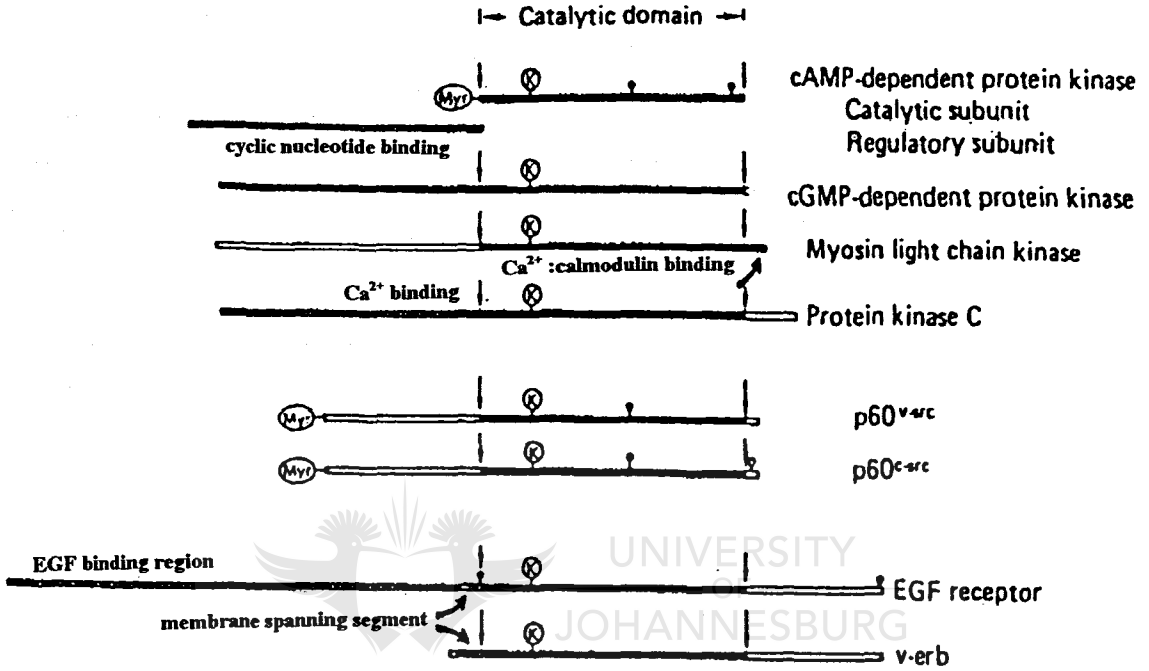



Figure 2.2.2 Comparison of regulatory and homologous catalytic regions of representative protein kinases (Taylor 1987). The homologous catalytic region that is shared by all kinases is indicated in black; known regulatory regions are cross-hatched; phosphorylations at serine/threonine or tyrosine are indicated by (●) and (⊗) respectively. Myr = site of attachment of myristic acid.

### 2.2.2.1 Evolution from a common precursor

Protein kinases vary in size and subunit structure, they phosphorylate different substrates, and they also require different ligands for activation (Flockhart & Corbin 1982). Within each category, there are frequently isoenzyme variants which are expressed differentially. Despite this multiplicity, these kinases can be grouped together collectively as protein kinases which transfer phosphate from ATP to either serine or threonine residues on their respective substrate protein. Homologies in amino-acid sequence indicate that all known protein kinases share a conserved catalytic core, and thus, belong to a related family of proteins that have evolved in part from a common ancestral origin. This conclusion is clear when the known primary structures of various protein kinases are compared. In some cases the primary structures have been determined by conventional protein sequencing, whereas, in other cases, the primary structures have been deduced from DNA sequences of cloned genes. Although these kinases differ in size, in cellular location, in mechanism of regulation, and in specificity, they are related proteins which share a common catalytic core that has evolved from a common ancestor. Figure 2.2.2 aligns this homologous catalytic core from variety of protein kinases. So far every enzyme known to have protein kinase activity has proved to be a homologous member of this family when sequence information has been available (Taylor 1987). This family includes cellular kinases, oncogenic viral kinases and their proto-oncogene counterparts, and growth factor receptors.

### 2.2.2.2 Nucleotide-binding site

Some common functions such as the binding of $Mg^{2+}$-ATP are shared by all protein kinases. The most detailed knowledge of the ATP binding-site of protein kinases is derived from structural studies of cAMP-dependent protein kinases.

Two structural features serve tentatively to define and localise that ATP binding site. The first derives from affinity labelling studies which established that covalent modification of the catalytic subunit with an ATP analogue, p-fluorosulphonylbenzoyl-adenosine (FSBA), led to inactivation of the kinase by the stoichiometric covalent modification of Lys 72 (Zoller et al. 1981). Although it was possible that this residue was simply in close proximity to the ATP binding site and not directly involved in catalysis, subsequent evidence has strengthened the likelihood that it is an essential residue. For example, the homologous lysine (Lys 295) is modified by FSBA in $p60^{v\text{-}src}$ (Kamps et al. 1984). Selective modification of this residue in $p60^{v\text{-}src}$ led to inactivation of protein kinase activity. Directed mutagenesis of cloned genes has been used to selectively alter this residue. Mutagenesis of Lys 295 in $p60^{src}$ also led to the formation of an inactive protein which was incapable of transformation (Kamps & Sefton 1986). That this residue is essential is also supported by the fact that it is invariant in every protein kinase.

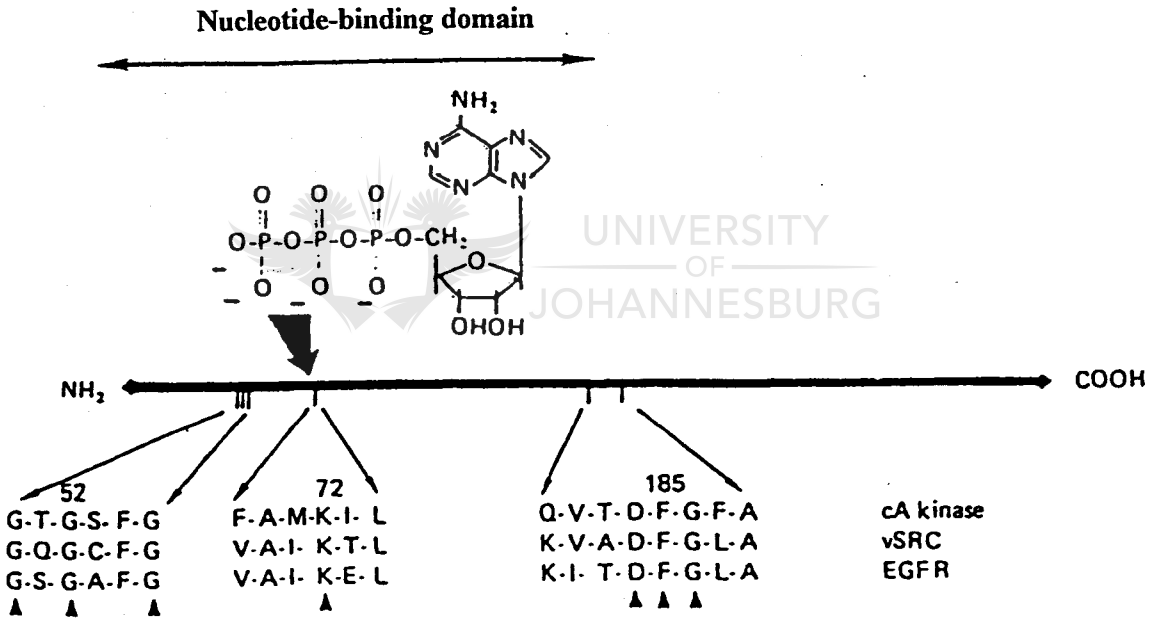



Figure 2.2.3 Conserved regions of amino acid sequence thought to be associated with $Mg^{2+}$-ATP binding.

Another striking feature of the kinase structures is the conservation of a triad of glycines which correspond to Gly 50, 52, and 55 in the C-subunit of cAMP-dependent protein kinase (Figure 2.2.3). These glycines are a conserved feature of most adenine nucleotide binding sites (Wierenga et al. 1986) and are part of a structure that was first recognised by Rossmann when he compared the NAD binding sites in the crystal structures of lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase (Rossman et al. 1974). He hypothesised that there was a common nucleotide fold that would be found in most adenine nucleotide binding proteins, and this prediction has been remarkably accurate as more crystal structures continued to be resolved. The structure in general consists of 6 parallel β-sheets with two α-helices above and below the plane formed by the β-sheets. Variations of this structure have been found consistently in nearly all adenine nucleotide binding proteins. These residues form a bend between β-strands, and the reason for this sequence invariance is two-fold. In one case, the glycine is

essential to accommodate the bend which is imposed by the secondary structure. Another glycine frequently comes in very close contact with the nucleotide so that any amino acid having a larger substituent would interfere with nucleotide binding.

In most cases, the nucleotide binding-fold includes approximately 150 amino acid residues and constitutes the amino-terminal or carboxy-terminal portion of the molecule. The localisation of these glycine residues in the C-subunit as well as the identification of Lys 72 as part of the ATP-binding site, all suggest that the amino-terminal portion of the C-subunit will constitute a nucleotide fold similar to those that have been previously characterised (Figure 2.2.3).

### 2.2.2.3 Catalytic site

The extensive sequence homologies that persist throughout the catalytic core strongly suggest that a common catalytic mechanism has been conserved. The kinetic studies of Walsh have established an ordered mechanism where the initial event is the binding of $Mg^{2+}$-ATP. Only when $Mg^{2+}$-ATP is bound is there a high affinity binding for peptide which then leads to the catalytic event (Whitehouse et al. 1983). NMR-studies of Mildvan and Kaiser have predicted a mechanism that involves a metaphosphate intermediate for the C-subunit of cAMP-dependent protein kinase, and it has been hypothesised that a nucleophilic group participates in enhancing the nucleophilic properties of the oxygen that will eventually serve as the phosphate group acceptor (Bramson et al. 1983). The identity of this nucleophile can only be speculated upon at this time. Although one of the two sulphydryl groups in the C-subunit is in close proximity to the active site and is a potential candidate, it is unlikely that this residue plays an essential role in catalysis. This Cys, furthermore, is not conserved in the other kinases (Bramson et al. 1983 ). It is more likely that one of the other invariant residues participate in catalysis, possibly Asp 166 or Asp 184 (Figure 2.2.3).



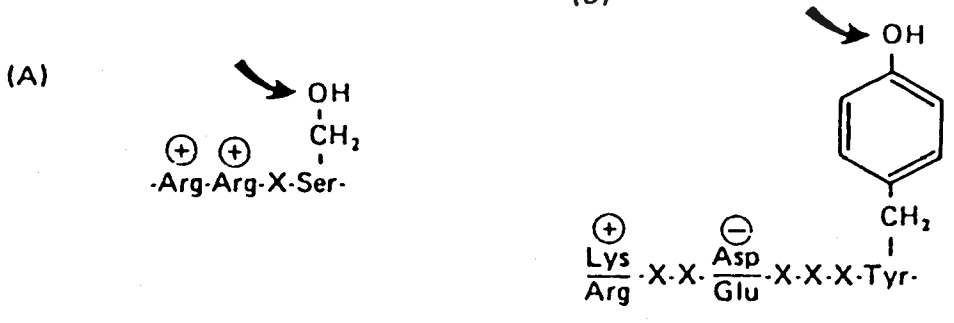



Figure 2.2.4 Requirements for peptide recognition by various protein kinases. The structural features that are required for peptide recognition by cAMP-dependent protein kinases (A) and by $p60^{src}$ and the EGF receptor (B) are summarised. The acceptor site for phosphate transfer is designated by the arrows.

### 2.2.2.4 Peptide recognition site

The peptide recognition site is the one region where differences are anticipated between the various kinases. Sequencing of naturally occurring phosphorylation sites and the subsequent synthesis of peptides corresponding to these regions has been a useful mechanism for defining peptide recognition sites. In the case of the C-subunit; very good peptide analogs have been synthesised, and variants of these peptides have been used to map the structural requirements that are necessary for peptide recognition (Bramson et al. 1983). These requirements are summarised in Figure 2.2.4A. The C-

subunit recognises two basic residues preceding the phosphorylation sites. There is a single intervening residue between the arginines and the serine which is usually small and hydrophobic. The serine hydroxyl group itself also appears to be important for peptide recognition since the inhibitor peptide, Leu-Arg-Arg-Ala-Ala-Leu-Gly, has a $K_i$ of 0.5 mM compared to a $K_m$ of 16 μM for the substrate peptide containing Ser at this position (Whitehouse et al. 1983). Ala has been substituted for Ser at position 5 in this peptide.

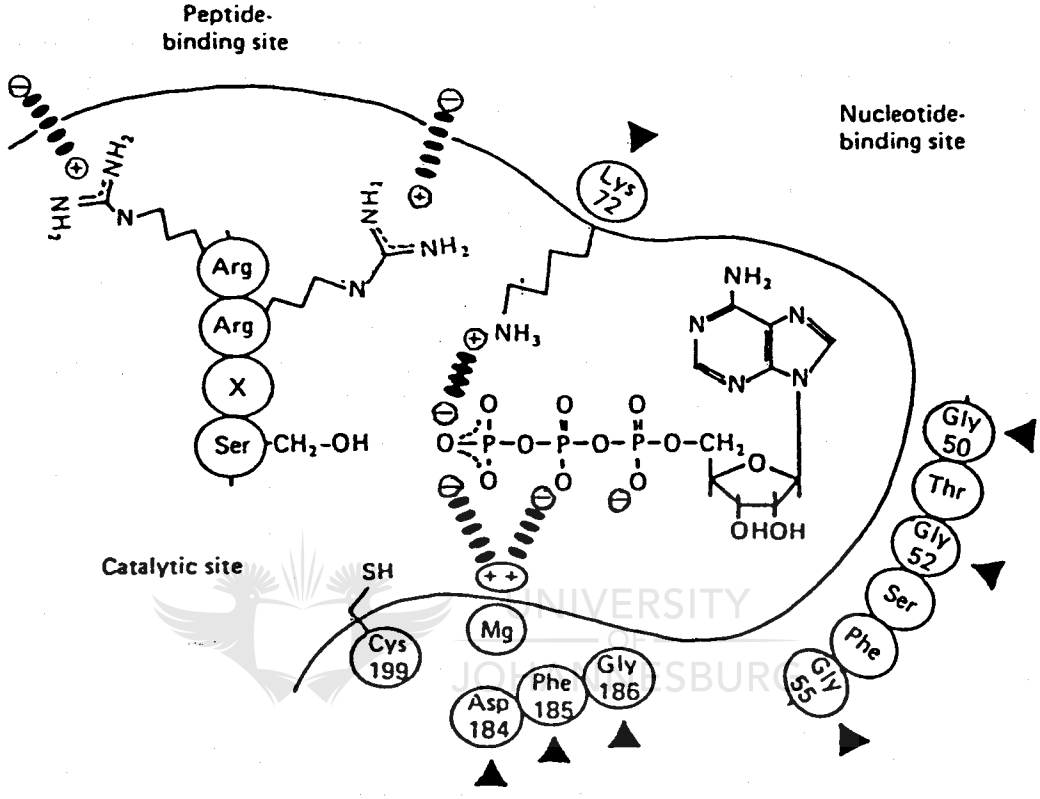



Figure 2.2.5 Model for the active site of cAMP-dependent protein kinase. Amino acids which are known to be in close proximity of the active site are indicated. Those residues which are conserved in all protein kinases are indicated by a triangle (Δ).

Similar approaches have been used to map the peptide recognition site for other kinases. For example, myosin light chain kinase, protein kinase C, and the proteolytically activated kinase are similar to cAMP-dependent protein kinase in that all recognise basic residues preceding the phosphorylation site, although the preference for one or two basic residues and also for Lys or Arg varies (Flockhart & Corbin 1982). Casein kinases, on the other hand, reflect a different specificity where acidic residues following the phosphorylation site are required (Meggio et al. 1984). Thus, there are subtle variations on specific recognition sites and also major variations perhaps similar to the specificity differences that have been structurally characterised in the serine proteases such as trypsin, chymotrypsin, and thermolysin.

Synthetic peptides have also been used to define the structural requirements that are recognised by the tyrosine-specific kinases. These kinases, such as the EGF receptor and $p60^{src}$, have similar recognition sequences although there is greater variability in the sequences that flank the phosphorylation site. As summarised in Figure 2.2.4B, there is a general requirement for an acidic residue four residues before the phosphorylation site and for a basic residue 8 residues before the phosphorylation site (Hunter 1991). The synthetic peptides that mimic these naturally occurring phosphorylation sites do serve as substrates; however, they are relatively poor substrates having $K_m$'s in the mM range suggesting that

more information than the immediate linear sequence surrounding the phosphorylation site is required for recognition of the target protein.

A model of the active site region of the catalytic subunit of cAMP-dependent protein kinase is shown in Figure 2.2.5 which also designates those features that are thought to be common to all protein kinases and those which are unique to the cAMP-dependent kinase.

### 2.2.2.5 Regulatory regions

The regions that are not included within the catalytic domain do not show homologies conserved throughout the larger family of protein kinases. The various kinases, thus, appear to represent products of creative gene splicing with the non-catalytic segments being important for at least two important functions:

- First, these non-catalytic regions play a major role in regulation of the kinase activity whether activation is mediated by peptide hormones, diacylglycerol, cyclic nucleotides or calcium.
- Second, the non-catalytic regions, in many instances, play a primary role in determining the correct cellular location of the kinase.

Kinase activation is mediated by a variety of mechanisms and usually requires an effector ligand. The diversity of these effector molecules accounts in large part for the structural differences that are seen within this family of kinases. General structural information at both the protein and DNA levels has identified regulatory regions such as those summarised in Figure 2.2.2. In the case of the cAMP-dependent kinase, the activator, cAMP, binds to a distinct regulatory subunit which then promotes dissociation of R- and C-subunits. This kinase is unique in that the regulatory site is part of a separate subunit and that activation involves subunit dissociation. The cGMP-dependent protein kinase is homologous but contains the regulatory and catalytic domains as part of a contiguous polypeptide chain (Takio et al. 1984). Myosin light chain kinase is regulated by $Ca^{2+}$-calmodulin, and sequencing of this kinase has identified a 20-residue peptide that clearly constitutes the calmodulin recognition site (Blumenthal et al. 1985). Protein kinase C also has a regulatory region which can be released proteolytically from the catalytic domain. Activation of protein kinase C requires both $Ca^{2+}$ and diacylglycerol, and the recent characterisation of cDNA clones for protein kinase C is consistent with the $Ca^{2+}$ and diacylglycerol binding regions lying in the amino-terminal region which precedes the catalytic site (Parker et al. 1986). The regulatory portion of the EGF-receptor is the EGF binding domain which lies in the amino terminal extracellular region of the molecule. This model, originally postulated on the basis of limited proteolysis, has been confirmed subsequently by sequencing several cDNA clones (Ullrich et al. 1984, 1985, Staros et al. 1985) and by site-directed mutagenesis. The transforming protein, *v-erb B*, has lost this regulatory region as well as the capacity to bind EGF (Staros et al. 1985).

The kinases also differ in terms of cellular location, although in all cases catalysis is an intracellular event. Those which play a major role in regulating glycolytic enzymes, for example, such as the cAMP-dependent protein kinase appear to be primarily soluble, cytoplasmic enzymes. Other kinases, such as the EGF receptor and the retrovirus kinase $p60^{src}$, are anchored in the plasma membrane. Protein kinase C appears to represent a kinase that is transiently associated with membranes. In each case, the portion of the molecule that is responsible for this cellular location is located in the non-catalytic region of the molecule. The major structural feature for localising the EGF receptor in the plasma membrane is the single membrane spanning segment that separates the cytoplasmic kinase domain from the extracellular EGF-binding domain (Figure 2.2.2.) The other hormone receptors such as the insulin receptor have a similar mechanism for localising the receptor and for segregating cytoplasmic and extracellular domains (Ullrich et al. 1985).

#### 2.2.2.6 Kinase regulation by phosphorylation (Auto-inhibitory domains)

The kinases themselves are also substrates for protein phosphorylation. In some cases, these phosphorylations are auto-catalytic events whereas in other cases there is communication between kinases. In addition, evidence is accumulating which demonstrates that these phosphorylations can represent important regulatory features of the enzyme (Soderling 1993). The proto-oncogene of $p60^{c\text{-}src}$, for example, differs from $p60^{v\text{-}src}$ by only the 19 amino residues at the carboxy-terminal end. The significance of this region has been correlated with phosphorylation of Tyr 527 in this region in $p60^{c\text{-}src}$. Phosphorylation of Tyr 527 in $p60^{c\text{-}src}$ reduces the kinase activity, and this Tyr is missing in $p60^{v\text{-}src}$ (Kamps et al. 1986). The loss of this regulatory site in $p60^{v\text{-}src}$ renders the molecule permanently active.

The EGF receptor is autophosphorylated, and this has been proposed to be the initial event in activation of the kinase in response to EGF binding (Bertics & Gill 1985). The EGF receptor also is phosphorylated and regulated by protein kinase C. Site-directed mutagenesis indicates that this phosphorylation may be required for proper processing of the receptor (Lin et al. 1986). Some of these phosphorylation sites are summarised also in Figure 2.2.2.

#### 2.2.2.7 Conclusion

Taylor et al. (1993) have described the essential features of cAMP-dependent protein kinase (cAPK) that can serve as a prototype for the large family of protein kinases. The close correlation of the crystal structure with all of the chemical and genetic information that preceded the structure solution is striking and provides a solid basis for beginning to understand how a protein kinase functions.



### 2.2.3 Protein tyrosine phosphorylation

Three remarkable discoveries, 15 years ago, generated considerable excitement in the field of cellular regulation by protein phosphorylation.

- First, the finding by the groups of Erickson (Collett & Erikson 1978) and Varmus and Bishop (Levinson et al. 1978) that the product of the *src* gene responsible for the oncogenicity of Rous sarcoma virus was a protein kinase they designated as $pp60^{src}$.
- Second, the unexpected report by Hunter and Sefton (1981) that this kinase, unlike all previously known enzymes, phosphorylated its protein substrates exclusively on tyrosyl residues.
- Third, the non-transforming homologue of *v-src*, that is the cellular *c-src*, was identified. *c-src* Encodes a product ($pp60^{c\text{-}src}$), which differs from its oncogenic viral counterpart by having, among other discrete mutations, a short extension at the C terminus. This extension carries a phosphotyrosyl residue that keeps the activity of the enzyme under control (Weinberg 1989). Today more than a dozen tyrosine kinases of cellular or viral origin are known, and their number continues to grow (Hunter 1991, Fischer 1993).
- Finally came the seminal discovery from the laboratory of Cohen that the receptor for epidermal growth factor was itself a tyrosine kinase whose activity was induced by binding of the ligand. Since then, many families of receptors with tyrosine kinase activities have been identified. They all have an external, ligand-binding domain, some with cystein-rich regions, a single transmembrane segment, and a cytoplasmic tyrosine kinase (PTK) domain (Yarden & Ullrich 1988, Fantl et al. 1993).

### 2.2.4 Protein phosphatases

Targeting subunits are particularly crucial for serine/ threonine phosphatases because these enzymes are not geared to recognise specific sequences, or structural determinants within their substrates. Furthermore, unlike the kinases, they consist of just a few types of enzymes that have broad and overlapping specificities (Cohen 1989, Cohen & Cohen 1989). Thus they have to depend on regulatory subunits or binding proteins to direct them toward particular compartments of the cell where they will encounter particular substrates. That is the case, for instance, of the type 1 phosphatase whose catalytic subunit can bind to a glycogen-recognising subunit, a myosin-recognising subunit, or an inhibitory molecule called Inhibitor-2. In each of these forms, the enzyme recognises a particular set of substrates. Formation or dissociation of these complexes is under hormonal control (Hubbard & Cohen 1989).

## 2.3 Signal perception and transduction in plants

There is particular need for plants to be able to register and adapt to a changing environment as they are generally unable to escape from a given location when conditions change and become unfavourable.

Cellular mechanisms exist that receive and interpret, information from the surrounding environment, inducing a cellular response. Closely controlled intracellular physico-chemical conditions are important for metabolic processes, as it could prove both hazardous, and indeed difficult, for a cell to allow external messages direct access to its interior. Cell : environment interactions have evolved in which signals are registered first at the exterior surface of the plasma membrane. A sequence of events - often referred to as signal transduction, then follow, in order for the signal to be conveyed to the appropriate response inside the cell. A number of different eukaryotic signal transduction pathways are currently known. Several of the most prominent pathways have a number of biochemical features in common. A schematic representation of such systems is given in Figure 2.3.1 (Drøbak 1992).

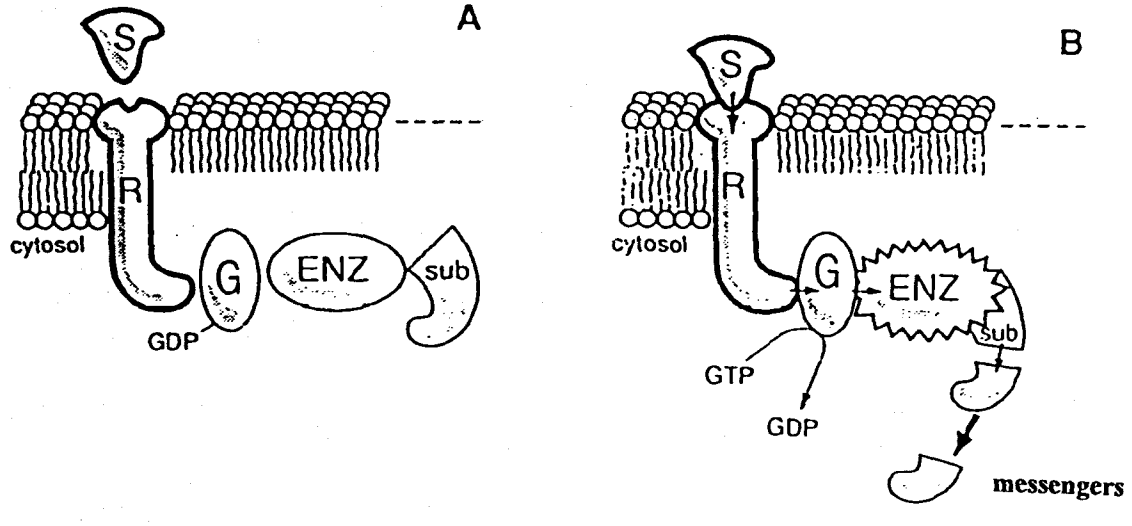



Figure 2.3.1 Outline of receptor-coupled transmembrane signalling processes common to several eukaryotic signal transducing systems. A. Unstimulated cell; B. Stimulated cell. Symbols: S, signal; R, receptor molecule; G, regulatory GTP-binding protein; ENZ, enzyme; sub, substrate.

The initial step in the cell's recognition of the arrival of a signal at the cell surface is achieved by a molecular interaction between the signal (S) and an appropriate membrane-associated receptor protein (R). This interaction may in many cases convert one or more membrane-associated GTP-binding proteins (G-proteins) into their active form(s). The interaction between the signal-receptor complex and the GTP-binding protein(s) leads to the activation of an enzyme(s) localised on the cytosolic face of the plasma membrane. As a result of this enzyme activation, molecules are formed which are capable of carrying the message conveyed by the extracellular signal to intracellular response-elements. Although these message-carrying molecules or ions, are produced relatively late in the transduction cascade, they are often referred to as "second messengers". Several second messengers are now known to be functioning in mammalian cells. These include calcium ions, cAMP, cGMP, 1,2-diacylglycerol (DG), inositol 1,4,5-trisphosphate [Ins(1,4,5)$P_3$] and probably inositol 1,3,4,5-tetrakisphosphate [Ins(1,3,4,5)$P_4$]. Of these only the $Ca^{2+}$ ion is known beyond any doubt to be involved in transmembrane signalling in plants (Poovaiah & Reddy 1987). However, evidence has been rapidly accumulating suggesting that Ins(1,4,5)$P_3$ also plays a role as a second messenger in plant cells (Coté & Crain 1993). In higher plants, no function for cAMP as second messenger has been demonstrated yet, although there is convincing evidence for its occurrence (Brown & Newton 1981).

## 2.3.1 Calcium as a second messenger in plants

The divalent nature of $Ca^{2+}$ and the lack of stringent crystal field requirements for $Ca^{2+}$ chelate formation allows this ion to interact with a wide range of biological molecules with very fast association/dissociation kinetics. The ability of $Ca^{2+}$, or $Ca^{2+}$ and calcium-binding protein complexes (e.g. $Ca^{2+}$-calmodulin) to modulate cellular processes has resulted in the development of signalling systems where $Ca^{2+}$ acts as a second messenger (Drøbak 1992).

The $Ca^{2+}$ concentrations surrounding most plant and mammalian cells are in the low millimolar region. The concentration (or more precisely, activity) of $Ca^{2+}$ in the cytosol needs to be kept very low in "unstimulated" cells to avoid constant activation of $Ca^{2+}$ dependent response elements. Studies using intracellular $Ca^{2+}$-sensitive dyes and microelectrodes have shown that the $Ca^{2+}$ activity in the cytosol of both mammalian and plant cells is maintained at around 50-200 nM when the cell is not being stimulated (Felle 1989). The very steep electrochemical gradient across the plasma membrane, and the fact that electrical driving forces for cytosolic $Ca^{2+}$ entry also exist, necessitate the presence of ion transporting systems with the ability of rapidly removing $Ca^{2+}$ from the cytosol. The $Ca^{2+}$transporting systems in plant cells believed to be of most importance for control of cytosolic $Ca^{2+}$ at low activities are, the plasma membrane and endoplasmic reticulum $Ca^{2+}$-ATPases and the tonoplast $H^+/Ca^{2+}$-antiport system.

Upon stimulation of cells by a wide range of extracellular signals a rapid increase in cytosolic $Ca^{2+}$ ensues. Since $Ca^{2+}$ can neither be synthesised nor broken down, the only mechanisms by which a change in $Ca^{2+}$ activity can be achieved is by altering the cellular $Ca^{2+}$ buffering capacity or by movement of $Ca^{2+}$ from one compartment to another.

The debate about the source of $Ca^{2+}$ mobilised during signalling events has been going on for a considerable length of time amongst researchers and is still not completely resolved. In the early 1980s the general view was that $Ca^{2+}$ was somehow introduced into the cytoplasm from the extracellular medium via signal-sensitive $Ca^{2+}$ channels located in the plasma membrane. However, the discovery around 1983 of the phosphoinositide signalling system by Berridge, Irvine, Michell and their co-workers has dramatically changed this view (Berridge & Irvine 1989) (See section 2.4.2.5).

It is a widespread phenomenon that extracellular signals alter cytosolic levels of $Ca^{2+}$ in plants and animals. The change in cytosolic $Ca^{2+}$ is considered to be the primary event in triggering the cellular response. The extracellular and environmental stimuli which alter cytosolic $Ca^{2+}$, includes hormones,

light, and gravity. In animals it is well documented that some external signals cause changes in cytosolic $Ca^{2+}$ (Berridge & Irvine 1989, Nishizuka 1984 a & b). Response in these systems is always preceeded by a rise in $Ca^{2+}$ level. Because of the paucity of information on direct $Ca^{2+}$ measurements in plants, changes in intracellular $Ca^{2+}$ concentrations (increase or decrease) in response to signals are not well understood. Indirect evidence suggest that some signals (e.g., cytokinins) may increase and some other signals (e.g., auxin) may decrease the cytosolic $Ca^{2+}$. Direct measurements of $Ca^{2+}$ in the cytosol in response to the signals would provide valuable information.

### 2.3.1.1. Calcium binding proteins

In most cases where $Ca^{2+}$ acts as the second messenger of a stimulus, the chemical signal that initiates the response is not $Ca^{2+}$ itself but the complex between $Ca^{2+}$ and any one of a number of proteins of a special class called calcium-binding proteins (CBP) (Kretsinger 1981). These binding proteins are defined as proteins that bind $Ca^{2+}$ with high-affinity (Kd $10^{-8}$ to $10^{-6}$ M) and thus undergo a conformational change leading to change in their ability to interact with other proteins and alter their functions. CBP show a high degree of homology (Cheung 1980). The chemical properties of $Ca^{2+}$-binding sites allow these proteins to have a high $Ca^{2+}$ to $Mg^{2+}$ selectivity ratio, which enables them to bind $Ca^{2+}$ preferentially despite the high intracellular concentrations of $Mg^{2+}$ (1000 times higher than that of $Ca^{2+}$). CBP are functionally inert in the absence of bound $Ca^{2+}$.

Calmodulin, the best known and more generally distributed $Ca^{2+}$-binding protein, mediates $Ca^{2+}$ messages in most eukaryotic cells (Cheung 1980, Cormier et al. 1980, Klee at al. 1980). Other CBP are found in vertebrates and, even in these animals, are confined to a small number of tissues or cells where they play restricted roles. These proteins include troponin C (in skeletal and cardiac muscles), parvalbumin (in skeletal muscle), and the vitamin-D-dependent $Ca^{2+}$-binding proteins (found in intestine, kidney, etc.)(Kretsinger 1981, Klee at al. 1980).



#### 2.3.1.1.1 Calmodulin

- Detection and isolation

Calmodulin was first discovered in animals by Cheung (1980) as an activator of brain cyclic nucleotide phosphodiesterase. Subsequently, calmodulin has been isolated and characterised from a large variety of animal tissues ranging from protozoans to mammals (Klee et al. 1980). In 1978, the presence of calmodulin-like activity was reported in plants and fungi (Cormier et al. 1980, Muto & Miyachi 1977, Anderson et al. 1980). Subsequently, Cormier's group purified calmodulin from plant sources using an elegant but simple fluphenazine-Sepharose affinity chromatography technique (Cormier et al. 1980, Anderson et al. 1980, Dubery & Schabort 1987). This procedure is based on the fact that anti-psychotic phenothiazine drugs (e.g., trifluoperazine and chlorpromazine) bind to calmodulin with high affinity in the presence of $Ca^{2+}$ and with much reduced affinity in the absence of $Ca^{2+}$. Comparison of the physical and biochemical properties of calmodulin isolated from various sources have revealed a high degree of conservation of the structural and functional properties of this protein during evolution. Its ubiquitous distribution and highly conserved structure suggest that it may play a fundamental role in mediating intracellular $Ca^{2+}$-dependent processes.

- Calcium binding to calmodulin

Examination of the tertiary structure of calmodulin revealed four roughly similar domains, each of which contains a $Ca^{2+}$-binding site (Marmé 1986). Recent studies on the three-dimensional structure of rat testis calmodulin have shown that calmodulin consists of two globular lobes connected by a long

exposed α-helix (Marmé 1986). Each lobe binds to two $Ca^{2+}$ ions through helix-loop-helix domain. Structural studies of animal calmodulin indicate either two classes of sites or negative cooperativity between the sites. Upon binding $Ca^{2+}$, calmodulin undergoes a large conformational change accompanied by a 5 to 10% increase in α-helix content. The $Ca^{2+}$-dependent conformational change explains why $Ca^{2+}$ converts the inactive form of calmodulin to an active one capable of interacting with the protein or enzyme that initiates the biological response. The mechanism of activation of several enzymes by calmodulin occurs in two steps (Poovaiah & Reddy 1987): (1) $Ca^{2+}$ binds to calmodulin resulting in a conformational change of calmodulin; and (2) the active conformer of calmodulin interacts with an inactive or partially active enzyme thereby producing a conformational change and activation of the enzyme. Klee et al. (1980) concluded that four different conformations of $Ca^{2+}$-calmodulin complex exist which can be recognised by different proteins, i.e., in a given conformation, calmodulin interacts with a particular enzyme or set of enzymes. In this way calmodulin could translate quantitative $Ca^{2+}$ signals of different amplitudes into qualitatively different cellular responses.

- Inhibitors of calmodulin activity

The activation of various enzymes by calmodulin can be inhibited by several classes of drugs, including phenothiazines (e.g., chlorpromazine, fluphenazine, and trifluoperazine), naphthalene sulfonamide derivatives (e.g., N-(6-aminohexyl) 5-chloro-l-naphthalene sulphonamide hydrochloride which is also called W-7), phenylbutylamine derivatives, and nitrogen mustard-based adrenergic antagonists (Poovaiah & Reddy 1987). These drugs have been widely used in both animals and plants to understand the involvement of calmodulin in various physiological processes. Although these drugs produce interesting effects on cells when used at noncytotoxic doses, it is known that other molecules can interact with these drugs (Nishizuka 1984a, Raghothama et al. 1985). In addition, little is known about the intracellular concentration of the drugs utilised in the various studies. Studies on the uptake of chlorpromazine by corn coleoptile segments, showed that uptake increased with time and nearly doubled from 2 to 4 hr of incubation (Raghothama et al. 1985). The activity of protein kinase C, a calmodulin-independent enzyme, has been shown to be profoundly inhibited by calmodulin inhibitors such as chlorpromazine, dibucaine, and trifluoperazine. Care must therefore be exerted in identifying calmodulin-dependent effects solely on the basis of inhibition by these drugs (Raghothama et al. 1985, Poovaiah & Reddy 1987). Despite the limited investigational utility of these drugs in calmodulin physiology, they have proved useful in mapping functional domains of calmodulin and in purifying calmodulin from various sources with phenothiazine affinity columns.

#### 2.3.1.1.2 Other calcium-binding proteins in plants

Apart from calmodulin, the occurrence of other $Ca^{2+}$-binding proteins has been reported in plants. Ranjeva (Ranjeva & Boudet 1987) have demonstrated a 63-kDa $Ca^{2+}$-binding protein called calciprotein that acts as a reversible subunit of quinate: $NAD^+$ oxidoreductase (QORase). In dark-grown cells this enzyme becomes oligomeric, $Ca^{2+}$-dependent, and contains a specific $Ca^{2+}$-binding moiety (63 kDa), whereas in light-grown cells it is monomeric and insensitive to $Ca^{2+}$. Calciprotein has been found to protect the QORase from dephosphorylation. The presence of a member of calcimedins, a newly described class of calcium-binding proteins in animal cells (Moore & Ackerman 1984), has also been demonstrated in plants (Dauwalder et al. 1986).

### 2.3.1.2 Calcium- and calmodulin-regulated enzymes

The $Ca^{2+}$-calmodulin complex can act in two ways: directly on an effector system or indirectly on a regulatory system, usually a protein kinase which through phosphorylation promotes or inhibits the

activity of other enzymes. These two modes of action allow fast and slow responses to be mediated by $Ca^{2+}$ and calmodulin. In plants, enzymes such as NAD kinase (Muto & Miyachi 1977, Anderson et al. 1980), $Ca^{2+}$ATPase (Marmé 1982, Dieter & Marmé 1980), $H^{+}$ATPase (Zocchi et al. 1983, Zocchi 1985), quinate: $NAD^{+}$ oxidoreductase (Ranjeva et al. 1986) and protein kinases (Poovaiah & Reddy 1987) have been shown to be regulated by $Ca^{2+}$ and calmodulin.

#### 2.3.1.3 External signals and cellular calcium

Considerable indirect evidence and some direct evidence indicates that a rise in cytosolic $Ca^{2+}$ occurs in many cells in response to extracellular stimuli (Nishizuka 1988, Berridge & Irvine 1989, Poovaiah & Reddy 1987, Lehle 1990, Coté & Crain 1993). How the primary stimuli bring about changes in cytosolic $Ca^{2+}$ is not well understood in plants. Depending on the cell and on the stimulus, cytosolic $Ca^{2+}$ levels increase either as a consequence of the inflow of extracellular $Ca^{2+}$ or of the release of $Ca^{2+}$ from intracellular stores (Berridge & Irvine 1989, Coté & Crain 1993). Present evidence in animals indicates that influx at the plasma membrane level and/or release from intracellular stores, especially from the endoplasmic reticulum, are involved in the process (Lehle 1990, Coté & Crain 1993). This process as well as the role of phosphoinositides is discussed in the following section.

#### 2.3.1.4 Concluding remarks

The second messenger role of $Ca^{2+}$ becomes more important since the efforts of plant physiologists to establish such a role for cAMP have not been successful. Although plants differ vastly from animals, there seems to be considerable similarity in their $Ca^{2+}$ messenger systems, except in their lack of cAMP messenger system. It is apparent that the $Ca^{2+}$ messenger system is more complex than the cAMP messenger system in two ways: (1) the means for translating changes in the amounts of cytoplasmic $Ca^{2+}$ into cellular events are more diverse than those by which cAMP acts, and (2) the metabolism of $Ca^{2+}$ by the cell is more complex than that of cAMP.

It is evident that the functions of $Ca^{2+}$in plants, as in animals, are diverse, multifaceted, and complex. The manner of $Ca^{2+}$ regulation of diverse physiological processes can be resolved if one considers the multiple mechanisms by which $Ca^{2+}$ regulates biochemical processes. Calcium regulation occurs through many distinct branches. First, altered $Ca^{2+}$ levels affect calmodulin, thereby regulating a number of enzymes including protein kinases. These protein kinases could further regulate biological properties of other enzymes. Second, $Ca^{2+}$ acts through protein kinase C which probably regulates the activities of a number of other proteins. In addition to these mechanisms, $Ca^{2+}$ could evoke different responses in different cells depending on the types of $Ca^{2+}$-regulated enzymes (e.g., protein kinases) and their substrates. Furthermore, $Ca^{2+}$ alone could directly modulate the activity of some of the enzymes. It is therefore apparent that insight into the molecular mechanisms mediating the diverse actions of $Ca^{2+}$ will arise from a multifaceted approach to this fundamental problem.

### 2.3.2 The phosphoinositide system

The phosphoinositide signalling system fits the general scheme outlined in Figure 2.3.1. A more detailed description of this system is given in Figure 2.3.2. Upon stimulation of mammalian cells by selected agonists known to induce a rise in intracellular $Ca^{2+}$, it was found that a minor inositol-containing phospholipid, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)$P_2$], localised in the inner leaflet of the plasma membrane, is hydrolysed by the enzyme phosphoinositidase C (phospolipase C). This results in the formation of two molecules, diacylglycerol (DG) and Ins(1,4,5)$P_3$, DG remains in the plasma membrane matrix where it activates a protein kinase (protein kinase C). Ins(1,4,5)$P_3$, which is highly polar, diffuses from the plasma membrane to intracellular membrane-bounded $Ca^{2+}$ stores (likely to be

associated with the endoplasmic reticulum) where it interacts with specific Ins(1,4,5)$P_3$ receptor molecules. The Ins(1,4,5)$P_3$-receptor interaction results in the opening of $Ca^{2+}$ channels and $Ca^{2+}$ is released into the cytosol. Thus, certain agonists are able to trigger a bifurcated cellular response involving both activation of protein kinase C and a rise in cytosolic $Ca^{2+}$. The use of two transduction strands opens the possibility for crosstalk and results in a high degree of flexibility in cellular interpretation. This may explain why the phosphoinositide signalling system is involved in the transduction of a large number of eukaryotes.

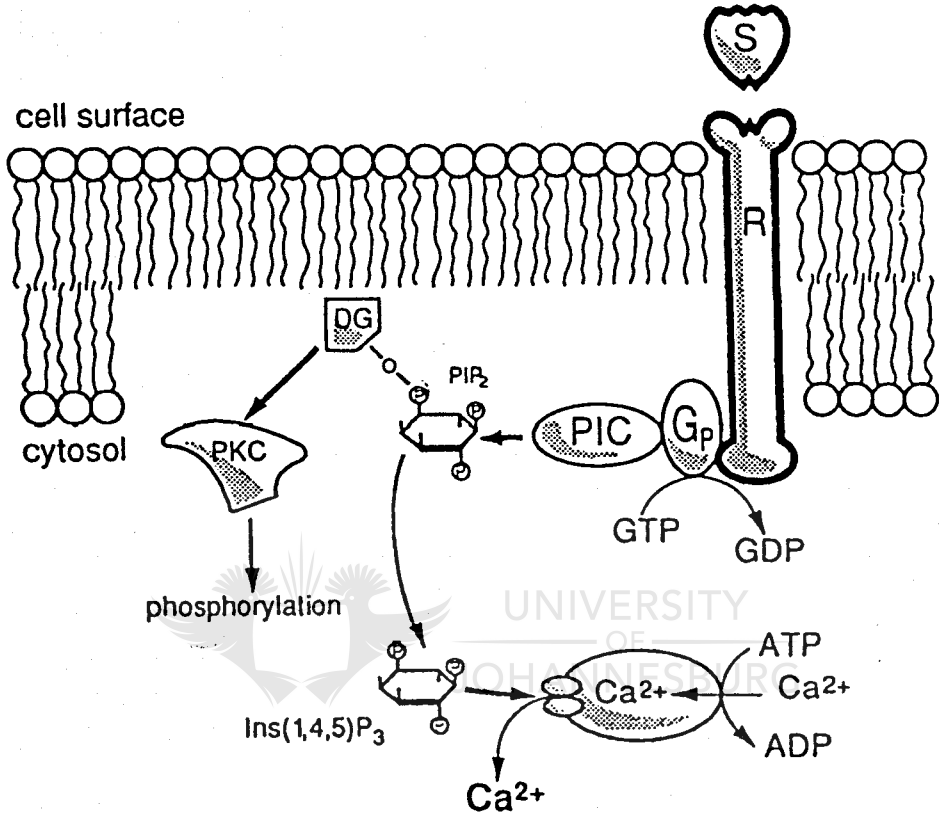



Figure 2.3.2 Outline of the phosphoinositide signalling system in mammalian cells

PtdIns(4,5)$P_2$ is formed by a two-step phosphorylation of phosphatidylinositol (PtdIns)(Figure 2.3.3). PtdIns is first phosphorylated in the 4 position of the inositol ring resulting in the formation of phosphatidylinositol 4-phosphate [PtdIns(4)P]. This lipid is then further phosphorylated in the 5 position yielding PtdIns(4,5)$P_2$. Two phosphohydrolases work concomitantly with the 4- and 5-hydroxykinases so PtdIns(4)P and PtdIns(4,5)$P_2$ are constantly being formed and degraded. This phosphoinositide cycle is not futile. Both DG and Ins(1,4,5)$P_3$ are rapidly hydrolysed, so removal of the signal causing the activation of phosphoinositidase C (PIC) results in deactivation of protein kinase C and a return of cytosolic $Ca^{2+}$ concentrations to unstimulated levels, courtesy of the $Ca^{2+}$ transport systems.

Since $Ca^{2+}$ also acts as a second messenger in plant cells it is an obvious possibility that fluxes are controlled by a system corresponding to the mammalian phosphoinositide signalling system. Direct evidence supporting this idea came from experiments summarised in Table 2.3.1. It has been suggested that the phosphoinositide signalling system may function in several important transduction events in plant cells, and evidence is accumulating that supports this view (McAinsh et al. 1990, Ettlinger & Lehle 1988, Morse et al. 1987).

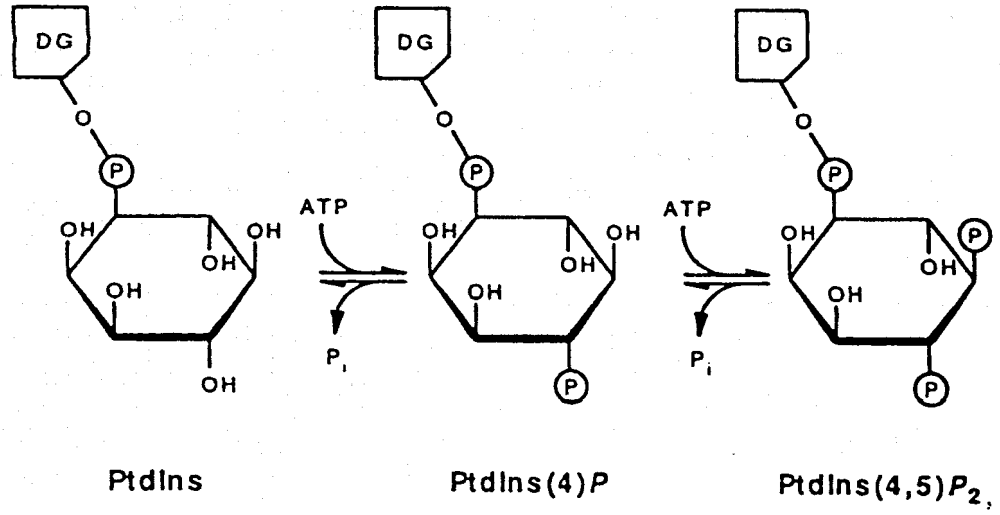



Figure 2.3.3 Interconversion between phosphatidylinositol and polyphosphoinositides.

Table 2.3.1. Identified components of the plant phosphoinositide system

| | |
|---|---|
| 1. Ability of Ins(1,4,5)$P_3$ to release $Ca^{2+}$ from intracellular stores | (Drøbak & Ferguson 1985, Schumaker & Sze 1987) |
| 2. Presence of phosphatidylinositol, phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate in plant membranes | (Drøbak et al. 1988, Irvine et al. 1989) |
| 3. Presence of kinases/phosphatases involved in polyphosphoinositide turnover | (Drøbak et al. 1988, Sommarin & Sandelius 1988) |
| 4. Presence of phospholipase(s) C capable of hydrolysing phosphatidyl inositol 4,5-bisphosphate | (McMurray & Irvine 1988) |
| 5. Presence of enzymes which in several respects resemble mammalian protein kinase C | (Drøbak et al. 1988) |
| 6. Presence of enzymes capable of rapidly metabolising Ins(1,4,5)P3 | (Drøbak et al. 1991, Joseph et al. 1989) |



### 2.3.2.1 Polyphosphoinositide metabolism

*In vitro* enzyme studies using isolated membrane fractions as enzyme source and [$\gamma$-$^{32}$P]ATP as phosphate donor have demonstrated that both PtdIns-4-hydroxykinase and PtdIns(4)P-5-hydroxykinase are present and can utilise both endogenous and exogenous substrates (Sommarin & Sandelius 1988). Such studies have identified the plasma membrane as one of the main sources of polyphosphoinositide-kinases, and it has been observed that the PtdIns-kinase is around 10-fold more active than the PtdInsP kinase under optimum assay conditions. In this regard the plant poly-phosphoinositide kinases are similar to the mammalian enzymes.

However, in *in vivo* labelling studies carried out by incubating plant cells or tissues with [$^{32}$P]orthophosphate it is found, after short incubation times, that a large percentage of total phospholipid label (approx. 30%) is present in the monoester phosphate of PtdIns(4)P, indicating that this lipid is metabolised very rapidly (i.e. the PtdIns 4-hydroxykinase is highly active). In contrast PtdIns$P_2$ only incorporates very small amounts of label after both short- and long-term incubations (Drøbak & Ferguson 1985). The incorporation of label into PtdIns$P_2$ is far lower than one would expect on the basis of *in vitro* studies of PtdInsP kinase activity. Explanations for the apparent anomaly is the following: The chemical level of PtdIns$P_2$ are extremely low, that however rapidly the

label is incorporated into $PtdIns_2$ the total amount of incorporated radioactivity will always appear very minor when compared to the label found in the total phospholipid pool (except after extremely short labelling times - but investigation of this possibility is very difficult).

Some researchers see the very low levels (or slow rate of metabolism) of $PtdInsP_2$ as an obstacle to $Ins(1,4,5)P_3$/DG formation. This is not necessarily the case, as only very small amounts of $PtdInsP_2$ are needed to fulfil the role as second messenger precursor.

Another possibility could be that $PtdInsP_2$ kinase may be under direct control of the signal-response coupling complex and may only be active when an agonist interacts with its receptor. Large quantities of $Ins(1,4,5)P_3$/DG could be produced in a short period of time without necessarily changing the chemical levels of $PtdInsP_2$. Simultaneous activation of phosphoinositidase C and the PtdInsP kinase would result in a very rapid flux through the $PtdInsP_2$ pool. The rate of flux is often the important feature in signalling events, not absolute chemical amounts.

The high rate of PtdInsP turnover and the considerable amount of adenylate energy expended by the PtdIns4-kinase in plant cells, indicate that the function of PtdInsP is not solely to act as precursor for $PtdInsP_2$ formation. Polyphosphoinositides are also capable of modulating the activity of the plasma membrane $H^+$ATPase in sunflower hypocotyls (Memon & Boss 1990) and several additional functions for polyphosphoinositides in plant cells are under investigation.

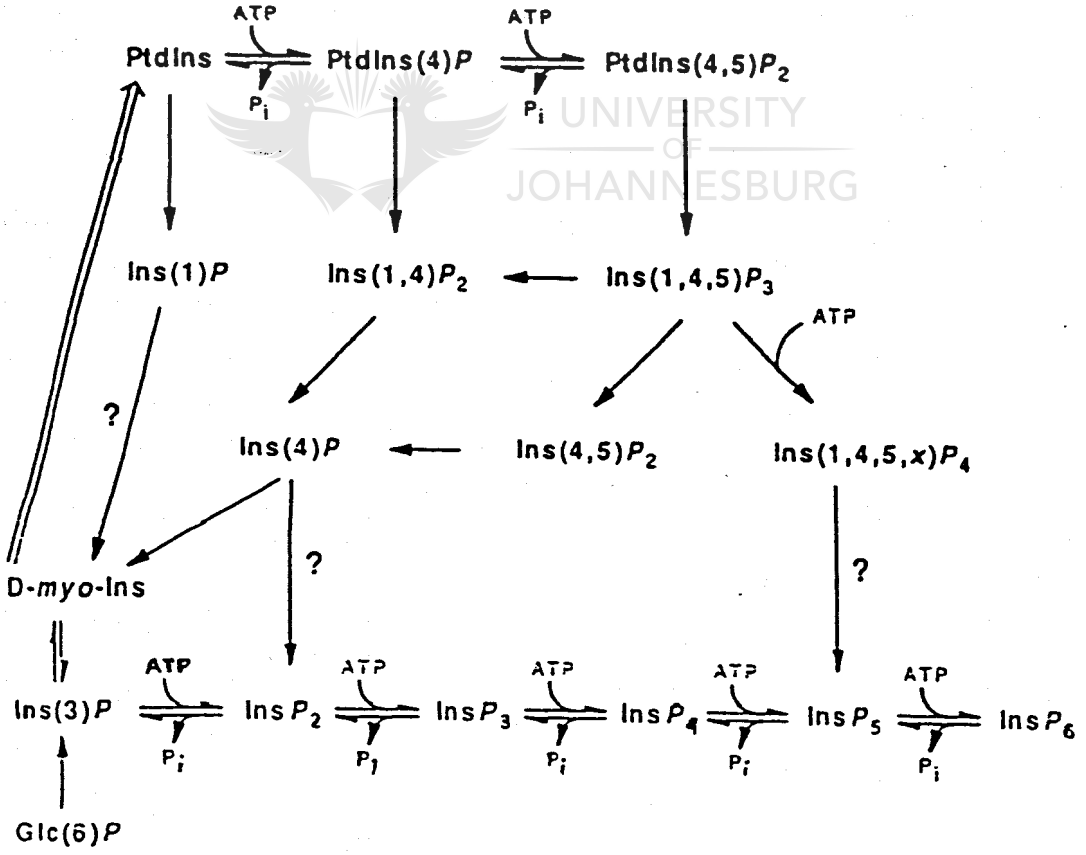



Figure 2.3.4 Likely pathways for inositol/inositol phosphate metabolism in plant cells. Arrows indicate enzymic steps which have been characterised *in vivo* or *in vitro*. All isomers are numbered using the D-configuration. Arrows marked with "?" indicate potential pathways which have not yet been firmly established. The diagram has been constructed on the basis of data from references sited by Drøbak (1992).

### 2.3.2.2 Metabolism of $Ins(1,4,5)P_3$

Sixty three different isomers of D-myo-inositol phosphates are theoretically possible and 12 have been found to be present in different mammalian cells. This array of inositol phosphates is causing problems for researchers in the mammalian field. Plant scientists are faced with the fact that inositol phosphate metabolism in plants is likely to have an even higher degree of complexity. In mammalian cells all known inositol phosphates are derived as a result of phosphoinositide hydrolysis. In plants cells at least one additional pathway for inositol phosphate production exists. This is the phytic acid (inositol hexakisphosphate) pathway. Little is known about the route of phytic acid formation and degradation in plants, except that D-myo-inositol 3-monophosphate probably is the precursor and that enzymes exist which are capable of stepwise phosphorylation of inositol phosphates and dephosphorylation of phytic acid (Loewus & Loewus 1983). It has recently been demonstrated that phytic acid in the slime mould *Dictyostelium* is formed by stepwise phosphorylation of myo-inositol and that this pathway bypasses the inositol phosphate isomers known to be involved in signal transduction (Stephens & Irvine 1990). Whether a similar biosynthetic route is followed in plant cells is currently not known. Studies have made it clear that enzyme systems are present in plants which are capable of rapidly metabolising $Ins(1,4,5)P_3$ using pathways not encountered in other eukaryotes. An overview of the possible pathways of inositol phosphate formation and degradation in plant cells is given in Figure 2.3.4.

### 2.4.2.5 What is the nature of the $Ins(1,4,5)P_3$-sensitive $Ca^{2+}$ pool?

Evidence from studies using patch-clamp and indicator dye techniques strongly points towards the vacuole as being the $Ins(1,4,5)P_3$-sensitive $Ca^{2+}$ store in plant cells (Alexandre et al. 1990). Much more information is needed before the vacuole can be accepted as the sole target for $Ins(1,4,5)P_3$-induced $Ca^{2+}$ release in plant cells. Investigations of the spatial distribution of $PtdIns(4,5)P_2$, phosphoinositidase C, $Ins(1,4,5)P_3$ tonoplast receptors and $Ins(1,4,5)P_3$-metabolising enzymes within the cell are clearly necessary before a detailed assessment of temporal aspects of signal-induced $Ca^{2+}$ release is possible. Although the distance between the plasma membrane and the tonoplast varies from cell type to cell type, and also within a cell, the average distance is often small (100-200 nm). $Ins(1,4,5)P_3$ could thus easily diffuse from plasma membrane to tonoplast in a short period of time. The vacuole occupies approx. 90% of the intracellular volume in many plant cells and the concentration of free $Ca^{2+}$ is typically 1-2 mM. The enormous difference in both $Ca^{2+}$ concentration and total $Ca^{2+}$ between the vacuole and the cytoplasm necessitates extremely meticulous cellular control over the opening and closure of $Ca^{2+}$ channels in the tonoplast. The failure to maintain very stringent control of $Ca^{2+}$ efflux will rapidly result in the build up of lethal $Ca^{2+}$ concentrations in the cytoplasm.

It is an open question why plant cells should have developed mechanisms for signal transduction which in many respects resemble those of mammalian cells and yet use a signal-sensitive $Ca^{2+}$ pool which is so different from that found in most other eukaryotes. As more is learnt about the details of $Ca^{2+}$ handling in plant cells it is likely that a much more complex picture with many nuances will emerge. That this is already happening is perhaps best illustrated by a recent study of abscisic acid-induced $Ca^{2+}$ fluxes in guard cells which points to the possibility of several signal-sensitive $Ca^{2+}$ pools operating in concert (Schroeder & Hagiwara 1990).

### 2.3.2.4 Enzymes of the phosphatidylinositol cycle

The existence of several enzymatic activities assumed to be involved in the phosphatidylinositol cycle has been described.

#### 2.3.2.4.1 Phosphoinositide kinases

In animals, a PtdIns kinase transfers a phosphate from ATP onto the 4 position of the inositol head-group to form PtdIns4P. A separate kinase adds another phosphate to the 5 position to give PdtIns(4,5)$P_2$ (Lehle 1990, Coté & Grain 1993). PtdIns and PtdInsP kinase activities have been detected in plasma membrane fractions from shoots and roots of wheat where their products are also found (Sommarin & Sandelius 1988). Phosphorylation of both endogenous or exogenous substrates requires ATP and $Mg^{2+}$ (but could not be substituted by $Ca^{2+}$). Furthermore, it has been shown that in *Catharanthus* cells PtdIns and PtdInsP kinase activities vary during the cell cycle with peak activities in the cell division phase (Heim & Wagner 1987, Heim et al. 1987).

2.3.2.4.2 Phospholipase C and G protein coupling

The agonist-induced hydrolysis of PtdIns(4,5)$P_2$ to give Ins(1,4,5)P, and DAG is catalysed by a phosphoinositide-specific phospholipase C, PLC (other terms for this enzyme are phospho-inositidase C, PIC, or phosphodiesterase, PDE). This is the key step in signal transduction (Coté & Crain 1993). There are a number of PLC isoenzymes in animals with unknown differences in structure and function (Downes et al. 1988, Meldrum et al. 1991, Rhee & Choi 1992).

Several lines of experimental evidence indicate the involvement of a guanine nucleotide-dependent protein (G protein) in PtdIns(4,5)$P_2$ hydrolysis, comparable with hormone receptor coupling to adenylate cyclase or light-activated rhodopsin coupling to cyclic GMP phosphodiesterase (Meldrum et al. 1991, Rhee & Choi 1992). The evidence for involvement of Gp proteins (the subscript p stands for phospholipid) is based primarily on the observation that GTP or non-hydrolysable analogues stimulate PtdIns(4,5)$P_2$ hydrolysis. Solubilisation of a receptor-G-protein-PLC complex has been achieved, indicating a physical association of these components (Aiyar et al. 1989).

The current state in plants is that PLC activities were detected in celery (McMurray & Irvine 1988), green alga *Dunaliella salina* (Einspahr et al. 1989) and wheat seedlings and, with regard to specificity and localisation, they fulfil criteria making them an element in signal transduction. Whether these PLC activities should be considered as an element of the PtdIns cycle remains uncertain. All the plant PLC activities revealed calcium stimulation in contrast to the enzymes from animal tissues.

Evidence for the presence of G proteins in plants is emerging, albeit still of circumstantial nature, and is based on experiments showing binding of GTP to crude extracts (Hasunuma & Funadera 1987) or microsomal membranes (Warpeha et al. 1991). Identification of G proteins in the plasma membrane, using antisera raised against conserved amino acid sequences of animal G proteins, has indicated that the plant proteins resemble their animal counterparts in some properties, while they differ in others (Blum et al. 1988). So far, a physiological role was not assigned in any of these studies. Regulation of PLC by heterotrimeric G proteins in plants will be conclusively established only when a purified plant PLC is reconstituted with a purified plant heterotrimeric G protein and activated (or inactivated) by activation of the G protein (Coté & Crain 1993).

2.3.2.4.3 Protein kinase C and diacylglycerol

Protein kinase C in animals is a phospholipid-dependent and $Ca^{2+}$-dependent serine/threonine protein kinase. It is now known to be a large family of proteins with multiple subspecies, possessing individual enzymatic characteristics and serving distinct functions as external stimuli (Kikkawa & Nishizuka 1986, Nishizuka 1988). The enzyme is activated by DAG, which increases the affinity for $Ca^{2+}$ and phosphatidyl serine. Activation is accompanied with physical translocation of PKC from the cytosol into the membrane (Bell 1986). The action of DAG can be mimicked by tumour-promoting phorbol esters (Castagna et al. 1982), by which PKC can be activated independently of the $InsP_3/Ca^{2+}$ pathway. PKC control through DAG is the other limb of the bifurcating signal pathway, as it is termed by Berridge (1987). In many cells, this pathway cooperates synergistically with the $InsP_3/Ca^{2+}$

pathway. Although a direct contribution of PKC to the final cellular response by phosphorylation of target proteins is assumed, its major role seems to be modulation of the calcium signal or, in a heterologous fashion, interaction with voltage-dependent channels, membrane potential, or cyclic nucleotide messenger pathways.

A partially purified protein kinase from zucchini has been shown to respond to $Ca^{2+}$, DAG and phospholipids, including phosphatidyl serine, phosphatidyl ethanolamine, but not to PtdInsP (Schäfer et al. 1985). Similarly, in an extract from *Amaranthus tricolor,* a calcium-dependent, phospholipid-stimulated kinase activity has been described, which is not affected by phorbol ester, (Elliot & Skinner 1986). Also a C-type kinase from *Neurospora crassa* has been reported not to respond to this drug (Favre & Turian 1987). However, activation by phorbol ester has been demonstrated in an enzyme fraction from wheat (Oláh & Kiss 1986). In addition, this kinase activity is stimulated by a mixture of either phosphatidyl serine plus phorbol ester or phosphatidyl serine plus diolein.

#### 2.3.2.4.4 Diacylglycerol kinase

This kinase converts DAG into phosphatidic acid (DAG + ATP → PA + ADP). The reaction may initiate again synthesis of phosphoinositides, as well as control the intracellular concentration of DAG. The enzyme has been detected in a variety of animal cells as an 80 kDa cytosolic protein (Yamada & Kanoli 1988) and becomes membrane-associated via phosphorylation by PKC (Besterman et al. 1986, MacDonald et al.1988). A membrane-associated DAG kinase of plant origin has been highly purified, the activity being assigned to a 51 kDa protein. The enzyme requires divalent cations and is completely devoid of activity without addition of phospholipid (Wissing et al. 1989). Its function in signal transduction remains to be shown.



#### 2.3.2.4.5 Hydrolysis of inositol phosphates

$Ins(1,4,5)P_3$ in animal tissues can be metabolised via two separate pathways. It is either dephosphorylated sequentially in a series of reactions to free inositol or enters an inositol tris/tetrakis-phosphate pathway to form $Ins(1,3,4,5)P_4$ (Irvine et al. 1986) and other inositol polyphosphates, which may have messenger functions as well (Irvine et al. 1988). The first degrading enzyme, a 5'-trisphosphatase, terminates the messenger action of $Ins(1,4,5)P_3$ and has thus some kind of key function in the pathway. In plants, a soluble fraction from *Nicotiana tabacum* had hydrolysing activity towards inositol (Joseph et al. 1989). Interestingly, two products, $Ins(1,4)P_2$ and $Ins(4,5)P_2$, were formed from $Ins(1,4,5)P_3$, whereas in mammalian cells the enzyme specifically removes the phosphate from the 5 position. In carrots, it was shown that a microsomal membrane fraction hydrolysed $Ins(1,4,5)P_3$ primarily to $InsP_2$, whereas the hydrolysis products with a soluble extract were $InsP_2$ and InsP, in about equal amounts. $Ca^{2+}$ enhanced $InsP_3$ dephosphorylation, but inhibited $InsP_2$ hydrolysis.

### 2.3.2.5 Concluding remarks

With the advent of new analytical and molecular genetic techniques, allowing detection of fast and minute changes of cellular metabolites, pH, ions, membrane potentials, or gene expression, it has become clear that plants respond very rapidly to environmental or hormonal stimuli. Taken together, the widely scattered reports discussed above provide suggestive evidence indicating that phosphoinositides may play a role in the coupling of such signals to convey them into an intracellular language. Although the progress in knowledge on phosphatidyl inositol metabolism in plants has been discussed in connection with work with animals, it should be emphasised that it is by no means certain that both pathways work in an identical way. The mere presence of an animal signal second messenger

does not prove that the compound has a similar function, as is the case for cAMP or acetylcholine (Lehle 1990).

While animal cells may provide useful parallels to guide plant studies, plants may be expected to have unique characteristics. In fact, these have already emerged, such as the vacuole as a source for calcium release, or the additional complexity of the phosphoinositide composition caused by lysoderivatives, or the alternate or additional way of inositol phosphate hydrolysis (Lehle 1990).

Future work will have to aim at carrying out a thorough biochemical analysis and characterisation of the metabolites and enzymes involved and to unify them into a scheme of stimulus/response coupling. On the other hand, other candidates than phosphoinositides for generation of second messengers as sporadically discussed in the literature, such as fatty acids or diacylglycerol released from phospholipids, acetylcholine or cytoplasmic pH changes should also be investigated (Lehle 1990).

## 2.3.3 Protein phosphorylation in plants: Enzymes, substrates and regulators

Voluminous information on protein phosphorylation is available in animal systems (Krebs 1993, Fischer 1993). In plants, protein phosphorylation may regulate unique processes, such as photosynthesis, photomorphogenesis, gravitropism and fruit ripening (Poovaiah & Reddy 1987, Allen 1992), in addition to pathways common to eukaryotes. Although the existence of protein kinases and their substrates is well established in plants (Ma 1993, Poovaiah & Reddy 1987, Ranjeva & Boudet 1987) much less information is available on this subject as compared to animal systems. The study of the physiological role and regulation of protein kinases as well as the characterisation of their specific substrates in plants has become an active area of research. Protein kinases that phosphorylate serine, threonine, and tyrosine residues have been detected in a number of plants (Ranjeva & Boudet 1987). They have been found in cytoplasmic and membrane fractions, chloroplasts, mitochondria, nuclei, and ribosomes. Protein kinases have been purified from various plant sources (Poovaiah & Reddy 1987, Ma 1993). Much of what is known about protein phosphorylation in plants comes from biochemical studies, and only very recently have genes involved in protein phosphorylation been isolated and characterised at the molecular level.

### 2.3.3.1 Calcium/calmodulin-dependent protein kinases

$Ca^{2+}$-promoted phosphorylation of soluble and membrane proteins was demonstrated in a variety of plant tissues (Poovaiah & Reddy 1987). The role of calmodulin in $Ca^{2+}$-promoted phosphorylation has been reported. Hetherington and Trewavas (1982) have shown that pea shoot membranes contain protein kinase(s) which are activated by physiological concentrations of $Ca^{2+}$. Polya et al. (1983) have partially purified a protein kinase from wheat germ whose activity is shown to be stimulated by $Ca^{2+}$ and calcium-calmodulin. The influence of $Ca^{2+}$ on phosphorylation of membrane proteins of zucchini hypocotyls has also been shown (Salimath & Marme 1983). Calcium enhanced the phosphorylation of about ten proteins, five of which showed increased phosphorylation in the presence of calmodulin. Calmodulin antagonists inhibited the calmodulin-dependent phosphorylation. Inhibition of phosphorylation of one polypeptide in the presence of $Ca^{2+}$ has also been observed. Veluthambi and Poovaiah (Poovaiah & Reddy 1987) have shown enhanced phosphorylation of several membrane and soluble polypeptides of corn and oat coleoptiles in the presence of $Ca^{2+}$. The $Ca^{2+}$ -promoted phosphorylation was further increased by calmodulin. Both $Ca^{2+}$ and $Ca^{2+}$-CaM-stimulated phosphorylation was inhibited by calmodulin inhibitors such as chlorpromazine and trifluoperazine. 1 μM free $Ca^{2+}$ was sufficient to promote phosphorylation of several polypeptides (Veluthambi & Poovaiah 1986) These results indicate physiological significance of $Ca^{2+}$-promoted phosphorylation since the $Ca^{2+}$ concentration required for promotion is close to the estimated cytosolic free $Ca^{2+}$ concentration of plant cells. Subsequently, $Ca^{2+}$- and calmodulin-regulated protein phosphorylation

has been reported in tomatoes, apples, *Neurospora crassa,* germinated pollen of *Nicotiana tabacum,* suspension cultured Italian rye grass endosperm cells, and amyloplasts isolated from sycamore suspension cultured cells (Reverences as Poovaiah & Reddy 1987). The pattern of $Ca^{2+}$-promoted phosphorylation in tomato changed from 10- to 50-day-old fruits in a stage-specific manner (Raghothama et al. 1985). Reduced $Ca^{2+}$- and calmodulin-dependent protein phosphorylation was observed in senesced apple membranes as compared to normal ones (Paliyath & Poovaiah 1984). All these results suggest the ubiquitous presence of $Ca^{2+}$ and calmodulin-dependent protein kinases in plants and indicate a key role for $Ca^{2+}$- and calmodulin-regulated protein phosphorylation. No $Ca^{2+}$- and calmodulin-dependent protein kinase has been purified to complete homogeneity and characterised from plant sources. Blowers et al. (1985) have partially purified a membrane-bound $Ca^{2+}$-activated protein kinase from pea buds. The authors have used the western blotting technique and have exploited the fact that many protein kinases autophosphorylate (Blowers & Trewavas 1989). Calcium-calmodulin-dependent autophosphorylation of this enzyme has been shown. Evidence that this enzyme is localised in the plasma membrane was provided by the phase partitioning technique.

### 2.3.3.2 Calcium-dependent protein kinases

In all the plant systems studied so far, phosphorylation of several polypeptides have been shown to be regulated by $Ca^{2+}$ in the absence of added calmodulin. These results suggest either the possible existence of protein kinases whose activity is stimulated by $Ca^{2+}$ alone or the involvement of endogenous calmodulin. The observation that only part of $Ca^{2+}$-stimulated protein kinase activity is inhibited by calmodulin inhibitors also indicates the possible presence of $Ca^{2+}$-dependent and calmodulin-independent protein kinases (Poovaiah & Reddy 1987, Harmon et al. 1987).

The first calcium-dependent calmodulin-independent protein kinase (CDPK) was discovered in plants three years ago (Harper et al. 1991), and CDPKs have since been shown to be prevalent in plants. The amino-terminal catalytic domain of CDPKs is similar to that of the calcium/calmodulin-dependent protein kinases (CaMPKs), while the carboxy-terminal domain is similar to calmodulin: these regions are separated by a junction domain. The CDPK catalytic domain has calcium-independent kinase activity *in vitro* (Harper et al. 1991), and the calmodulin-like domain binds $Ca^{2+}$ (Ma 1993). Furthermore, recombinant CDPK that has only the catalytic and junction domains cannot be activated by $Ca^{2+}$ (Harper et al. 1991), indicating that the calmodulin-like domain is required for the $Ca^{2+}$-dependent activation of the enzyme (Ma 1993). The sequence of the junction domain resembles that of the auto-inhibitory region of CaMPKs: this region may act to inhibit CDPK activity, since synthetic peptides that match junction domain sequences inhibit CDPK kinase activity *in vitro* (Harper et al. 1991). Denatured recombinant CDPK containing only the junction and calmodulin-like domains binds labelled calmodulin (Harper et al. 1991), suggesting that in the native protein the calmodulin-like domain may bind the junction domain in an intramolecular interaction. One interesting observation is that the *Arabidopsis* membrane-associated CDPK (AK1) is stimulated by both $Ca^{2+}$ and lipids, and that this stimulation is synergistic (Harper et al. 1991). Finally, red light appears to induce a calcium-dependent kinase activity in etiolated wheat leaf cells, giving a hint that a CDPK might play a role in the phytochrome signalling pathway (Park & Chae 1990).

### 2.3.3.3 Calcium/Phospholipid dependent protein kinases (PKC-like)

Putative protein kinases C in plants have been reported. A partially purified protein kinase from zucchini has been shown to respond to $Ca^{2+}$, DAG and phospholipids, including phosphatidyl serine, phosphatidyl ethanolamine, but not to PtdInsP (Schäfer et al. 1985). Similarly, in an extract from *Amaranthus tricolor,* a calcium-dependent, phospholipid-stimulated kinase activity has been described and partially purified, which is not affected by phorbol ester, (Elliot & Skinner 1986, Elliott & Kokke 1987). Also a C-type kinase from *Neurospora* has been reported not to respond to this drug (Favre & Turian 1987). However, activation by phorbol ester has been demonstrated in an enzyme fraction from

wheat (Olàh & Kiss 1986). In addition, this kinase activity is stimulated by a mixture of either phosphatidyl serine plus phorbol ester or phosphatidyl serine plus diolein. It was shown in *Nitella* cells that TPA (12-O-tetradecanoylphorbol-13-acetate) modulates voltage-gated plasmalemma $Ca^{2+}$ channels and that polymyxin B, an inhibitor of PKC, blocks these *in vivo* (Zherelova 1989). Thus, the failure to demonstrate phorbol ester activation *in vitro* appears to be of a technical problem with the isolated enzyme rather than a fundamental difference with animal systems. Nevertheless, plant PKC may reveal a different specificity, since DAG from plants seems not to be esterified by arachidonic acid at the C-2 position of the glycerol moiety, as is the case in animals (Helsper et al. 1987, Mudd 1980).

DAG affects the physiology of plant cells. It mimics the effects of blue light by hyperpolarising guard cell protoplasts, possibly through stimulation of the $H^+$ ATPase, and by opening stomata in leaf epidermal peels (Lee & Assmann 1991). These effects are inhibited by the protein kinase inhibitor H-7. There are no reports indicating that DAG activates protein kinase in intact plant cells. However, an overall stimulatory effect of DAG and $Ca^{2+}$ on phosphorylation of soybean membrane proteins has been observed *in vitro* (Morré et al. 1984). From a general point of view, such a DAG stimulation of the kinase in membrane fractions may be difficult to detect, because DAG levels present in membranes could be high enough to give already maximum activity (Coté & Crain 1990, Ha & Thompson 1992, Quarmby et al. 1992).

#### 2.3.3.4 Receptor-like protein kinases

Both receptor protein tyrosine kinases and receptor serine/threonine kinases are important signal transducers in animals (Hunter & Cooper 1985, Ullrich & Schlessinger 1990, Massaguè 1992). In plants, receptor-like serine/threonine kinases have been discovered recently - these include the self-incompatibility (S-) locus receptor kinases (SRKs), which have a cysteine-rich extracellular domain (Walker & Zhang 1990, Stein et al. 1991, Goring & Rothstein 1992). Mutations in genes that encode SRKs are associated with defects in self-incompatibility (Ma 1993), indicating that functional SRK is indeed required for self-incompatibly. Genes that encode SRK-type kinases (Walker & Zhang 1990) are also present in self-compatible plants such as maize (ZmPKS) and *Arabidopsis* (RLKs), where they are expressed in various parts of the plant, including leaves and roots, suggesting that they function in processes other than pollen-stigma interaction. Another type of receptor-like kinase, discovered in *Arabidopsis,* contains an extracellular domain with leucine-rich repeats, believed to be involved in protein-protein interactions. Examples of this type of kinase are TMKI (Chang et al. 1992) and RLK5 (Ma 1993). Both TMK1 and RLK5 autophosphorylate on serines and threonines *in vitro*, and the intracellular carboxy-terminal domain of TMK1 acts as a competitive substrate for this phosphorylation. A gene encoding a kinase containing two repeat motifs similar to epidermal growth factor has also been isolated recently from *Arabidopsis* (Kohorn et al. 1992).

#### 2.3.3.5 Cyclin-dependent kinases

Cyclin-dependent protein kinases (CDKs) are homologues and relatives of the $p34^{cdc2}$ protein kinase, a key regulator of cell cycle, and plant CDKs are now being studied by many investigators (References as cited by Ma 1993). Four distinct CDKs were found in pea (Ma 1993); in synchronised cells from the root apical meristem, CDK activity was found to peak at S phase and, to a lesser extent, at the G2/M transition. In a separate study (Colasanti et al. 1991), the functional maize $p34^{cdc2}$ homologue was shown to colocalise with the microtubules of the preprophase band, which predicts the plane of cell division, in the dividing cells of root tips and the stomatal complexes. Therefore, *cdc2* may be involved in the regulation of position and orientation of plant cell division. Molecular analysis of the *Arabidopsis* genes encoding *cdc2a* (one of two homologues) and cyclins (Ma 1993) showed that *cdc2a* is produced not only in dividing cells, but also in proliferation-competent cells such as those of the root pericycle, whereas the cyclin component (regulatory subunit) of the kinase is only produced in dividing

cells. The phenotypes of transgenic plants that carry dominant mutations in the *Arabidopsis cdc2a* gene indicate that normal *cdc2* function is important for proper plant development (Ma 1993).

### 2.3.3.6 Casein-2 protein kinases

Casein kinase is a ubiquitous cyclic nucleotide-independent protein kinase so called because conventionally it has been assayed using casein as its substrate (Pinna 1990). Considerable interest in casein kinase 2 has arisen in the past few years because it has been reported to be activated in response to mitogens including serum, the tumour promoter 12-O-tetradecanoylphorbol 13-acetate, insulin, insulin-like growth factor I and epidermal growth factor (References as cited in review Meek & Street 1992). The cellular targets of casein kinase 2 are nuclear, DNA-binding, regulatory proteins including CREB (cyclic AMP responsive element binding protein), *Myc* and the tumour suppresser protein p53. Casein kinase 2 has a broad substrate specificity and targets many nuclear proteins which have been implicated in growth control, some of which are discussed by Meek & Street (1992). Casein kinase 2 is thus labelled a growth-related kinase.

Most casein kinases 2 in animals and plants are heteromeric, high-molecular weight proteins with a complex quaternary structure specific for ATP and GTP which modify serine and threonine residues of protein substrates, and have a noncatalytic (β) subunit between 25 and 29 kDa. For example, Li and Roux (1992) purified a casein kinase 2 from pea nuclei that has a regulatory subunit of 29 kDa and has a catalytic subunit of 36 kDa. A characteristic feature of casein kinases 2 is their capability of being inhibited by the stoichiometrical amounts of polyanions (heparin, RNA, etc.). These enzymes are capable of self-phosphorylation, but the biological meaning of this effect remains obscure (Pinna 1990).

Casein kinase 2-type protein kinases have been isolated from *Verticillium dahliae* (Kandror et al. 1990), pea nuclei (Li & Roux 1992) and broccoli (Klimczak et al. 1992). Casein kinase 2 is one of few plant protein kinases known to represent close biochemical counterparts of animal kinases ( the others being casein kinase 1 and $p34^{cdc2}$ protein kinase). The high degree of protein sequence conservation among the cloned casein kinases 2 (human, Drosophila, and yeast) suggests that some fundamental function of the enzyme must have been conserved throughout evolution (Klimczak et al. 1992).

### 2.3.3.7 Other kinases

Several other kinases were also described, including MAP kinases (Duerr et al. 1993), a ribosome S6 kinase (Walker & Zhang 1990), and a *raf* homologue (Kieber et al. 1993). In particular, Briggs and co-workers presented evidence that a blue-light induced kinase is present in the membranes of pea seedlings (Short et al. 1993, Reymond et al. 1992). This kinase, which autophosphorylates, can be activated by blue light even in solubilised membranes and after separation on a non-denaturing gel. This suggests that the blue-light receptor, kinase and substrate comigrate, perhaps even as a single component.

### 2.3.3.8 Substrates of protein phosphorylation

A number of proteins have been found to serve as substrates for protein kinases under *in vitro* and *in vivo* conditions (Ranjeva & Boudet 1987, Poovaiah & Reddy 1987, Ma 1993). Phosphorylation of ribosomal proteins, soluble and membrane proteins, nuclear proteins, and chloroplast and mitochondrial proteins has been reported in plants, although the physiological significance in most of these cases remains to be evaluated. The activity of the following enzymes/proteins has been shown to be regulated by phosphorylation: pyruvate dehydrogenase complex (Rao & Randall 1980, Ma 1993), $H^+$ATPase (Zocchi et al. 1983, Zocchi 1985, Schaller & Sussman 1988, Suzuki et al. 1992), quinate: $NAD^+$oxidoreductase (Ranjeva et al. 1986), phosphoenolpyruvate (PEP) carboxylase (McNaughton et

al. 1991, Quy & Champigny 1992, Ogawa et al. 1992, Ma 1993), light-harvesting chlorophyll-binding protein (Allen 1992, Bennett 1983) small subunit of ribulose bisphosphate carboxylase/oxygenase (Muto & Shimogawara 1985), 3-hydroxymethyl glutaryl Co A reductase (Ranjeva & Boudet 1987), cytokinin binding proteins (Polya et al. 1983), pyruvate Pi dikinase (Edwards et al. 1985), phytochrome (Wong et al. 1986), protein synthesis initiation factor-2 (Ranu 1980) and nitrate reductase (Huber et al. 1992, Ma 1993). Moreover, two membrane proteins, *Nod26* and *TIP* that have similarity to membrane transport channel proteins in bacteria and animals were shown to be phosphorylated (Ma 1993). *Nod26* is found on the symbiosome membrane that surrounds the bacteroids within legume nodule cells, and is phosphorylated on Ser-262 by a calcium-dependent protein kinase. *Nod26* phosphorylation has been correlated with malate uptake. Similarly, *TIP* is also phosphorylated by a calcium-dependent kinase. When one of the protein isoforms, *Y-TIP*, is expressed in *Xenopus* oocytes it forms efficient water channels, as does a related mammalian protein, *CHIP28* (Ma 1993).

Wong et al. (1986) have reported that a polycation-dependent protein kinase is associated with the purified preparation of *Avena* phytochrome. They have also shown that the red-absorbing form of phytochrome (P*r*) serves as a good substrate for endogenous protein kinase and three other mammalian protein kinases (cAMP-dependent protein kinase, cGMP-dependent protein kinase, and protein kinase C). However, the far-red absorbing form of phytochrome (P*fr*) acts as a good substrate only to cAMP-dependent protein kinase, whereas the other three kinases poorly phosphorylate the P*fr* form. The differential phosphorylation of two forms of phytochrome raises the possibility that phosphorylation may play an important role in the regulation of phytochrome-mediated biochemical changes in plants.

In plants, enzymes such as NAD kinase (Muto & Miyachi, 1977, Anderson et al. 1980), $Ca^{2+}$ATPase (Marmé 1986, Dieter & Marmé 1980), $H^{+}$ATPase (Zocchi et al. 1983, Zocchi 1985), quinate: $NAD^{+}$ oxidoreductase (Ranjeva & Boudet 1987) and protein kinases (Poovaiah & Reddy 1987) have been shown to be regulated by $Ca^{2+}$ and calmodulin. One enzyme, a ribulose-1,5-bisphosphate carboxylase/ oxygenase small subunit, have been shown to be regulated by $Ca^{2+}$- and phospholipid-dependent protein kinase (Muto & Shimogawara 1985). Quinate: $NAD^{+}$oxido-reductase (EC 1.1.1.24, hereafter referred to as QORase) catalyzes the reversible oxidation of quinic acid into dehydroquinic acid, an intermediate of the shikimate pathway that leads to aromatic amino acids and phenolic compounds. QORase from light-grown carrot cell suspension cultures has been shown to be activated by phosphorylation and inactivated by dephosphorylation (Ranjeva & Boudet 1987). The reactivation is completely blocked by EGTA and can be restored by the addition of $Ca^{2+}$. Calmodulin-depleted fractions containing QORase cannot be reactivated even in the presence of ATP and $Ca^{2+}$. However, QORase can be reactivated under these conditions by adding calmodulin. Based on these results, it has been concluded that QORase is activated by $Ca^{2+}$- and calmodulin-dependent protein kinase. Depletion of cellular $Ca^{2+}$ from carrot cell suspension by $Ca^{2+}$ ionophore and EGTA results in decreased QORase activity (Ranjeva & Boudet 1987). The reduction in enzyme activity is correlated with $Ca^{2+}$ efflux and little or no phosphorylation of certain proteins. The loss in the activity of QORase in $Ca^{2+}$-deprived extracts is recovered upon incubation with $Ca^{2+}$ and ATP. These results provide evidence that intracellular $Ca^{2+}$ may act *in vivo* as a regulator of plant metabolism through protein phosphorylation.

### 2.3.3.9 Phosphoprotein phosphatases

The state of phosphorylation of any protein depends on the relative activities of phosphoprotein phosphatases in addition to the activities of protein kinases (Cohen 1985). In animal cells there are four protein phosphatases (protein phosphatase 1, 2A, 2B, and 2C) with broader specificities (Cohen 1985). Furthermore, at least two of these enzymes are controlled by second messengers. Protein phosphatase 2B (also known as calcineurin) activity is regulated by $Ca^{2+}$ and calmodulin (Cohen 1985).

Phosphoprotein phosphatases have been identified and purified from soybean hypocotyls (Poovaiah & Reddy 1987), but there is no conclusive evidence for $Ca^{2+}$- and calmodulin-regulated protein

phosphatases. However, preliminary evidence for $Ca^{2+}$- and calmodulin-dependent protein phosphatase has been presented by Hetherington and Trewavas (1982). There are still many unanswered questions concerning $Ca^{2+}$- and calmodulin-regulated protein kinases and phosphatases. Purification of $Ca^{2+}$ and calmodulin-dependent protein kinases, phosphatases, and their substrates, and identification of the function of the substrate proteins would certainly provide valuable information towards an understanding of the mechanisms involved in $Ca^{2+}$-regulated processes.

#### 2.3.3.10 Prospects

By use of various approaches, a number of plant proteins have been identified that modulate protein phosphorylation or are regulated by phosphorylation. Molecular genetic and physiological analyses (Kieber et al. 1993) have already identified a kinase in the ethylene response pathway of *Arabidopsis*. It is now important to identify the signals that regulate specific protein kinases and phosphatases, the role of signal transduction pathways such as those mediated by G-proteins in this regulation, the substrate targets of individual enzymes and the cellular responses that result from regulation through protein phosphorylation. Molecular genetic and physiological analyses will continue to be important in elucidating the role of protein phosphorylation in plant responses *in vitro*. On the other hand, biochemical studies will be crucial in determining the mechanisms by which specific proteins function, including those proteins identified using molecular and genetic approaches. We are now seeing the beginning of rapid advances in this field (Ma 1993).

### 2.3.4 Effect of external signals on protein kinase activity

Extracellular signals transmit information to the cell interior by activating transmembrane signalling systems that control production of a relatively small number of chemical mediators or second messengers (Poovaiah & Reddy 1987). These substances regulate the activity of protein kinases and phosphatases, thereby altering the phosphorylation status of many intracellular proteins.



#### 2.3.4.1 Ethylene signal is transduced via protein phosphorylation events in plants

Cellular transduction of the external signal provided by ethylene is presumed to be an ordered process of consecutive events. One component in the pathway was recently shown to require calcium (Raz and Fluhr 1992). Other possible components such as protein phosphorylation are thought to play a key role in diverse biological signal transduction systems (for reviews, see Cohen 1982, Hardie 1990). In plants, protein phosphorylation was shown to play a direct regulatory role in light response (for reviews, see Bennett 1983, Allen 1992) and circadian rhythms (Carter et al. 1991). Correlations between protein phosphorylation and physiological responses were demonstrated in pollen embryogenesis (Kyo and Harada 1990) and fungal elicitor treatment of cultured cells (Grab et al. 1989, Dietrich et al. 1990, Grosskopf et al. 1990, Felix et al. 1991, Schwacke and Hager 1992).

Labelling experiments in leaves showed that specific protein phosphorylation events are induced by ethylene. α-AB (α-aminobuteric acid), which, induces a pathogenesis response in a pathway that requires ethylene, which induced profiles of transient polypeptide phosphorylation similar to that obtained with ethylene. The phosphorylation of these polypeptides was inhibited by kinase inhibitors K-252a and H-7. In addition, K-252a and H-7 inhibited the ethylene-induced accumulation of PRB-lb transcript and chitinase protein. Thus, rapid polypeptide phosphorylation is associated with ethylene application, and phosphorylation events are necessary for ethylene-activated signal transduction that results in the induction of pathogenesis response (PR) gene expression and microlesion formation. The fact that both K-252a and H-7 had the same effect on PR protein expression and protein phosphorylation may indicate that both drugs block kinase activity of the same enzyme, as has been suggested for their inhibition of protein kinase C activation of serotonin secretion in animal cells (Gat-

Yablonski and Sagi-Eisenberg 1990). Protein phosphorylation of cultured plant cells was shown to be affected by fungal elicitor treatments (Dietrich et al. 1990, Grosskopf et al. 1990, Felix et al. 1991, Schwacke and Hager 1992). The kinetics of phosphorylation induced by the elicitors was rapid (within minutes), transient (Dietrich et al. 1990), and negatively regulated by K-252a (Grosskopf et al. 1990, Felix et al. 1991). To what extent nondifferentiated cell cultures reflect normal signal transduction is unknown. Indeed, in tobacco, the regulation of PR proteins must be studied in leaves as they are constitutively expressed in cell cultures (Antoniw et al. 1981).

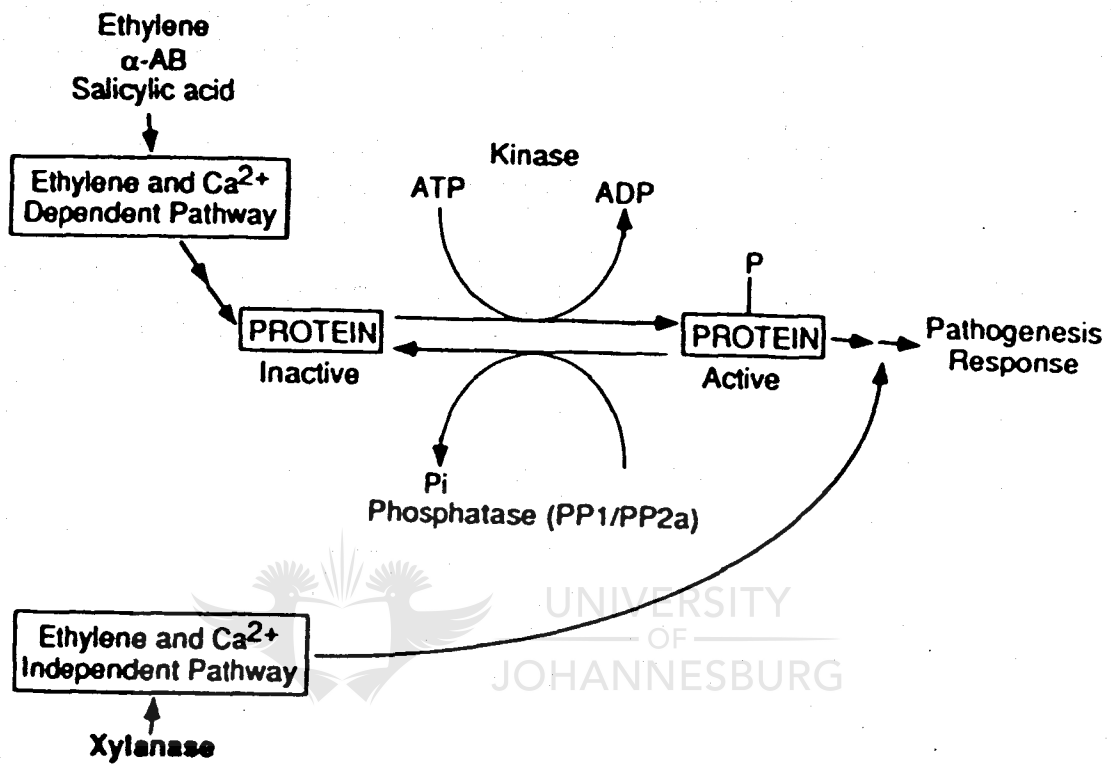



Figure 2.3.5. A scheme for the involvement of phosphorylation events in the induction of ethylene-dependent pathogenesis response.

Okadaic acid, a specific inhibitor of animal and plant protein phosphatases type 1 (PP1) and type 2 (PP2A), was shown to increase the general level of protein phosphorylation in intact leaves, similar to its effect in animal cell cultures (Cohen 1989, Heystead et al. 1989). In addition, it elicited the rapid accumulation of a PR-specific transcript and PR proteins in intact leaves. In animal systems, okadaic acid has been shown to be capable of inducing specific genes at the transcriptional level (Nagamine and Ziegler 1991, Guy et al. 1992). In leaves, the induction of PR gene expression by okadaic acid was blocked by the presence of kinase inhibitors, suggesting that the transduction events requiring kinase and phosphatase activity are probably acting through the same pathway. Two types of protein phosphorylation kinetics have been detected, a stable increase induced by okadaic acid and a transient increase induced by ethylene. The transient increase is consistent with attenuation of an initial receptor response to a signal molecule, while the stable increase is expected from a constitutive biochemical type of inhibition. Hence, at least one stage of the transduction pathway requires kinase activity, which is modified by a mechanism that maintains phosphorylation equilibrium, as shown schematically in Figure 2.3.6. Alternatively, or in addition, the induction of PR proteins by ethylene could be transduced via negative regulation of PPl/PP2A phosphatase activity.

Elicitors of pathogenesis utilise at least two pathways for the induction of PR proteins, which differ in their requirement for ethylene (Lotan and Fluhr 1990) and calcium (Raz and Fluhr 1993). Raz & Fluhr (1993) showed that the ethylene-independent pathway, which is induced by xylanase, was not affected by kinase inhibitors. Thus, these pathways differ in their initial cellular signal-transducing machinery as well. Apparently, downstream from the transduction events depicted here, the pathways leading to gene activation converge to give, in each case, co-ordinate regulation of PR protein accumulation (Figure 2.3.5).

Ethylene, α-AB, and salicylic acid utilise the ethylene- and calcium-dependent pathway while xylanase utilises a different pathway (Raz and Fluhr 1993). The transducing activity of a putative control protein is determined by its phosphorylation level. The level of phosphorylation is determined by the kinetics of kinase to phosphatase activity.

Studies implicate the ubiquitous cellular PPI/PP2A phosphatases in PR protein induction. A component in the transduction pathway of ethylene has been isolated recently using *Arabidopsis* mutants. It was shown to be a *raf*-like protein kinase that may negatively regulate ethylene-motivated triple response (Kieber et al. 1993). The possibility of isolating mutants in the core PPI/PP2A complex itself using genetic methods is unlikely, due to its participation in the regulation of many pathways. Future studies should involve differentiating between the two phosphatases and looking for their specific substrate interactions.

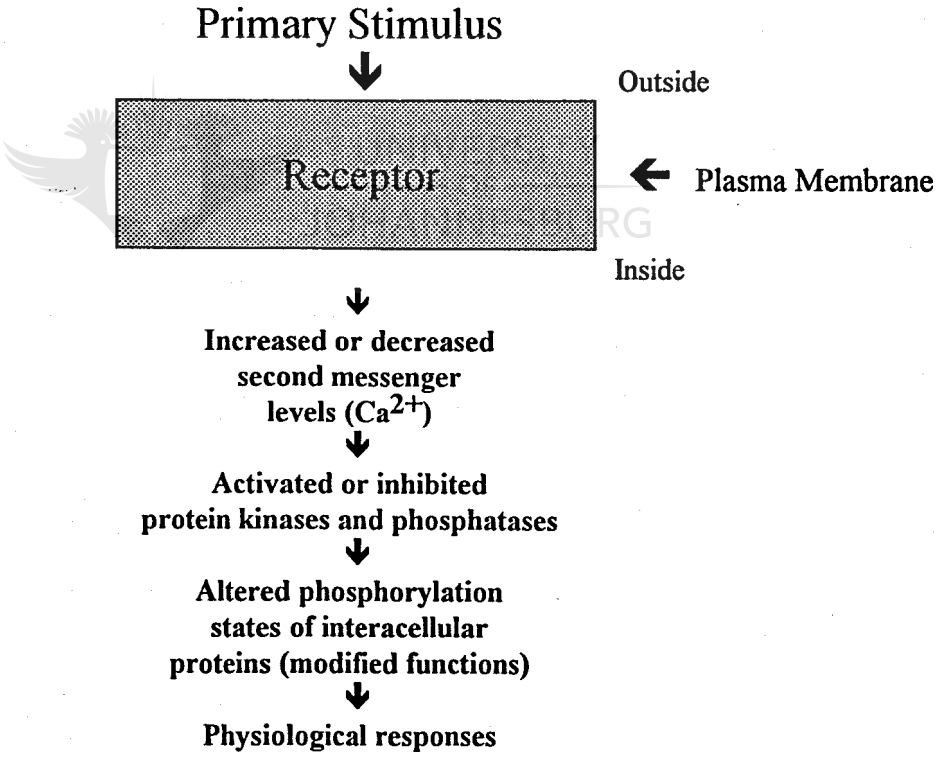



Figure 2.3.6 Illustration of the mechanism by which primary signals regulate physiological processes through $Ca^{2+}$-regulated protein phosphorylation and dephosphorylation.

### 2.3.4.2 The effect of auxin, cytokinins, and GA on phosphorylation

The effect of auxin, cytokinins, and gibberellic acid (GA) on phosphorylation *in vitro* has been studied (Poovaiah & Reddy 1987). However, it is very critical that these studies be carried out *in vivo* rather

than *in vitro*, as the latter studies do not allow evaluation of signal-induced changes mediated by messengers such as $Ca^{2+}$. Auxin-treated pea epicotyls have shown reduced phosphorylation of certain molecular weight polypeptides and a slight promotion of some other molecular-weight polypeptides (Poovaiah & Reddy 1987).

Many researchers have documented the involvement of $Ca^{2+}$ in auxin-induced elongation. Auxin-induced elongation can be inhibited by manipulating tissue $Ca^{2+}$ concentrations using $Ca^{2+}$ chelators or channel blockers (Poovaiah & Reddy 1989). In addition, auxin has been shown to increase $Ca^{2+}$ efflux from various tissues and plasma-membrane preparations (Hanson 1984). These findings suggested that auxin may alter cytosolic $Ca^{2+}$ thereby altering $Ca^{2+}$- and $Ca^{2+}$-calmodulin-dependent enzyme activity, including protein kinases. Chlorpromazine (calmodulin inhibitor)-induced changes in protein phosphorylation were strikingly similar to auxin-induced changes, indicating the involvement of calmodulin in auxin-induced changes in protein phosphorylation. Friedmann & Poovaiah (1991) showed that gravity induced rapid changes in phosphorylation in intact corn root tips within 3 min. This seemed to be among the quickest responses to gravity, which further suggested that phosphorylation may be one of the primary biochemical responses to this stimulus. Such changes in phosphorylation could then lead to the development of processes involved in the gravity response. The involvement of $Ca^{2+}$ in the phosphorylation response suggests the presence and activation by gravity of a $Ca^{2+}$-dependent protein kinase in corn root tips. Figure 2.3.6 illustrates the sequential steps by which primary signals regulate physiological processes through $Ca^{2+}$-regulated protein phosphorylation.

The role of other hormones (cytokinin and gibberellic acid) on protein phosphorylation *in vivo* is yet to be explored.

# CHAPTER 3

## Phosphoproteins in Ripening Mango Fruit Tissue: Effect of Gamma-Radiation and Various Effectors on Protein Phosphorylation during the Climacteric Rise, Climacteric Peak and Post-Climacteric Stages of Ripening.

# List of Figures and Tables

## Figures

## Tables



# CHAPTER 3

# Phosphoproteins in Ripening Mango Fruit Tissue: Effect of Gamma-Radiation and Various Effectors on Protein Phosphorylation during the Climacteric Rise, Climacteric Peak and Post-Climacteric Stages of Ripening.

## 3.1 Introduction

The transduction of environmental and hormonal signals into biochemical and physiological responses in living organisms is an area of ever-increasing interest (Cohen 1982, Poovaiah & Reddy 1987, Ma 1993). In many cases, signal transduction has been shown to involve transient increases in intracellular $Ca^{2+}$ concentrations and changes in $Ca^{2+}$-dependent protein phosphorylation, which are thought to be among the primary responses (Cohen 1982, Hepler & Wayne 1985, Poovaiah & Reddy 1987, Ranjeva & Boudet 1987, Ma 1993).

Gamma-radiation alters the biochemical balance in fruit tissue and leads to a delay in the onset of ripening and senescence (Surendranathan & Nair 1980). We showed that irradiation does not cause a true delay in the onset of ripening of fully mature mango fruit, but rather distorts certain biochemical processes so that senescence is delayed (Dubery et al. 1984). It is known that gamma-irradiation treatment causes a stress condition in the fruit that, depending on the dose, may lead to browning of the tissue or necrotic decay (Frylinck et al. 1987). Mangoes were used as experimental material because these fruit are important South African export products. A longer shelf life is of prime concern for the producers.



In order to adapt efficiently to the changing environment, all living organisms have built-in regulatory mechanisms that can be controlled in response to external stimuli. Calcium affects several physiological processes in plants. The participation of calcium in regulatory systems in plants may be inferred from the fact that calcium plays a major role in membrane structure and function (Jones & Lunt 1967). Studies on leaf senescence abscission (Poovaiah & Leopold 1973(a)), membrane leakage (Poovaiah & Leopold 1973(b)) and fruit ripening (Poovaiah & Leopold 1976) have indicated that by increasing calcium levels, a number of senescence processes can be delayed (Poovaiah 1979). Protein phosphorylation emerged as the major mechanism by which calcium and calmodulin regulate biochemical events inside the plant cell in response to external stimuli (Poovaiah & Reddy 1987, Ma 1993).

One of the main products generated during the radiolysis of aerobic aqueous solutions is hydrogen peroxide (Allen 1961). It is reported that hydrogen peroxide stimulates tyrosine phosphorylation in intact hepatoma cells (Koshio et al. 1988) and that protein kinase C is involved in lipid peroxidation and cell membrane damage induced by oxygen-based radicals in hepatocytes (Von Ruecker et al. 1989). Gamma-radiation has short-term effects on the stimulation of ethylene production and ethylene related enzymes like 1-aminocyclopropane-1-carboxylic acid synthase (Larrigaudière et al. 1989). Recently, Raz and Fluhr reported that protein phosphorylation stimulates ethylene signal transduction events in plants (Raz & Fluhr 1993). These findings infer the interrelationship of all these above mentioned events.

Biochemical actions of calcium in general has been shown to be mediated by calmodulin (Cheung 1980). This protein modulates the action of many enzymes and proteins after binding with and being activated by calcium (Cheung 1980, Comier et al. 1980, Dieter & Marmé 1980). Added exogenous

calmodulin reduced increased lipoxygenase activity associated with senescence in pea leaves (Leshem et al. 1992). Therefore, the modulation of calmodulin related processes could have an important role in the regulation of developmental processes. The occurrence of calmodulin has been well established in many higher plant systems (Dieter & Marmé 1980, Anderson et al. 1980, Watterson et al. 1980, Dubery & Schabort 1987).

From the literature it became clear that the delay in senescence as a result of gamma-irradiation, could involve the biochemical action of calcium and calmodulin. It would be of interest to ascertain whether gamma-irradiation could lead to $Ca^{2+}$-dependent changes in protein phosphorylation. This would suggest that gamma-irradiation may involve transient increases in cytoplasmic $Ca^{2+}$ resulting in the activation of $Ca^{2+}$-dependent kinases that in turn would set in motion processes leading to the delay of ripening and senescence.

The effect of gamma-radiation on the *in vitro* phosphorylation of soluble and membrane proteins was thus studied in relation to ripening and senescence of mango fruit. The role of various effectors like calcium, calmodulin, cAMP and phospholipids and calmodulin antagonists, calmidazolium and trifluoperazine, was investigated. Qualitative changes in the pattern of protein phosphorylation at different developmental stages are discussed.

## 3.2 Experimental procedures

### 3.2.1 Plant material

Mature, but unripe, Kent mangoes were obtained from orchards in the Tzaneen area. The fruits were picked one day before start of the experiment and carefully selected for uniformity of size and colour. Half of these mangoes were irradiated in a *Gamma beam* 650 (AECL)-$^{60}Co$ of the Chemistry department, Atomic Energy Corporation at a dose of 1 kGy that falls in the commercial dose range. The control and irradiated fruit were washed and wiped with ethanol. This fruit was kept at constant room temperature and ripened naturally. Groups of mangoes were selected according to ripening stage on the basis of colour and firmness i.e. climacteric rise, climacteric peak and post-climacteric stage. Depending on the experiment being performed, they were treated in the following ways: The fruit was frozen whole and kept at -25°C or acetone powders and membrane fractions were immediately prepared from fresh fruit and then frozen at -25°C. These procedures were necessary because of the seasonal availability of the fruit and seamed to give the best results.

### 3.2.1 Reagents

[$\gamma$-$^{32}P$]ATP (3000 Ci/mmol) was obtained from Amersham. PMSF, phosphatidylserine (PS) and diolein were purchased from Sigma. 3 MM filter paper was from Whatman. READY SOLV $^{TM}$EP was from Beckman. Calmodulin, calmidazolium, cAMP, were obtained from Boehringer Mannheim. $\beta$ ME, Triton X-100 $MgCl_2$, $CaCl_2$, TRIS, EDTA and EGTA were from Merck. Acetone, TCA, sucrose, NaF, Na azide and Na pyrophosphate were obtained from Saarchem and were analytical grade. All other chemicals were of analytical grade, obtained from ordinary commercial sources. Phosphatidylserine and diolein were stored as stock solutions in $CHCl_3$-MeOH (2:1) in the dark at -15° C. Before use an aliquot was dried down under $N_2$ and sonicated in buffer to give a clear solution (Olàh & Kiss 1986).

### 3.2.3 Extraction procedures

### 3.2.3.1 Extraction of total extractable proteins

3.2.3.1.1 Preparation of acetone powders (Nason, 1955)

Three fruits per sample and three samples per group were used. The fruit was cut into small pieces and powdered in liquid nitrogen with a mortar and pestle. The powder was kept at -20°C until it was used. 1.5 g Polyethylene glycol (MM 4000) and 90 ml acetone (-20°C) were added to each of 15 g frozen tissue sample. This preparation was then homogenised for 30 s with an Ultra Turrax homogeniser. The homogenate was filtered through a scinter glass funnel under suction and washed with an additional 100 ml acetone (-20°C). The powder was then dried on filter paper, weighed and stored at -20°C.

3.2.3.1.2 Extraction of acetone powders

One preparation of above mentioned powder was suspended in 5 ml cold reaction buffer (see Section 3.2.4) and centrifuged at 27 750 x g for 15 min at 0°C to remove any insoluble matter. The pellet was resuspended in a further 5 ml reaction buffer and centrifuged as above. The supernatants were combined and the pH was adjusted to 7. The protein concentration was determined immediately as described under section 3.3.4. Aggregation of proteins in day-old preparations during protein determination procedures was a problem. This preparation of total extractable proteins was then used for protein kinase activity experiments as described under Section 3.2.4.

### 3.2.3.2 Preparation of membrane fractions

A modified method of Salimath and Marmé (1983) was used for the preparation of membrane fractions. 200 g Fresh mango fruit tissue was cubed in small pieces at 4°C and homogenised in (1 g fresh mass per ml) 50 mM TRIS-HCl buffer, pH 7.8 containing 0.25 M sucrose, 3 mM EDTA, 1 mM $MgCl_2$ and freshly added 1 mM PMSF and 2 mM β-ME. The resulting crude extract was filtered through muslin and centrifuged at 130 000 g for 20 min at 4°C. The pellet was washed with 1:10 volumes homogenising buffer and centrifuged as before. The pellet was then resuspended in weighed Eppendorf tubes in 10 mM TRIS-HCl buffer, pH 7.8 containing 15% (m/v) sucrose, 0.02% Na azide and fresh 2 mM β-ME. The protein concentration was determined as described under section 3.3.4. and stored at -20°C. These membrane fractions, contained approximately 10 - 12 mg protein per ml and were used in SDS-PAGE protein phosphorylation studies as described under Section 3.2.5.

## 3.2.4 Protein kinase assay

Protein kinase activity was determined by measuring incorporation of labelled phosphate from [γ-$^{32}$P]ATP into endogenous protein substrates using a modified method of Muszynska et al. (1983) Concentrations and radioactive specificity were essentially the same as that used by Muszynska et al. (1983) for maize. The reaction buffer consists of 50 mM TRIS-HCl, pH 7.1, containing 10 mM $MgCl_2$, 0.2 mM EGTA, 2 mM β-ME, 0.5 mM PMSF, 10 mM NaF, 5% sucrose, 0.2% azide and 0.3% Triton X-100. 120 µg Protein suspended in 0.5 ml reaction buffer plus effectors (as described under Section 3.2.5) was incubated at 25°C for 2 min. For the preparation of a control, 50 µl were withdrawn and added to 50 µl 20% TCA-solution, containing 20 mM Na pyrophosphate and 10 mM EDTA in an Eppendorf tube and 1 µl [γ-$^{32}$P]ATP (6.6 µM ATP) was then added. The reaction was started by adding 9 µl [γ-$^{32}$P] ATP (6.6 µM ATP) to the remaining reaction mixture. 50 µl Aliquots were withdrawn with intervals of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 10.0 minutes and added to 50 µl 20% TCA-solution, containing 20 mM Na pyrophosphate and 10 mM EDTA in an Eppendorf tube.

The protein concentration used in the assays was of the same order as that described by Muszynska et al. (1983). A constant protein concentration (120 µg) was used in all the tests as specified under the contents of the reaction mixture.

The precipitates were filtered on Whatman 3 MM (1.5 x 1.5 cm) squares under suction. The squares were placed in separate test-tubes with 5 ml 10% TCA solution added and shaked over night. The squares were washed with 5% TCA solution and heated to 90°C in 10% TCA solution for 15 min and then washed 3 times with 5% TCA solution. The squares were finally washed in warm ethanol/ether (3:1 v/v) and dried (Hetherington & Trewavas 1982). The squares were added to scintillation vials containing 5 ml READY SOLV $^{TM}$EP and counted in a $^{32}$P liquid scintillation counter (Fox & Brian 1976).

For autoradiogram assays, the reactions were performed as above with the exception that the protein concentration was adjusted to 30 µg per 50µl reaction mixture and the reaction was terminated after 10 min. The samples were boiled for 2 min and electrophoresed as described under section 3.3.4. The sample buffer used for membrane fractions contained 0.1% w/v Triton X-100 (Weber et al. 1983).

1U of protein kinase activity is defined as the amount of enzyme that catalyses the incorporation of 1ρ mol terminal phosphogroup from [$\gamma$-$^{32}$P]ATP into a protein substrate per min at 25°C. Specific activity is defined as U/mg protein.

## 3.2.5 Determination of the effect of various effectors on protein kinase activity

Various effector combinations were tested on total extractable soluble proteins and membrane fractions isolated from each mango group, i.e. control mangoes and gamma-irradiated mangoes each of climacteric rise, climacteric peak and post-climacteric ripening stages. The reaction variations were:

- No EGTA or calcium was added.
- 0.2 mM EGTA was added and calcium was omitted.
- 1 mM calcium was added.
- 1 mM calcium as well as 1 µM calmodulin were added.
- 1 µM calmodulin was added and calcium was omitted.
- 1 mM calcium as well as phospholipids (20 µg/ml diolein and 100 µg/ml phosphatidylserine (PS)) were added.
- Phospholipids, 5 µg/ml diolein and 20 µg/ml phosphatidylserine (PS) (Schäfer et al. 1985) were added and calcium was omitted.
- 5 µM cAMP as well as 0.2 mM EGTA were added.
- 1 mM calcium and 1 µM calmidazolium or trifluoperazine were added.

## 3.2.6 SDS-PAGE and autoradiography

Electrophoresis in 10% acrylamide gels was performed according to a modified method of Laemmli (1970). The samples were heated to 80 to 90°C for 2 min in denaturing electrophoresis sample buffer as described under section 3.3.2. After electrophoresis, protein bands were visualised by both the silver-staining method as described by Merril et al. (1981) using the Amersham Quick-silver kit and Coomassie blue staining method (de Moreno et al. 1985).

The dried radio-labelled gels were autoradiographed at -20°C (Henke et al. 1988) for two weeks using at first Kodak X-Omat AR films and later Hyperfilm-beta-max (Amersham).

### 3.2.7 Determination of protein concentration

Protein concentrations were measured by using a dye binding protein assay kit (Bio-Rad) based on the method of Bradford (1976), with BSA as standard. The protein binds with Coomassie blue in a phosphoric acid medium, resulting in a blue colour complex that absorbs at 595 nm.

## 3.3 Results and Discussion

### 3.3.1 Extraction procedures

The preparation of acetone powder is a classical method to remove lipids and phenolic compounds with an excess volume of acetone at -20°C. The resulting dehydrated powder can be stored for long periods before use. The simultaneous use of polyethylene glycol 4000 (PEG) as phenol-binding agent, removes excess phenols that could be a problem in plant material (Scopes 1982). Detergents were not added at this stage because the purpose was not to isolate membrane proteins. It could be expected that some membrane proteins would dissolve under these experimental conditions for the next reasons:

- Acetone removes most lipids that associate with membrane proteins.
- The ripening stage of the isolation material resulted in a natural disintegration of membranes.
- The addition of EDTA in the extraction buffer would dissolve proteins that normally associate with divalent cations in the membrane, because they remove these cations from the membrane by chelation (Olàh & Kiss 1986).

The addition of calcium resulted in a lower total membrane yield, for the same reason mentioned above.



### 3.3.2 Optimisation of protein kinase determination method

#### 3.3.2.1 Sodium fluoride concentration

Biological systems possess phosphoprotein phosphatase activity to dephosphorylate proteins and thus provide a fine regulation of biochemical processes (Cohen 1982). NaF, an inhibitor of phosphoprotein phosphatases (Katko & Bishop 1972, Canaani et al. 1984) promoted protein kinase activity in the soluble and membrane fractions isolated from ripening mango fruit tissue. The phosphorylated proteins in these soluble and membrane fractions were thus being dephosphorylated by phosphoprotein phosphatases. NaF was increased in the assay mixture from 0 to 20 mM and resulted in an activity increase of approximately 2.5 fold at 10 mM NaF (Figure 3.1).

#### 3.3.2.2 Magnesium concentration

$Mg^{2+}$ is one of the most important divalent cations necessary for protein kinase activity. Most researchers reported optimum concentrations of 5 to 10 mM (Ranu 1980).

#### 3.3.2.3 Effect of EGTA and $Ca^{2+}$ on kinase-activity

There have been many reports on $Ca^{2+}$-dependent and $Ca^{2+}$-independent protein kinases in plant tissues (Poovaiah & Reddy 1987, Ma 1993). Various aspects of the involvement of $Ca^{2+}$ in plant metabolism were extensively discussed under Chapter 2. Based on measurements of cytosolic $Ca^{2+}$ in plant cells and by analogy with animal cells, it seems likely that cytosolic $Ca^{2+}$ concentration, in plant cells, is in the range of 0.1 to 1 μM (Poovaiah & Reddy 1987). The free calcium concentration in the protein kinase and autophosphorylation assays was controlled by a $Ca^{2+}$/EGTA buffer (final

concentrations: 0.2 mM EGTA, 10 mM $MgCl_2$, 50 mM TRIS-HCl buffer pH 7.2, and various amounts of calcium stock solution). The results are shown in Figure 3.2, indicating that a 0.5 mm concentration resulted in optimal activity.

### 3.3.2.4 Calmodulin-dependency

Calmodulin is an important effector in protein phosphorylation (See discussion under Section 2.3.1.2). Calmodulin is a small acidic protein and the molecular weight was estimated to be in the range of 17 kDa to 19 kDa. Bovine brain calmodulin is similar to zucchini calmodulin in having the same number of $Ca^{2+}$-binding sites, similar secondary structure and $Ca^{2+}$-induced conformational changes (Poovaiah & Reddy 1987). This calmodulin is also more readily available. Mostly it seemed as if calmodulin either had no effect on the calcium-dependent phosphorylation of proteins or inhibited this phosphorylation by complexating with $Ca^{2+}$ leaving a lower free $Ca^{2+}$ concentration. $Ca^{2+}$-dependent but calmodulin-independent phosphorylation has been observed in corn membrane proteins (Veluthambi & Poovaiah 1984) and has been suggested to result from the phosphorylation caused by the incomplete removal of calmodulin from proteins where calmodulin is a subunit, as in phosphorylase kinase (Cohen 1982). The endogenous calmodulin level could also be sufficient to modulate calcium-promoted phosphorylation.

Exogenous calmodulin was added to the assay mixture to investigate whether the promotion of protein kinase activity by $Ca^{2+}$ also involved calmodulin. A concentration of 1 µM bovine brain calmodulin was used in the reaction mixtures as indicated under Section 3.2.5. The results are discussed under Sections 3.3.3, 3.3.4 and 3.3.5.

Limited studies were performed on calmodulin inhibition by adding 1 µM calmidazolium or 1 µM trifluoperazine to the reaction mixture (See Section 3.2.5). There is some measure of concern among plant scientists regarding the use of calmodulin antagonists. All the evidence obtained from studies with calmodulin antagonists for the involvement of calmodulin should be considered suggestive because of their reported non-specific effects (Nishizuka 1984, Hepler & Wayne 1985, Raghothama et al. 1985).

### 3.3.2.5 Phospholipid-dependency

Evidently inositol phospholipids play a role in signal transduction in plants (Poovaiah & Reddy 1987, Morse et al. 1989, Coté & Crain 1993). Some protein kinase activities are stimulated in the presence of 4 µg/ml diolein and 20 µg/ml phosphatidylserine as effectors (Schäfer et al. 1985). These concentrations were used in reaction mixtures as indicated under Section 3.2.5. The results are summarised in Figures 3.7 to 3.12 and discussed under Section 3.3.5.

### 3.3.2.6 Cyclic nucleotide-dependency

Although there is convincing evidence for the existence of cAMP in higher plants (Brown & Newton 1981), there is no evidence for its physiological role as a second messenger (Marmé 1986, Veluthambi & Poovaiah 1984). Limited studies were conducted to determine the effect of cAMP on the activity of protein kinases in mango fruit tissue. cAMP-dependent protein kinases in animals are usually stimulated by 5 µM cAMP (Gopalakrishna & Anderson 1985). The same concentrations were used in the reaction mixtures as indicated under Section 3.2.5. The results are summarised in Figure 3.3. In the absence of $Ca^{2+}$ it seemed as if cAMP had no discernable effect on the phosphorylation of mango fruit proteins.

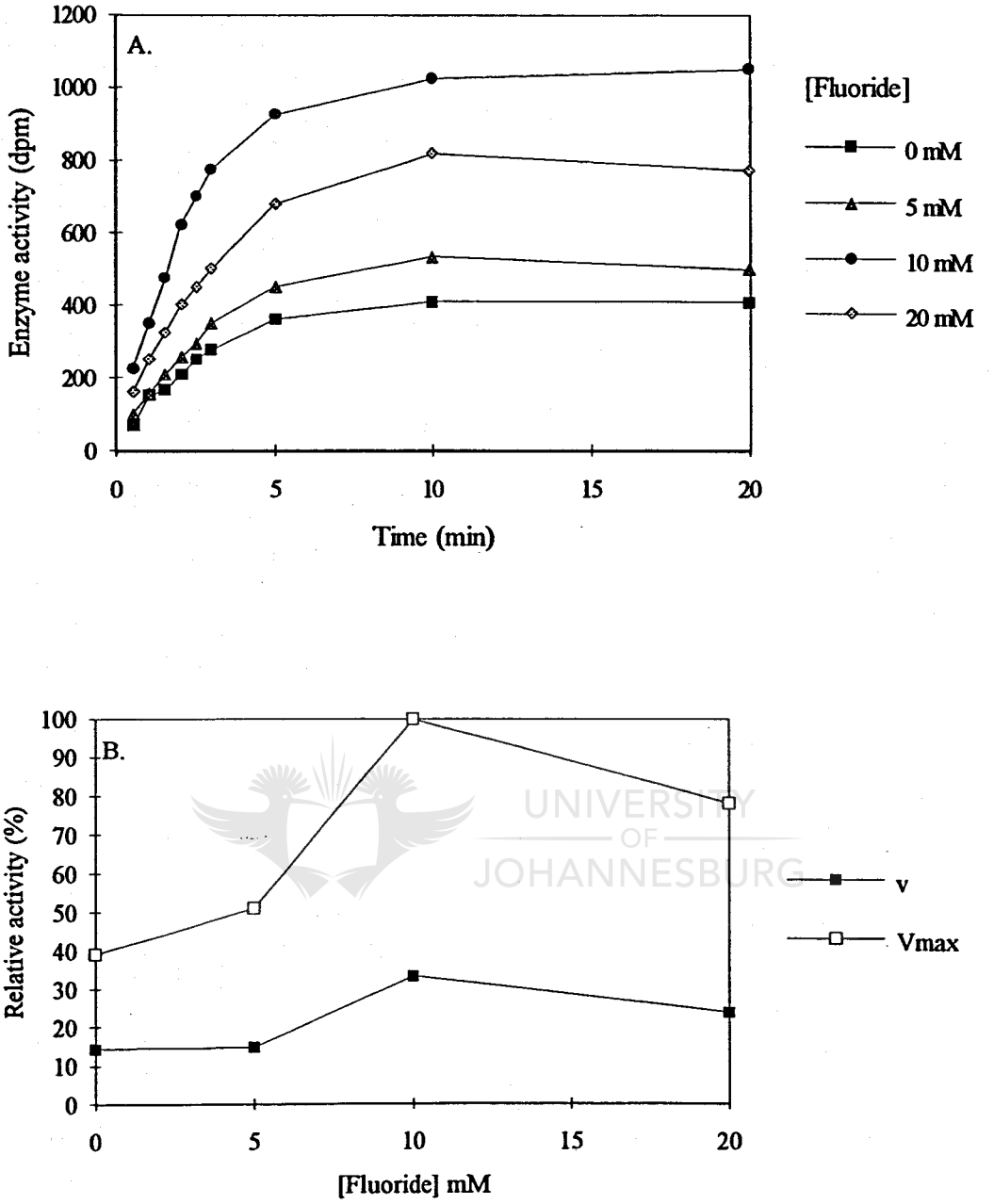





Figure 3.1 Effect of fluoride concentration on the protein kinase activity of soluble proteins isolated from ripening fruit tissue. A: Time course of phosphorylation of endogenous proteins from 0 to 20 minutes with different fluoride concentrations. The phosphorylation was determined by the protein kinase assay system described in Section 3.2.4. B: Relative initial activity, (v) and maximal activity, ($V_{max}$) of the phosphorylation of endogenous proteins with different fluoride concentrations. A 2.5 fold increase in activity was observed after an increase of 0 to 10 mM NaF to the reaction buffer containing 6.6 mM ATP (3.0 Ci/mmol) and 120 mg protein.

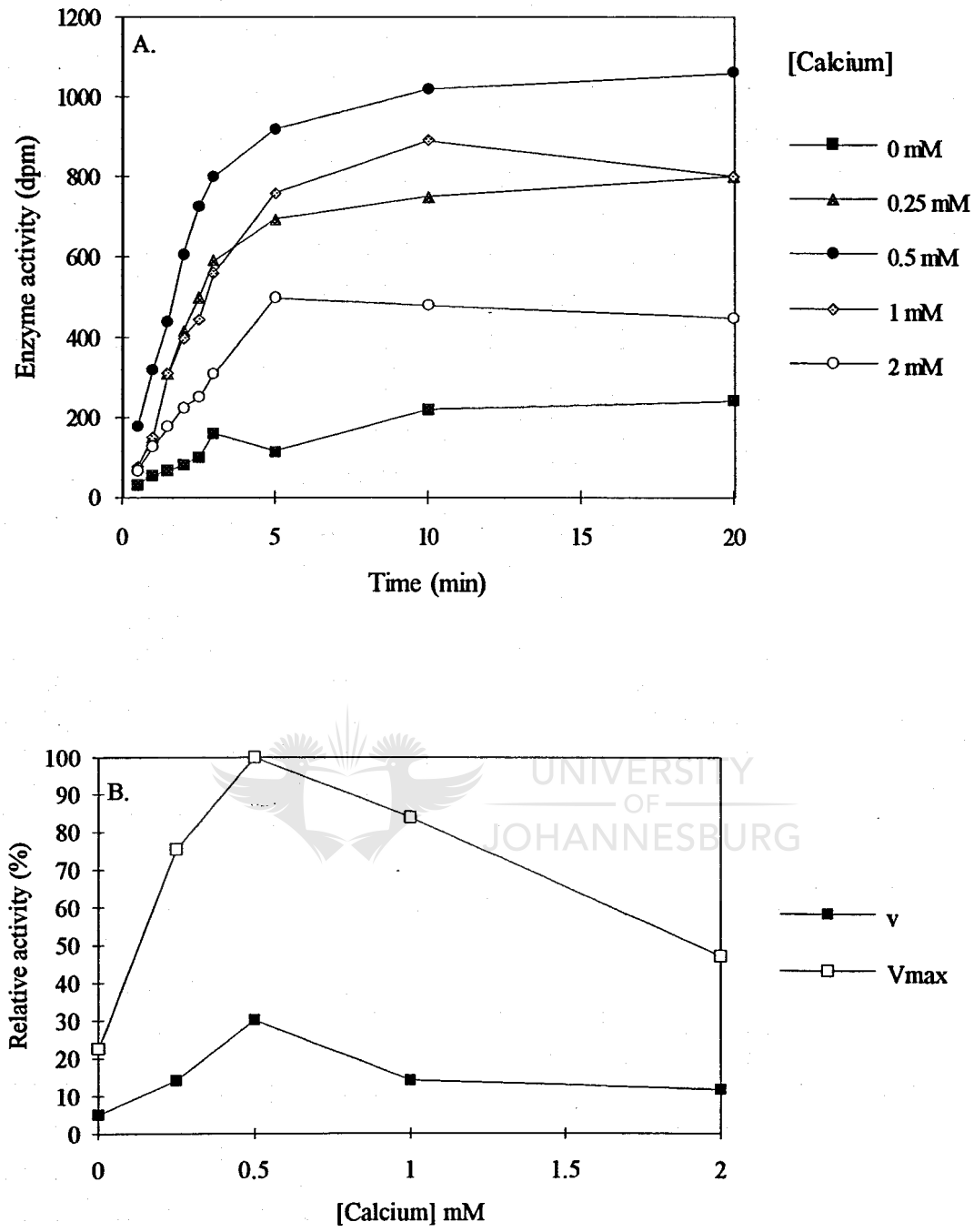





Figure 3.2 Effect of calcium concentration on the protein kinase activity of soluble proteins isolated from ripening fruit tissue. A: Time course of phosphorylation of endogenous proteins from 0 to 20 minutes with different calcium concentrations. The phosphorylation was determined by the protein kinase assay system described in Section 3.2.4. B: Relative initial activity, (v) and maximal activity, ($V_{max}$) of the phosphorylation of endogenous proteins with different calcium concentrations. A 4.4 fold increase in activity was observed after an increase of 0 to 0.5 mM $CaCl_2$ to the reaction buffer containing 6.6 mM ATP (3.0 Ci/mmol) and 120 mg protein.

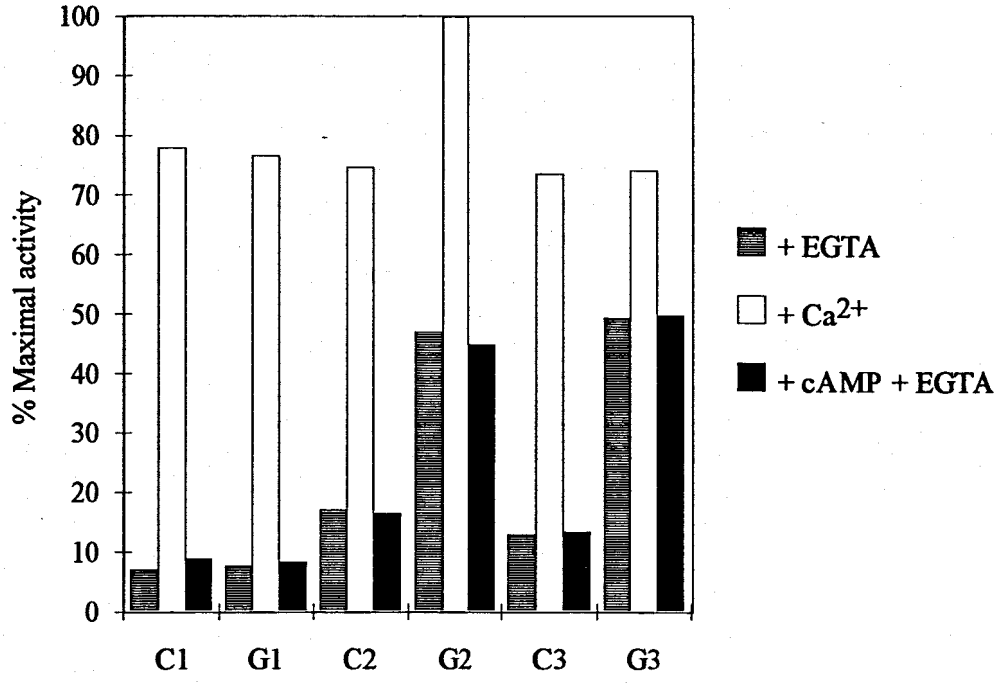



Figure 3.3 Effect of cAMP on the phosphorylation of proteins isolated from the soluble fraction of control (C) and gamma-irradiated (G) climacteric rise (1), climacteric peak (2) and post-climacteric (3) stages of ripening mango fruit tissue. The soluble fractions were incubated with 1) 0.2 mM EGTA, 2) 1 mM calcium, and 3) with 5 µM cAMP plus 0.2 mM EGTA, as described under Section 3.2.5 for 20 min. The left vertical axis represents % maximal activity of total proteins.



## 3.3.3 Endogenous protein kinase activities of soluble proteins

Soluble proteins were isolated from 1 kGy irradiated (G) and control (C) mangoes (see Section 3.2.3.1) at three different stages of ripening; i.e. climacteric rise (1), climacteric peak (2) and post-climacteric stage (3). Phosphorylation of proteins was performed in the presence of [$\gamma$-$^{32}$P]ATP (see Sections 3.2.4 and 3.2.5). The effects of EGTA, endogenous calcium, exogenous calcium and calmodulin on protein kinase activity are shown and compared in Figures 3.4 and 3.5. The same trends in results were repeatedly observed in similar experiments.

The calcium-dependent protein phosphorylation remained relatively high and in the same order throughout the natural ripening process. The calcium-independent phosphorylation remained much lower through out the natural ripening process, although it seems to increase towards the climacteric peak stage. The calcium-dependent protein phosphorylation was significantly higher than the calcium-independent protein phosphorylation. Phosphorylation in the absence of any added effectors (endogenous calcium) seemed to increase slightly towards the post-climacteric ripening stage. Calmodulin seemed to inhibit calcium-dependent phosphorylation towards the end of ripening.

Gamma-irradiation caused significant changes in the phosphorylation patterns of ripening fruit. In the corresponding climacteric peak stage the calcium-dependent protein phosphorylation became even higher (See data of the G2 group in Figure 3.4 and 3.5). The very significant effect of gamma-irradiation could be seen in the calcium-independent phosphorylation of endogenous proteins. At the climacteric rise stage this phosphorylation was in the same order of that of the corresponding control groups (C1, G1), but as from the corresponding climacteric peak stage (C2, G2) the calcium-

independent phosphorylation increased drastically to the same order as the calcium-dependent phosphorylation.

The endogenous calcium-activated phosphorylation also increased in the same way as the calcium-independent phosphorylation. Though it suggests that the endogenous calcium increased as a result of gamma-irradiation, the result could rather involve the same enzymes than those responsible for the calcium-independent phosphorylation. As will be discussed in future chapters, there are enzymes in mango fruit tissue, that can phosphorylate proteins in the absence of calcium, but are more active at very low calcium concentrations. Thus this result does not necessarily mean that the endogenous calcium increased during the climacteric period.

The obvious higher endogenous calcium- and calcium-independent protein kinases probably play an important role in the delayed ripening of mango fruit. The corresponding irradiated fruit of the post-climacteric ripening stage is still metabolically active. In fact, these fruits are physiologically not yet in the post-climacteric stage. Calcium- and calmodulin-promoted phosphorylation of membrane proteins is one of the possible indicators of the functional status of the membranes (Poovaiah & Reddy 1987).

Clearly endogenous calcium levels and calcium-dependent protein kinase activity play a major role during the natural ripening process. It seems that gamma-irradiation result in the activation, or synthesis, or release from the membrane of certain protein kinase activities that influence the natural ripening process.

Calmodulin, tested at a concentration of 1 μM, either had no effect on the protein kinase activity or had an inhibiting effect. See discussion under Section 3.3.2.4. Veluthambi and Poovaiah (1984) also found that calmodulin inhibited calcium-dependent phosphorylation. The importance of the reduced protein phosphorylation by calmodulin is not clear but may be the result of decreased available calcium concentrations due to the complexation by calmodulin.



cAMP, tested at a concentration of 5 μM, had no effect on the protein kinase activity as shown in Figure 3.3 (see Section 3.3.2.6).

Phospholipids, tested at concentrations of 20 μg/ml diolein and 100 μg/ml phosphatidylserine, were tested but the results were indecisive and are thus not shown.

The results obtained with crude extracts are difficult to interpret. Calcium activates and inhibits different protein kinase enzymes at the same concentrations. Working with a mixture of protein kinases, one would not know whether the mediator effects cancel each other. The same situation could apply to the calmodulin effects.

The total extractable proteins increased with ripening stage in the control as well as gamma-irradiated fruit. The protein concentration in the gamma-irradiated fruit was always higher than in the corresponding control fruit. Irradiation at 1 kGy causes a degree of damage with the result of a more porous membrane and the release or leakage of more soluble proteins (Frylinck et al. 1987).

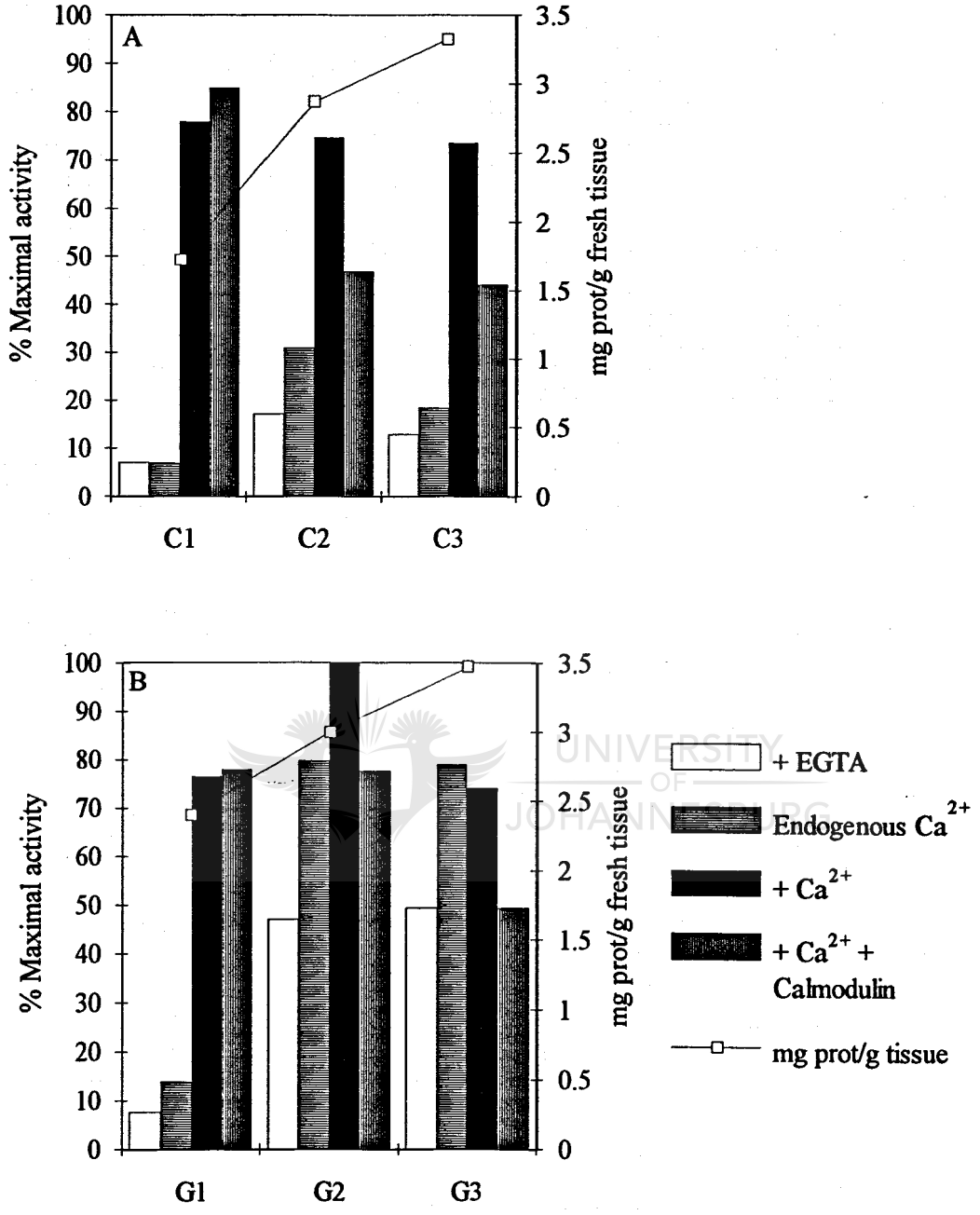



Figure 3.4 The effect of calcium and calmodulin on the phosphorylation of proteins isolated from the soluble fraction of A: control (C) and B: gamma-irradiated (G) climacteric rise (1), climacteric peak (2) and post-climacteric (3) stages of ripening mango fruit tissue. The soluble fractions were incubated with 1) 0.2 mM EGTA, 2) endogenous calcium, 3) 1 mM calcium, and 4) with 1 mM calcium and 1 µM calmodulin, as described under Section 3.2.5 for 20 min. The left vertical axis represents % maximal activity of total proteins and the right vertical axis of the overlap plot represents the mg protein isolated per gram fresh tissue.

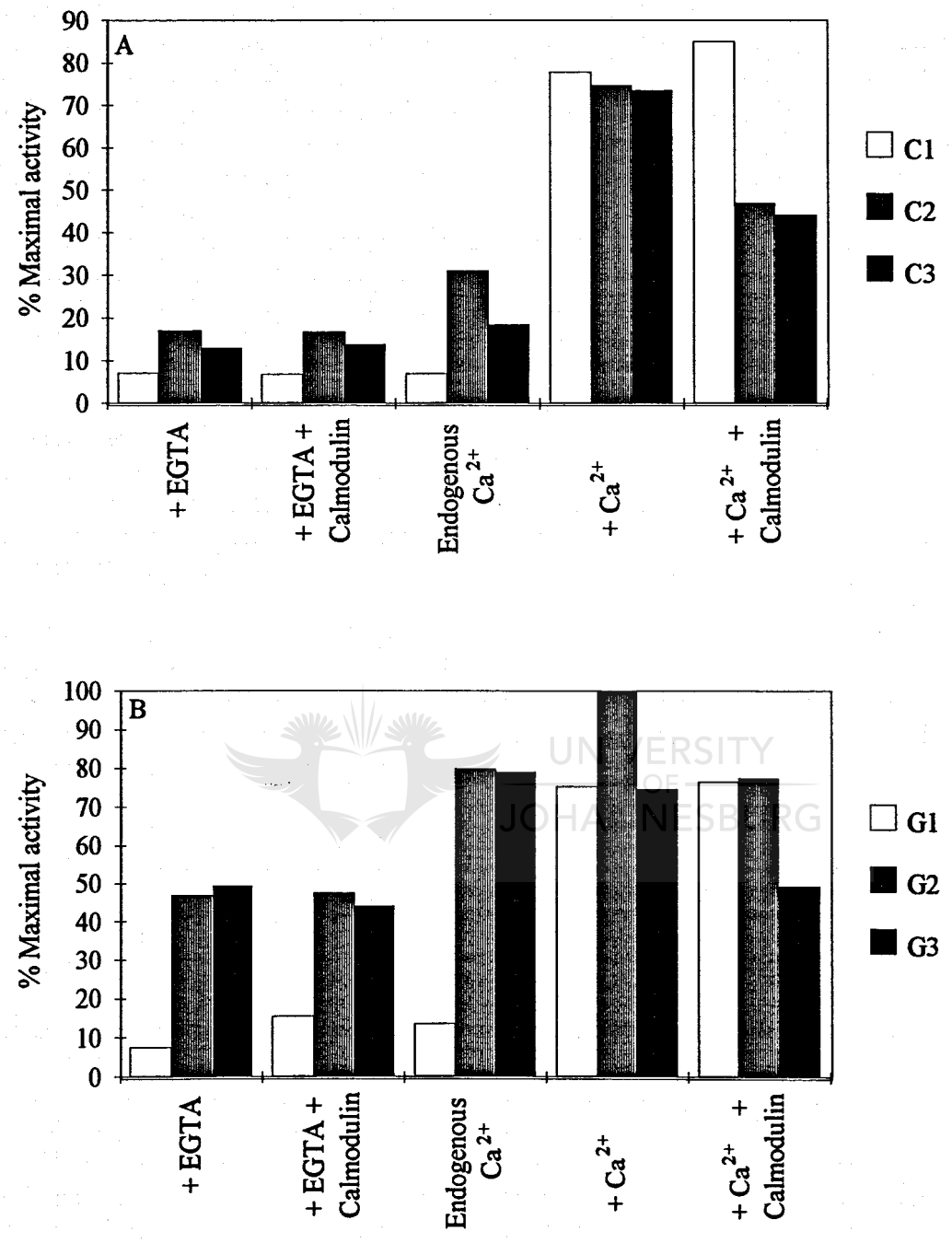



Figure 3.5 The effect of calcium and calmodulin on the phosphorylation of proteins isolated from the soluble fraction of A: control (C) and B: gamma-irradiated (G) climacteric rise (1), climacteric peak (2) and post-climacteric stage (3) stages of ripening mango fruit tissue. The soluble fractions were incubated with 1) 0.2 mM EGTA, 2) 0.2 mM EGTA and 1 μM calmodulin, 3) endogenous calcium, 4) 1 mM calcium, and 5) with 1 mM calcium and 1 μM calmodulin, as described under Section 3.3.3 for 20 min. The left vertical axis represents % maximal activity of total proteins.

## 3.3.4 Phosphorylation of soluble proteins.

Phosphorylation of endogenous proteins was measured in cytosolic fractions prepared from 1 kGy irradiated (G) and control (C) mangoes (see Section 3.2.3.1) at three different stages of ripening; i.e. climacteric rise (1), climacteric peak (2) and post-climacteric stage (3). Phosphorylated proteins, reduced and dissociated, were analysed by polyacrylamide gel electrophoresis in buffers containing sodium dodecyl sulphate. Labelled proteins were detected by autoradiography and shown in Figure 3.6. The detailed explanations of the procedures are given under Section 3.2.6. The effects of calcium and calmodulin were tested according to conditions set out under Section 3.2.5. Thus, three aspects were under consideration;

- the changes of phosphoprotein patterns along with the natural ripening process,
- how irradiation effects these patterns and
- how the absence and presence of calcium and calmodulin as second effectors would influence these patterns.

The six different groups (C1, G1, C2, G2, C3, G3) were all treated in the same way;

- first calcium was omitted and EGTA was added to chelate all the free calcium that was present in the preparation,
- secondly calcium was added and EGTA omitted and
- thirdly calcium in combination with calmodulin was added.

The phosphorylation patterns with added EGTA and calmodulin were identical to that with only EGTA and will not be discussed.

A total of eleven different soluble proteins or groups of proteins were phosphorylated during this experiment. The molecular weights were determined as >200 kDa, 104.7 kDa, 69.2 kDa, 64.6 kDa, 47.9 kDa, 42.7 kDa, 38 kDa, 30.9 kDa, ~8 kDa, ~7 kDa, and ~3 kDa. The results are represented in Tables 3.1 and 3.2. The phosphorylation of all the proteins, except the 104.7 kDa, 63.1 kDa, 42.7 kDa and 38 kDa proteins, was calcium dependent.



### 3.3.4.1 Calcium- and calmodulin-promoted phosphorylation of soluble proteins during the climacteric rise of ripening mango fruit.

- control

Minimal phosphorylation of proteins was observed in the absence of added calcium except in the non-specific (calcium-independent) phosphorylation of the 64.6 kDa protein.
Addition of calcium resulted in the promotion of phosphorylation of the 64.6 kDa protein as well as some phosphorylation of the 42.7 kDa and 38 kDa proteins. Additionally extensive phosphorylation of low molecular weight proteins in the range of approximately 7 kDa was promoted.
The further addition of calmodulin seamed to slightly inhibit the phosphorylation of the 64.6 kDa, 42.7 kDa and 38 kDa proteins. Surprisingly the phosphorylation of the 7 kDa proteins disappeared completely, but the extensive phosphorylation of a new low molecular weight protein group (ca. 3 kDa) was promoted (see C1 column in Table 3.2 and C1 lanes in Figure 3.6). The molecular weight of the last mentioned phosphorylated proteins was estimated to be approximately half the size of the phosphoproteins that disappeared. We suspect that the addition of calmodulin could in some way affect the molecular weight of the these proteins and that it was the same proteins being phosphorylated.

- gamma irradiated

Three proteins, 64.6 kDa, 42.7 kDa and 38 kDa, were slightly phosphorylated in the absence of calcium. Addition of calcium resulted in the promotion of the phosphorylation of these proteins. The calcium-dependent phosphorylation of the low molecular weight ~ 8 kDa and ~7 kDa proteins were promoted even more extensively. The addition of calmodulin had the same inhibiting effect on the

phosphorylation of the 64.6 kDa protein. It also had the same effect on the 7 kDa protein as pointed out before, namely the change of molecular weight to 3.5 kDa (see G1 column in Table 3.2 and G1 lanes in Figure 3.6).

### 3.3.4.2 Calcium- and calmodulin-promoted phosphorylation of soluble proteins during the climacteric peak ripening stage of mango fruit.

- control

A minimal non-specific (calcium-independent) phosphorylation of the 64.6 kDa, 42.7 kDa and 38 kDa proteins is apparent. The addition of calcium resulted in a phosphorylation increase of all these proteins (64.6 kDa, 42.7 kDa and 38 kDa) as well as the phosphorylation of the 30.9 kDa protein and the ~7 kDa low molecular weight protein. The further addition of calmodulin seamed to slightly inhibit the phosphorylation of the 64.6 kDa protein, but the 42.7 kDa and 38 kDa phosphoproteins remained unaffected. The slight phosphorylation of the 30.9 kDa protein disappeared completely. The extensive phosphorylation of the ~7 kDa low molecular weight protein disappeared and was replaced with the ~8 kDa low molecular weight protein (see C2 column in Table 3.2 and C2 lanes in Figure 3.6).

- gamma irradiated

The usual phosphorylated proteins, 64.6 kDa, 42.7 kDa and 38 kDa increased extensively in this mango group, even without the addition of calcium. New phosphoproteins, 104.7 kDa, 69.2 kDa and 47.9 kDa appeared in relative low intensity. Calcium addition activated the phosphorylation of the 30.9 kDa and the low molecular weight ~8 and 7 kDa proteins. The addition of calcium inhibited the phosphorylation of the 104.7 kDa and 47.9 kDa proteins. The 69.2 kDa, 64.6 kDa and 42.7 kDa proteins remained unaffected. Calcium also promoted the phosphorylation of these proteins in the previous groups discussed. Irradiation might result in the release of enough internal calcium (more than could be chelated by the addition of 0.2 mM EGTA) to activate these proteins. Irradiation obviously resulted in the release of more proteins and calcium from the usual insoluble structures like the membranes. A calcium-independent phosphorylation of a new high molecular weight protein (>200 kDa) appeared. The further addition of calmodulin inhibited the phosphorylation the ~7 kDa protein and activated the phosphorylation of the 69.2 kDa protein. The rest remained unaffected (see G2 column in Table 3.2 and G2 lanes in Figure 3.6).

### 3.3.4.3 Calcium- and calmodulin-promoted phosphorylation of soluble proteins during the post-climacteric ripening stage of mango fruit.

- control

The only calcium-independent phosphorylated protein at this stage is the 64.6 kDa protein. The phosphorylation of this protein was quite substantial. The addition of calcium activated the phosphorylation of the >200 kDa proteins extensively, as well as the 69.2 kDa, 47.9 kDa, 42.7 kDa, 30.9 kDa and ~7 kDa proteins. Calmodulin increased the phosphorylation of the 69.2 kDa protein. The 64.6 kDa protein remained unaffected. The phosphorylation of the >200 kDa, 47.9 kDa and 42.7 kDa proteins were decreased (see C3 column in Table 3.2 and C3 lanes in Figure 3.6).

- gamma irradiated

Increased calcium-independent phosphorylation of the same proteins (104.7 kDa, 69.2 kDa, 64.6 kDa, 47.9 kDa, 42.7 kDa and 38 kDa) as the previous gamma-irradiated climacteric peak mangoes occurred. The addition of calcium activated the >500 kDa proteins as well as the 30.9 kDa, ~8 kDa and ~7 kDa

proteins and decreased the phosphorylation of the 104.7 kDa, 69.2 kDa and 38 kDa proteins. The rest (64.6 kDa, 47.9 kDa and 42.7 kDa) were unaffected. The addition of calmodulin decreased the phosphorylation of about all the proteins except that of the >200 kDa, 69.2 kDa and 64.6 kDa proteins (see G2 column in Table 3.2 and G2 lanes in Figure 3.6).

### 3.3.4.4 Effect of calcium and calmodulin on the phosphorylation patterns of gamma-irradiated and control ripening mango fruit tissue.

The >200 kDa protein group was present in the gamma-irradiated climacteric peak and post-climacteric peak, as well as the control post-climacteric peak preparations. The high molecular weight and the presence in these preparations where one expects membrane disintegration, support the idea of these proteins as being released membrane proteins.

The calcium-independent phosphorylation of the 104.7 kDa protein was inhibited by the addition of calcium. This protein was only present in the gamma-irradiated climacteric peak and post-climacteric peak preparations. This phosphoprotein eventuates as a result of gamma-irradiation.

The calcium-dependent phosphorylation of the 69.2 kDa protein was present in the gamma-irradiated climacteric peak and post-climacteric peak, as well as the control post-climacteric preparations. This phosphoprotein could also be a product of normal membrane disintegration.

The 64.6 kDa calcium-independent phosphoprotein was not a gamma-irradiation product. Although the protein concentration increased with time, it was present in all the preparations. The phosphorylation of this protein was unaffected by calcium.

The 47.9 kDa calcium-dependent phosphoprotein was a natural membrane degradation product. It was present in the post-climacteric gamma-irradiated and control preparations.



Although the protein concentration increased along with ripening and senescence, the 42.7 kDa calcium-dependent phosphoprotein was present in all the preparations. It seemed to lose its calcium dependency in the gamma-irradiated post-climacteric preparation. Irradiation could result in the release of enough internal calcium (more than could be chelated by the addition of 0.2 mM EGTA) to activate the phosphorylation of this protein. There are enzymes in mango fruit tissue, that can phosphorylate proteins in the absence of calcium, but are more active at very low calcium concentrations (see future chapters). This protein kinase substrate appears to be also a natural membrane degradation product.

The 38 kDa low concentration calcium-dependent phosphoprotein was moderately present in all the preparations, except in that of the control post-climacteric peak mangoes. The concentration of this phosphoprotein is increased as a result of gamma-irradiation. This protein was definitely not a degradation protein and could play a role in the control of ripening and even help to delay ripening.

The phosphorylation of the 30.9 kDa protein was totally calcium dependent. This protein could have been present in all the preparations, although it was only visible in the gamma-irradiated and control, climacteric peak and post-climacteric preparations.

The phosphorylation of the low molecular weight proteins (~8 kDa, ~7 kDa and ~3 kDa) was all totally calcium dependent. The ~3 kDa phosphoproteins were only present in the gamma-irradiated and control, climacteric rise preparations and was totally dependent on calmodulin. The ~7 kDa phosphoproteins were present in all the preparations. Calmodulin totally inhibited the phosphorylation of these proteins. The ~8 kDa phosphoproteins were present in all the preparations, except in that of the control climacteric rise mangoes. The phosphorylation of these proteins was totally dependent on addition of calmodulin. These low molecular weight phosphoproteins could be all interconnected in some way. The significance of this is still unknown.

As mentioned above endogenous calcium and calcium-dependent protein kinase activity plays a major role during the natural ripening process. It seems that gamma-irradiation result in the activation, or synthesis, or release from the membrane of certain protein kinase activities that influence the natural ripening process.

Leshem et al. (1984) reported results that support the involvement of intracellular $Ca^{2+}$ in senescence, by activating calmodulin. Treatment of the foliage with the $Ca^{2+}$ ionophore (A23187) during the senescence-induction period promoted a lateral phase separation of the bulk lipids in microsomal membranes indicating that internalisation of $Ca^{2+}$ facilitates membrane deterioration. In addition, microsomal membranes from ionophore-treated tissue displayed an increased capacity to convert 1-aminocyclopropane-1-carboxylic acid to ethylene and an increased propensity to produce the superoxide anion ($O_2^-$). Treatment of the tissue with fluphenazine during the senescence-induction period, which prevents binding of the $Ca^{2+}$:calmodulin complex to enzymes, delayed membrane deterioration as measured by these criteria. It also proved possible to simulate these *in situ* effects of the $Ca^{2+}$ ionophore on ethylene production and $O_2^-$ formation by treating microsomal membranes isolated from young tissue with phospholipase $A_2$ and $Ca^{2+}$:calmodulin, and these effects of phospholipase $A_2$ and $Ca^{2+}$:calmodulin were inhibited by calmodulin antagonists. The observations collectively suggest that internalised $Ca^{2+}$ promotes senescence by activating calmodulin, which in turn mediates the action of phospholipase $A_2$ on membranes.

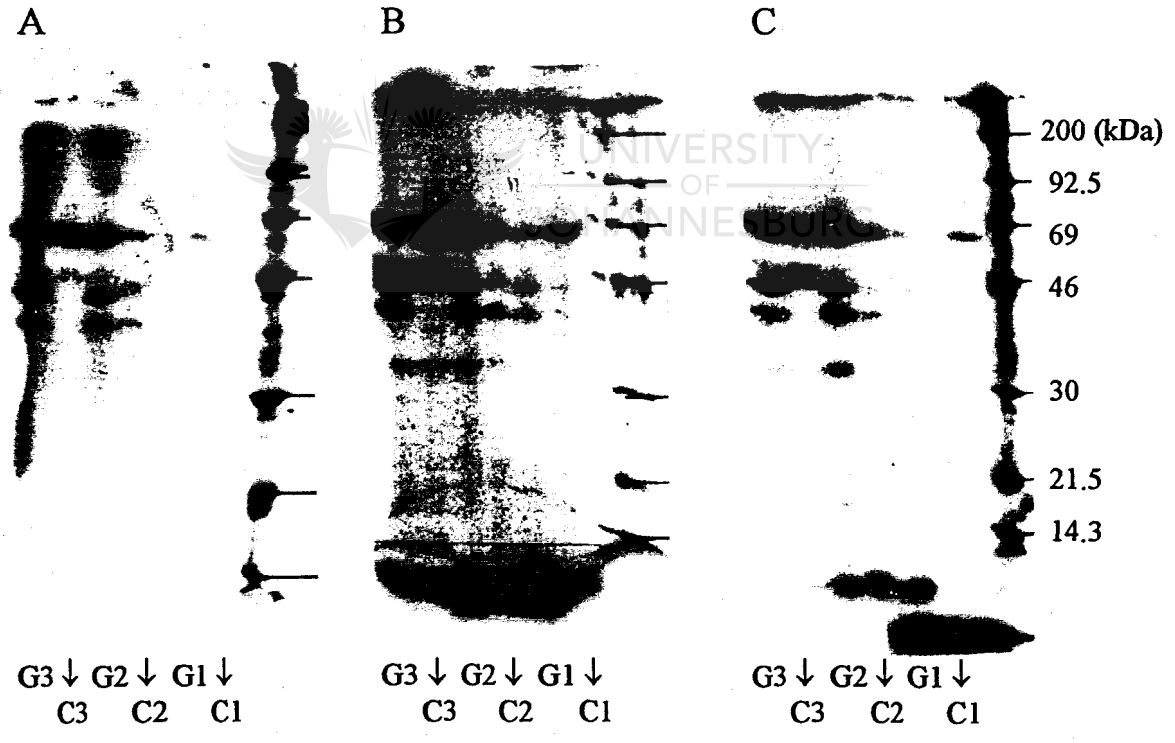



Figure 3.6 Effect of calcium and calmodulin on the phosphoproteins separated by SDS-polyacrylamide gels isolated from the soluble fraction of the six test groups; i.e. control (C) and gamma-irradiated (G) climacteric rise (1), climacteric peak (2) and post-climacteric (3) stages of ripening mango fruit tissue. A: EGTA added. B: Exogenous calcium added. C: Exogenous calcium and calmodulin added. The determined molecular weights of the phosphorylated proteins were tabulated under Table 3.1 and the relative quantities of phosphorylated proteins in the test groups are compared under Table 3.2.

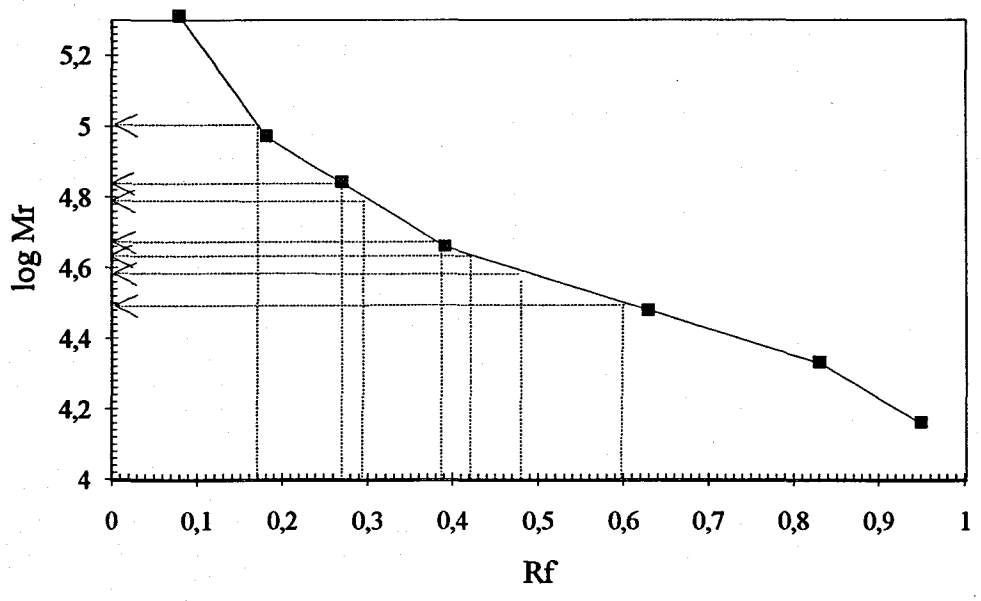



Figure 3.7 Plot of $R_f$ *versus* log $M_r$ for the determination of the $M_r$'s of the phosphorylated proteins isolated from the soluble fraction of mango fruit tissue, separated by SDS-PAGE. The molecular weight markers (in kDa) used for standardisation were myosin, 200; phosphorylase b, 92.5; bovine serum albumin, 69; ovalbumin, 46; carbonic anhydrase, 30; trypsin inhibitor, 21.5; and lysozyme, 14.3. The determined relative molecular weights of the different phosphoproteins were tabulated under Table 3.1



Table 3.1 Tabulation of the determined weights of the phosphorylated proteins separated in Figure 3.6

| Phosphoprotein | $R_f$ | log $M_r$ | $M_r$ | |
|---|---|---|---|---|
| 1 | 0.01 | 7.01 | >>200000 | |
| 2 | 0.17 | 5.02 | 104700 | * |
| 3 | 0.27 | 4.84 | 69200 | |
| 4 | 0.29 | 4.81 | 64600 | * |
| 5 | 0.39 | 4.68 | 47900 | |
| 6 | 0.42 | 4.63 | 42700 | * |
| 7 | 0.48 | 4.58 | 38000 | * |
| 8 | 0.61 | 4.49 | 30900 | |
| 9 | 1.06 | 3.93 | 8500 | |
| 10 | 1.08 | 3.86 | 7200 | |
| 11 | 1.18 | 3.46 | 2900 | |

* = Calcium-independent

+ EGTA

| $M_r$ of phosphorylated protein | C1 | G1 | C2 | G2 | C3 | G3 |
|---|---|---|---|---|---|---|
| >>200000 | 0 | 0 | 0 | 0 | 0 | 0 |
| 104700 | 0 | 0 | 0 | ++ | 0 | ++ |
| 69200 | 0 | 0 | 0 | + | 0 | + |
| 64600 | + | <+ | + | +++ | +++ | +++ |
| 47900 | 0 | 0 | 0 | <+ | <+ | ++ |
| 42700 | 0 | <+ | + | ++ | 0 | +++ |
| 38000 | 0 | <+ | + | ++ | 0 | +++ |
| 30900 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8500 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7200 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2900 | 0 | 0 | 0 | 0 | 0 | 0 |

+ Calcium

| $M_r$ of phosphorylated protein | C1 | G1 | C2 | G2 | C3 | G3 |
|---|---|---|---|---|---|---|
| >>200000 | 0 | 0 | 0 | + | +++ | + |
| 104700 | 0 | 0 | 0 | 0 | 0 | 0 |
| 69200 | 0 | 0 | 0 | ++ | ++ | ++ |
| 64600 | ++ | + | ++ | +++ | +++ | +++ |
| 47900 | 0 | 0 | 0 | 0 | +++ | +++ |
| 42700 | + | + | + | ++ | +++ | +++ |
| 38000 | + | + | + | ++ | 0 | ++ |
| 30900 | 0 | 0 | <+ | <+ | + | + |
| 8500 | 0 | + | 0 | ++ | 0 | + |
| 7200 | +++ | ++++ | ++++ | ++++ | ++ | ++ |
| 2900 | 0 | 0 | 0 | 0 | 0 | 0 |



+Calcium + Calmodulin

| $M_r$ of phosphorylated protein | C1 | G1 | C2 | G2 | C3 | G3 |
|---|---|---|---|---|---|---|
| >>200000 | 0 | 0 | 0 | + | + | + |
| 104700 | 0 | 0 | 0 | 0 | 0 | 0 |
| 69200 | 0 | 0 | 0 | ++ | ++ | ++ |
| 64600 | <++ | + | <++ | +++ | +++ | +++ |
| 47900 | 0 | 0 | 0 | 0 | ++ | + |
| 42700 | >+ | + | + | ++ | 0 | ++ |
| 38000 | >+ | + | + | ++ | 0 | + |
| 30900 | 0 | 0 | 0 | ++ | + | + |
| 8500 | 0 | ++ | ++ | ++ | <+ | <+ |
| 7200 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2900 | ++++ | ++++ | 0 | 0 | 0 | 0 |

Table 3.2 Tabulation of different phosphoproteins separated on SDS-PAGE of analysed mango tissue isolated from six test groups; i.e. C1 (climacteric rise - control group), G1 (climacteric rise - gamma-irradiated group), C2 (climacteric peak - control group), G2 (climacteric peak - gamma-irradiated group), C3 (post-climacteric stage - control group), and G3 (post-climacteric stage - gamma-irradiated group). The symbols 0, <+, +, <++, ++, <+++ and +++ represents the quantity of phosphoprotein present varying between 0 (not present) to +++ (highest quantity present).

## 3.3.5 Phosphorylation of membrane proteins

Membrane proteins were isolated from 1 kGy irradiated (G) and control (C) mangoes (see Section 3.2.3.2) at three different stages of ripening; i.e. climacteric rise (1), climacteric peak (2) and post-climacteric stage (3). Phosphorylation of proteins was performed in the presence of [$\gamma$-$^{32}$P]ATP. Phosphorylated proteins were analysed by polyacrylamide gel electrophoresis in buffers containing sodium dodecyl sulphate. Labelled proteins were detected by autoradiography as shown in Figures 3.8 to 3.13 (See the detailed explanations of the procedures under Section 3.2.6).

Nine different reaction conditions were tested i.e.;

- lane 1, endogenous calcium in the presence of 5 mM $Mg^{2+}$,
- lane 2, the absence of calcium (0.2 mM EGTA) plus 5 mM $Mg^{2+}$,
- lane 3, the absence of calcium (0.2 mM EGTA) plus 10 mM $Mg^{2+}$,
- lane 4, the presence of calcium (5 mM) plus 5 mM $Mg^{2+}$,
- lane 5, the presence of calcium (5 mM) plus 10 mM $Mg^{2+}$,
- lane 6, the presence of calcium (5 mM) plus 1 µM calmodulin and 5 mM $Mg^{2+}$,
- lane 7, the presence of calcium (5 mM) plus 0.1 µM calmidazolium/DMSO and 5 mM $Mg^{2+}$,
- lane 8, the presence of calcium (5 mM) plus 0.1 µM trifluoperazine and 5 mM $Mg^{2+}$, and
- lane 9, the presence of calcium (5 mM) plus phospholipids (20 µg/ml diolein and 100 µg/ml PS and 5 mM $Mg^{2+}$.

Fifteen different molecular weight membrane proteins or groups of proteins were phosphorylated; i.e. >200 kDa, 100 kDa, 86 kDa, 70.3 kDa, 59.1 kDa, 51.5 kDa, 48 kDa, 45.4 kDa, 40.2 kDa, 36.3 kDa, 31.6 kDa, 27.9 kDa, 25.8 kDa, ~14 kDa and ~7 kDa. The phosphorylation of the >400 kDa, 100 kDa, 59.1 kDa, 51.5 kDa, 40.2 kDa, 36.3 kDa, 31.6 kDa, 25.8 kDa and ~7 kDa proteins was calcium independent (Figures 3.8 to 3.13). The addition of 5 mM calcium inhibited the phosphorylation of most of these proteins. The phosphorylation of the 86 kDa, 70.3 kDa, 45.4 kDa, 27.9 kDa, and ~18 kDa proteins was calcium dependent. Having more experience, the researcher knows now that 5 mM $Ca^{2+}$ is in some cases be too high a concentration for calcium-dependent protein kinases, as this concentration range has an inhibiting effect on some protein kinases (see Figure 3.2 for $Ca^{2+}$ dependency for soluble protein kinases). Apparently the addition of 10 mM $Mg^{2+}$ meets the requirements for protein phosphorylation, and the addition of 5 mM $Mg^{2+}$ was to low.

The addition of exogenous calmodulin had negligible effects on the phosphorylation patterns. Endogenous calmodulin could be enough for maximal activity. The addition of calmidazolium in DMSO affected the phosphorylation of almost all the proteins. On the other hand the addition of trifluoperazine had no effect on the phosphorylation patterns. A DMSO control (8% in reaction mixture) showed no inhibition of protein phosphorylation. A definite conclusion on the calmodulin dependency could not be made (see Section 3.3.2.4).

The addition of phospholipids had also negligible effects on the results. Being membrane proteins, they are most probably in contact with enough endogenous membrane lipids to meet their demand for activation. Working with a mixture of proteins, make it difficult to identify the different protein kinases involved. The different kinds of protein kinases have different activation requirements that can inhibit other protein kinases. It is almost impossible to set conditions to satisfy the requirements of all these protein kinases.

The phosphorylation of >200 kDa proteins increased from C1, the climacteric rise stage of ripening to C2, the climacteric peak stage of ripening in the control fruit. The phosphorylation of these proteins was much less in the post-climacteric stage. These proteins were also present in the gamma-irradiated membrane groups, climacteric peak and post-climacteric peak stages of ripening (G2, G3), but in a much lower concentration. These size proteins would probably be most affected by the natural

degradation of membranes during senescence as well as the degradation caused by gamma-irradiation. High lipid and protein complexes could break down to low molecular weight protein fractions. Depending on the hydrophilic or hydrophobic character of the protein molecules they could become more soluble and be isolated with the soluble fractions.

The calcium-dependent phosphorylation of the 86 kDa and 70.2 kDa proteins was present in all the preparations, but seem to lose its calcium dependency in the gamma-irradiated preparations. Because irradiation causes the membrane to release more calcium from its stores (Köteles 1982, Sato et al. 1979), the intrinsic calcium concentration could increase and could explain this behaviour.

The 59.5 kDa phosphoprotein was totally absent from the climacteric rise and climacteric peak stages of the control fruit (C1, C2). It appeared in the post-climacteric ripening stage of the control mango fruit (C3). The phosphorylation of this protein was quite high in the gamma-irradiated climacteric rise group (G1) and almost disappeared in the climacteric peak and post-climacteric stages G2, G3). This protein was subjected to a calcium-dependent mobility shift (60.2 kDa to 59.5 kDa). In this instance both calmidazolium and trifluoperazine inhibited the phosphorylation of the 59.5 kDa protein. It seemed that this phosphorylation could be calmodulin dependent.

The calcium-dependent phosphorylation of the 51.5 kDa protein was high in both the climacteric rise and climacteric peak of the control fruit (C1, C2), but almost disappeared in the post-climacteric stage (C3). The phosphorylation of this protein was absent in the gamma-irradiated climacteric rise group (G1) and high in both the climacteric peak and post-climacteric stages of gamma-irradiated fruit (G2, G3) and thus the synthesis of this protein seems to be delayed.

The 48.1 kDa and 45.4 kDa phosphoproteins seem to be degradation products of the membranes, because they became apparent only in the later stages or ripening, G2, C3, G3 (post-climacteric stage - control and gamma-irradiated groups as well as the gamma-irradiated climacteric peak). These phosphorylation processes were, as mentioned before, calcium dependent.



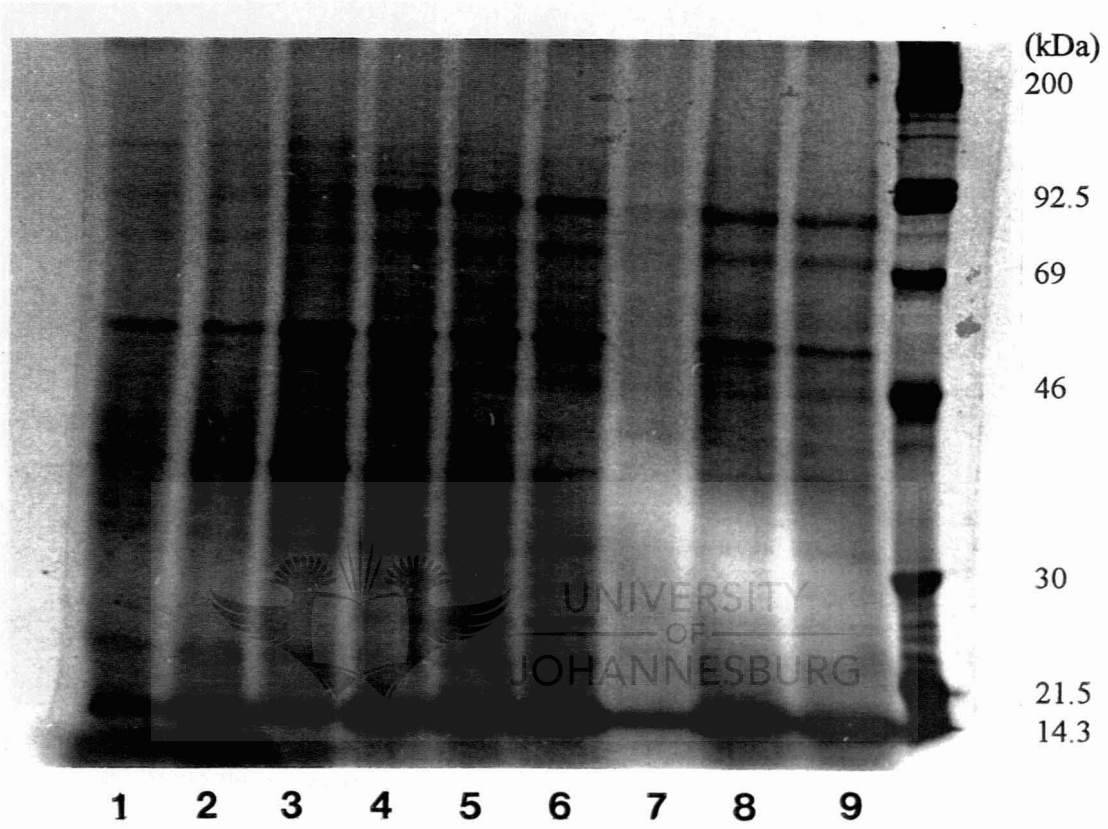
There are traces of the phosphorylation of 40.3 kDa, 31.6 kDa and 27.9 kDa proteins throughout - not necessarily calcium-dependent processes.

The phosphorylation of the 36.3 kDa and 25.8 kDa proteins seems to be calcium independent. The 36.3 kDa phosphoprotein is most prominent in the climacteric rise ripening stage (C1) and the 25.8 kDa phosphoprotein is the highest during the climacteric peak stage (C2) of ripening of the control mango group. Calcium inhibits the phosphorylation of these proteins. In the other preparations these phosphoproteins were absent or decreased.

Two basic low molecular weight phosphoprotein groups, ~14 kDa and ~7 kDa, were present in the membrane fractions. The phosphorylation of the former protein seemed to be calcium dependent and that of the latter, calcium independent. They were both present in elevated concentrations in all the ripening stages of the control mango group. The ~14 kDa phosphoprotein is present in the gamma-irradiation groups in much the same concentrations, except for the post-climacteric stage were the phosphorylation seemed lower. In contrast, the ~7 kDa phosphoprotein group was totally absent in the climacteric rise stage, and much decreased in the post-climacteric stage of the gamma-irradiated mango groups. The climacteric peak stage remained much the same as in the control groups.

Though equal amounts of membrane proteins were used from all the groups tested, the phosphorylation of proteins promoted by $Ca^{2+}$ was considerably reduced in membrane proteins from senesced mangoes. Decreased calcium-promoted protein phosphorylation was also found in membranes from senesced apples (Paliyath & Poovaiah 1984). The phosphorylation patterns in the post-climacteric stage of gamma-irradiated mango fruit were of the same intensity compared to the others. In contrast the phosphorylation of the soluble proteins of this group increased extensively.

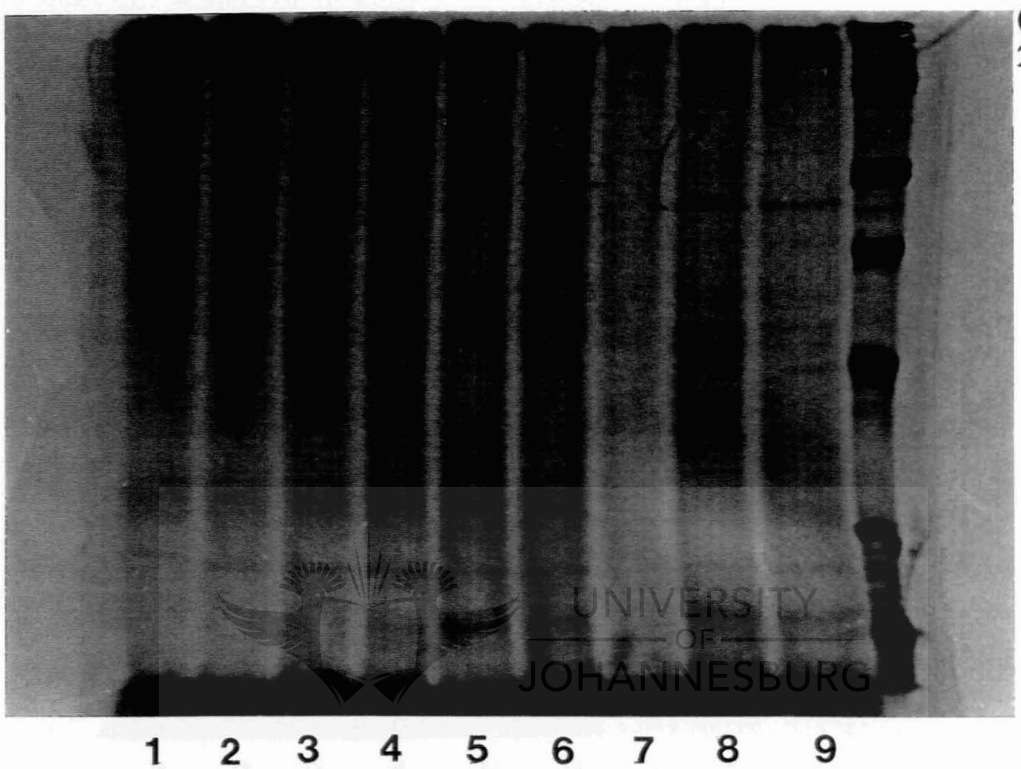
Figure 3.8 Analysis of the phosphorylated proteins isolated from membranes of control climacteric rise ripening stage of mango fruit tissue (C1) separated on SDS-PAGE. Each lane represents a different reaction composition; i.e. L1 (Endogenous $Ca^{2+}$ 5 mM $MgCl_2$), L2 (-$Ca^{2+}$, 5 mM $MgCl_2$), L3 (-$Ca^{2+}$, 10 mM $MgCl_2$), L4 (+ $Ca^{2+}$, 5 mM $MgCl_2$), L5 (+$Ca^{2+}$, 10 mM $MgCl_2$), L6 (+$Ca^{2+}$ + Calmodulin), 5 mM $MgCl_2$), L7 (+$Ca^{2+}$ + Calmidazolium), 5 mM $MgCl_2$), L8 (+$Ca^{2+}$ + Trifluoperazine, 5 mM $MgCl_2$), and L9 (+$Ca^{2+}$ + Phospholipids, 5 mM $MgCl_2$). The symbols 0, <+, +, <++, ++, <+++, +++, etc. represents the quantity of phosphoprotein present, varying between 0 (not present) to ++++++ (highest quantity present).






| $M_r$ of phosphorylated protein | L1 | L2 | L3 | L4 | L5 | L6 | L7 | L8 | L9 |
|---|---|---|---|---|---|---|---|---|---|
| >200000 | + | + | + | <+ | <+ | <+ | 0 | 0 | 0 |
| 110000 | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 |
| 86000 | <+ | <+ | <+ | ++ | ++ | ++ | <+ | ++ | ++ |
| 70300 | <+ | <+ | <+ | + | + | + | 0 | + | + |
| 59000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 51500 | ++ | ++ | +++ | ++ | ++ | ++ | 0 | ++ | ++ |
| 48000 | <+ | <+ | <+ | <+ | <+ | <+ | 0 | <+ | <+ |
| 45400 | <+ | <+ | ++ | + | + | + | 0 | <+ | <+ |
| 41300 | <+ | <+ | <+ | <+ | <+ | <+ | <+ | <+ | <+ |
| 36300 | + | + | +++ | + | + | + | 0 | + | + |
| 31600 | 0 | 0 | 0 | <+ | <+ | <+ | 0 | 0 | 0 |
| 27800 | 0 | 0 | 0 | + | + | + | 0 | <+ | <+ |
| 25800 | + | + | + | + | + | + | 0 | <+ | <+ |
| 18000 | ++ | ++ | +++ | ++++++ | ++++++ | ++++++ | ++ | ++++++ | ++++++ |
| 7000 | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |

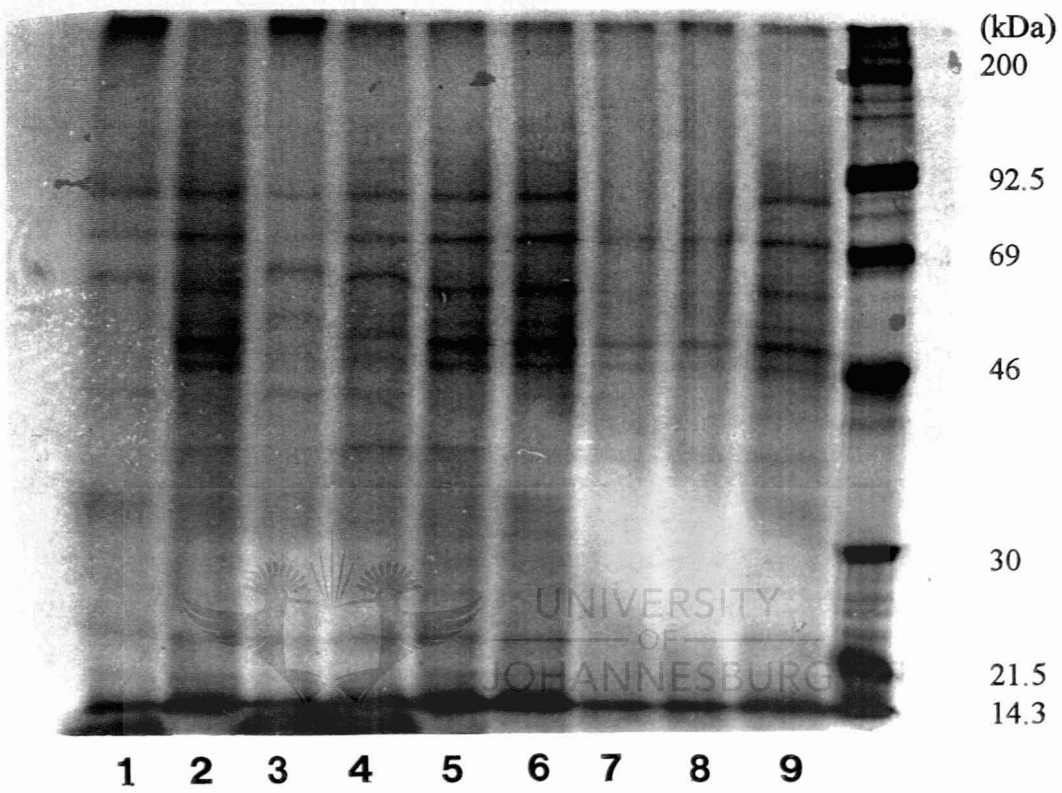
Figure 3.9 Analysis of the phosphorylated proteins isolated from membranes of control climacteric peak ripening stage of mango fruit tissue (C2) separated on SDS-PAGE. Each lane, (L1 to L9) represents a different reaction composition same as indicated under Figure 3.8. The symbols 0, <+, +, <++, ++, <+++, +++, etc. represents the quantity of phosphoprotein present, varying between 0 (not present) to ++++++ (highest quantity present).






| $M_r$ of phosphory-lated protein | L1 | L2 | L3 | L4 | L5 | L6 | L7 | L8 | L9 |
|---|---|---|---|---|---|---|---|---|---|
| >200000 | +++ | +++ | +++ | ++ | ++ | ++ | + | + | 0 |
| 110000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | + |
| 86000 | + | + | + | + | + | + | <+ | + | + |
| 70300 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 59000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 51500 | + | + | +++ | ++ | ++ | ++ | 0 | ++ | ++ |
| 48000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45400 | 0 | 0 | 0 | <+ | <+ | <+ | 0 | <+ | <+ |
| 41300 | 0 | 0 | 0 | <+ | <+ | <+ | 0 | <+ | <+ |
| 36300 | 0 | 0 | 0 | <+ | <+ | <+ | 0 | <+ | <+ |
| 31600 | 0 | 0 | <+ | <+ | <+ | <+ | 0 | 0 | 0 |
| 27800 | 0 | 0 | 0 | <+ | <+ | <+ | 0 | <+ | <+ |
| 25800 | ++ | +++ | +++ | <+ | <+ | <+ | 0 | <+ | <+ |
| 18000 | ++++++ | ++++++ | ++++++ | ++++++ | ++++++ | ++++++ | +++ | ++++++ | ++++++ |
| 7000 | ++++++ | ++++++ | ++++++ | ++++++ | ++++++ | ++++++ | ++++++ | ++++++ | ++++++ |

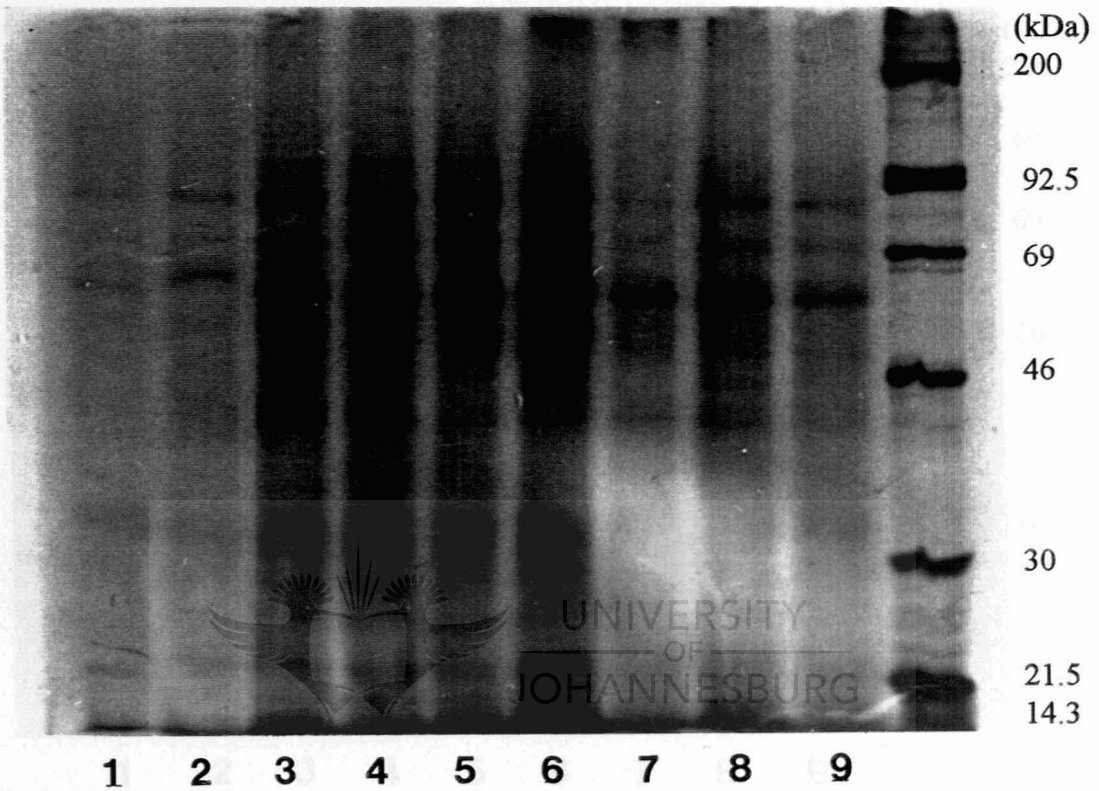
Figure 3.10 Analysis of the phosphorylated proteins isolated from membranes of control post-climacteric ripening stage of mango fruit tissue (C3) separated on SDS-PAGE. Each lane, (L1 to L9) represents a different reaction composition same as indicated under Figure 3.8. The symbols 0, <+, +, <++, ++, <+++, +++, etc. represents the quantity of phosphoprotein present, varying between 0 (not present) to ++++++ (highest quantity present).






| $M_r$ of phosphorylated protein | L1 | L2 | L3 | L4 | L5 | L6 | L7 | L8 | L9 |
|---|---|---|---|---|---|---|---|---|---|
| >200000 | ++ | 0 | ++ | 0 | 0 | 0 | 0 | 0 | 0 |
| 110000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 86000 | <+ | + | <+ | 0 | ++ | ++ | 0 | 0 | ++ |
| 70300 | <+ | ++ | + | ++ | ++ | ++ | <+ | + | ++ |
| 59000 | + | ++ | 0 | + | ++ | ++ | 0 | 0 | + |
| 51500 | 0 | <+ | 0 | 0 | <+ | <+ | 0 | 0 | <+ |
| 48000 | 0 | ++ | 0 | <+ | ++ | ++ | <+ | <+ | ++ |
| 45400 | 0 | + | 0 | <+ | + | + | <+ | <+ | <+ |
| 41300 | <+ | 0 | <+ | <+ | <+ | <+ | 0 | 0 | <+ |
| 36300 | 0 | + | 0 | <+ | <+ | <+ | 0 | 0 | <+ |
| 31600 | 0 | 0 | 0 | <+ | <+ | <+ | 0 | 0 | 0 |
| 27800 | 0 | 0 | 0 | <<+ | <<+ | <<+ | 0 | 0 | <<+ |
| 25800 | <+ | + | + | + | + | + | 0 | <+ | <+ |
| 18000 | ++ | ++++ | +++ | +++++ | ++++++ | ++++++ | ++ | ++ | ++++ |
| 7000 | ++++++ | 0 | ++++++ | ++++++ | 0 | 0 | 0 | 0 | 0 |

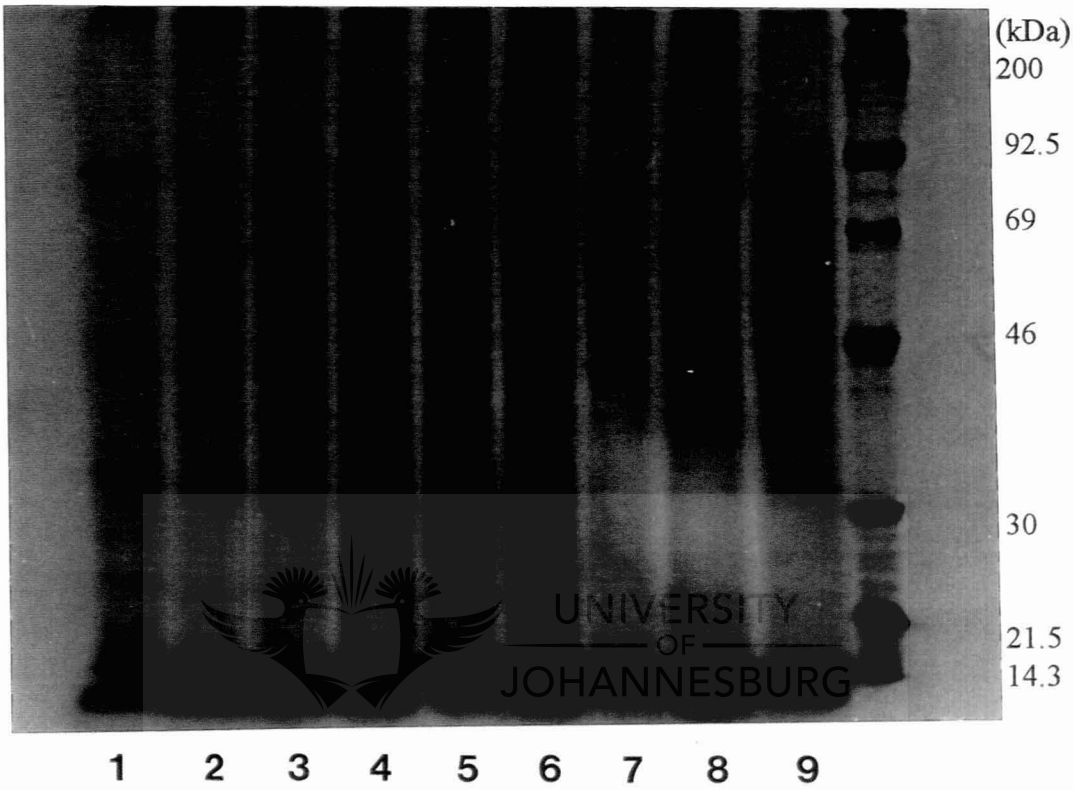
Figure 3.11 Analysis of the phosphorylated proteins isolated from membranes of gamma-irradiated climacteric rise ripening stage of mango fruit tissue (G1) separated on SDS-PAGE. Each lane, (L1 to L9) represents a different reaction composition same as indicated under Figure 3.8. The symbols 0, <+, +, <++, ++, <+++, +++, etc. represents the quantity of phosphoprotein present, varying between 0 (not present) to ++++++ (highest quantity present).






| $M_r$ of phosphorylated protein | L1 | L2 | L3 | L4 | L5 | L6 | L7 | L8 | L9 |
|---|---|---|---|---|---|---|---|---|---|
| >200000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 110000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 86000 | <+ | + | ++ | ++ | ++ | ++ | <+ | + | + |
| 70300 | <+ | <+ | ++ | ++ | ++ | ++ | 0 | + | + |
| 59000 | <+ | <+ | ++ | ++ | ++ | ++ | + | + | + |
| 51500 | 0 | 0 | 0 | 0 | + | + | <+ | + | <+ |
| 48000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 45400 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 41300 | 0 | 0 | + | + | + | + | <+ | <+ | <+ |
| 36300 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 31600 | <+ | <+ | + | + | + | ++ | <+ | <+ | <+ |
| 27800 | 0 | 0 | <+ | <+ | <+ | <+ | 0 | 0 | 0 |
| 25800 | <+ | <+ | + | + | + | + | 0 | <+ | <+ |
| 18000 | ++ | ++ | ++++ | ++++ | ++++ | ++++ | ++ | ++++ | ++++ |
| 7000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

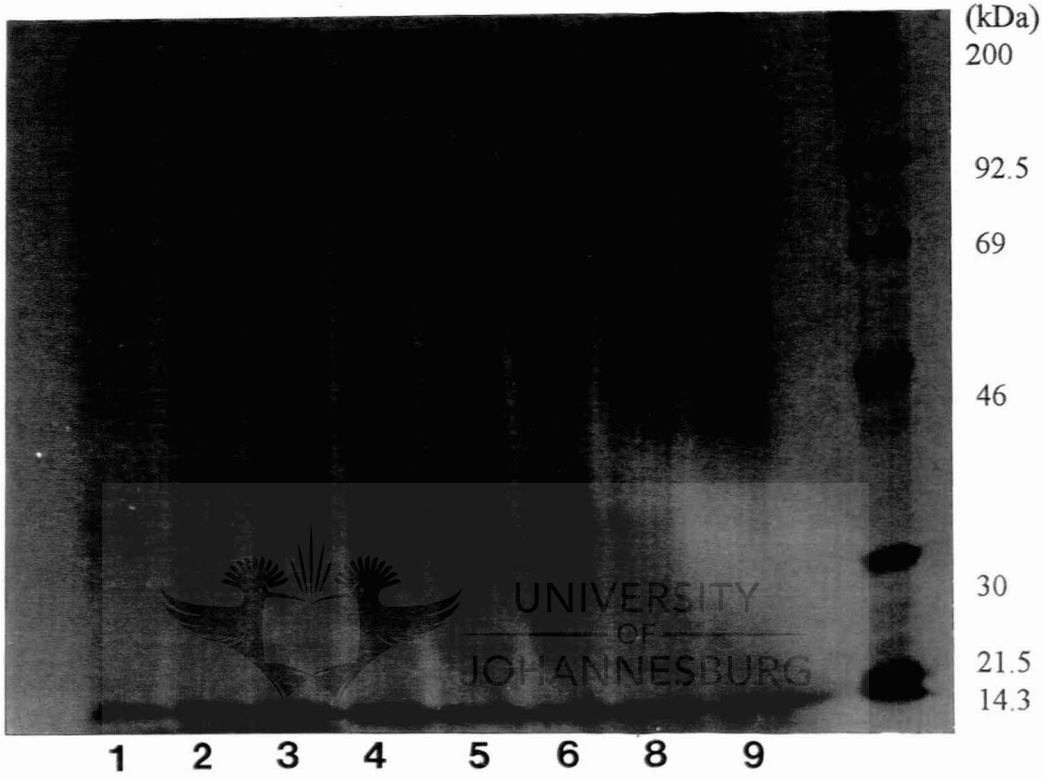
Figure 3.12 Analysis of the phosphorylated proteins isolated from membranes of gamma-irradiated climacteric peak ripening stage of mango fruit tissue separated (G2) on SDS-PAGE. Each lane, (L1 to L9) represents a different reaction composition same as indicated under Figure 3.8. The symbols 0, <+, +, <++, ++, <+++, +++, etc. represents the quantity of phosphoprotein present, varying between 0 (not present) to ++++++ (highest quantity present).






| $M_r$ of phosphorylated protein | L1 | L2 | L3 | L4 | L5 | L6 | L7 | L8 | L9 |
|---|---|---|---|---|---|---|---|---|---|
| >200000 | + | + | + | 0 | 0 | 0 | 0 | 0 | 0 |
| 110000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 86000 | + | + | + | ++ | ++ | ++ | <+ | ++ | ++ |
| 70300 | <+ | 0 | 0 | + | + | + | 0 | + | + |
| 59000 | 0 | 0 | <+ | <+ | <+ | <+ | 0 | <+ | <+ |
| 51500 | + | + | <+ | ++ | ++ | ++ | 0 | ++ | <+ |
| 48000 | 0 | 0 | <+ | ++ | ++ | ++ | <+ | ++ | + |
| 45400 | 0 | 0 | 0 | ++ | ++ | ++ | <+ | ++ | + |
| 41300 | <+ | <+ | <+ | <+ | <+ | <+ | <+ | <+ | <+ |
| 36300 | <+ | <+ | + | <+ | <+ | <+ | 0 | <+ | <+ |
| 31600 | 0 | 0 | 0 | <+ | <+ | <+ | 0 | <+ | <+ |
| 27800 | 0 | 0 | 0 | <+ | <+ | <+ | 0 | <+ | <+ |
| 25800 | + | + | + | <+ | <+ | <+ | 0 | <+ | <+ |
| 18000 | ++ | ++ | +++ | ++++++ | ++++++ | ++++++ | +++ | ++++++ | ++++++ |
| 7000 | ++++++ | ++++++ | ++++++ | +++++ | +++++ | ++++++ | ++ | ++++++ | +++ |

Figure 3.13 Analysis of the phosphorylated proteins isolated from membranes of gamma-irradiated post-climacterium ripening stage of mango fruit tissue separated (G3) on SDS-PAGE. Each lane, (L1 to L9, here L7 was left out) represents a different reaction composition same as indicated under Figure 3.8. The symbols 0, <+, +, <++, ++, <+++, +++ etc. represents the quantity of phosphoprotein present, varying between 0 (not present) to ++++++ (highest quantity present).






| $M_r$ of phosphorylated protein | L1 | L2 | L3 | L4 | L5 | L6 | L8 | L9 |
|---|---|---|---|---|---|---|---|---|
| >200000 | + | + | + | + | + | + | + | + |
| 110000 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 86000 | 0 | 0 | 0 | <+ | <+ | <+ | ++ | ++ |
| 70300 | <+ | <+ | <+ | <+ | <+ | <+ | + | + |
| 59000 | 0 | 0 | <+ | <+ | <+ | <+ | 0 | 0 |
| 51500 | 0 | 0 | <+ | + | + | + | ++ | ++ |
| 48000 | 0 | 0 | <+ | <+ | <+ | <+ | <+ | <+ |
| 45400 | 0 | 0 | <+ | <+ | <+ | <+ | <+ | <+ |
| 41300 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 36300 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 31600 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 27800 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 25800 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18000 | ++ | ++ | +++ | ++++ | ++++ | ++++ | +++ | +++ |
| 7000 | +++ | +++ | +++ | 0 | 0 | 0 | 0 | 0 |

Irradiation causes a degree of damage with the result of a more porous membrane and the release of more soluble proteins (McKersie et al. 1978). Gamma irradiation therefore could cause the disruption of the normal compartmentation in the cells.

It appears that the decrease in membrane protein phosphorylation during senescence and as a result of gamma-irradiation could be due to:

- selective degradation of protein kinases and substrates or,
- inhibition of enzyme activities by unfavourable micro-environment.

## 3.4 Conclusion

The importance of protein phosphorylation and dephosphorylation in the regulation of $Ca^{2+}$-dependent enzyme activities in plants are well understood (Poovaiah & Reddy 1987, Ma 1993). $Ca^{2+}$ and calmodulin-dependent protein kinases occur in membrane (Ladror & Zielinski 1989) and soluble fractions (Raghothama et al. 1987) from a wide variety of tissues such as coleoptiles, hypocotyls, cell suspensions and fruits. Observations have been made in a number of light and hormone induced processes (Hepler and Wayne 1985, Owen 1988, Poovaiah & Reddy 1987, Roux & Serlin 1987, Friedmann & Poovaiah 1991). All these findings suggest that such a phosphorylation system could be of general importance as a biochemical mechanism for calcium action. Along with protein kinases, these membranes also contain a wide array of substrates whose functions ultimately determine the physiological status of the cell, tissue or the plant.

In all the plant systems studied, phosphorylation of several polypeptides was stimulated by $Ca^{2+}$ in the absence of exogenously added calmodulin. These results suggest two possibilities (Veluthambi & Poovaiah 1984):

- $Ca^{2+}$ alone could stimulate protein kinase activity or
- endogenous calmodulin remaining bound to the proteins or trapped in membrane vesicles was involved in the activation.

In the latter case, it is also possible that calmodulin could be a subunit of the protein kinase as in the case of phosphorylase kinase (Cohen 1982). Attempts to purify $Ca^{2+}$-calmodulin-dependent protein kinases and their substrates should help to understand the mode of action of these enzymes and their role of protein phosphorylation in the ripening process.

Earlier studies showed that calcium treatment delayed the senescent breakdown of post-climacteric Golden Delicious apples (Poovaiah & Shekhar 1978). During investigations on membrane properties of apples the fluorescence polarisation of membrane embedded biphenyl hexatriene increased in membranes from senesced apples as compared to normal cold-stored apples (Paliyath et al. 1982). Increase in micro viscosity of membranes has been noted with senescence (McKersie et al. 1978). There was no significant difference in the phospholipid content between senesced and normal apple membranes. The change could be in the protein pattern or in their functions. Such changes could alter the micro environment of proteins thus affecting their conformation and function. Inhibition of calmodulin activity by endogenous compounds was found to increase during senescence in apples (Paliyath and Poovaiah 1984). The results showed that with senescence there was a decrease in the membrane protein content as observed in other senescing systems (Marinos 1962, Thimann 1980). An increase in the low molecular weight protein (~18 kDa) in membranes from senesced mangoes, as in apples (Paliyath & Poovaiah 1984), may be of some significance. Synthesis of new proteins has also been observed in ripening tomatoes (Rattanapanone et al. 1978).

As previously mentioned it has been reported that application of exogenous $Ca^{2+}$ delays senescence, apparently by preventing the large increase in ethylene production that occurs during the very early stages of senescence (Lieberman & Wang 1982, Poovaiah & Leopold 1973a). However, this has been attributed to an extra-cellular effect whereby $Ca^{2+}$ associates with the outside surface of the plasma

membrane and by acting as a divalent ligand, stabilises the membranes. Parallel ESR studies have shown that the levels of $Ca^{2+}$ found to delay senescence is able to rigidify membrane surfaces (Legge et al. 1982). Accordingly, inasmuch as the senescence delaying effect of $Ca^{2+}$ appears to manifest an effect of external $Ca^{2+}$ it is not inconsistent with the contention that internalised $Ca^{2+}$ promotes senescence through a calmodulin-mediated effect (Leshem et al. 1984).

Gamma-irradiation distort the normal biochemical patterns during the normal phases of ripening and senescence (Frylinck et al. 1987). A number of events could cause this distortion of natural physiological processes that result in the delay in senescence. These events could include the following:

- The enzymes involved in triggering the ripening and senescence process, like phospholipase $A_2$ (Leshem et al. 1984, 1992), could be inactivated by gamma-irradiation.
- Early release of peroxidase and catalase from the membranes as a result of gamma-irradiation damage could reduce natural as well as irradiation produced radicals (Goodenough et al. 1985, Frylinck et al. 1987).
- Activation or synthesis (Rattanapanone et al. 1978) of protein kinases that phosphorylate important proteins needed to preserve the physiological processes that keep cells active, e.g. the low calcium concentration dependent phosphorylation of the 37.7 kDa soluble protein.

Isolation and characterisation of the protein kinases and their substrates could add further insight into their function and mode of regulation and should greatly enhance our understanding of how protein phosphorylation can be involved in signal transduction (Ranjeva & Boudet 1987). Protein kinases are known to phosphorylate themselves, called autophosphorylation (Soderling 1993). Some of the phosphorylated proteins, e.g. the 37.7 kDa phosphoprotein, could thus be protein kinases. One of the protein kinases isolated and discussed in future chapters, has a similar molecular weight and kinetic and regulatory properties.

# CHAPTER 4

## Protein Kinase Activities in Ripening Mango Fruit Tissue: Classification, Purification and Biochemical Characterisation

# List of Figures and Tables

## Figures

## Tables

# Chapter 4

# Protein Kinase Activities in Ripening Mango Fruit Tissue: Classification, Purification and Biochemical Characterisation

## 4.1. Introduction

Protein kinases (EC 2.7.10, EC 2.7.11) are important components of eukaryotic signal transducing systems. They control diverse cellular processes by phosphorylating specific proteins. Various protein kinases respond to different signals and serve in different regulatory roles in cells. They share the analogous function of controlling substrate proteins by transferring the terminal phosphate of ATP to specific serine, threonine, or tyrosine residues. They also share a basic structure for their analogies in function; the catalytic domains, including ATP-binding and phosphorylation sites, are generally conserved among protein kinases studied to date (Hanks et al. 1988, Soderling 1990).

The present study was carried out to purify protein kinases from Kent mango fruit tissue at the climacteric peak stage of ripening. The main purpose was to characterise the protein kinase activities active during ripening and senescence.

There are more and more reports of protein kinases that apparently can phosphorylate both tyrosine and serine/threonine, suggesting that a class of dual-specificity protein kinases exists (Lindberg et al. 1992, Hirayama & Oka 1992). The physiological significance of this protein kinase group is not yet known. It may be that the acceptor infidelity is kinetically inconsequential or it may result from the use of non-physiological assay systems or substrates. The fact that some enzymes phosphorylate both alcohol and phenol groups suggests they should form a distinct class, which are referred to as dual-specificity protein kinases.

The isolation and characterisation of a possible dual-specificity $Ca^{2+}$-stimulated protein kinase from ripening mango tissue are described under Section 4.3.2.

A considerable fraction of total phosphoprotein transferase activity of cell extracts is accounted by the so called casein kinases. The isolation procedure of the enzymes for mammalian cells has been developed and their properties studied (Kandror et al. 1990). Casein kinases 2 are high-molecular weight proteins with a complex quaternary structure, specific for ATP and GTP, that modify serine and threonine residues of protein substrates. A characteristic feature of casein kinases 2 is their capability of being inhibited by the stoichiometrical amounts of polyanions (heparin, RNA, etc.) and of being stimulated by polyamines. These enzymes are capable of self-phosphorylation, but the biological meaning of this effect remains obscure (Kandror et al. 1990). Interest in CK-2-type kinases in plants has been enhanced by recent reports that this type of protein kinase participates in controlling the binding of the AT-1 nuclear factor to a consensus sequence in certain light-regulated promoters (Datta & Cashmore 1989, Li & Roux, 1992). The casein kinases of plants and lower eukaryota are poorly studied.

The isolation and characterisation of a possible casein kinase 2 from ripening mango tissue is described under Section 4.3.3. Despite the evolutionary remoteness from animals, plants also contain casein kinase II that exactly falls under the conventional classification (Hunter 1987).

The existence of different $Ca^{2+}$-dependent protein kinases (CDPK) have been known for a long time in plants (Salimath & Marme 1983, Schäfer et al. 1985) but few of them have been purified and characterised. A $Ca^{2+}$-dependent but calmodulin-independent protein kinase was purified from soybean

(Harmon et al. 1987). Two proteins of 46 and 51 kDa were identified to have CDPK activity after renaturing enzyme activity in SDS gels. These protein kinases were capable of autophosphorylation.

Under Section 4.3.4 the partial purification of a $Ca^{2+}$-dependent protein kinase from ripening mango fruit tissue is described. This enzyme shows similarities to CDPK purified from soybean (Harmon et al. 1987), alfalfa (Bögre et al. 1988) and green alga, *Dunaliella tertiolecta* (Yuasa & Muto 1992).

## 4.2 Experimental Procedures

### 4.2.1 Plant material

Mature but unripe Kent mangoes were obtained from orchards in the Tzaneen area. The fruits were carefully selected for uniformity of size and colour. The fruit was kept at constant room temperature and ripened naturally. At the climacteric peak stage of ripening the mangoes were frozen whole at -25°C. Before use the mangoes were thawed slightly, peeled and sliced. This procedure was necessary because of the seasonal availability of the fruit and gave the best results.

### 4.2.2 Reagents

[$\gamma$-$^{32}$P]ATP (3000 Ci/mmol) was obtained from Amersham. DIECA, PMSF, dephosphorylated casein, calf thymus histone III-S (lysine-rich histone H-I), histone II-S, and chlorpromazine were purchased from Sigma. P-81 filter paper was from Whatman. READY SOLV $^{TM}$EP was from Beckman. Anti-phosphotyrosine, Anti-mouse Ig-AP, Fab fragments, BSA, ATP(TRIS salt), calmodulin, calmidazolium, cAMP and $\beta$-mercaptoethanol were obtained from Boehringer Mannheim. $(NH_4)_2SO_4$, $MgCl_2$, $MnCl_2$, $CaCl_2$, TRIS, EDTA and EGTA were from Merck. Acetone, TCA, sucrose, Na azide and Na pyrophosphate were obtained from Saarchem and were analytical grade. All other chemicals were of analytical grade, obtained from ordinary commercial sources. Pharmalyte (pH 3-10), DEAE-Sephacel, Cibacron Blue Sepharose CL-6B, Phenyl-Sepharose CL-6B, Sephadex G-200F and Sephadex G-75SF were purchased from Pharmacia. Toyopearl HW-55F were purchased from TosoHaas. Calibration proteins for SDS-gel electrophoresis were obtained from Boehringer Mannheim Biochemica (range 20000 to 340000) and Amersham (Rainbow$^{TM}$ range 2350 to 46000 and $^{14}$C-labelled range 14300 to 200000). Calibration proteins for gel chromatography (range 12500 to 450000) were purchased from Boehringer Mannheim Biochemica. Marker proteins for pI-determination were purchased from Sigma. PVDF (Polyvinyl-difluoride) membranes were purchased from MilliPore.

Phosphatidylserine and diolein were stored as stock solutions in $CHCl_3$-MeOH (2:1) in the dark at -15° C. Before use an aliquot was dried down under $N_2$ and sonificated in buffer to give a clear solution (Olàh & Kiss 1986).

### 4.2.3 Protein kinase enzyme assay system

The system is based upon the protein kinase transfer of the $\gamma$-phosphate group of adenosine-5'-triphosphate to a protein substrate (endogenous and exogenous) which is specific for the specific type of protein kinase tested. This method has been optimised to exhibit maximum protein kinase activity.

#### 4.2.3.1 Contents of the assay system

**Buffer A:** 50 mM HEPES containing 0.02% w/v sodium azide. pH as specified between 7 and 8.

1. **DTT buffer:** 30 mM dithiothreitol in buffer A. Store in freezer.

2. **EGTA buffer:** 36 mM EGTA in Buffer A was used to control the calcium concentration or **Calcium buffer:** 12 mM $CaCl_2$ in Buffer A. (Various amounts of calcium stock solution were used as specified under Results and Discussion).

3. **Substrate buffer** - One of the following substrates were used as specified under Results and Discussions of the various substrate specificity studies, kinetic studies and routine assays:
- histone II-S or III-S in Buffer A (concentrations as specified),
- dephosphorylated casein in Buffer A (0.12 g per 10 ml = 2 mg/ml final concentration),
- endogenous substrate (substrate present in sample being tested) or
- isolated substrate, e.g. malic enzyme.

4. **Effectors or inhibitors** - One of the following effectors or inhibitors was used as specified under Results and Discussions of the studies on the regulation of protein kinase activity:
- calmodulin (1 µM) in buffer A
- calmidazolium (250 µM) in DMSO
- chlorpromazine (250 µM) in buffer A
- cAMP (10 µM) in buffer A

5. **Sample** - 6 to 15 times dilution of protein sample was used as specified.

6. **Magnesium [$\gamma^{32}$P]ATP buffer** - 10 µl [$\gamma$-$^{32}$P]ATP (~ 3000 Ci/mmol, 2 mCi/ml) or 1 µl [$\gamma$-$^{32}$P]ATP (~ 3000 Ci/mmol, 10 mCi/ml) in 1 ml 150 µM ATP and 45 mM $MgCl_2$ in buffer A.



**Stop reagent** - 1 mM ATP

**Peptide binding papers** - Sheets of P-81 peptide binding paper (Whatman) containing numbered assay squares (2 cm x 2 cm).

#### 4.2.3.2 Materials and equipment required

The following equipment was required; pipettes or pipetting equipment with disposable polypropylene tips, capable of pipetting 4 to 200 µl, disposable Eppendorf polypropylene microfuge tubes (1.5 ml capacity), water bath at 25°C, suitable dishes or trays for washing binding papers, 2.5 litres of 10% TCA containing 20 mM sodium pyrophosphate and 10 mM EDTA for washing binding papers, 10 ml scintillation counting vials, scintillation counter suitable for $^{32}$P, and liquid scintillation cocktail (for example READY SOLV $^{TM}$EP Beckman).

#### 4.2.3.3 Incubation conditions

It was essential that the temperature remained constant throughout the incubation procedure and therefore all reagents reached the incubation temperature of 25°C before starting the reaction. Pre-incubation periods of greater than five minutes resulted in loss of enzyme activity.

Incubation time was varied between 1 and 15 minutes as specified in separate experiments.

### 4.2.3.4 Assay procedure

**Blanks and controls**

Apparent activity might be raised due to the binding of endogenous [$\gamma$-$^{32}$P]-phosphorylated peptides and proteins present in samples. A suitable control was carried out when preparing the component mixture. Substrate buffer was substituted with Buffer A. Any apparent binding observed in samples tested using this modified component mixture may be due to endogenous phosphorylation.

**Table 1. Assay procedure:**

| | Volume (µl) |
|---|---|
| Reagent 1 | 25 |
| Reagent 2 | 25 |
| Reagent 3 | 25 |
| Reagent 4 | 25 |
| Sample 5 | 25 |
| $Mg^{2+}$ [$\gamma^{32}$P]ATP (start each reaction with 2.22 x $10^6$ c.p.m./150 µl or 4.44 x $10^5$ c.p.m./30 µl) | 25 |
| Total volume | 150 |

The reaction was started by the addition of 25 µl of magnesium [$\gamma$-$^{32}$P]ATP buffer, mixed and incubated at 25°C for 0 (blank), 1, 5, 10 to 15 minutes. The reaction was terminated by pipetting 30 µl aliquots in 10 µl cold 1 mM ATP.



**Separation procedure**

Twenty µl of the terminated reaction was pipetted onto a numbered 2 $cm^2$ P-81 square of binding paper. The solution was allowed to completely soak into the paper. The binding paper was twice washed in 200 ml ice cold 10% TCA, containing 20 mM sodium pyrophosphate and 10 mM EDTA for 20 minutes. The binding paper was now washed once in 200 ml ice cold 5% TCA as above at room temperature, once in ethanol and dried in diethyl ether. The binding paper was cut in squares and placed in individual scintillation vials. Five ml of scintillate was added to the vials. These vials were counted for [$^{32}$P] in a liquid scintillation counter. For autoradiography and immunodetection of phosphotyrosine proteins, see procedure for reaction termination for SDS-PAGE as described under Section 4.2.6.

### 4.2.3.5 Calculation of results

The [$^{32}$P] incorporated into the protein substrate bound to the binding paper was quantitatively measured by liquid scintillation counting. The result obtained should be corrected for any non-specific effects using an appropriate blank. The extent of phosphorylation and thus protein kinase activity could therefore be determined directly as follows:

**Calculation of specific radioactivity (R) of 150 µM magnesium [$\gamma^{32}$P]ATP**

10 µl of 150 µM ATP contains 1.5 nmoles

$$R = \frac{\text{cpm per 10 µl magnesium [}^{32}\text{P]ATP-Mg}}{1.5} \quad \text{cpm/nmole}$$

$= \frac{4.44 \times 10^5 \text{ cpm}}{1.5}$

$= 2.96 \times 10^5$ cpm/nmole
$= 296000 \times 10^3$ cpm/pmole

**Calculation of total phosphate (T) transferred to peptide**

20 µl of 40 µl assay volume spotted onto binding paper

T = (Sample cpm - blanks cpm) x 2*

* This factor is derived from the total terminated assay volume divided by the volume applied to the binding paper.

**Calculation of pmoles phosphate (P) transferred per minute**

$P = \frac{T \times 10^3}{I \times R}$ pmole/minute

R in cpm/nmole

Where I = incubation time (min)

**Calculation of pmoles phosphate transferred per minute per ml enzyme**



| enzyme/150 µl | dilution factor |
|---|---|
| 25 µl | x 6 |
| 20 µl | x 7.5 |
| 15 µl | x 10 |
| 12.5 µl | x 12 |
| 10 µl | x 15 |

P x dilution factor = pmoles phosphate transferred per minute per 30 µl enzyme.
P x dilution factor x 33.33 = pmoles phosphate transferred per minute per ml enzyme.

**Calculation of enzyme units and specific activities**

One International Unit (1U) of enzyme is that amount that catalyses the formation of 1 µmole of product per minute under defined conditions.

Thus 1U = 1 µmole phosphate transferred per minute
$= 1 \times 10^6$ pmole phosphate transferred per minute

One katal is that amount of enzyme that catalyses the conversion of one mole of substrate per second.

1U = 1/60 µkatal
= 16.67 nkatal

One katal = $6 \times 10^7$ Units

## 4.2.4 Control and determination of calcium concentration

The free calcium concentration in the protein kinase and autophosphorylation assays was controlled by a $Ca^{2+}$/EGTA buffer (final concentrations: 0.2 mM EGTA, 10 mM $MgCl_2$, 50 mM HEPES, pH 7.2, and various amounts of calcium stock solution). The amount of contaminating $Ca^{2+}$ in the assay components was determined by plasma emission spectroscopy to be < 1μM.

## 4.2.5 Removal of calmodulin

Calmodulin usually co-purified with protein kinase activity through the most chromatography steps. Calmodulin separates from protein kinase-activity on Cibacron Blue-Sepharose. Calmodulin does not bind to the resin and could be recovered in the flow-through fractions (Harmon et al. 1987).

Another method was used to remove calmodulin from protein kinase preparations. The purified protein kinase preparations were incubated in a buffer containing 3 mM EGTA for 1 hour to favour the dissociation of calmodulin. The preparations were subjected to centrifugal-ultrafiltration through a 20 kDa cut off filter and washed with 2 volumes of buffer containing 3 mM EGTA.

## 4.2.6 SDS-PAGE and autoradiography

Proteins were electrophoresed on SDS-polyacrylamide gels as described by Laemmli (1970) to determine the following;

- the homogeneity of the protein kinases after each fractionation step,
- the existence of subunits,
- homogeneous or heterogeneous subunits,
- relative molecular weight of protein kinase protein subunits,
- autophosphorylation of protein kinases,
- relative molecular weight of endogenous substrates of protein kinases and
- immunodetection of phosphotyrosine proteins.

In SDS separations, migration is determined not by intrinsic electrical charge of polypeptides but by molecular weight (Shapiro 1967). Sodium dodecylsulphate (SDS) is an anionic detergent that denatures proteins by wrapping around the polypeptide backbone. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length. When treated with SDS and a reducing agent, the polypeptides became rods of negative charges with equal charge densities or charge per unit length. $M_r$s were calculated from a plot of $R_f$ *versus* log $M_r$ for the following marker proteins (Amersham Rainbow™ protein calibration kit $^{14}$C-labelled): myosin (200 kDa), phosphorylase b (92.5 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.3 kDa). When radio-labelling was not required, a calibration kit of Boehringer Mannheim was used containing α2-macroglobulin (340 kDa, non-reduced; 170 kDa, reduced), phosphorylase b (97.4 kDa), glutamate dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa) and trypsin inhibitor (20.1 kDa).

For endogenous substrates (phosphotyrosine protein and phosphoserine/threonine protein determination) and autophosphorylation assays, the protein kinases were incubated for 15 min at 25°C in a total volume of 150 μl. The reaction contained $Ca^{2+}$/EGTA buffer, 50 μM [γ-$^{32}$P]ATP (3000 Ci/mmol) and various concentrations of calcium. Effectors and inhibitors were added as specified. The reaction was terminated by addition of electrophoresis sample buffer (2% SDS, 1 mM EGTA, 2% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue, final concentrations). The protein samples were either boiled for 2 min or incubated for 5 min at 55 °C. The samples were electrophoresed in either 10% or 15% (0.15 x 14 x 12 cm) gels. Either way the results did not differ. Proteins were detected by staining

with Coomassie brilliant blue R. Dried radio-labelled gels were autoradiographed at -20°C for 2 weeks using Amersham Beta max film.

## 4.2.7 Agar isoelectric focusing (IEF)

Analytical fractionation of proteins according to their isoelectric points by means of agar isoelectric focusing (IEF) was used to determine the homogeneity of the enzymes as well as characterisation by means of their pI values. Rapid near equilibrium isoelectric focusing is accomplished with the Hoefer SE250 Mighty Small vertical gel unit. The protocol was developed as a quick IEF screening procedure, using non denaturing vertical slab agarose gels cast on GelBond polyester support film. After the run proteins are fixed, dried onto the support film and then stained for easy permanent storage. The technique was done as described in the Hoefer Scientific Instruments, Technical Bulletin #134, based on the method described by Robertson et al. (1987). Carrier ampholytes (Pharmalyte, pH range 3 - 10) were used with the following standard proteins with known pI values: C-phycocyanin (pI = 4.75, 4.85), azurin (pI = 5.65), trifluoroacetylated myoglobin (pI = 5.9), myoglobin Met (porcine) (pI = 6.45), myoglobin Met (equine) (pI = 7.3), myoglobin Met (sperm whale) (pI = 8.3) and cytochrome C (horse heart) (pH = 10.6). The agar was 0.75 mm thick. Separation started at 200 volts for 30 min. The voltage was then turned to 600 volts for approximately one hour. The agarose gel was fixed and stained as described in the same Technical Bulletin #134. The pI's of the unknown protein bands were determined by means of a calibration curve.

## 4.2.8 Analytical gel chromatography

The technique described by Andrews (1970) was used to determine the molecular weight of the protein kinases in relation to a range of known standard proteins.



A Sephadex G-200F column (2.5 cm x 76 cm) was equilibrated with a 0.1 M TRIS HCl buffer, pH 8.5 containing 0.6 M NaCl. The bed volume of the column was 373 ml. A flow rate of 8 ml/h (1.63 cm/hr) was used and 3.0 ml fractions were collected. The included volume ($V_t$) of the column, determined by blue dextrin, was 120 ml and the void volume ($V_0$) of the column, determined by β-ME, was 405 ml. PK-I and PK-III were detected by their activity. The column was standardised by blue dextrin (1 000 kDa), ferritin (450 kDa), aldolase (158 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), lysozyme (14.4 kDa), cytochrome C (12.5 kDa) and β-ME (0.081 kDa). The molecular weight ($M_r$) of the kinases was determined from a plot of elution volume ($V_e$) *versus* log $M_r$ for the same standard proteins.

A Toyopearl HW-55F column (2.5 x 32 cm), equilibrated with a 0.1 M TRIS HCl buffer, pH 8 containing 0.6 M NaCl, was used to determine the molecular weight of PK-II. The bed volume of the column was 157 ml. A flow rate of 26 ml/h (5.3 cm/hr) was used and 3.0 ml fractions were collected. The column was standardised by ferritin (450 kDa), aldolase (158 kDa), ovalbumin (45 kDa) and cytochrome C (12.5 kDa). The molecular weight ($M_r$) of PK-II was determined as described above.

## 4.2.9 Phosphoamino acid studies

### 4.2.9.1 Detection of alkali-resistant phosphoproteins in SDS-PAGE gels

Alkali-resistant phosphoproteins in SDS-polyacrylamide gels were detected by the method described by Bourassa et al. (1988). Following their separation by SDS-polyacrylamide gel electrophoresis, labelled proteins obtained from various purification steps of mango fruit tissue incubated with [$\gamma$-$^{32}$P]ATP were

subjected to alkali treatment, a method usually used to detect phosphotyrosine-containing proteins. The proteins within the gels were cross linked within the gel by gluteraldehyde and thereby eliminated protein losses and did not alter the efficiency of phosphoester bond hydrolysis by alkali treatment. Consequently, the time required to detect alkali-resistant phosphoproteins by autoradiography was greatly reduced. Ripening mango fruit tissue were shown to contain phosphotyrosine proteins, indicating the presence of tyrosine protein kinase activity in the tissue.

It has been shown that the phosphorylation of tyrosyl residues of specific proteins is closely linked to the cell response to growth factors (Fantl et al. 1993). In protein hydrolysates from normal cells, phosphotyrosine represents less than 0.1% of the phosphate content, the remaining phosphoamino acids being phosphoserine and phosphothreonine (Cooper et al. 1983). Since the phosphoryl groups linked to tyrosine and to a certain extent to threonine are more resistant to alkali treatment than the phosphoryl group linked to Ser-residues, incubation of polyacrylamide gels in alkali is currently used to detect the $^{32}$P-labelled phosphotyrosylated cellular proteins (Hunter & Cooper 1985).

Alkali treatment of $^{32}$P-labelled proteins in polyacrylamide gels: Solubilised phosphoproteins were subjected to gel electrophoresis as described above. The unstained gels were fixed overnight (18 h) at room temperature in 10% methanol-7% acetic acid, soaked in a 10% (w/v) glutaraldehyde solution for 30 min at room temperature with constant and gentle agitation, and rinsed in water. The gels were immersed in a preheated solution (56°C) of 1.0 M KOH (300 ml/30 ml of gel), and incubated at 56°C for 3 h with occasional gentle agitation. The gels were then immersed in 10% acetic acid solution and washed with gentle agitation at room temperature for 3 h with two changes of the acidic solution.

Parallel gels were treated as described above except that the alkali treatment steps were omitted. All the gels were stained with Coomassie blue and dipped in 3% acetic acid-3% glycerol solution, dried, and then submitted to autoradiography with Amersham Hyperfilm beta max at -20°C as described under Section 4.2.6.



**4.2.9.2 Immunodetection of phosphotyrosine proteins after transfer to PVDF membranes**

Anti-phosphotyrosine is a monoclonal antibody to phosphotyrosine from mouse-mouse hybrid cells. The antibody specifically binds to proteins which contain phosphorylated tyrosine residues. It does not cross react with any other phosphorylated amino acids, including phosphoserine, phosphothreonine, and phosphohistidine, nor with other phosphorylated molecules, such as ribose phosphate and nucleotides. The antibody is particularly useful for purifying phosphotyrosyl growth factor receptors, as well as phosphotyrosyl transforming proteins and their substrates. Anti-phosphotyrosine (1G2) obtained from Boehringer Mannheim, was used for Western blot analysis.

Following the termination of protein kinase reactions (Sections 4.2.3 and 4.2.6), proteins were electrophoresed on 15% SDS-polyacrylamide gels according to Laemmli (1970) as described under Section 4.2.7, with Rainbow protein markers as standards to eliminate protein staining. Electroblotting was carried out as described in the Hoefer Scientific Instruments manual according to the method of Pratt et al. (1986). PVDF (Polyvinyl-difluoride) was used as transfer membrane.

Immunodetection of protein blots were according to the method described by Walker & Gaastra (1988). The non-specific binding sites of the PVDF membrane were blocked in 50 mM TRIS, pH 10.3, 0.15 M NaCl, 0.5% Tween 20 and 4% BSA for longer than 30 min. The membrane was now treated in a solution of first antibody (anti-phosphotyrosine) for 30 min and washed 4 times for 5 min each in rinsing buffer (10 mM TRIS, 1.0 mM NaCl, 1% Tween 20, pH 8.0). The membrane was subsequently treated in a solution of second antibody (anti-mouse Ig-AP) for longer than 30 min and washed in substrate buffer 4 times for 5 min each to remove all traces of unbound antibody. Finally, the sheets were washed in the buffer used for the enzyme substrate (Veronal acetate, pH 9.6). The sheets were

washed in freshly prepared substrate solution (5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT) and $MgCl_2$ prepared according to Blake et al., 1984). Coloured indigo bands appeared on the PVDF membrane within 5 to 30 min. The hydrogen ion released from dimerisation reduces the NBT salt to the corresponding diformazan, which is intensely purple. When sufficiently coloured, the sheets were washed in distilled water and blotted dry between two sheets of filter paper.

## 4.2.10 Measurement of protein concentration

Protein concentrations were measured by using a dye binding protein assay kit (Bio-Rad) based on the method of Bradford (1976), with BSA as standard. The protein bind with Coomassie blue in a phosphoric acid medium, resulting in a blue colour complex that absorbs at 595 nm. Protein concentrations of relatively pure preparations were also measured spectroscopically by absorption at 280 nm and 230 nm.

## 4.2.11 Extraction and chromatographic resolution of protein kinase activities

All steps were carried out at 4°C. Protein kinase activity and protein concentration was determined as described under Sections 4.2.3 and 4.2.10.

### 4.2.11.1 Extraction

Six hundred grams sliced, frozen mango tissue was added to 800 ml Buffer A (50 mM TRIS-HCl buffer, pH 8 containing 5 mM EGTA, 3 mM EDTA, 10 mM $MgCl_2$, 1 mM diethyldithiocarbamate (DIECA) and 0.02% Na azide). 14 mM 2-mercaptoethanol (β-ME), 1 mM phenylmethyl-sulphonylfluoride (PMSF), 10 g policlar AT (soaked overnight in 40 ml of buffer A) and 2 g pectinase were added directly. This mixture was homogenised and the pH was determined and adjusted to 4.5 with 1 M HCl. The homogenate was stirred for a further 10 min at 4°C to allow for pectin hydrolysis. The pH was now adjusted to 7.2 with 2 M TRIS base. The homogenate was centrifuged at 39 000 x g for 15 min at 4°C. The pH of the resulting supernatant was adjusted to 7.2.

### 4.2.11.2 Ammonium sulphate precipitation

The optimum percentage ammonium sulphate saturation needed for the precipitation of the protein kinases was determined. The first range tested was 0-20%, 20-40%, 40-60% and 60-80%. Secondly the 0-30%, 30-50%, 50-70% and 70-90% range was tested. Powdered ammonium sulphate was slowly added with constant stirring until the appropriate saturation was reached. The preparations were left for at least one hour at 4°C to allow for the completion of the precipitation. The precipitates were collected by centrifugation at 39 000 x g for 15 min at 4°C. The precipitates were resuspended in buffer A and dialysed against three volumes of the extraction buffer. The total specific activities of all the fractions were determined. The results are represented in Figure 4.3.1.1. The highest yield of protein kinase activity was obtained with the 30% to 70% saturated ammonium sulphate fractionation.

The 30-70% saturated ammonium sulphate precipitate of the crude extract supernatant was routinely suspended in 100 ml 20 mM TRIS HCl buffer, pH 8.5. The suspension was dialysed (cut off of 12 to 14 kDa) against three 2 l volumes of the same buffer containing 5 mM β-ME and 0.5 mM PMSF over a period of 12 hours. The suspension was centrifuged at 39 000 x g for 15 min at 4°C.

#### 4.2.11.3 DEAE ion exchange chromatography

The dialysed 30-70% ammonium sulphate fraction (~100 ml) was applied to a DEAE-Sephacel column (2.5 x 20 cm) equilibrated with 20 mM TRIS HCl buffer, pH 8.5 at a flow rate of 35 ml/h (7.13 cm/h). After washing with equilibration buffer, bound proteins were eluted with a 0-0.4 M NaCl gradient in equilibration buffer. The 3 ml fractions were immediately tested for calcium-dependent and calcium-independent protein kinase activity. The results are demonstrated in the elution profiles in Figures 4.3.1.2 and 4.3.1.3. There are four to five major protein kinases in the crude 30 - 70% ammonium sulphate fraction of mango extracts. They were named PK-0, PK-I, PK-II, PK-III and PK-IV (Figure 4.3.1.2).

### 4.2.12 Purification of PK-I

#### 4.2.12.1 DEAE ion exchange chromatography

The dialysed 30-70% ammonium sulphate fraction (~100 ml) was routinely fractionated on a DEAE-Sephacel column (2.5 x 20 cm) equilibrated with 20 mM TRIS HCl buffer, pH 8.5 at a flow rate of 35 ml/h (7.13 cm/h). The first successful separation of PK-I from PK-II was accomplished by a step wise elution with buffer C containing 0.075 M NaCl. The PK-I activity peak appeared immediately. The PK-II activity peak's appearance was delayed and to an extent still associated with the column (Figure 4.3.2.1). Hereafter PK-I and PK-II were eluted and separated by a 0 - 0.15 M NaCl gradient over 500 ml. The 3 ml fractions were immediately tested for calcium-dependent (0.1 mM and 1 mM $Ca^{2+}$) and calcium-independent (in the presence of EGTA) protein kinase activity. The results are shown in the elution diagram in Figure 4.3.2.2. The active fractions of peak 1 (PK-I) were combined as indicated in Figure 4.3.2.2.



#### 4.2.12.2 Cibacron blue affinity chromatography

The combined PK-I active fractions from the previous DEAE column were adjusted to 5.5 mM $MgCl_2$, 2.5 mM EDTA and 2 mM β-ME. It was loaded on a Cibacron blue-Sepharose CL-6B column (7 cm x 1.5 cm) equilibrated in 20 mM TRIS HCl buffer, pH 8 containing 5.5 mM $MgCl_2$, 2.5 mM EDTA and 2 mM β-ME. Flow rates ranged from 1 to 2 ml/min (~50 cm/h). The column was washed with 3 bed volumes of equilibration buffer containing 0.6 M NaCl, and thereafter with 3 bed volumes of equilibration buffer containing 1.5 M KCl. PK-I was separated from contaminating proteins by elution with 1 M NaSCN and the column was washed with a further 4 bed volumes equilibration buffer containing 3 M NaSCN. The results are shown in the elution diagram in Figure 4.3.2.3. PK-I and PK-II reacted the same towards Cibacron blue Sepharose under these conditions and it became important to separate them before reaching this step. PK-I was inactive in 1 M NaSCN. To be able to determine protein kinase activity, it was necessary to first pool the 1 M NaSCN peak and then remove the NaSCN by means of dialysis or centrifugal-ultrafiltration through a 30 kDa cut off filter.

#### 4.2.12.3 Hydrophobic interaction chromatography

Before it was posible to separating PK-I and PK-II on the DEAE-Sephacel column, it was necessary to make use of a Phenyl-Sepharose column to separate PK-I from PK-II. The protein kinase pool was adjusted to 0.6 M NaCl and 1 mM PMSF and 2 mM β-ME were added. The pooled protein kinase fractions were now adsorbed onto a phenyl-Sepharose column (5 cm x 1.5 cm) equilibrated with 10 mM TRIS HCl buffer, pH 8 containing 1.5 M NaCl. Flow rates ranged from 1 to 2 ml/min (~50 cm/h). The column was washed with 3 bed volumes equilibration buffer without the NaCl. It was possible to elute

PK-I from the column with equilibration buffer containing 0.5 M NaSCN, but the PK-II activity could not be recovered (Figure 4.3.2.4).

#### 4.2.12.4 Exclusion chromatography

The Cibacron blue-Sepharose CL-6B pool of PK-I activity was adjusted to 0.6 M NaCl and 1 mM PMSF and 2 mM β-ME were added, and was subsequently concentrated to a volume of 4 ml in an centrifugal-ultrafiltration filter with a cut off of 20 kDa. After addition of glycerol to a final concentration of 10%, the concentrated enzyme was chromatographed on a Sephadex G-75SF column (1.5 cm x 57 cm) equilibrated in 100 mM TRIS HCl buffer, pH 8, containing 0.6 M NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol and 0.02% $NaN_3$. The flow rate was approximately 15 ml/h (8.5 cm/h). The results are shown in the elution diagram in Figure 4.3.2.5. At this advanced stage of purification the protein kinase activity was very unstable and it was necessary to determine the activity immediately and stabilise the enzyme. The active fractions of peak 1 (PK-I) were combined and immediately concentrated by means of dehydration in a dialysis bag against powdered sucrose before being analysed.

The purification procedure of PK-I is summarised in Table 4.3.2.1 and the results are discussed under Section 4.3.2.

### 4.2.13 Purification of PK-II

#### 4.2.13.1 DEAE ion exchange chromatography



The dialysed 30-70% ammonium sulphate fraction (~100 ml) was fractionated on a DEAE-Sephacel column (2.5 x 20 cm) equilibrated with 20 mM TRIS HCl buffer, pH 8.5 at 35 ml/h (7.13 cm/h). After washing with equilibration buffer, bound proteins were eluted with a 0-0.4 M NaCl gradient in buffer B. The 3 ml fractions were immediately tested for calcium-dependent and calcium-independent protein kinase activity. PK-II was identified as the second activity peak that appeared after the onset of the salt gradient. The results are shown in the elution profiles in Figure 4.3.1.2. At the time of the purification of PK-II, the PK-I content of the mangoes was relatively low (compare Figures 4.3.1.2 and 4.3.1.3). Although we could not manage to separate PK-II entirely from PK-I, the presence of this protein kinase was so low that, at that stage it was not realised that it was in fact a separate protein kinase. The active fractions of the second protein kinase peak (PK-II), then thought to be the only peak, were combined.

#### 4.2.13.2 Cibacron blue affinity chromatography

The combined PK-II active fractions from the DEAE-Sephacel column was adjusted to 5.5 mM $MgCl_2$, 2.5 mM EDTA and 2 mM β-ME and was loaded on a Cibacron blue-Sepharose column (15 cm x 1.5 cm) equilibrated in 20 mM TRIS HCl buffer, pH 8 containing 5.5 mM $MgCl_2$, 2.5 mM EDTA and 2 mM β-ME. A preceding concentration step was not necessary. Flow rates ranged from 1 to 2 ml/min (~50 cm/h). The column was washed with 3 bed volumes of equilibration buffer containing 0.6 M NaCl, and thereafter with 3 bed volumes of equilibration buffer containing 1.5 M KCl. After an extensive study to determine the optimum NaSCN concentration to use (see Figure 4.3.3.1), the PK-II was separated from contaminating proteins by elution with 1 M NaSCN and the column was washed with a further 4 bed volumes equilibration buffer containing 3 M NaSCN. The results are shown in the elution profiles in Figure 4.3.3.2. PK-I and PK-II reacted the same towards Cibacron blue Sepharose under these conditions and eluted together. After the discovery of a high amount of PK-I in the new batch of mangoes used, it became important to separate them before reaching this step. PK-II was inactive in 1 M NaSCN. To be able to determine protein kinase activity, it was necessary to first pool

the 1 M NaSCN peak and then remove the NaSCN by means of dialysis or centrifugal-ultrafiltration through a 30 kDa cut off filter.

#### 4.2.13.3 Exclusion chromatography

The Cibacron blue-Sepharose pool of PK-II activity was adjusted to 0.6 M NaCl and 1 mM PMSF and 2 mM β-ME were added, which was subsequently concentrated to a volume of 4 ml in an ultrafiltration filter with a cut off of 30 kDa. After addition of glycerol to a final concentration of 10%, the concentrated enzyme was chromatographed on a Toyopearl HW-55F column (2.5 cm x 32 cm) equilibrated in 100 mM TRIS HCl buffer, pH 8, containing 0.6 M NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol and 0.02% $NaN_3$. The flow rate was approximately 26 ml/h (5.3 cm/h). The results are shown in the elution profile in Figure 4.3.3.3. The Toyopearl HW-55F pool of PK-II activity was concentrated to a volume of 2 ml in an ultrafiltration filter with a cut off of 30 kDa.

Subsequently the concentrated PK-II was chromatographed on a Sephadex G-75SF column (1.5 cm x 57 cm) equilibrated in 100 mM TRIS HCl buffer, pH 8, containing 0.6 M NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol and 0.02% $NaN_3$. The flow rate was approximately 10 ml/h (5.7 cm/h). The results are shown in the elution profile in Figure 4.3.3.4. At this stage the protein kinase activity was very unstable and it was necessary to determine the activity immediately and stabilise the enzyme. The PK-II active fractions were combined. The Sephadex G-75SF pool of PK-II activity was concentrated to a volume of 2 ml in an ultrafiltration filter with a cut off of 30 kDa.

The purification procedure of PK-II was summarised in Table 4.3.3.1 and results discussed under Section 4.3.3.



### 4.2.14 Purification of PK-III

#### 4.2.14.1 DEAE ion exchange chromatography

The dialysed 30-70% saturated ammonium sulphate precipitate enzyme preparation (~100 ml) was fractionated on a DEAE-Sephacel column (2.5 cm x 20 cm) equilibrated with 20 mM TRIS HCl buffer, pH 8.5 at 35 ml/h (7.13 cm/h). After washing with equilibration buffer, bound proteins were eluted with a 0-0.4 M NaCl gradient in equilibration buffer. The 3 ml fractions were immediately tested for calcium-dependent and calcium-independent protein kinase activity. PK-III was identified as the third activity peak that appeared after the onset of the salt gradient. The results are shown in the elution profiles in Figures 4.3.1.2 and 4.3.1.3.

#### 4.2.14.3 Hydrophobic interaction chromatography

One mM PMSF and 2 mM β-ME were added to the 0.3 M NaCl DEAE-Sephacel PK-III activity pool, which was subsequently added to a phenyl-Sepharose column (5 cm x 1.2 cm) equilibrated with 10 mM TRIS HCl buffer, pH 8 containing 1.5 M NaCl. Flow rates ranged from 1 to 2 ml/min (~50 cm/h). The column was washed with 3 volumes equilibration buffer without the NaCl. An ethylene glycol gradient of 0 to 50% was passed through the column. The PK-III active fractions were collected at approximately 30% ethylene glycol, pooled and concentrated to a volume of 4 ml with powdered sucrose. The results are shown in the elution profile in Figure 4.3.4.1.

#### 4.2.14.2 Cibacron blue affinity chromatography

The combined PK-III active phenyl-Sepharose pool was dialysed against three volumes of 20 mM TRIS HCl buffer, pH 8 containing 5.5 mM $MgCl_2$, 2.5 mM EDTA and 2 mM β-ME. The PK-III retentate was loaded on a Cibacron blue-Sepharose column (16 cm x 1.5 cm) equilibrated in 20 mM TRIS HCl buffer, pH 8 containing 5.5 mM $MgCl_2$, 2.5 mM EDTA and 2 mM β-ME. Flow rates ranged from 1 to 2 ml/min (~50 cm/h). After washing with equilibration buffer, PK-III was separated from other contaminating protein kinases by elution with 0.6 M NaCl. Another prominent protein kinase (PK-IV) contaminant remained on the column and only eluted by means of 1 M NaSCN, similar to PK-I and PK-II. The results are shown in the elution profile in Figure 4.3.4.2.

#### 4.2.14.4 Exclusion chromatography

The Cibacron blue-Sepharose pool of PK-III activity was adjusted to 0.6 M NaCl and 1 mM PMSF and 2 mM β-ME were added, which was subsequently concentrated to a volume of 2 ml in an ultrafiltration apparatus with a filter with a cut off of 30 kDa. After addition of glycerol to a final concentration of 10%, the concentrated enzyme was chromatographed on a Toyopearl HW-55F column (2.5 cm x 32 cm) equilibrated in 100 mM TRIS HCl buffer, pH 8, containing 0.6 M NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol and 0.02% $NaN_3$. The flow rate was approximately 26 ml/h (5.3 cm/h). The results are shown in the elution profile in Figure 4.3.4.3. The Toyopearl HW-55F pool of PK-III activity was concentrated to a volume of 1 ml with ultrafiltration using a filter with a cut off of 30 kDa.

Subsequently the concentrated PK-III was chromatographed on a Sephadex G-75SF column (1.5 cm x 71.5 cm) equilibrated in 100 mM TRIS HCl buffer, pH 8, containing 0.6 M NaCl, 2 mM EDTA, 2 mM EGTA, 10% glycerol and 0.02% $NaN_3$. The flow rate was approximately 10 ml/h (5.7 cm/h). The results are shown in the elution profile in Figure 4.3.4.4. At this advanced stage of purification the protein kinase activity was very unstable and it was necessary to determine the activity immediately and stabilise the enzyme. The active fractions of PK-III were combined.

The purification procedure of PK-III was summarised in Table 4.3.4.1 and results discussed under Section 4.3.4.

## 4.2.15 Concentration and stabilisation

Although the protein kinases were inactive in higher concentrations of NaSCN (> 1 M), they were stabilized by this reagent. To determine their activity, the NaSCN had to be removed by dialysis and ultrafiltration. Micro dialysis was a useful method to remove among others, NaSCN from small protein samples.

Unnecessary concentration procedures were avoided by using the separating columns in the right sequence. The enzymes were stabilized with 10 to 20% glycerol or sucrose and stored at -20°C. Under these conditions the enzymes were not frozen (Scopes 1982).

Centrifugal-ultrafiltration was conducted with 30 kDa and 20 kDa cut off filters at 4000 x g for 35 to 60 min. This procedure led to aggregation (precipitation/denaturation) of the purified enzymes and preference was given to sucrose concentration methods.

## 4.2.16 Determination of enzymological characteristics

#### 4.2.16.1 pH optimum

The optimum pH of the transfer of the γ-phosphate group of adenosine-5'-triphosphate to suitable protein substrates by the protein kinases (PK-I, PK-II and PK-III) was determined by variation of the reaction pH between 5.74 and 10.04. The buffers were prepared from BIS-TRIS (pKa = 6.5), HEPES (pKa = 7.33) and CHES (pKa = 9.5). The buffers have different inhibiting or activating effects on protein kinase activety and it was necessary to extrapolate the determined pH activity curves to give one curve (see figures 4.3.2.11, 4.3.3.8 and 4.3.4.8).

### 4.2.16.2 Temperature studies

Most chemical reactions proceed at a faster velocity as the temperature is raised. An increase in T imparts more kinetic energy to the reactant molecules resulting in more productive collisions per unit time. Enzyme catalysed reactions behave similarly, up to a point. Enzymes are complex protein molecules. Their catalytic activity results from a precise, highly ordered tertiary structure that juxtaposes specific amino acid R-groups in such a way as to form the stereo specific substrate binding sites and the catalytic centre. The tertiary structure of an enzyme is maintained primarily by a large number of weak non-covalent bonds (Segel 1975). An enzyme molecule is a very delicate and fragile structure. If the molecule absorbs to much energy, the tertiary structure will disrupt and the enzyme will be denatured, the expected increase in v resulting from increased E + S collisions is offset by the increasing rate of denaturation (Segel 1975). A plot of v *versus* T usually shows a peak, reverred to as the "optimum temperature." The true "optimum" temperature for an assay is the maximum temperature at which the enzyme exhibits a constant activity over a time period at least as long as the assay time (Segel 1975). This was established for the purified protein kinases, by pre-incubating the enzymes at different temperatures for one to two minutes and then measuring the activity at temperatures between 10 and 50 °C.



The relationship between the rate constant of a reaction, $k$ and the activation energy, $E^*$ is given by the Arrhenius equation:

$$k = Ae^{-E^*/RT}$$

and

$$\log k = \log A - E^*/2.303RT$$

The activation energy, $E^*$ of the catalytic action of the protein kinases was calculated from the enzyme activities as a function of temperature from the slope of a linear plot of log v *versus* $10^3/T$:

$$E^* = 2.303 \times R \times \text{slope}$$

The reaction rate, v, is directly proportional to the rate constant, $k$ under experimental conditions where the substrate concentration is high (Segal 1975, Laidler & Bunting 1973). The activation energy, $E^*$ required by molecules before they can react, is lowered by enzymes, thereby enabling the reaction to proceed more rapidly (Segel 1975).

For molecules to collide with sufficient energy, they must be correctly oriented in order to react. The transition-state theory (Cornish-Bowden 1976) attempts to relate the rates of chemical reactions to the thermodynamic properties of a particular high-energy state of the reaction molecules, known as the transition state of activation complex (X*). During a catalytic process, the enzyme-substrate complex is converted to the activation complex, which can then change to products (P) and free enzyme (Q):

$$A + B \xrightarrow{K^*} X^* \rightarrow P + Q$$

where K* is the transition state equilibrium constant. The concentration of X* is assumed to be governed by the laws of thermodynamics, thus [X*] = K*[A][B], where K* is given by

$$\Delta G^* = -RT \ln K^* = \Delta H^* - T\Delta S^*$$

where $\Delta G^*$, $\Delta H^*$ and $\Delta S^*$ are the free energy, enthalpy and entropy of formation of the transition state (activation complex) from the reactants, A being the enzyme (based on active site concentration), and B, the substrate concentration in the reaction mixture.

Enthalpy and entropy of activation provide valuable information about the nature of the transition state and the reaction mechanism. Large enthalpy of activation, $\Delta H^*$ indicates that a large amount of squeezing, stretching or even breaking of chemical bonds is necessary for the formation of the transition state. Entropy of activation provides a measure of inherent probability of the transition state formation, apart from energetic considerations. A large, negative $\Delta S^*$ indicates that the formation of the transition state requires that the reaction molecules adopt precise conformations, and approach one another with specific orientation.

Catalysts can increase a reaction rate by either reducing $-\Delta S^*$ or reducing $\Delta H^*$, or both. It is likely that both effects are important in enzyme catalysis (Cornish-Bowden 1976). Free energy ($\Delta G^*$) must be added to the enzyme-substrate complex to generate the transition state. Efficient catalysis requires lowering of the $\Delta G^*$ for activation.

The free energy of formation ($\Delta G^*$) for the catalytic action of the protein kinases was calculated in a similar manner as the activation energy, $E^*$ from the enzymes activities as a function of temperature from the slope of a linear plot of log (v/T) *versus* $10^3/T$. The free energy of formation equation only holds true under conditions of constant temperature and pressure and the effect of the varying temperature was thus circumvented. The reaction rate, v, is directly proportional to the rate constant, $k$ under experimental conditions where the substrate concentration is high (Segel 1975, Laidler & Bunting 1973).From the equation,

$$k = RT/Nh \exp(\Delta S^*/R) \exp(-\Delta H^*/RT)$$

where R is the gas constant, N is Avogadro's number and $h$ is Planck's constant, it follows that

$$-\Delta H^* = 2.303 \times R \times \text{slope}$$

and

$$\Delta S^* = (\text{intercept} - \log (R/Nh)) \times 2.303 \times R$$

$\Delta G^*$ ($\Delta G^* = \Delta H^* - T\Delta S^*$) can now be calculated if it is assumed that T is constant at 297.15°K.

#### 4.2.16.2 Kinetic studies

The assays for the kinetic studies were conducted as described under Section 4.2.3. Because the phosphotransferase reaction is to be studied at a single pH only, it is recommended that a pH of 8.0 be used with free metal ion held constant at a high, non-inhibitory concentration (Morrison, 1979). The free metal ion used for PK-I and PK-II was $Mg^{2+}$ and its concentration was kept constant at 8 mM. The free metal used for PK-III was $Mn^{2+}$ and its concentration was kept constant at 2 mM. These optimum concentrations were determined in preliminary experiments and are presented in Figures 4.3.2.15, 4.3.3.12 and 4.3.4.12. At pH 8.0 the nucleotide species in solution are limited to mostly

$MgATP^{2-}$ and to a lesser extent $ATP^{4-}$. The other nucleotide species $MgHATP^{-}$ and $HATP^{3-}$ are not present in significant concentrations under these conditions.

At lower pH values, with a divalent metal ion ($M^{2+}$) held at a fixed noninhibitory concentration, variation of the concentration of $MATP^{2-}$ will cause the concentration of $ATP^{4-}$ to vary in constant ratio with the $MATP^{2-}$ concentrations. This follows from the fact that

$$K_1 = \frac{(M)(ATP)}{(MATP)} \quad \text{so} \quad \frac{K_1}{(M)} = \text{constant} = \frac{(ATP)}{(MATP)}$$

If the value of $K_1/(M)$ is such that $ATP^{4-}$ is present as a significant proportion of $MATP^{2-}$ and functions as a linear competitive inhibitor with respect to the nucleotide substrate, then the determined values for the maximum velocity and Michaelis constant of $MATP^{2-}$ will be in error. A plot of $v^{-1}$ against the reciprocal of the total ATP concentration would be non-linear, the non-linearity being also a function of the concentrations of the free nucleotide species as well as the values for their inhibition constants (Morrison 1979).

Initial velocity studies, in the absence of products, were performed over a wide range of ATP and casein concentrations, 2.36 to 26 µM and 0.22 to 2 mg/ml, respectively. The $K_m$ATP, $K_m$Casein or $K_m$Histone and $V_{max}$ for PK-I, PK-II and PK-III were determined from the slopes and intercepts of the secondary replots of the Lineweaver-Burk plots, *versus* 1/[$Mg^{2+}$-ATP] or 1/[casein] or 1/[histone]) by means of the following equations (Segel 1975):

For secondary plot of slopes *versus* 1/[casein] or 1/[histone]

$$\text{Slope} = \frac{K_i\text{ATP} \times K_m\text{Casein or } K_m\text{Histone}}{V_{max}}$$



Intercept on y-axis = $K_m$ATP / $V_{max}$

Intercept on x-axis = -1 / $K_m$Casein or -1 / $K_m$Histone

For secondary plot of slopes *versus* 1/[$Mg^{2+}$ATP]

$$\text{Slope} = \frac{K_m\text{ATP} \times K_i\text{Casein or } K_i\text{Histone}}{V_{max}}$$

Intercept on y-axis = $K_m$Casein or $K_m$Histone / $V_{max}$

Intercept on x-axis = -1 / $K_m$ATP

Product inhibition studies were conducted by measuring initial velocity in the presence of the product (ADP) of the reaction. $K_i$ADP for PK-I, PK-II and PK-III was determined from the x-intercepts of the secondary replots of the slopes of the Lineweaver Burk plots, *versus* [$Mg^{2+}$-ADP] by means of the following equation (Segel 1975):

$$K_i\text{ADP} = -\text{x-axis}$$

## 4.3 Results and Discussion

### 4.3.1 Identification of Protein Kinases in Ripening Mango Fruit Tissue

#### 4.3.1.1 Optimising extraction and ammonium sulphate precipitation conditions

The mango fruit tissue used for purifying protein kinase enzymes posed particular difficulties. The reasons for quality variation with season are multiple. Changes in temperature, micro-nutrients, storage and transport conditions after picking, and correct evaluation of ripening stage are but a few reasons for different basal enzyme concentrations.

Fruit cells start off to be highly compartmented; the bulk of the volume is vacuolar space, which contains quite acidic solutions, proteases, and a variety of other detrimental compounds. There is also a large amount of cell wall (cellulose), starch granules (depending on ripening stage), and other organelles occupying much of the cytoplasmic space. The cytoplasm and membrane fractions that contain most of the proteins, consist of approximately 2% of the total cell volume in fruit (Scopes 1982). Consequently fruit extracts are very low in protein even if very little fluid is added when making the extracts. Mangoes are only available in season and it was found that whole frozen fruit ensured the best protein kinase yield. Freezing cause the formation of ice crystals that are very destructive for membranous layers and organelles, but do not normally upset the enzymes directly. Freezing is also utilised to ensure proper cell breakage and effective protein release (Scopes 1982). Calcium increased the proportion of protein kinase fractionating with cell membranes while divalent cation specific chelators released the proteins from membranes (Oláh & Kiss 1986, Battey 1990). For this purpose the homogenisation medium contained 5 mM EDTA and 3 mM EGTA. Additionally EGTA inhibits metalloproteinases. The addition of powdered policlar AT was beneficial in adsorbing phenols and β-ME was used as reducing sulfhydryl agent. PMSF is a non-reversible inhibitor of serine and thiol proteases, as well as some carboxypeptidases (Scopes 1982). Pectin is a major substance in ripening mango fruit extracts that lead to a thick viscose suspension. Pectinase was added, and pH (4.5) and temperature (4 °C) was changed to favour the degradation of pectin (see Section 4.2.11.1 for the precise method conducted). Although precautions were taken to reduce protease activity, prompt work was still important as protease activity reduced the ultimate yield of protein kinase activity.

One great advantage of ammonium sulphate fractionation over virtually all other techniques ais the stabilisation of proteins (Scopes 1982). The high salt concentration also prevents proteolysis and bacterial action. The optimal yield of protein kinase activity was obtained between 30% and 70% saturated ammonium sulphate as determined from step-wise fractionation of crude extracts (Figure 4.3.1.1 and Section 4.2.11.2). In the fraction precipitated by ammonium sulphate, both $Ca^{2+}$-dependent and $Ca^{2+}$-independent protein kinase activities were detectable.

During the early stages of purification, it is very difficult to evaluate all the possible sources of error (i.e., ATPase activity of the crude extract, other kinase activities, not related to the one that is purified, endogenous inhibitor(s)) and therefore the values of $\gamma$-$^{32}$P-incorporation should be taken with some caution. For this reason it is difficult to interpret recovery and the degree of purification should be calculated only after the DEAE-Sephacel chromatography step. Only after this stage it is possible, to an extent, to separate and identify the different protein kinases from each other.

#### 4.3.1.2 Analyses of various $Ca^{2+}$-dependent and $Ca^{2+}$-independent protein kinases separated by DEAE ion exchange chromatography

Figure 4.3.1.2 illustrates an elution pattern where the 36 000 x g supernatant of the ammonium sulphate precipitation fraction was subjected to DEAE-Sephacel chromatography. The eluted fractions were

assayed for protein kinase activity using dephosphorylated casein as exogenous substrate. When assays were performed in the presence of EGTA, four peaks of kinase activity were observed. The first (PK-0) appeared in the flow through buffer before applying the salt gradient (0 to 0.4 M NaCl). At 0.05 M NaCl the second protein kinase activity peak (PK-I), formed a shoulder with the third protein kinase activity peak (PK-II) that eluted at 1.0 M NaCl. The fourth distinct calcium-independent protein kinase activity (PK-IV) was observed forming a shoulder with PK-II at 0.25 mM NaCl. In the presence of 0.1 mM $Ca^{2+}$ another kinase activity was revealed overlapping the PK-IV activity at 0.25 M NaCl. This protein kinase activity (PK-III) was $Ca^{2+}$ dependent. 0.1 mM $Ca^{2+}$ stimulated the PK-I and PK-II activities, the PK-0 activity remained unaffected, and the PK-IV-activity was far overlapped by PK-III and could not be detected. These studies revealed an intricate network of protein kinases and everything pointed towards the fact that it was just the tip of the iceberg. Feng and Kung (1991) reported the cloning, sequencing and characterisation of eight rice protein kinases from rice (*Oryza sativa L.*). The use of genomic DNA as a template and different sets of primers at different annealing temperatures for PCR, may reveal a larger set of members of the rice protein kinase family, including tyrosine kinases. Feng and Kung suggested that there is great complexity in the protein kinase gene family in plants and that protein phosphorylation may play an as important role in plants as in other eukaryotes. This phenomenon of protein kinase gene multiplicity agrees with the view that protein kinase genes in mammals, *Drosophila* and yeast are complex families containing multiple genes of like classes of enzymes (Hunter 1987).

Figure 4.3.1.3 also illustrates an elution pattern where the 36 000 x g supernatant of the ammonium sulphate precipitation fraction was subjected to DEAE-Sephacel chromatography of the ripening mango fruit tissue. Using a new batch of mangoes resulted in a lower yield of PK-II and an increased amount of PK-I. The basal amounts of each enzyme differed in the different mango batches used from year to year. This could be as a result of different growing conditions, different areas where they were grown, temperature and stress condition after the fruit were picked and ripening stage (attempts were made to select fruit in the climacteric peak stage but accuracy was impossible). This situation was at first confusing, but the further the research progressed the results became clearer. The complexity of the protein kinase network is being emphasised.



### 4.3.1.3 Identification of endogenous phospho-tyrosyl- and phospho-serine/threonine-protein substrates of $Ca^{2+}$-dependent and $Ca^{2+}$-independent protein kinase activities

During the first stages of the development of the isolation procedures, the phosphorylation of endogenous proteins was measured at various purification stages as set out in Figure 4.3.1.4. Alkali-resistant phosphoproteins in SDS-PAGE gels were detected as described by Bourassa et al. (1988) (See Section 4.2.9.1) in order to detect if there are any Phospho-Tyr-protein kinases detectable in mango fruit extracts.

A total of 16 phosphoproteins, including exogenously added casein (32.4 kDa) were detected in the normally acid treated SDS-PAGE gel (Figure 4.3.1.4 and Table 4.3.1.1). From these 16 phospho-proteins, 3 were identified as phospho-Tyr-proteins; i.e. 67.6 kDa, 38 kDa and 30.2 kDa molecular weight proteins. The other 12 phosphoproteins, 224 kDa, 126 kDa, 89 kDa, 83.2 kDa, 77.6 kDa, 58.9 kDa, 45.7 kDa, 41.7 kDa, 35.5 kDa, 27.5 kDa, 15.8 kDa and 13.2 kDa must then be phosphorylated on either a Ser or a Thr. It is also evident that casein is phosphorylated on either a Ser or Thr (Figure 4.3.1.4 lanes 3, 6 and 7). This phospho-Tyr activity was only present in the ammonium sulphate precipitation fraction and it seemed to be calcium dependent.

This method of phospho-Tyr protein determination is very insensitive and there could still be other phospho-Tyr-proteins. A more advanced technique, i.e. anti-Phospho-Tyr antibody screening in fact revealed a larger spectrum of phospho-Tyr proteins as demonstrated under Section 4.3.2.3.8 and Figure 4.3.2.21.

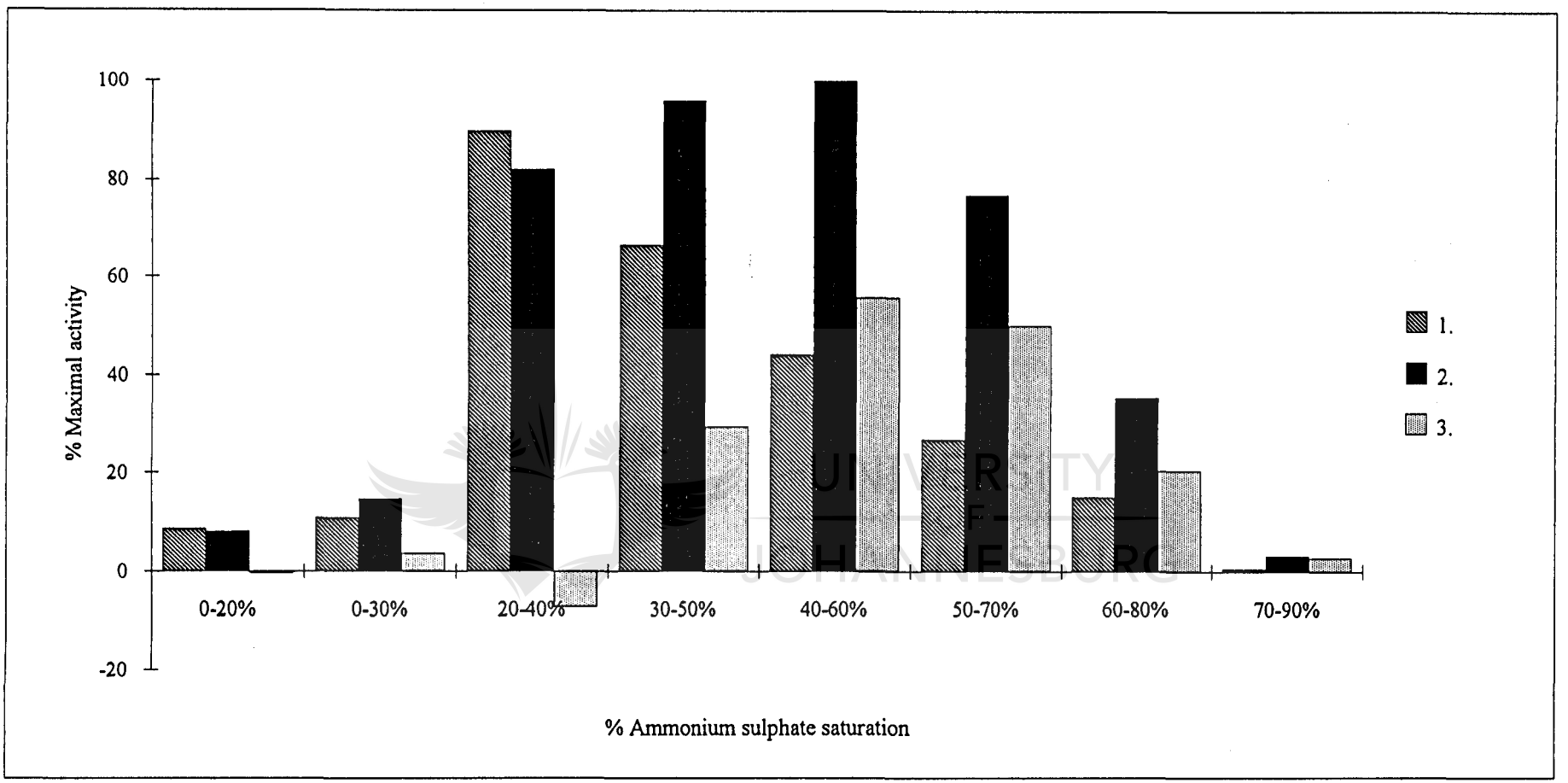



Figure 4.3.1.1 Step-wise fractionation of crude extracts of mango fruit tissue by differential ammonium sulphate precipitation to determine the saturation percentage range needed to collect the optimal yield of protein kinase activity. 1. Protein kinase activity was measured in the presence of 6 mM EGTA (monitoring calcium-independent protein kinase activity). 2. Protein kinase activity was measured in the presence of 1 mM $CaCl_2$ (monitoring calcium-independent plus calcium-dependent protein kinase activity). 3. Protein kinase activity determined in 1. and 2. was subtracted from each other (monitoring calcium-dependent protein kinase activity). The effect of calcium inhibition was not taken into account.

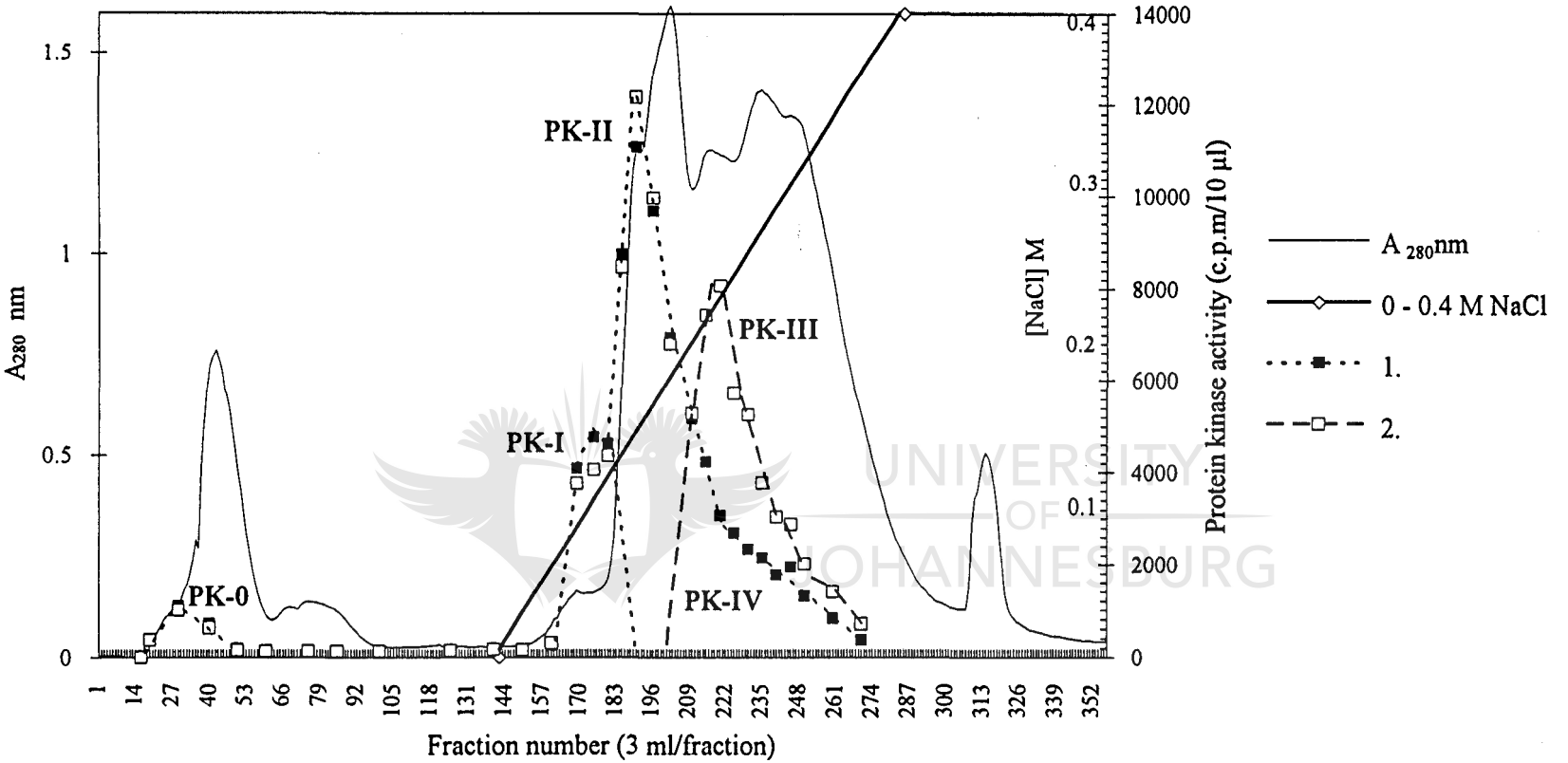



Figure 4.3.1.2 Separation of protein kinases from 36 000 x g supernatant of ammonium sulphate (30 -70%) precipitation preparation from ripening mango fruit tissue with high basal PK-II activity, by DEAE-Sephacel chromatography as described under Section 4.2.11.3. Protein was eluted with a gradient of increasing NaCl concentration (0 to 0.4 M). Protein content of eluted fractions was determined by absorption at 280 nm. Activity of protein kinase was measured, using dephosphorylated casein as substrate in 1: the absence of $CaCl_2$ (plus 6 mM EGTA), and 2: the presence of $CaCl_2$ (1 mM) in the incubation medium.

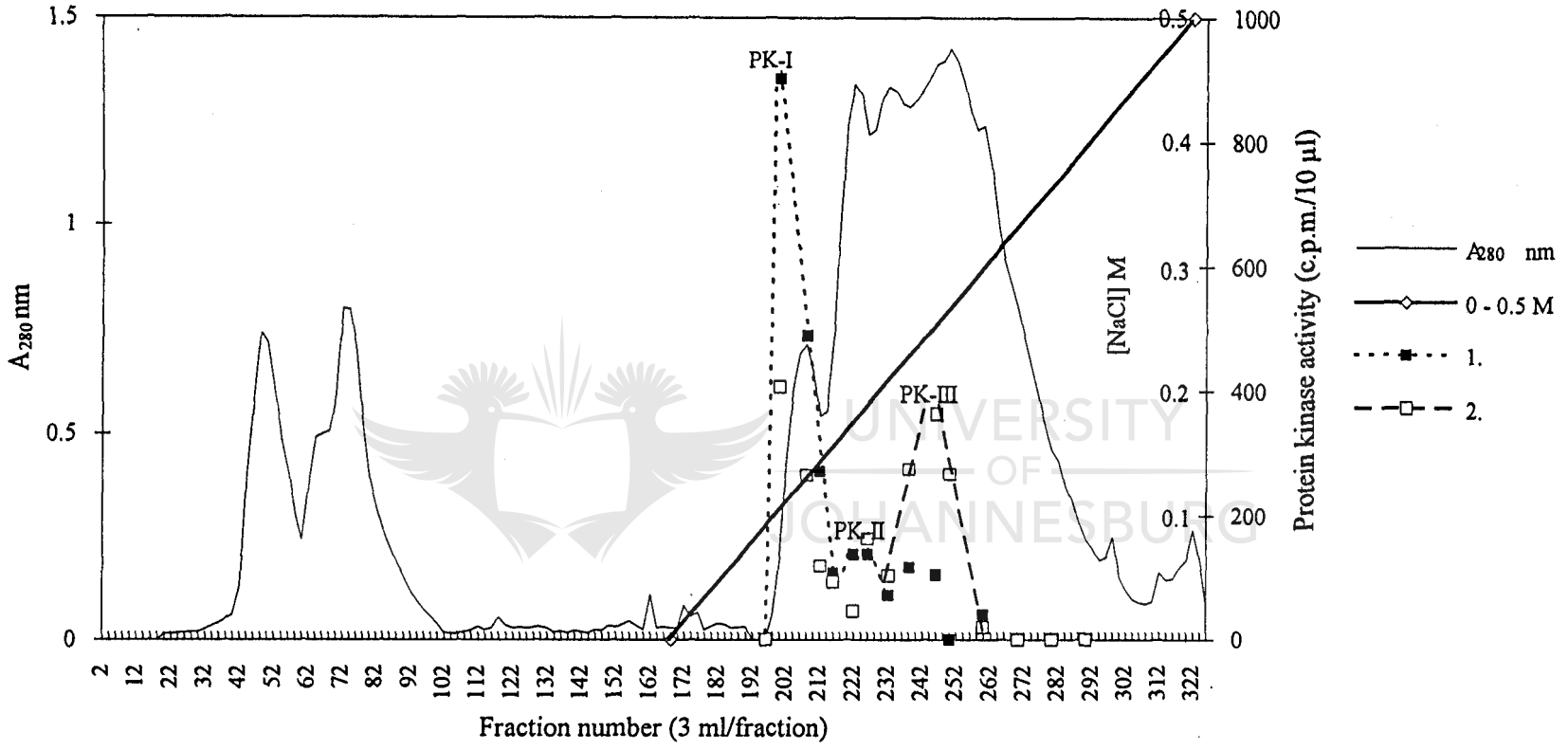



Figure 4.3.1.3 Separation of protein kinases from 36 000 x g supernatant of ammonium sulphate (30 -70%) precipitation preparation from ripening mango fruit tissue with high basal PK-I activity, by DEAE-Sephacel chromatography as described under Section 4.2.11.3. Protein was eluted with a gradient of increasing NaCl concentration (0 to 0.5 M). Protein content of eluted fractions was determined by absorption at 280 nm. Activity of protein kinase was measured, using dephosphorylated casein as substrate in 1: the absence of $CaCl_2$ (plus 6 mM EGTA), and 2: presence of $CaCl_2$ (1 mM) in the incubation medium.

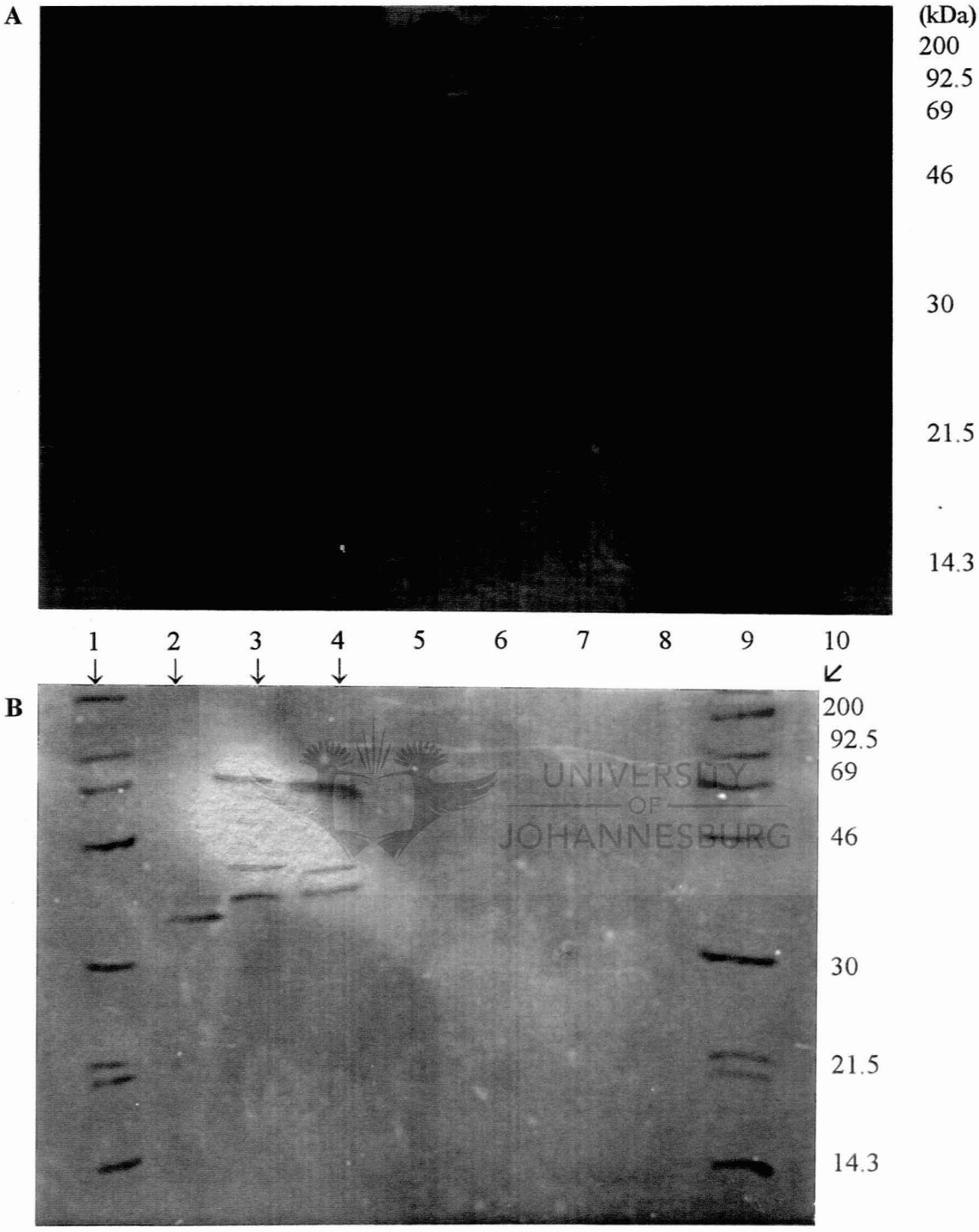



Figure 4.3.1.4 Effect of alkali treatment on labelled proteins. Lanes 1 and 10 contained the following molecular weight markers (in kDa): myosin 200; phosphorylase b, 92.5; BSA, 69; ovalbumin, 46; carbonic anhydrase, 30; trypsin inhibitor, 21.5; and lysozyme, 14.3. The other lanes show protein pools of purification stages of protein kinase activities from ripening mango fruit tissue that were radio labelled with [$\gamma$-$^{32}$P]ATP: lane 2, ammonium sulphate precipitation pool - $Ca^{2+}$; lane 3, ammonium sulphate precipitation pool + $Ca^{2+}$ + casein; lane 4, ammonium sulphate precipitation pool + $Ca^{2+}$; lane 5, Sephadex G-150 PK-II pool + $Ca^{2+}$; lane 6, Sephadex G-150 PK-II pool + $Ca^{2+}$ + casein; lane 7, Sephadex G150 PK-III pool plus casein + $Ca^{2+}$; lane 8, DEAE-Sephacel PK-III pool + $Ca^{2+}$; and lane 9, DEAE-Sephacel PK-III pool - $Ca^{2+}$. Electrophoretic separation through 10% polyacrylamide gels were performed as described under Section 4.2.9.1. A: The gel was treated with acid and B: the gel was treated with alkali as described under Section 4.3.4. The results were summarised under Table 4.3.1.1 and discussed under Section 4.3.1.3.

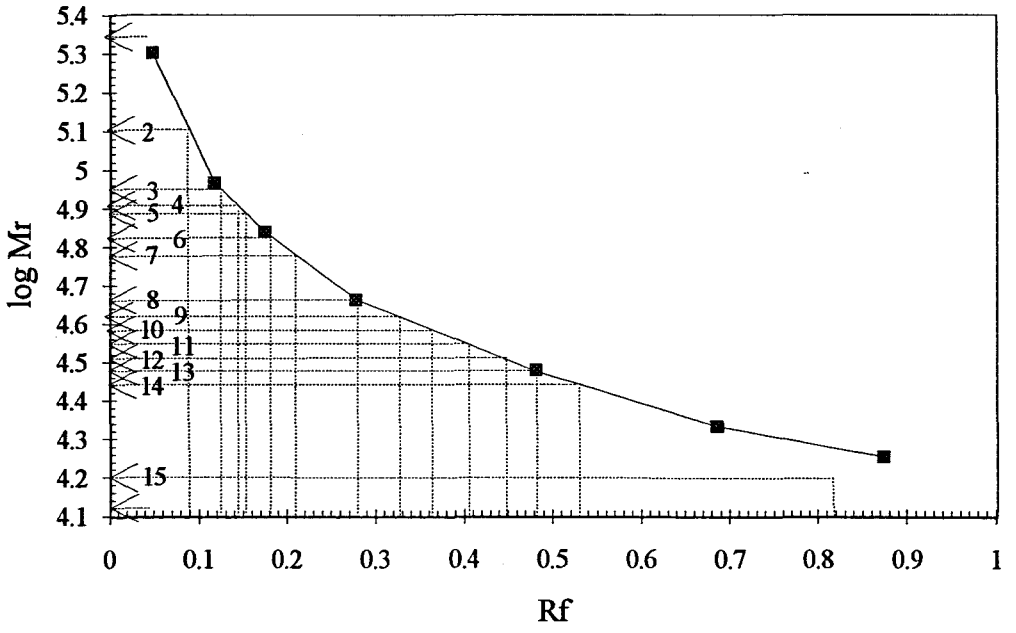



Figure 4.3.1.5 Plot of log $M_r$ *versus* $R_f$ to determine the $M_r$ of the phosphorylated proteins separated by SDS-PAGE (Figure 4.3.1.4)



| Phosphoprotein | $M_r$ (Da) | log $M_r$ | $R_f$ |
|---|---|---|---|
| 1. | 223900 | 5.35 | 0.015 |
| 2. | 125900 | 5.1 | 0.091 |
| 3. | 89100 | 4.95 | 0.125 |
| 4. | 83200 | 4.92 | 0.144 |
| 5. | 77600 | 4.89 | 0.154 |
| 6. (Phospho-Tyr) | 67600 | 4.83 | 0.183 |
| 7. | 58900 | 4.77 | 0.212 |
| 8. | 45700 | 4.66 | 0.279 |
| 9. | 41700 | 4.62 | 0.327 |
| 10. (Phospho-Tyr) | 38000 | 4.58 | 0.365 |
| 11. | 35500 | 4.55 | 0.404 |
| 12. (Casein) | 32400 | 4.51 | 0.447 |
| 13. (Phospho-Tyr) | 30200 | 4.48 | 0.482 |
| 14. | 27500 | 4.44 | 0.529 |
| 15. | 15800 | 4.2 | 0.817 |
| 16. | 13200 | 4.12 | 0.894 |

Table 4.3.1.1. Tabulation of phospho-Tyr- and phospho-Ser/Thr-proteins separated by SDS-PAGE (Figure 4.3.1.4).

## 4.3.2 Purification and Characterisation of Protein Kinase I (A Dual-Specificity Protein Kinase)

### 4.3.2.1 Purification and homogeneity of PK-I

The purification of PK-I from ripening mango fruit tissue (climacteric peak stage) was summarised in Table 4.3.2.1. The standard purification procedure as described under Section 4.2.11 and 4.2.12, allowed recovery of lower than 1% of the total available kinase activity, and yielded approximately 60 µg of purified PK-I from 600 g of starting material (Table 4.3.2.1). The final specific activity, determined as described under Section 4.2.3 in the presence of 0.1 mM $Ca^{2+}$, was consistently 1-5 nmol/min/mg. PK-I was analysed by SDS-PAGE to assess its purity (Figure 4.3.2.6) and subunit structure. PK-I consists of a monomeric structure (see discussion under Section 4.3.2.2.1). It was shown below that PK-I was not $Ca^{2+}$-dependent, but rather $Ca^{2+}$-stimulated.

#### 4.3.2.1.1 Chromatographic properties of PK-1

Figure 4.3.2.1 shows the step-wise elution of the different protein kinases as discussed under Section 4.3.1.2, by means of anion exchange chromatography on a DEAE-Sephacel column. At 0.075 M NaCl PK-I eluted almost directly from the column while PK-II was delayed on the column eluting very slowly. It was decided to rather use a very low NaCl gradient (0 to 0.15 M over 500 ml). As an after thought the step-wise approach might be better, preventing over-dilution of the enzyme, leading to denaturation.

Four column chromatography steps and an ammonium sulphate precipitation step were ultimately used to purify PK-I over a period of a week. The elution profiles of the chromatographic steps are shown in Figures 4.3.2.2, 4.3.2.3, 4.3.2.4 and 4.3.2.5. The two calcium-stimulated casein phosphorylating activity peaks were eluted from the DEAE-Sephacel with a 0 to 0.15 M NaCl gradient over 500 ml of elution buffer. PK-I eluted at a NaCl concentration of 0.05 M NaCl (Figure 4.3.2.2).

The previous problem with purifying PK-I was that the other calcium stimulated protein kinases (PK-II and PK-IV) had the same characteristics on Cibacron blue-Sepharose, i.e. eluted with 1 M NaSCN (Figures 4.3.3.1 and 4.3.3.2), and could not be separated from each other. This characteristic made it necessary to include a phenyl-Sepharose column (Figure 4.3.2.4) where PK-I eluted with 0.5 M NaSCN and PK-II and IV bound tighter, thus being more hydrophobic than PK-I. After the lower NaCl gradient DEAE-Sephacel column approach, PK-I and PK-II were separated to a greater extent and the convenience of the blue-Sepharose separation could be used optimally to purify PK-I without contamination with PK-II.

The activity peak from Blue-Sepharose was concentrated by means of centrifugal-ultrafiltration in a 20 kDa filter as discussed under Section 4.2.15. The concentration by centrifugal-ultrafiltration had a detrimental effect on the activity yield (only 9% - Table 4.3.2.1). Being highly hydrophobic, the pure protein kinases molecules tend to aggregate, forming an insoluble precipitate leading to high losses.

Gel filtration chromatography on Sephadex G75 (Figure 4.3.2.5) had a detrimental effect on the activity of the enzyme. The hydrophobic character of PK-I may lead to non-specific adsorption and aggregation leading to high activity losses (Table 4.3.2.1). Since the activities of partially purified PK-I are almost completely lost upon gel filtration, this step proved ineffective for improving purification. Appreciable recoveries however can be obtained if gel filtration is performed with more crude preparations, i.e. after the DEAE-Sephacel step. At this stage PK-I eluted from Sephadex G200 as a single peak with a molecular weight of 29.5 kDa (Figure 4.3.2.10).

4.3.2.1.3 Homogeneity

SDS-PAGE was used to determine the homogeneity, molecular weight and subunit structure of the purified enzyme as described under Section 4.2.6 and the results discussed under Section 4.3.2.2.1. After the examination of SDS-PAGE (Figure 4.3.2.6), it could be assumed that PK-I was at least 90 % pure. Only the 30 kDa PK-1 band, although faint itself, can be seen in lane 4, the G-75 activity pool. Agar isoelectric focusing of PK-I according to its pI characteristics (see Section 4.2.7) confirmed the homogeneity of this enzyme (one Coomassie stained protein band at pI = 5.1 in Figure 4.3.2.8, lane 3).

As the other protein kinases isolated, PK-I was highly hydrophobic and PK-I could also be a membrane associated protein. The ripening process results in a natural breakdown in membranes and thus makes membrane associated proteins more available. The high concentration EGTA (3 mM) in the extraction medium also attributed to the release of membrane associated proteins (see Section 4.3.1.1).

As can be seen from the discussion above, the purified PK-I was very labile. The loss of activity can be partly prevented by storing the enzyme in buffer containing 25% glycerol at -20°C.

### 4.3.2.2 Physico-chemical properties of purified/isolated PK-I

4.3.2.2.1 SDS-PAGE: Determination of the subunit structure and molecular weight

SDS-PAGE was used to determine the homogeneity, molecular weight and subunit structure of the purified enzyme as described under Section 4.2.6. The experimental information of the standardisation process and the determined results were given in Figure 4.3.2.6. Purified PK-I, which eluted as a single peak of activity during molecular exclusion chromatography (Figure 4.3.2.5), resolved into a single protein band of molecular weight 30 kDa on 15% SDS gels (Figure 4.3.2.6, lane 4). The molecular weight of 30 kDa agrees well with the size of 29.5 kDa determined by analytical gel chromatography (Section 4.3.2.2.3 and Figure 4.3.2.10). This indicates that the active form of the kinase is a monomer.

4.3.2.2.2 IEF: Determination of the isoelectric point

Agar isoelectric focusing of PK-I according to its pI characteristics (see Section 4.2.7) confirmed the homogeneity of this enzyme. The pI is the point at which the protein or its subunits are stationary and uncharged within an electric field and where its pI corresponds to a point in the appropriate pH gradient. The experimental information of the standardisation process and the determined results are given in Figures 4.3.2.8 and 4.3.2.9. Purified PK-I, resolved into one Coomassie stained band with pI 5.1 (Figure 4.3.2.8, lane 3).

4.3.2.2.3 Analytical gel chromatography: Determination of the molecular weight

Analytical gel chromatography (Andrews 1970) on Sephadex G200 (described under Section 4.2.8) was used to determine the molecular weight of PK-I. The experimental information of the standardisation process and the determined results are given in Figure 4.3.2.10. The molecular weight of PK-I isolated from mango fruit tissue was approximately 28.2 kDa.

The molecular weight of 28.2 kDa agrees well with the weight of 30 kDa of the PK-I band determined in 15% SDS gels (Figure 4.3.2.6).

### 4.3.2.3 Enzymatic properties of PK-1

#### 4.3.2.3.1 Effect of pH on catalysis

Tests of the pH dependence of PK-I (Figure 4.3.2.11) showed that the enzyme was active between pH 6 and 8 and the optimum pH was 6.5 under assay conditions used (Section 4.2.3). Relative to its activity at pH 6.5, its activity at pH 6.0 and 8.0 was approximately 50%.

#### 4.3.2.3.2 Effect of temperature on catalysis

The effect of temperature on PK-I enzyme activity was determined between 10 and 50 °C (Figure 4.3.2.12). The optimum reaction temperature under standard reaction conditions described under Section 4.2.3 with casein as exogenous substrate was 35 °C.

The activation energy of the catalytic reaction (E*), the free energy (ΔG*), enthalpy (ΔH*) and entropy of formation (ΔS*) of the transition state (activation complex) from the reactants, was determined as described under Section 4.2.16.2.

The Arrhenius-plot of log v *versus* 1/T (Figure 4.3.2.13) showed a definite change in slope. This phenomenon is found when at some temperature a different step becomes rate-limiting. The values of 15.04 and 8.07 kcal/mole were calculated from slopes (1) and (2) of this Arrhenius-plot for E*(1) and E*(2) for the two rate-limiting steps respectively, as described under Section 4.2.16.2. The E* did thus not stay constant throughout the temperature range tested.

The values of 8.5 kcal/mole and -36.28 cal/mole/K for ΔH* and ΔS* were only calculated from slope (2) and intercept of the modified Arrhenius-plot of log v/T versus 1/T (Figure 4.3.2.14) as described under Section 4.2.16.2. From these two known values ΔG* was calculated as 19.32 kcal/mole.

#### 4.3.2.3.3 Regulatory properties

- $Mg^{2+}$ and $Mn^{2+}$ dependency

The enzyme required $Mg^{2+}$ ($Mg^{2+}$-ATP) for its activity, and $Mn^{2+}$ could not substitute for $Mg^{2+}$ (Figure 4.3.2.15). In a plot of [$Mg^{2+}$] and [$Mn^{2+}$] *versus* kinase activity under standard assay conditions, except varying the $Mg^{2+}$- and $Mn^{2+}$ concentration from 0 to 20 mM, the optimum $Mg^{2+}$ concentration was 8 to 12 mM (Figure 4.3.2.15). At a 15 to 20 mM $Mg^{2+}$ concentration inhibition of PK-I started to occur.

- $Ca^{2+}$ stimulation

Figure 4.3.2.16 shows the determination of $Ca^{2+}$ stimulation of PK-I in the presence of casein at pH 8. The purified PK-I was stimulated to approximately 42% of the base EGTA activity by 1 to 5 μM $Ca^{2+}$. Calmodulin did not have a significant effect on the activity. The activation of the enzyme by $Ca^{2+}$ was not due to the presence of contaminating amounts of calmodulin (see Section 4.3.4.3.3). 1 mM $Ca^{2+}$ inhibits the activity of PK-I to approximately 50% of the basal $Ca^{2+}$-independent ($Ca^{2+}$ removal by EGTA chelation) activity (Figure 4.3.2.16).

- Effector and inhibitor studies

Using casein as an artificial substrate, the sensitivity of PK-I to various potential inhibitors and activators were tested (Table 4.3.2.3 and Figure 4.3.2.17). PK-I activity was insensitive to such inhibitors of $Ca^{2+}$/calmodulin-regulated protein kinases as chlorpromazine and calmidazolium (Figure 4.3.2.18). Calcium chelation by EGTA did not inhibit activity and exogenous calmodulin had no effect on its activity (Figure 4.3.2.16). $Ca^{2+}$ stimulated PK-I to approximately 40% higher than its basal $Ca^{2+}$-independent activity. As shown in Figure 4.3.2.17 heparin (30 µg/ml) inhibited the phosphorylating activity of PK-I with approximately 30%. It is well known that heparin, as one of the negative charged polysaccharides, has diverse effects on the activity of various protein kinases (Sakai et al. 1988). Casein kinase 2, one of the most abundant serine-threonine kinases, is inhibited by heparin (Hathaway et al. 1980, Yamamoto et al. 1979). The effect of heparin on PK-I is similar to that on CK-2 in which the inhibitory action by heparin is rather specific for the enzyme. There seem to be some structural similarities between these kinases.

On the other hand, heparin is a potent activator of phosphorylase kinases (Krebs et al. 1964, Chrisman et al. 1981) and Wong and Goldberg (1984) have found that the activity of p75 kinase, a cytosolic protein-tyrosine kinase from rat liver is enhanced about 75% by the addition of 0.1 mg/ml of heparin. Brunati and Pinna (1988) have reported four protein-tyrosine kinases from rat spleen particulate fractions. Two of them are activated by heparin, one is inhibited by heparin and the last one is neither stimulated nor inhibited by heparin. The effect of heparin on the cytosolic protein-tyrosine kinase from porcine spleen (CPTK-40) isolated by Sakai et al. (1988) was not identical with that of all four types of protein-tyrosine kinases from rat spleen particulate fractions. Although the effect of heparin on PK-I was similar to that on CPTK-40, the inhibition by heparin is not a specific characteristic of cytosolic protein-tyrosine kinases. PK-I activity was very sensitive to inhibition by 17 µM cAMP but the significance hereof is not known. cAMP probably competes with ATP for the active centre of PK-I.



4.3.2.3.4 Substrate specificity

Under the assay conditions chosen, the PK-I favoured casein and phosvitin as artificial substrates, but it could not phosphorylate histone III-S, histone II-S, protamine and BSA at 0.4 mg/ml (Table 4.3.2.4 and Figure 4.3.2.19). Phosvitin was reported to be a relatively good substrate for protein kinase C (Kikkawa & Nishizuka 1986) and protein kinase A (Tao et al. 1980).

The substrate concentration dependency of histone II-S, histone III-S and casein phosphorylation by PK-II is shown in Figure 4.3.2.20. The phosphorylation of casein preparations showed non-linear progression curves with no substrate inhibition at higher concentrations. The upper limit of enzyme activity for dephosphorylated casein was obtained at a concentration of 2 mg/ml. PK-I had no affinity for histone III-S and histone II-S.

4.3.2.3.5 Endogenous substrates

The endogenous proteins phosphorylated by PK-I on tyrosine residues as determined by anti-P-Tyr antibodies (Section 4.2.9.2), had molecular weights of 90.2 kDa, 77.6 kDa, 61.7 kDa, 50.1 kDa, 38.9 kDa, 34.7 kDa, 32.4 kDa and 28.2 kDa (Figures 4.3.2.21 and 4.3.2.22). Because the DEAE-PK-I activity pool was contaminated with PK-II, and the DEAE-PK-II activity pool was contaminated with PK-I and PK-III, the 34.7 kDa and the 50.1 kDa phospho-Tyr-proteins could be Tyr-phosphorylated PK-II and PK-III respectively. It has been reported that phospho-Tyr-kinases could control the activity of Ser- and Thr-protein kinases (Krebs et al. 1993).

4.3.2.3.6 Autophosphorylation

The 32.4 kDa phospho-Tyr protein band was the most abundant phospho-Tyr protein present in lane 2 and 3. It is possible that the phosphorylation of this protein could cause a structural change, resulting in a mobility shift from 30 kDa to 32.4 kDa, so that this protein could represent an autophosphorylated PK-I (Figures 4.3.2.21 and 4.3.2.22).

4.3.2.3.7 Kinetic properties

- Initial velocity studies

Initial velocity studies, in the absence of products, were performed over a wide range of ATP and casein concentrations (2.36 to 26 µM and 0.22 to 2 mg/ml, respectively), to adequately define both the kinetic mechanism and the kinetic constants of the reaction. In Figures 4.3.2.23 and 4.3.2.24 velocity is plotted *versus* $Mg^{2+}$-ATP or casein concentration respectively (Michaelis-Menten plot A), reciprocal velocity is plotted *versus* the reciprocal of $Mg^{2+}$-ATP or casein concentration respectively (Lineweaver-Burk plot B) and the slopes determined from the double-reciprocal plots is plotted *versus* the reciprocal of casein and $Mg^{2+}$-ATP concentrations respectively (Secondary plots C). The kinetic constants obtained from these plots are $K_m$ATP = 20 µM and $K_m$casein = 0.2 mg/ml. The $V_{max}$ for both was ca. 0.38 nmol/min/mg protein.

- Product inhibition studies

The results of initial velocity measurements made in the presence of the product (ADP) of the reaction are presented in Figures 4.3.2.25 and 4.3.2.26. As indicated, ADP was a competitive inhibitor *versus* ATP and a simple linear non-competitive inhibitor *versus* casein. Product inhibition by phospho-casein was not tested. The inhibition constant for ADP obtained from the x-axis ($K_i$ = x-axis) is $K_i$ADP = 9 µM.

- Enzyme mechanism

The initial velocity pattern studies of PK-I follow a sequential, rather than a Ping-Pong mechanistic pathway. That is, both of the substrates bind to the enzyme prior to product release. The product inhibition patterns observed are entirely consistent with a steady state ordered Bi-Bi kinetic mechanism with the $Mg^{2+}$-ATP binding first. This mechanism is in concurrence with the findings of Granot et al. (1981) and Whitehouse et al. (1983) who have shown binding of nucleotide to enzyme in the absence of peptide substrate and concluded that if the reaction mechanism is ordered, ATP must be the first substrate to bind. The kinetic scheme can be represented by the following:

$$\begin{array}{ccccccc} & A & & B & & P & Q \\ & k_1 \downarrow k_{-1} & & k_2 \downarrow k_{-2} & & k_{-3} \uparrow k_3 & k_{-4} \uparrow k_4 \\ E & \text{———} & EA & \text{———} & (EAB \Leftrightarrow EPQ) & \text{———} & EQ \text{———} E \end{array}$$

where A and B represent $Mg^{2+}$-ATP and casein, and P and Q the products, phospho-casein and $Mg^{2+}$-ADP, respectively. The competitive inhibition by $Mg^{2+}$-ADP, when $Mg^{2+}$-ATP was the varied substrate, supports the assignment of $Mg^{2+}$-ATP as the first substrate to bind, and if there are no unusual rate constants, suggests that the mechanism is not Random. The product inhibition patterns also exclude the possibility that the reaction mechanism might be nonrapid equilibrium random sequential since, if it was, both products should have been competitive inhibitors with their substrate counterparts (Segel 1975).

4.3.2.3.8 Identification of amino acids phosphorylated by PK-I

To detect phospho-tyrosine proteins the endogenous and exogenous phosphorylated substrates separated by SDS-PAGE were reacted with anti-P-Tyr antibody as described under Section 4.2.9.2 and the results are presented in Figures 4.3.2.21 and 4.3.2.22. All the preparations were active at the time of protein kinase specific reactions as it can be verified by the presence of $\gamma$-$^{32}$P-labelled endogenous and exogenous substrates in all the preparations tested (autoradiograph in Figure 4.3.2.21B as well as some results not included in the thesis). Phospho-Tyr proteins were detected in lanes 2, 3, 6 and 7 on the SDS-PAGE transferred PVDF membranes. All these lanes contained PK-I activity, but also PK-II and to a lesser extent PK-III (lane 3) contamination. On the other hand lanes 4 and 8, and lanes 5, 9 and 10 contained purified PK-II and PK-III respectively without contamination of PK-I. This phospho-Tyr activity can thus only be as a result of PK-I activity. Lanes 3 and 7 contained additional endogenous phosphotyrosine substrates because this preparation contained more proteins that were separated from the fraction tested in lanes 2 and 6, that could serve as endogenous substrates. Lanes 6 to 10 contained a further addition of exogenous substrate, i.e. casein to PK-I, PK-II and PK-III and histone III-S (lane 10) to PK-III. Increased protein kinase activity could be seen on the corresponding autoradiograms (results not presented), but the intensity of the phospho-Tyr protein bands remained the same. The results indicate that PK-II and PK-III had definitely no phospho-Tyr activity. The 32.4 kDa phospho-Tyr protein band was the most abundant phospho-Tyr protein present in lane 2 and 3. If the phosphorylation of this protein caused a structural change so that the protein underwent a mobility shift from 30 kDa to 32.4 kDa, this protein could represent an autophosphorylated form of PK-I. (The 34.7 kDa phosphotyrosine protein could in turn represent a tyrosine phosphorylated PK-II)

After the blue Sepharose step PK-I could still phosphorylate casein thus PK-I had Ser/Thr-specific protein kinase activity as well. According to Brunati and Pinna (1988) casein can not be phosphorylated on a tyrosine, and this is a confirmation for Ser/Thr-specific protein kinase activity.

It was widely accepted that protein kinases can either phosphorylate tyrosine or serine/threonine residues but not both, and that the amino acid specificity of a protein kinase can be predicted from its primary sequence. This dual-specificity phenomenon of protein kinases is discovered by more and more researchers (Lindberg et al. 1992, Hirayama & Oka, 1992) and what seemed totally impossible became quite normal (see discussion under Chapter 2). The result that PK-I might possess phospho-Tyr activity, was quite unexpected. The enzyme was however never assayed with phospho-Tyr specific substrates. That the most abundant phospho-Tyr protein band (32 kDa) in the preparations corresponded with the molecular weight of PK-I does count in favour of the theory that PK-I possess phospho-Tyr activity. It is known that most protein kinases have the ability to autophosphorylate (Soderling 1990, Soderling 1993). The possibility still exists that PK-I could be contaminated by a Tyr-protein kinase and follow up experiments with the more purified enzyme are necessary to confirm this result.

### 4.3.2.4 Conclusion

The anti-P-Tyr antibody screening of expression libraries has become a common way to uncover enzymes that phosphorylate tyrosine, which may not be highly related to known phospho-tyrosine kinases (Lindberg et al. 1992). Detection of phosphotyrosine by anti-P-Tyr antibodies is extremely sensitive in bacteria where phosphoproteins are rare, particularly tyrosine phosphorylated proteins (Lindberg et al. 1992). The same reasoning could be applied to plant fruit tissue.

Cellular feedback of 'checkpoint' mechanisms maintain the order of completion of essential cell-cycle related functions (Hartwell & Weinert 1989). For example in fission yeast, the cdc25 and cdc2 gene products influence the ability of cells to delay mitosis in response to the inhibition of DNA synthesis (Enoch & Nurse 1990). Wee1, isolated from *Schizosaccharomyces pombe*, one of the most convincing dual-specificity protein kinases, is required for $\gamma$-radiation-induced mitotic delay that involves one of

above mentioned checkpoint controls (Rowley et al. 1992). There is strong evidence that this protein kinase needs its dual-specificity capability to perform its physiological function (Lindberg et al. 1992). Being a rapidly dividing organ, fruit cells may need similar protein kinases for the control of cell division and ripening control. PK-I may be one such an enzyme fulfilling a similar physiological role in mango fruit cells as wee1.

PK-I is a highly hydrophobic protein as can be concluded from its chromatographic characteristics. Several lines of evidence suggest that many protein-tyrosine kinases are associated with plasma membranes (Hunter & Cooper 1985, Fantl et al. 1993). Until now, animal models have been considered as reference systems. However, it is reasonable to conclude that plants may use the phosphorylation/dephosphorylation process as a regulatory device at least as frequently as animals do. The first phospho-Tyr-kinase activity in higher plants was found in pea plantlets by Torruella et al. (1986). Phosphotyrosine is a minor component of animal cells, but cells stimulated to divide by certain factors may contain 5- to 10-fold more phosphotyrosine than that of the resting cells (Hunter & Cooper 1985).

Since tyrosine phosphorylation also seems to be involved in cellular proliferation and transformation in animal tissues, it was tempting to speculate that it may play a similar role in the development of mature and ripening fruit cells. It is therefore possible that PK-I can phosphorylate the other isolated protein kinases (PK-II and PK-III) thereby controlling the activity of PK-II and PK-III in the cell. It is reported that phospho-Tyr kinases could control the activity of Ser and Thr protein kinases (Krebs et al. 1993).

It should be born in mind that some protein kinases already classified as being serine/threonine or tyrosine protein kinases, could prove to be dual-specificity if they were in fact tested (Lindberg et al. 1992)

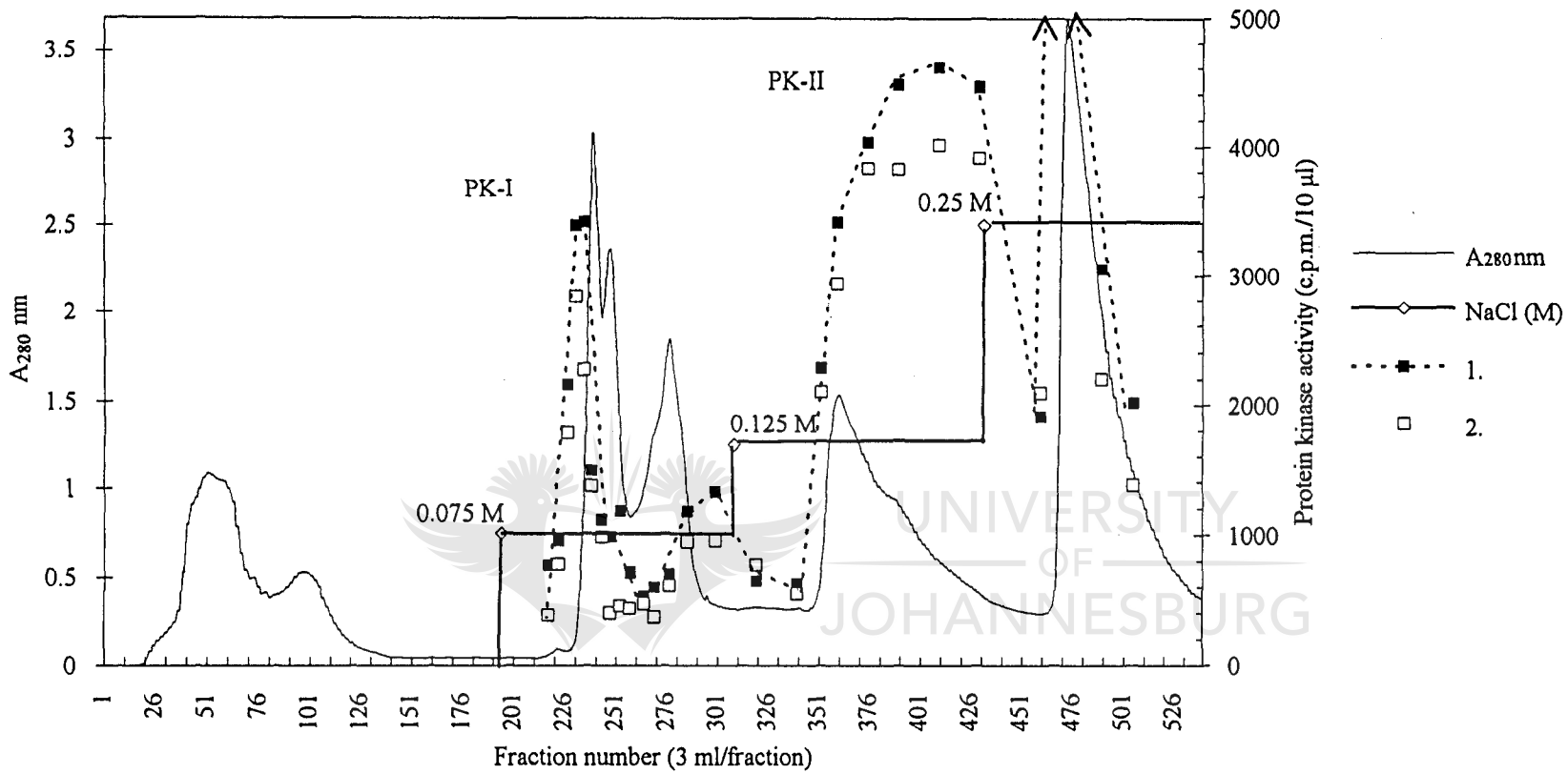



Figure 4.3.2.1 Step-wise elution of protein kinases from the 36 000 x g supernatant of the ammonium sulphate precipitation preparation from ripening mango fruit tissue used to isolate PK-I by DEAE-Sephacel chromatography. NaCl concentration was increased from 0 to 0.075 M, from 0.075 M to 0.125 M, and from 0.125 to 0.25 M. Protein content of eluted fractions was determined by absorption at 280 nm. Activity of protein kinase was measured, using dephosphorylated casein as substrate in 1: the presence of 0.1 mM $CaCl_2$ and 2: the presence of 1 mM $CaCl_2$, in the incubation medium.

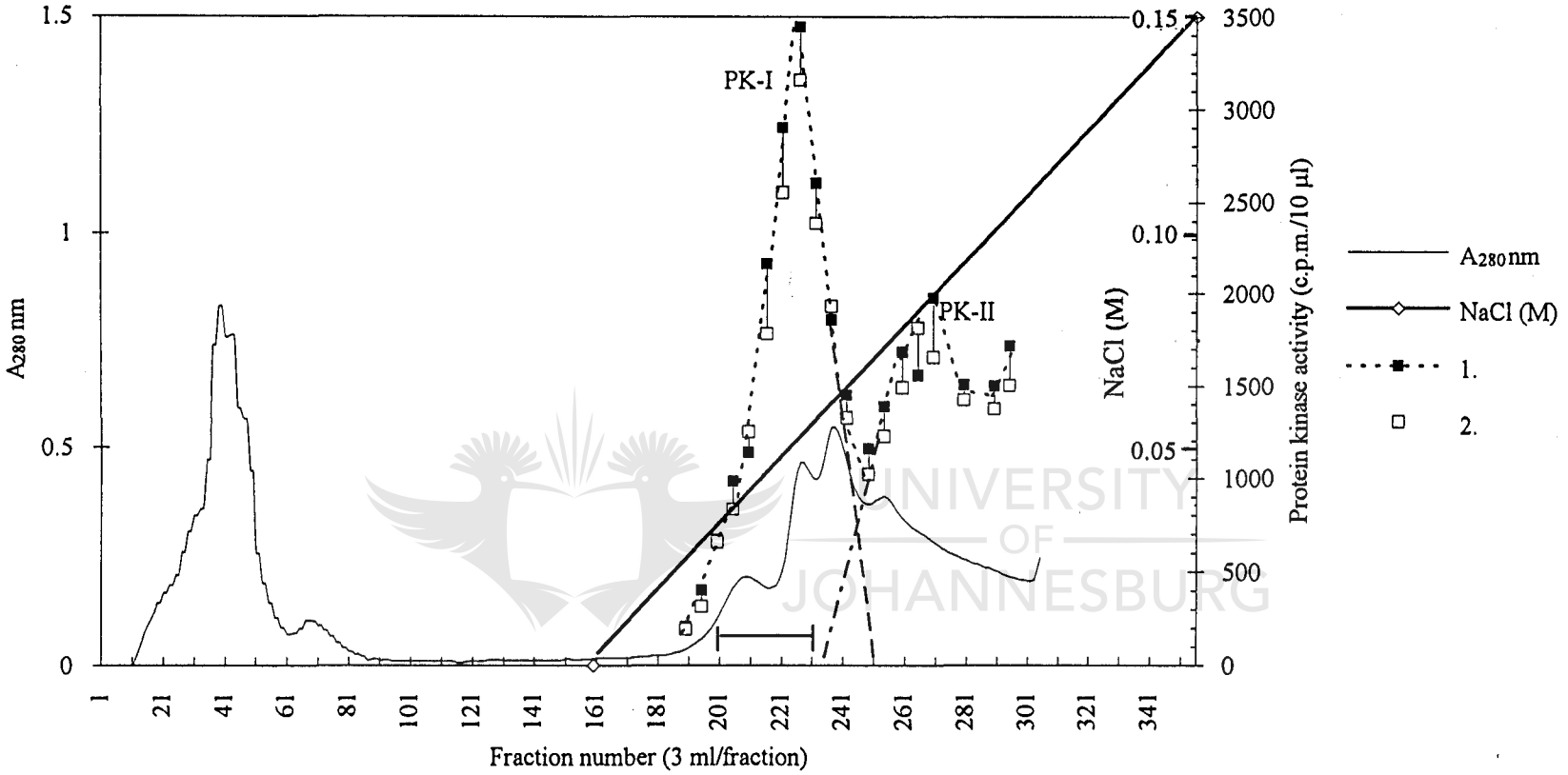



Figure 4.3.2.2 Separation of PK-I and PK-II by means of anion exchange chromatography on a DEAE-Sephacel column. The experimental conditions are described under Section 4.2.11.3. Protein was eluted with a gradient of increasing NaCl concentration (0 to 0.15 M). Protein content of eluted fractions was determined by absorption at 280 nm. Activity of protein kinase was measured, using dephosphorylated casein as substrate in 1: the presence of 0.1 mM $CaCl_2$, and 2: the presence of 1 mM $CaCl_2$ in the incubation medium.

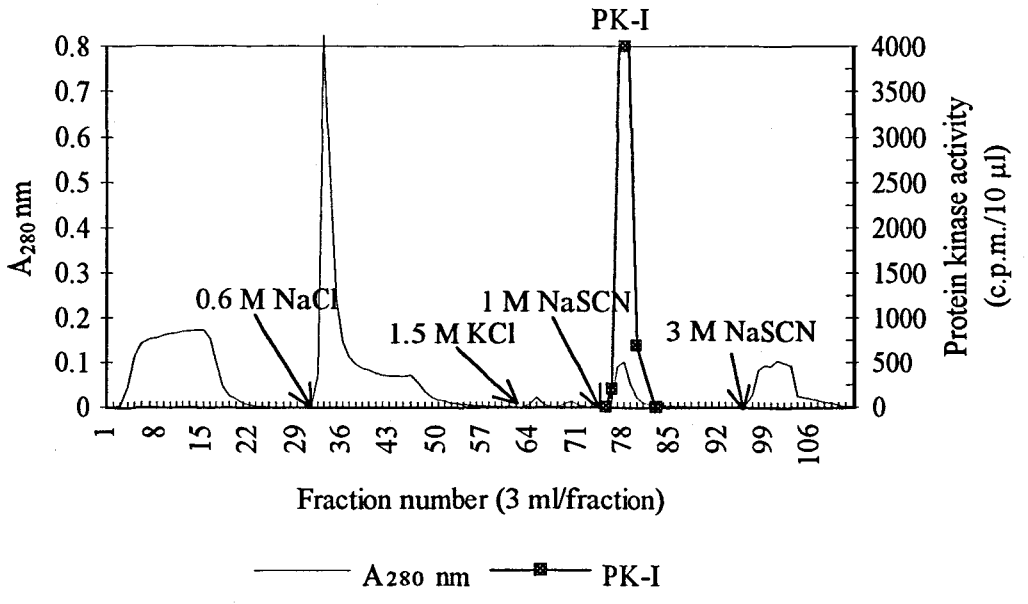



Figure 4.3.2.3 Elution profile of PK-I from a Cibacron Blue-Sepharose column after affinity chromatography. The experimental conditions are described under Section 4.2.12.3. Protein content of eluted fractions was determined by absorption at 280 nm. Activity of PK-I was measured, using dephosphorylated casein as substrate in the presence of 0.1 mM $CaCl_2$. Fractions 77 to 82 were pooled.



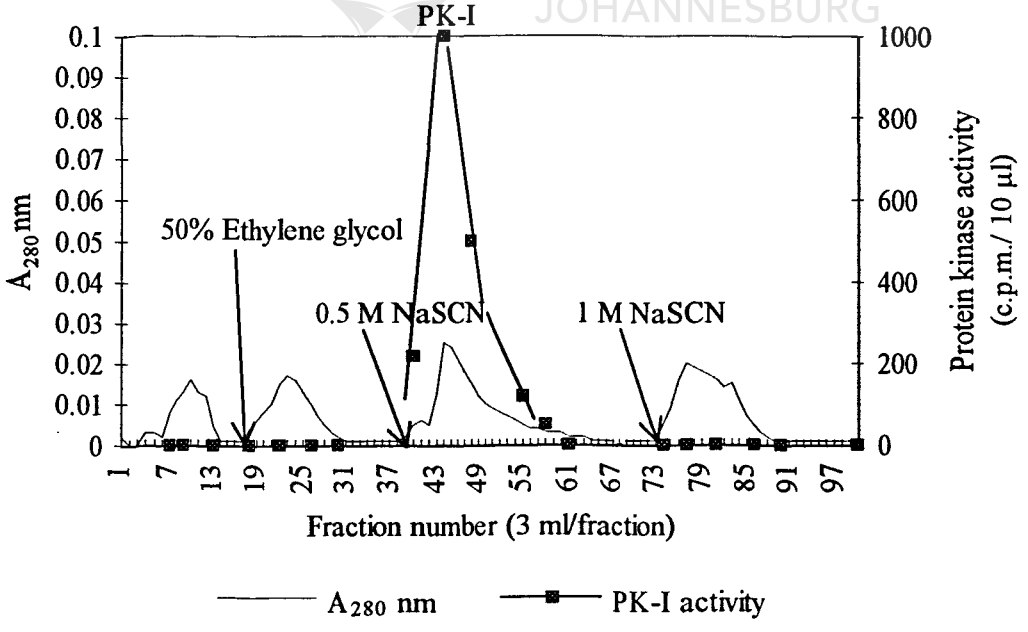



Figure 4.3.2.4 Elution profile of PK-I from a phenyl Sepharose CL-6B column after hydrophobic interaction chromatography. The experimental conditions are described under Section 4.2.12.2. Protein content of eluted fractions was determined by absorption at 280 nm. Activity of PK-I was measured, using dephosphorylated casein as substrate in the presence of 0.1 mM $CaCl_2$. Fractions 40 to 55 were pooled.

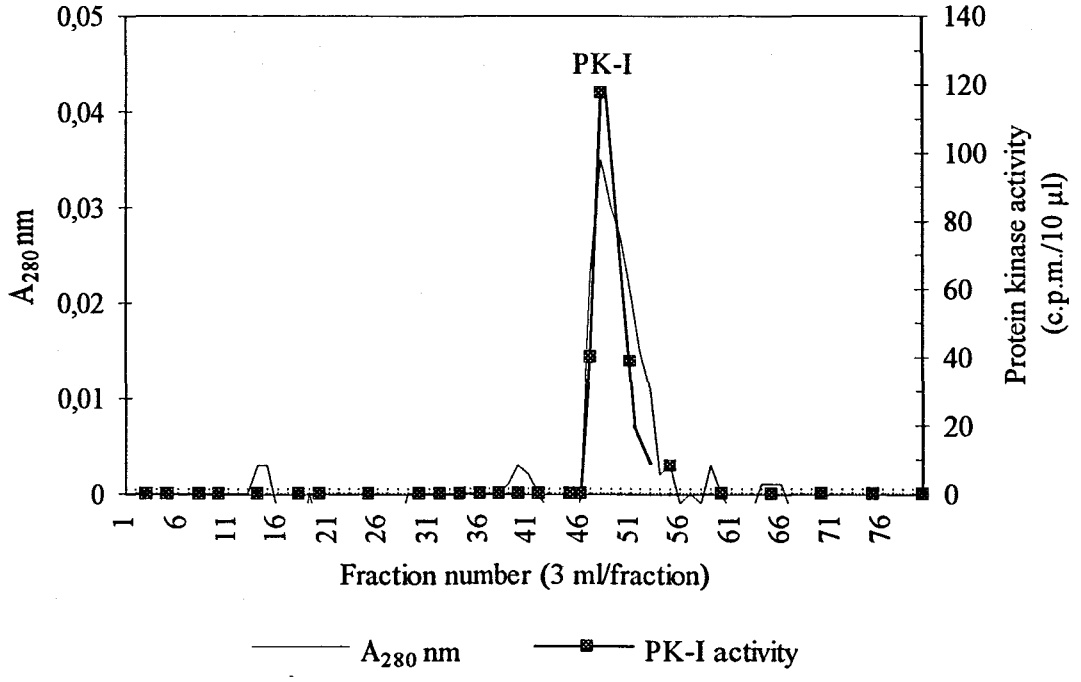



Figure 4.3.2.5 Elution profile of PK-I from a Sephadex G75SF column after exclusion chromatography. The experimental conditions are described under Section 4.2.12.4. Protein content of eluted fractions was determined by absorption at 280 nm. Activity of PK-I was measured, using dephosphorylated casein as substrate in the presence of 0.1 mM $CaCl_2$. Fractions 47 to 52 were pooled.

Table 4.3.2.1 Summary of purification of the PK-I from ripening mango fruit tissue

| Purification step | Volume (ml) | [Protein] (mg/ml) | Total protein (mg) | Activity/ml (pmol /min/ml) | Total activity (pmol/min) | Specific activity (pmol/min/mg) | Yield (%) | Purification (x-fold) |
|---|---|---|---|---|---|---|---|---|
| 1. Crude homogenate # | 1340 | 1 | 1340 | 42 | 56280 | 42 | 100 | 1,00 |
| 2. Ammonium sulphate precipitation # | 56 | 3 | 268 | 1576 | 88256 | 329 | 157* | 7,84 |
| 3. DEAE-Sephacel PK-I # | 14,7 | 2,63 | 38,661 | 788 | 11584 | 300 | 21 | 7,13 |
| 4. Blue-Sepharose PK-I | 3 | 1,5 | 4,5 | 1600 | 4800 | 1067 | 9 | 25,40 |
| 5. Phenyl-Sepharose | 2 | 0,1 | 0,2 | 500 | 1000 | 5000 | 2 | 119,05 |
| 6. Sephadex-G75 | 10 | 0,006 | 0,06 | 10 | 100 | 1667 | 0,18 | 39,68 |

# These preparations also contained PK-II activity and therefore the yield and purification factors cannot be accurately determined.

* The removal of protein kinase inhibiting substances by previous purification steps result in a higher apparent yield of PK-I.

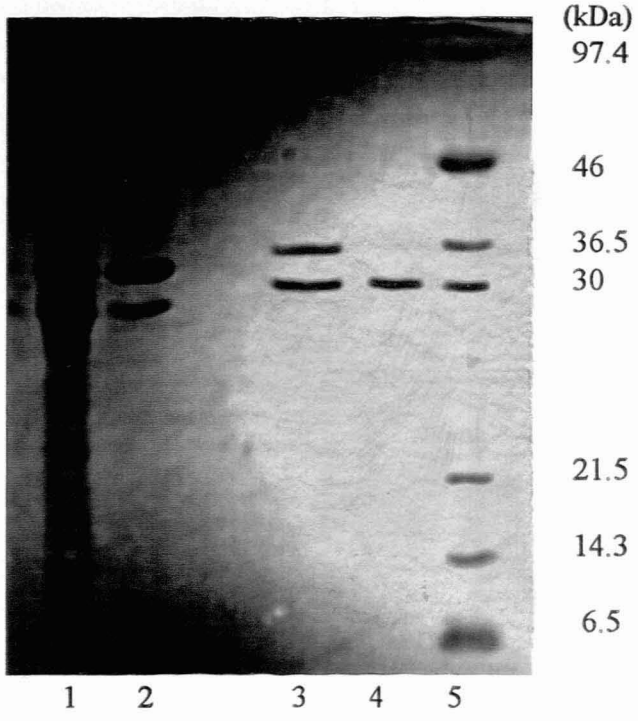



Figure 4.3.2.6 SDS-Polyacrylamide gel electrophoretic analysis of proteins from each stage of purification of PK-I. Aliquots from each stage of purification were treated as described in Section 4.2.6 and resolved in a 15% polyacrylamide gel in the presence of SDS and stained with Coomassie blue. Lane 5 contained the following molecular weight markers (kDa): phosphorylase b, 97.4; ovalbumin, 46; lactate dehydrogenase, 36.5; carbonic anhydrase, 30; trypsin inhibitor, 21.5; lysozyme, 14.3 and aprotinin 6.5. Other lanes show the following fractions from each step of the purification procedure: lane 1, 30 -70% ammonium sulphate precipitation pool; lane 2, DEAE pool; lane 3, Blue-Sepharose pool; and lane 4, Sephadex G75 pool.

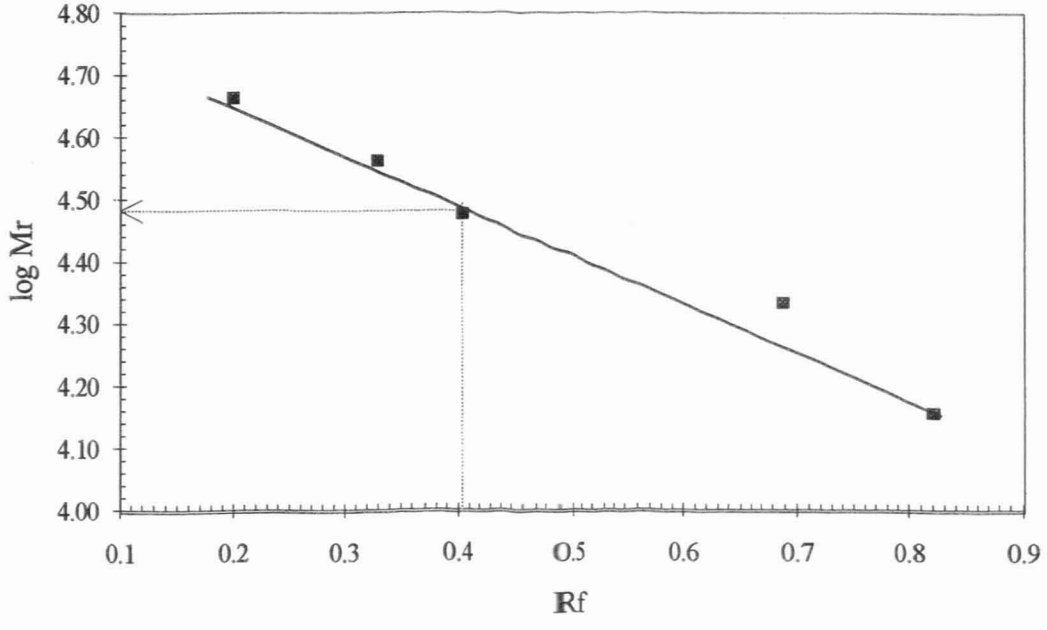



Figure 4.3.2.7 Plot of $R_f$ (Electrophoretic mobility) *versus* log $M_r$ (Molecular weight) for the determination of the $M_r$ of PK-I. The determined weight in kDa was 30.

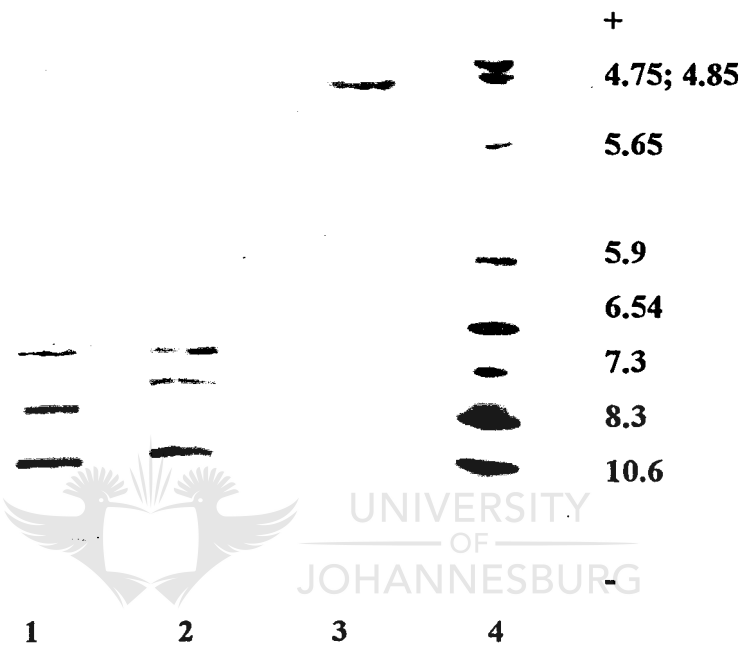





Figure 4.3.2.8 Analytical agar gel isoelectricfocusing (pH 3-10 gradient) for the determination of the purity and pI values of PK-I, PK-II and PK-IV isolated from ripening mango fruit tissue. The lanes are as follows: 1. PK-IV; 2. PK-II; 3. PK-I and 4. pI calibration standards: C-phycocyanin, 4.75 and 4.85; azurin, 5.65; trifluoroacetylated myoglobin, 5.9; myoglobin Met (porcine), 6.45; myoglobin Met (equine), 7.3; myoglobin Met (sperm whale), 8.3 and cytochrome C (horse heart), 10.6. All enzyme preparations were in the Blue Sepharose purification stage. At the time of this experiment a suitable PK-III preparation was not available.

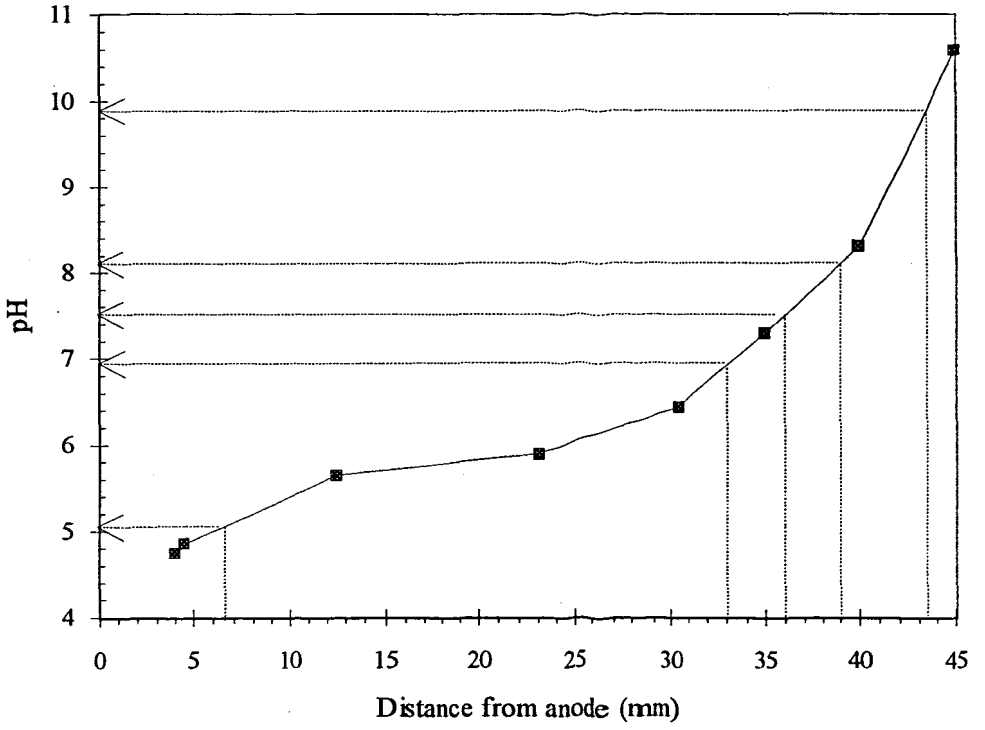



Figure 4.3.2.9 Calibration curve of standard proteins analysed by analytical agar gel isoelectric-focusing in the pH range of 3 to 10 for the determination of the pI values of PK-I, PK-II and PK-IV isolated from ripening mango fruit tissue. Experimental conditions were described under section 4.2.7. The pI's of standards and protein kinases are tabulated below.



Table 4.3.2.2 pI's of calibration standards and protein kinases separated by means of analytical agar gel isofocusing.

| Standards | Distance from anode (mm) | pI |
|---|---|---|
| C-Phycocyanin | 4 | 4.75 |
| | 4.5 | 4.85 |
| Azurin | 12.5 | 5.65 |
| Trifluoroacetylated Myoglobin | 23 | 5.9 |
| Myoglobin Met (Porcine) | 30.5 | 6.45 |
| Myoglobin Met (Equine) | 35 | 7.3 |
| Myoglobin Met (Sperm Whale) | 40 | 8.3 |
| Cytochrome C (Horse Heart) | 45 | 10.6 |
| PK-I | 6.5 | 5.1 |
| PK-II (1) | 33 | 6.95 |
| PK-II (2) | 36 | 7.5 |
| PK-II (3) | 43.5 | 9.9 |
| PK-IV (1) | 33 | 6.95 |
| PK-IV (2) | 39 | 8.1 |
| PK-IV (3) | 43.5 | 9.9 |

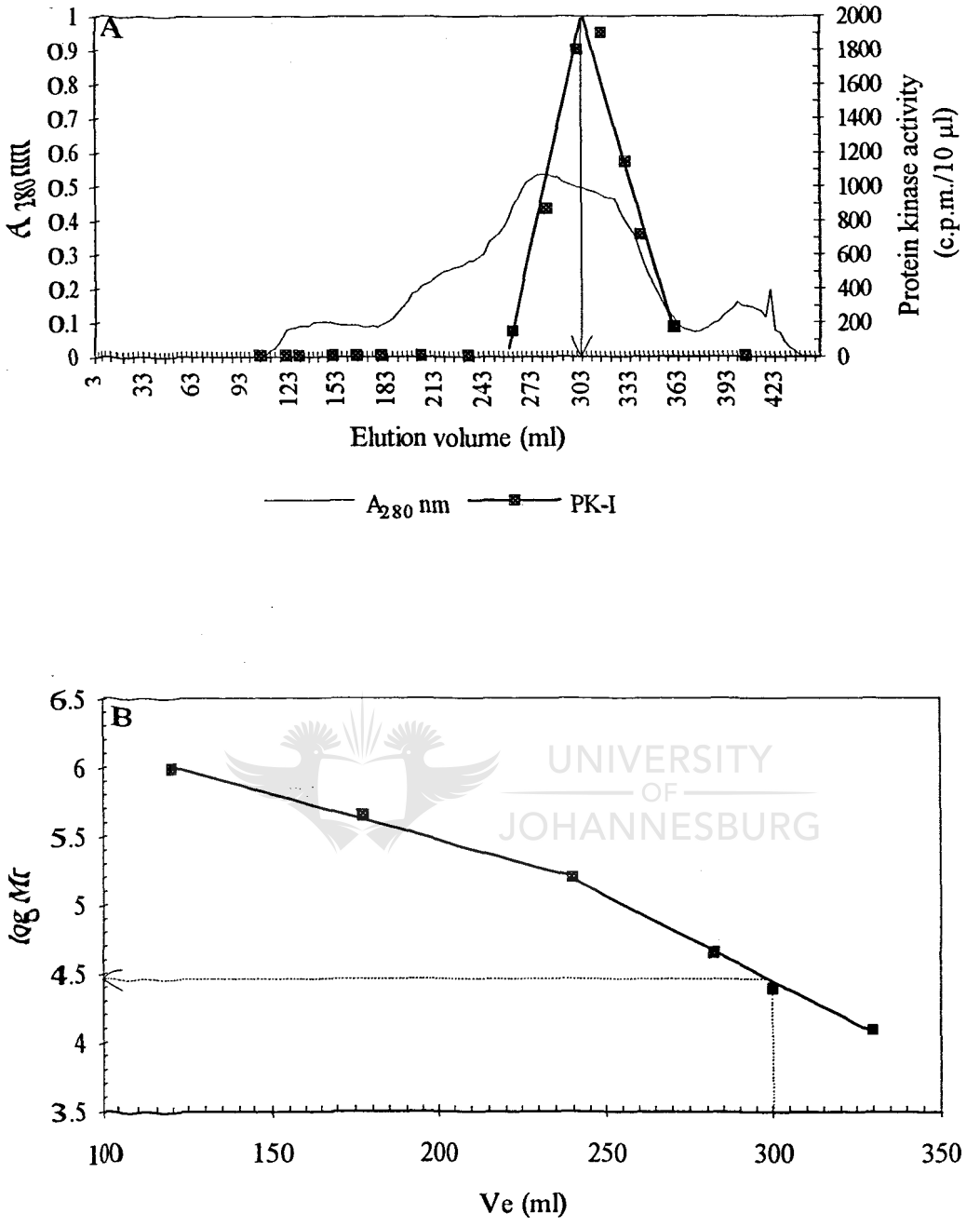



Figure 4.3.2.10 A: Separation of PK-I by means of gel filtration chromatography on a Sephadex G200 column. The experimental conditions are described under Section 4.2.8. The bed volume of the column was 373 ml, the flow rate was 8 ml/h and 3 ml fractions were collected. B: Plot of eluation volume ($V_e$) *versus* molecular weight (log $M_r$) for the determination of the molecular weight of PK-I by means of analytical gel chromatography on Sephadex G-200. Standards used were blue dextran (1000 kDa), ferritin (450 kDa), aldolase (158 kDa), ovalbumin (45 kDa), chymotrysinogin A (25 kDa), lysozyme (14.4 kDa) and β-ME (0.078 kDa). The $M_r$ of PK-I is ca. 28.2.

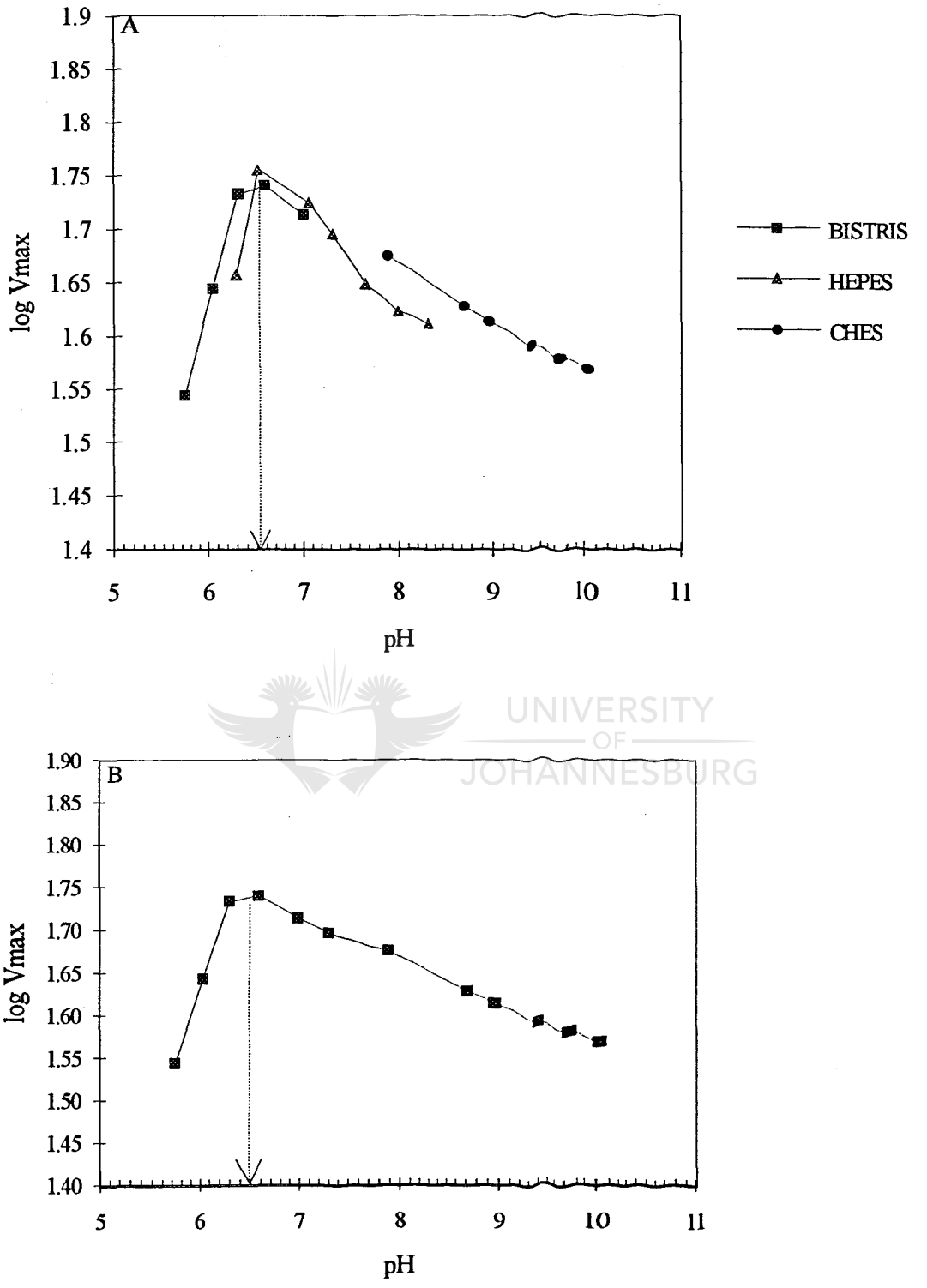





Figure 4.3.2.11 pH dependence of PK-I. Plots of log $V_{max}$ *versus* pH. For reaction conditions see Section 4.2.3 and 4.2.16.1. The value of each point is the average of two determinations in duplicate. The pH optimum = pH 6.5. A: Activities of PK-I were determined in three different buffers namely, 100 mM BISTRIS (pKa = 6.5), 100 mM HEPES (pKa = 7.33) and 100 mM CHES (pKa = 9.5). B: The pH-activity curves determined in the different buffers were extrapolated to give one curve.

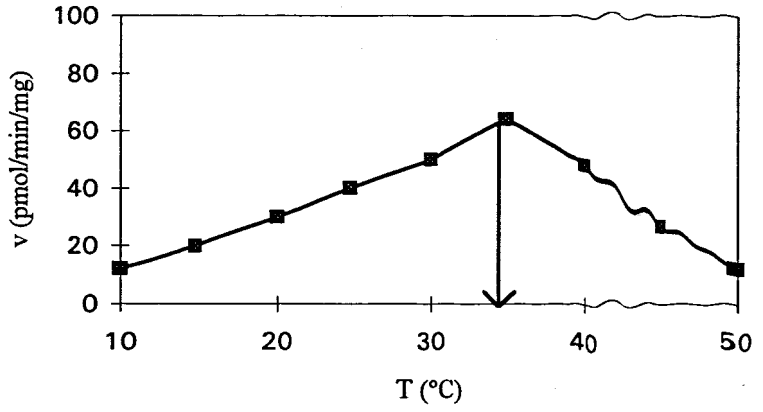



Figure 4.3.2.12 Effect of temperature on PK-I activity. The optimum reaction temperature at the standard reaction conditions as described under Section 4.2.3 was ca. 35°C. Casein was used as exogenous substrate. v = Initial reaction rate.

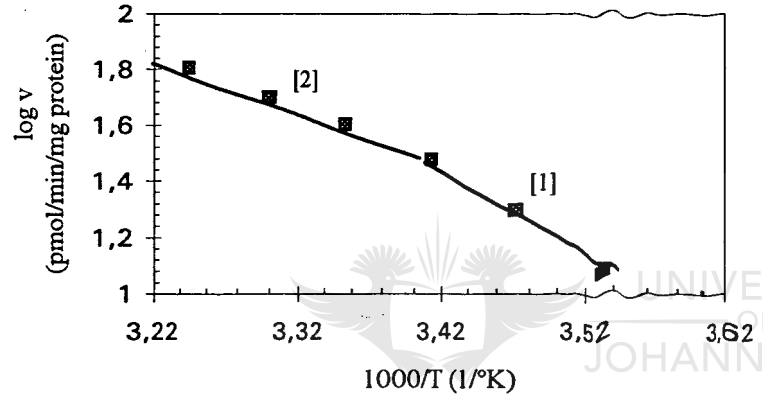



Figure 4.3.2.13 Arrhenius-plot for the determination of the activation energy of PK-I in HEPES buffer (100mM, pH 7.3) at saturated levels of ATP (26 µM) and casein (0.8 mg/ml). v = Initial reaction rate. Slope (1) = -3286, slope (2) = -2000 and calculated activation energy E* (1) = 15.04 kcal/mole and E* (2) =8.07 kcal/mole.

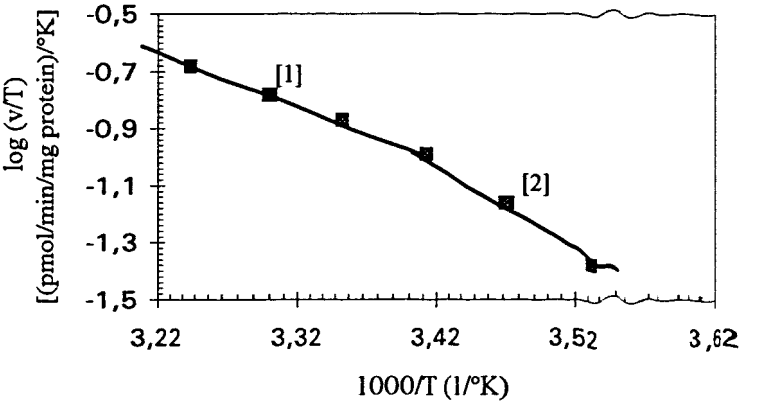



Figure 4.3.2.14 Modified Arrhenius-plot for the determination of the free energy, enthalpy and entropy of formation of the transition state of the PK-I reaction. v = Initial reaction rate. Slope (1) = -1857, Slope (2) = -3428 and intercept = -0.63. Calculated $\Delta G$ = 19.32 kcal /mole, $\Delta H$ = 8.5 kcal/mole, and $\Delta S$ = -36.28 cal/mole/K.

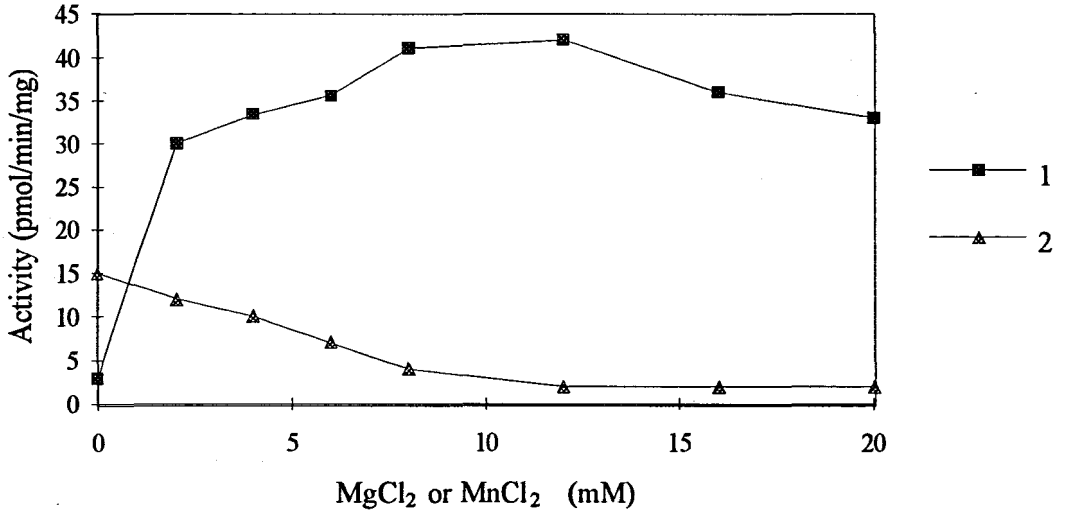



Figure 4.3.2.15 Effect of 1) $MgCl_2$ and 2) $MnCl_2$ on PK-I activity. PK-I assays were performed as described under Section 4.2.3 at pH 8 with 2 mg/ml casein as exogenous substrate and 0.1 mM $CaCl_2$. The value of each point is the average of two determinations in duplicate.

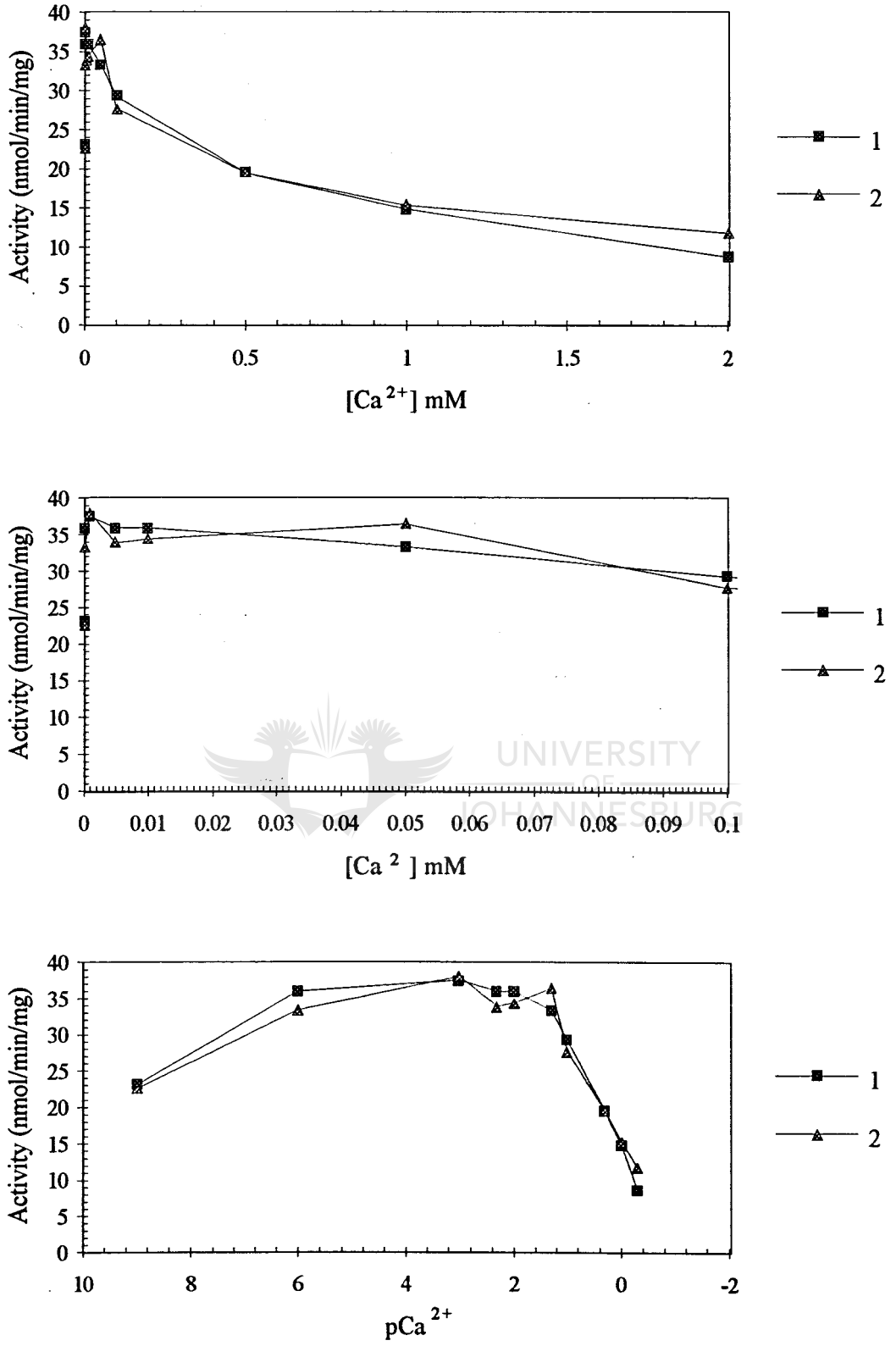





Figure 4.3.2.16 Effect of calcium on PK-I activity. Assays were performed as outlined under Section 4.2.3 in a calcium/EGTA buffer. Values are averages of duplicates. A: Plot of activity vs. $[Ca^{2+}]$, 0 - 2 mM range. Casein was used as exogenous substrate and 8mM $MgCl_2$ was present in the assay mixture at pH 8. B: Plot of activity vs. $[Ca^{2+}]$, 0 - 0.1 mM range. C: Plot of activity vs. $pCa^{2+}$. The different curves 1 and 2 represents the presence of 1) 1 μM exogenous calmodulin and 2) no exogenous calmodulin added.

Table 4.3.2.3 Effects of various agents on PK-I activity. Values are the means of duplicate determinations. The enzyme activity was determined as described under Section 4.2.3 in the presence of 0.1 mM $CaCl_2$ under standard assay conditions with 2 mg/ml casein as exogenous substrate.

| Agent | Concentration | Maximal activity (%) |
|---|---|---|
| Heparin | 30 µg/ml | 70 |
| Calmidazolium | 250 µM | 104 |
| Chlorpromazine | 112 µM | 99 |
| cAMP | 17 µM | 54 |
| Calmodulin | 1 µM | 101 |
| DMSO | 16% | 99 |
| Control | | 100 |

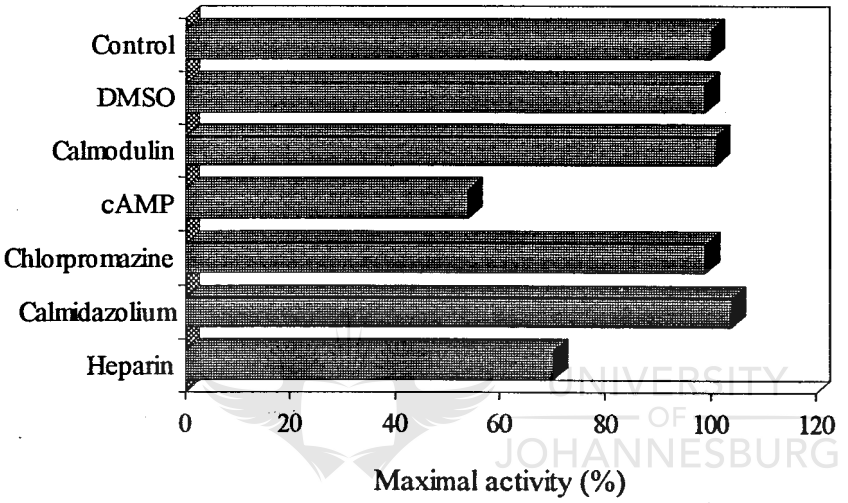





Figure 4.3.2.17 Graphical representation of the effects of the various effectors and inhibitors tabulated in Table 4.3.2.3 on PK-I activity.

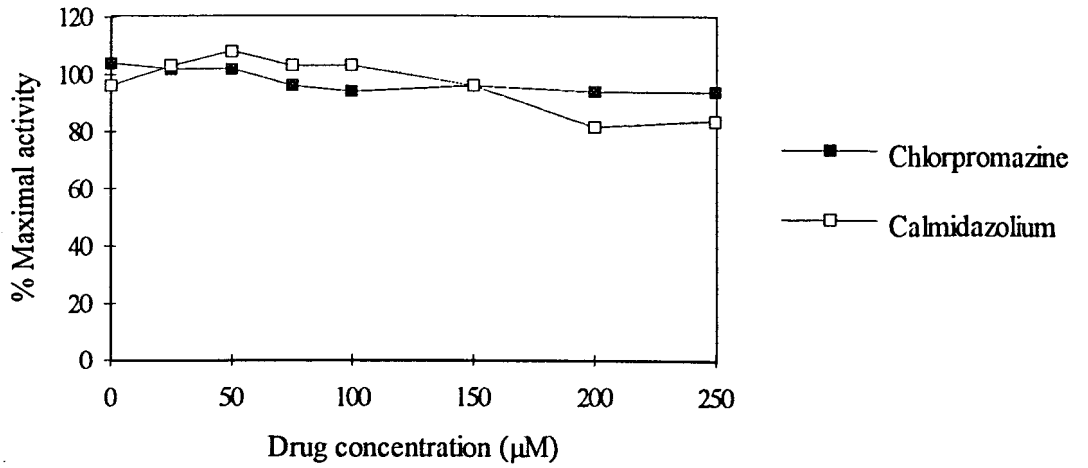



Figure 4.3.2.18 Effect of chlorpromazine and calmidazolium on PK-I activity. Values are the means of duplicate determinations. The enzyme activity was determined as described under Section 4.2.3 in the presence of 8mM $MgCl_2$, 5 µM $CaCl_2$ and 2 mg/ml casein as exogenous substrate.

Table 4.3.2.4 Substrate specificity of PK-I from ripening mango fruit tissue. Values are the means of duplicate determinations. The enzyme activity was determined as described under Section 4.2.3 in the presence of 0.005 mM $CaCl_2$ with 0.4 mg/ml of each of the exogenous substrates tested and 66µg PK-1. 100% activity was 18 nmol/min/mg.

| Protein | Activity nmol/min/mg | Relative activity (%) |
|---|---|---|
| Casein | 18 | 100 |
| BSA | 3 | 17 |
| Phosvitin | 18 | 100 |
| Protamine | 1 | 6 |
| Histone type II-S | 1 | 6 |
| Histone type III-S | 1 | 6 |
| No substrate | 2 | 11 |

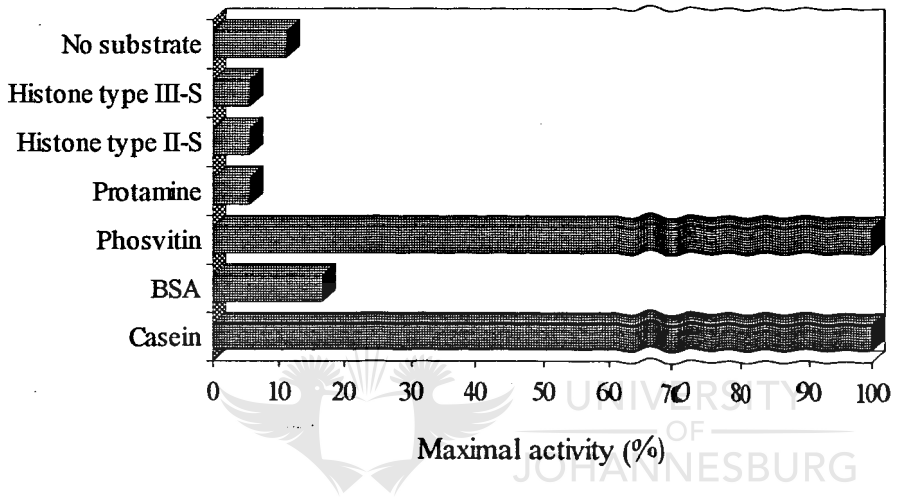



Figure 4.3.2.19 Graphical representation of the substrate specificity of PK-I tabulated in Table 4.3.2.4.

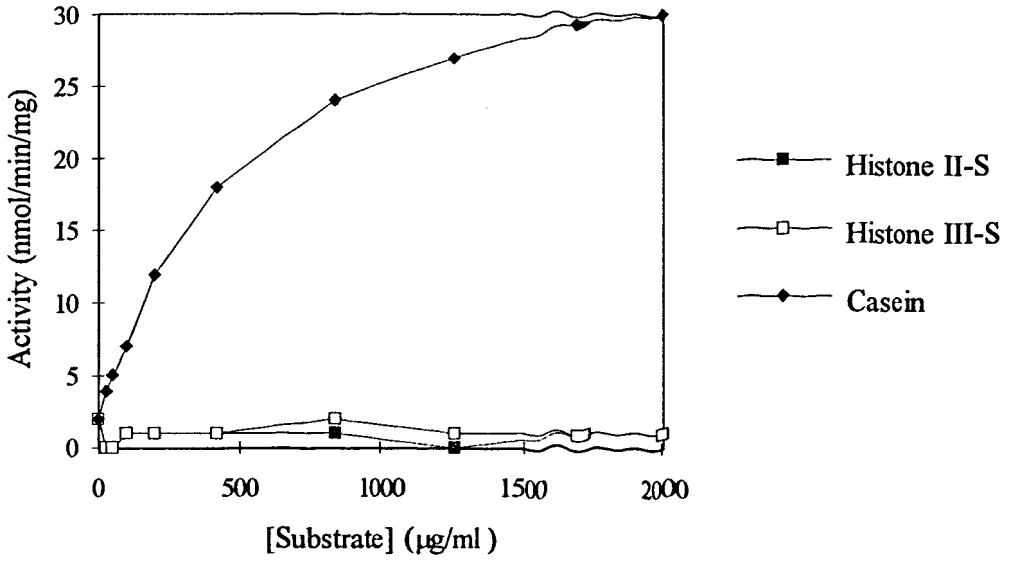



Figure 4.3.2.20 Substrate concentration dependency of histone II-S, histone III-S and casein phosphorylation by PK-I isolated from ripening mango fruit tissue. Histone II-S, histone III-S and casein were incubated with purified enzyme fractions in the standard incubation media in the presence of 0.005 mM $CaCl_2$ and 2 mM $MgCl_2$. Values are the means of duplicate determinations.

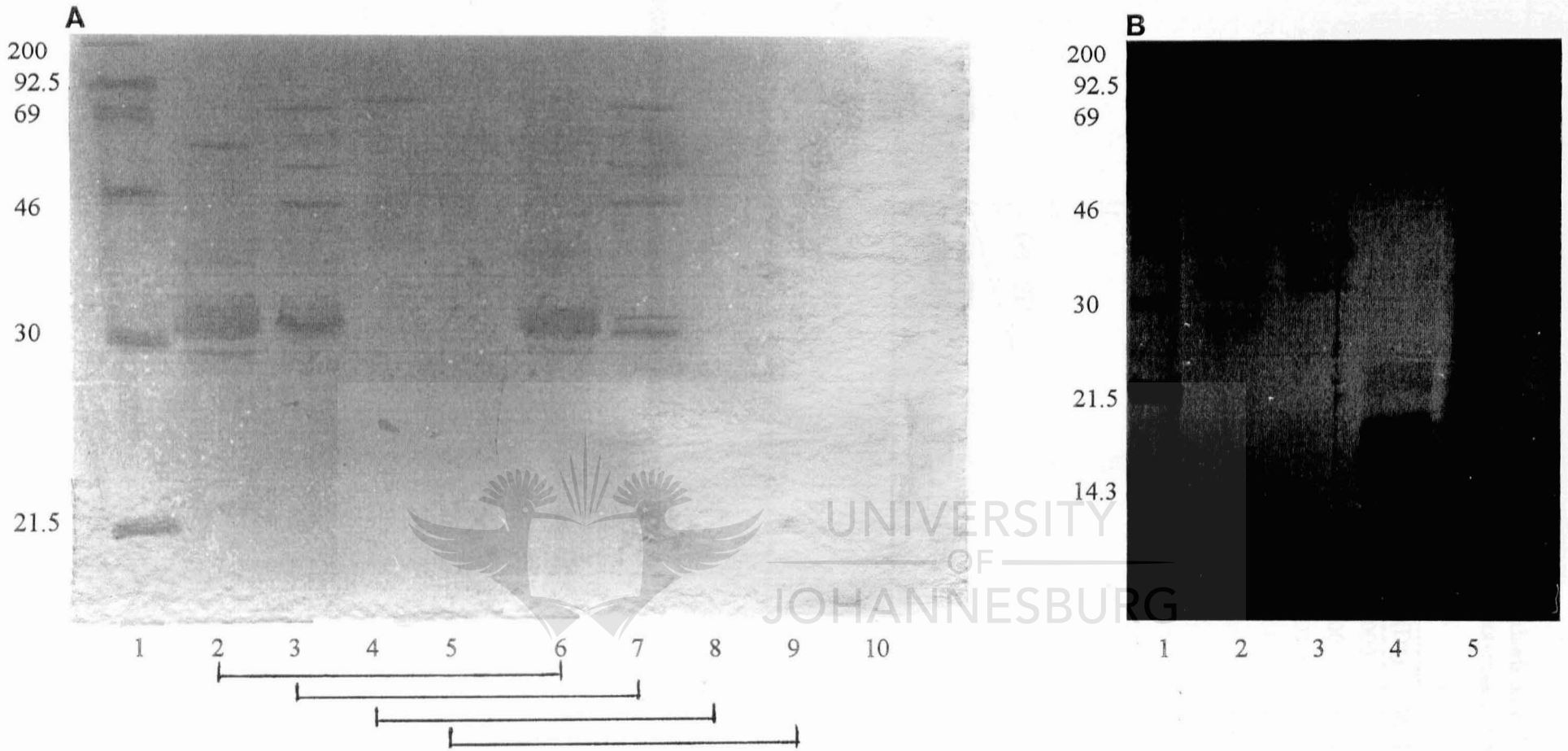



Figure 4.3.2.21 A: Proteins recognised by the anti-P-Tyr antibody in the following purified protein kinase fractions; lane 2, DEAE-Sephacel peak 1 (PK-I); lane 3, DEAE-Sephacel peak 2 (PK-I and PK-II); lane 4, purified PK-II from Cibacron blue Sepharose step; lane 5, purified PK-III from the phenyl-Sepharose step; lane 6, DEAE-Sephacel peak 1 (PK-I) plus casein; lane 7, DEAE-Sephacel peak 2 (PK-I and PK-II) plus casein; lane 8, purified PK-II from Cibacron blue Sepharose step plus casein; lane 9. purified PK-III from the phenyl-Sepharose step plus casein; lane 10, purified PK-III from the phenyl-Sepharose step plus histone III-S. The proteins were separated electrophoretically on 15% SDS-polyacrylamide gels and transferred to PVDF membranes. The blotted proteins were then probed with the anti-P-Tyr antibody. Lane 1 contained coloured molecular weight markers that are expressed in kilodaltons. The phospho-Tyr proteins were tabulated in Table 4.3.2.5. B: Autoradiogram of the same preparations as stated in A showing the total phosphorylation of the endogenous substrate proteins of the various protein kinases present; i.e. phospho-Tyr- as well as phospho-Ser/Thr-proteins. Experimental conditions are described under Section 4.2.9.2. The results are discussed under Sections 4.3.2.3.8, 4.3.3.3.8 and 4.3.4.3.8.

Table 4.3.2.5 Tabulation of calibration standards as well as the molecular weights of the determined phosphotyrosine proteins.

| Standards | $R_f$ | $M_r$ (Da) | log $M_r$ |
|---|---|---|---|
| Myosin | 0.055 | 200000 | 5.301 |
| Phosphorylase b | 0.1 | 92500 | 4.966 |
| BSA | 0.145 | 69000 | 4.839 |
| Ovalbumin | 0.255 | 46000 | 4.663 |
| Carbonic anhydrase | 0.427 | 30000 | 4.477 |
| Trypsin inhibitor | 0.555 | 21500 | 4.332 |
| Lysozyme | 0.68 | 14300 | 4.155 |
| P-Tyr- proteins: | | | |
| 1. | 0.104 | 90 200 | 4.955 |
| 2. | 0.14 | 77 600 | 4.89 |
| 3. | 0.18 | 61 700 | 4.79 |
| 4. (PK-III?) | 0.23 | 50 100 | 4.7 |
| 5. | 0.32 | 38 900 | 4.59 |
| 6. (PK-II?) | 0.382 | 34 700 | 4.54 |
| 7. (PK-I?) | 0.4 | 32 400 | 4.51 |
| 8. | 0.455 | 28 200 | 4.45 |



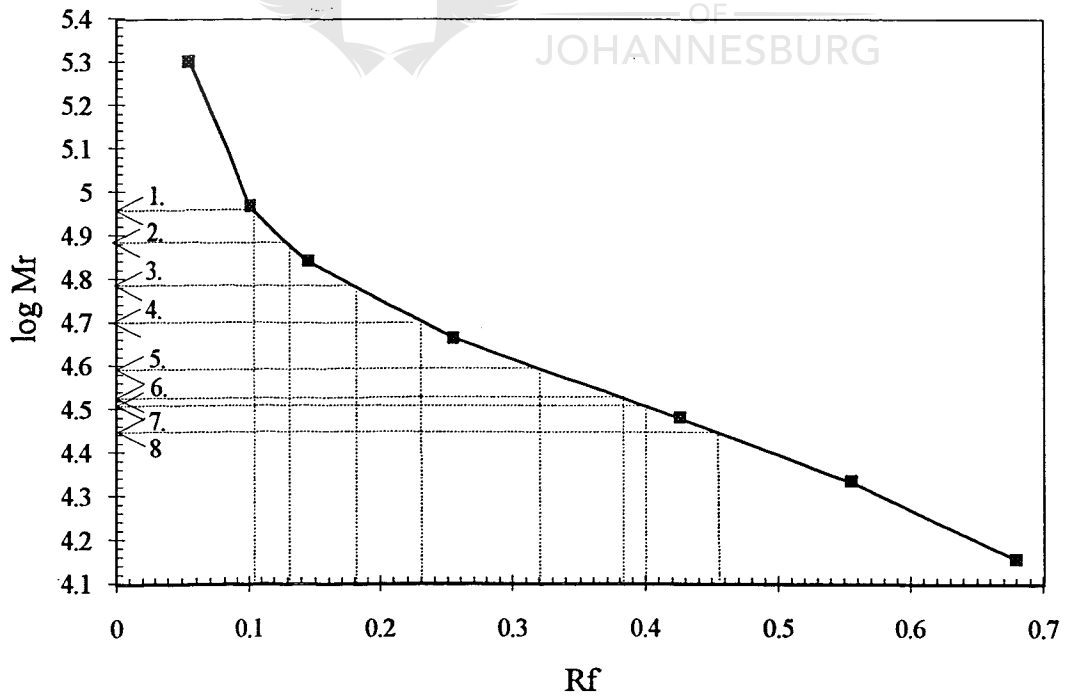



Figure 4.3.2.22 Plot of $R_f$ (electrophoretic mobility) *versus* log $M_r$ (molecular weight) for the determination of the $M_r$'s of the phosphotyrosine-proteins substrates of PK-I. The determined weights in Da are tabulated above.

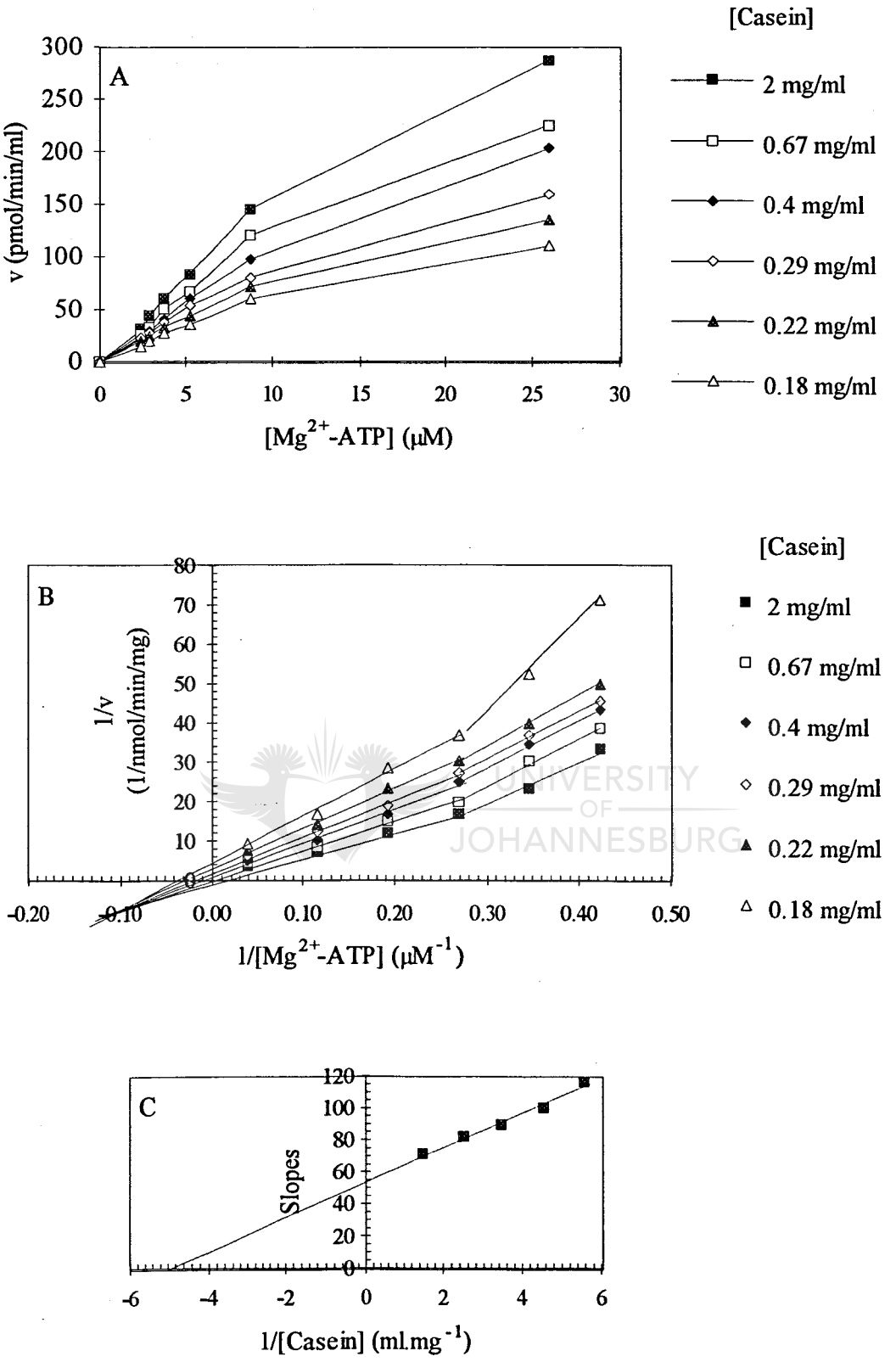



Figure 4.3.2.23 Initial velocity patterns for PK-I with varying ATP and casein concentrations. A: Michaelis-Menten plot of v *versus* [$Mg^{2+}$-ATP] at different fixed concentrations of casein. B: Double reciprocal plots of 1/v *versus* 1/[$Mg^{2+}$-ATP] at different fixed concentrations of casein. C: Secondary plot of slopes determined from plot B *versus* 1/[casein]. Conditions were as described under Section 4.2.3.

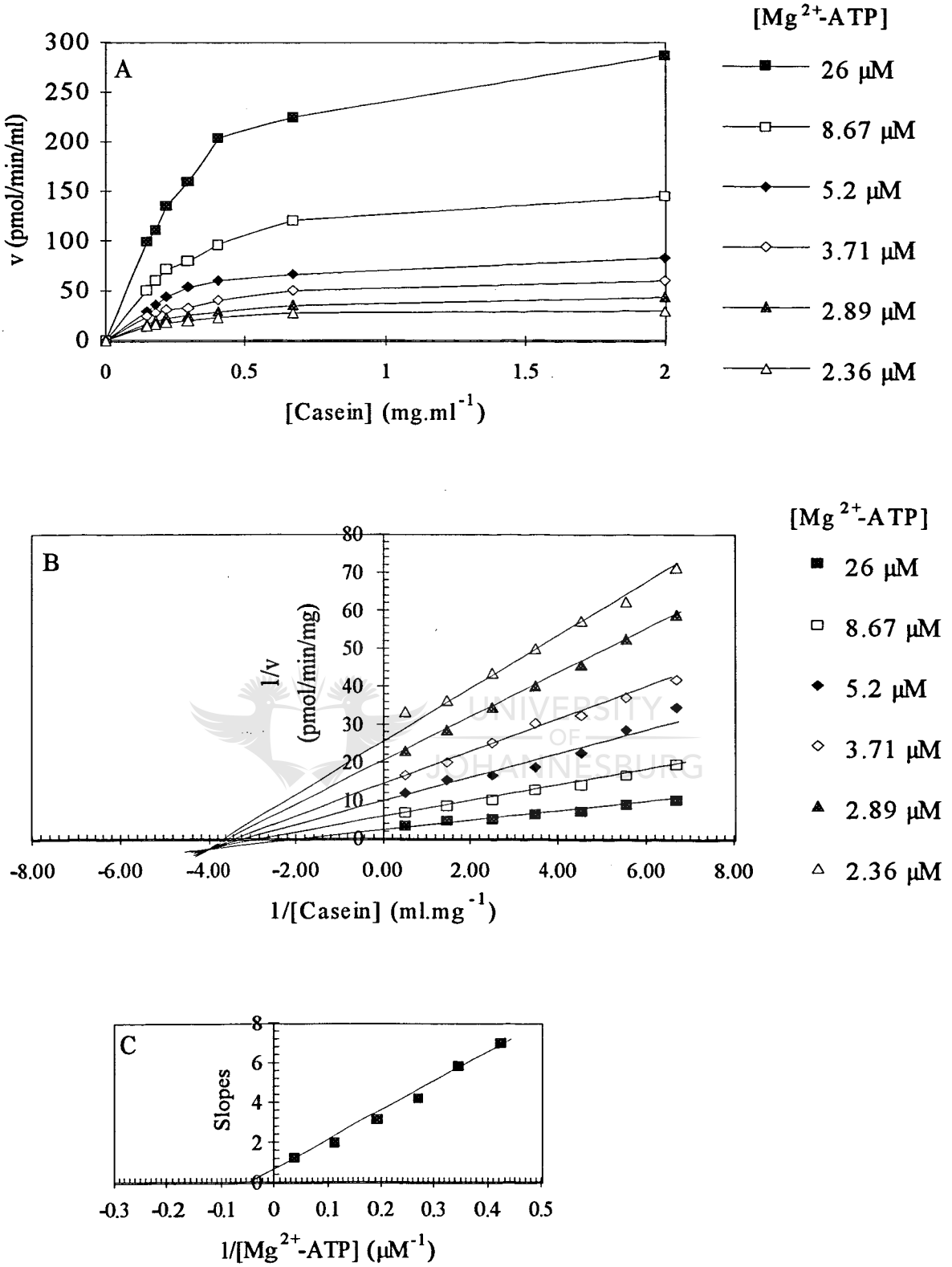



Figure 4.3.2.24 Initial velocity patterns for PK-I with varying ATP and casein. A: Michaelis-Menten plot of v *versus* casein at different fixed concentrations of [$Mg^{2+}$-ATP]. B: Double reciprocal plots of 1/v *versus* 1/casein at different fixed concentrations of [$Mg^{2+}$-ATP]. C: Secondary plot of slopes determined from plot B *versus* 1/[$Mg^{2+}$-ATP]. Conditions were as described under Section 4.2.3.

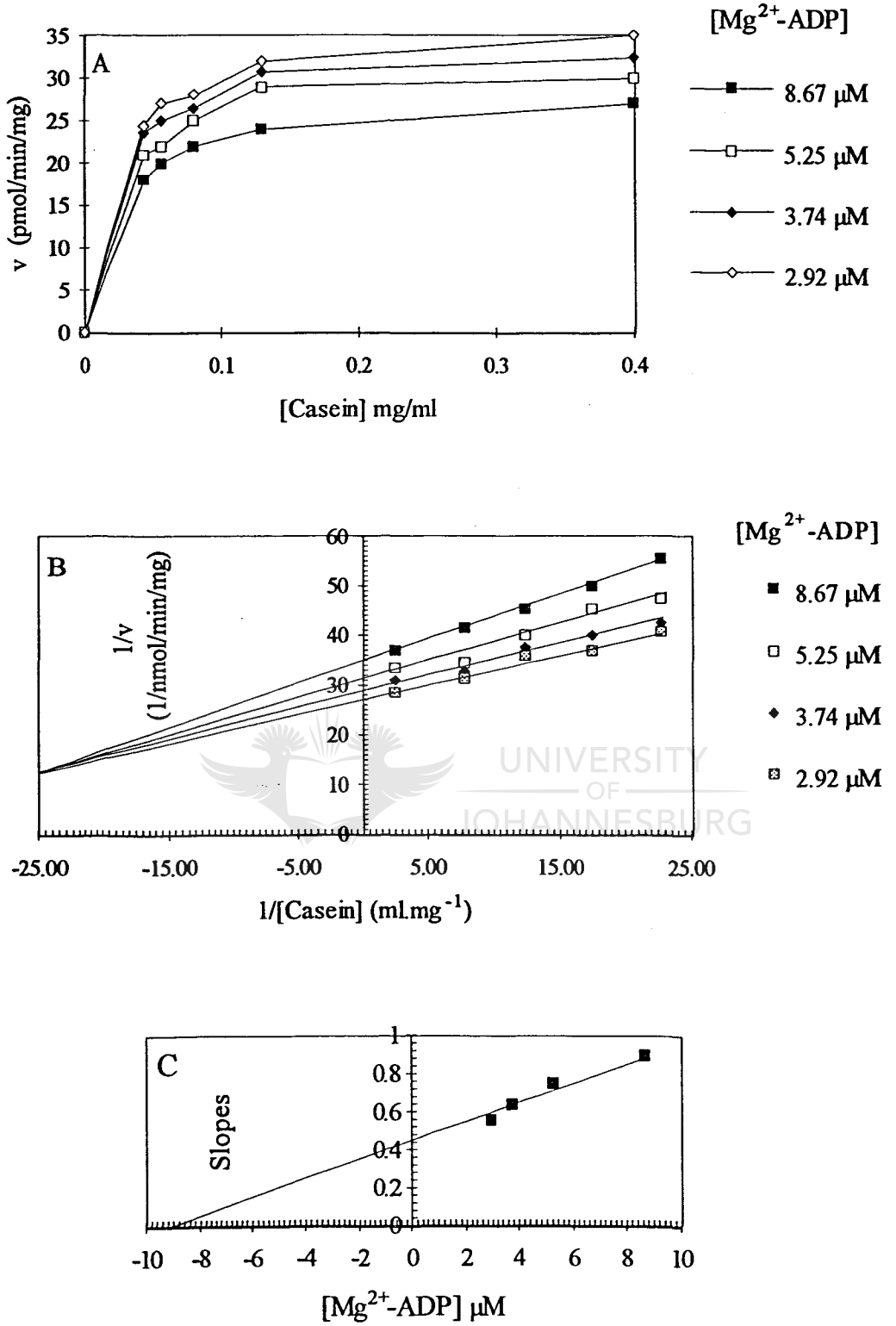



Figure 4.3.2.25 Product inhibition patterns of PK-I by ADP. A: Michaelis-Menten plot of v *versus* [casein] at 26 µM [$Mg^{2+}$-ATP] with varying ADP concentration; B: Double reciprocal plots of 1/v *versus* 1/[casein] at 26 µM [$Mg^{2+}$-ATP] with varying ADP concentration; C: Secondary plot of slopes determined from plot B *versus* [$Mg^{2+}$-ADP]. Conditions are as described under Section 4.2.3.

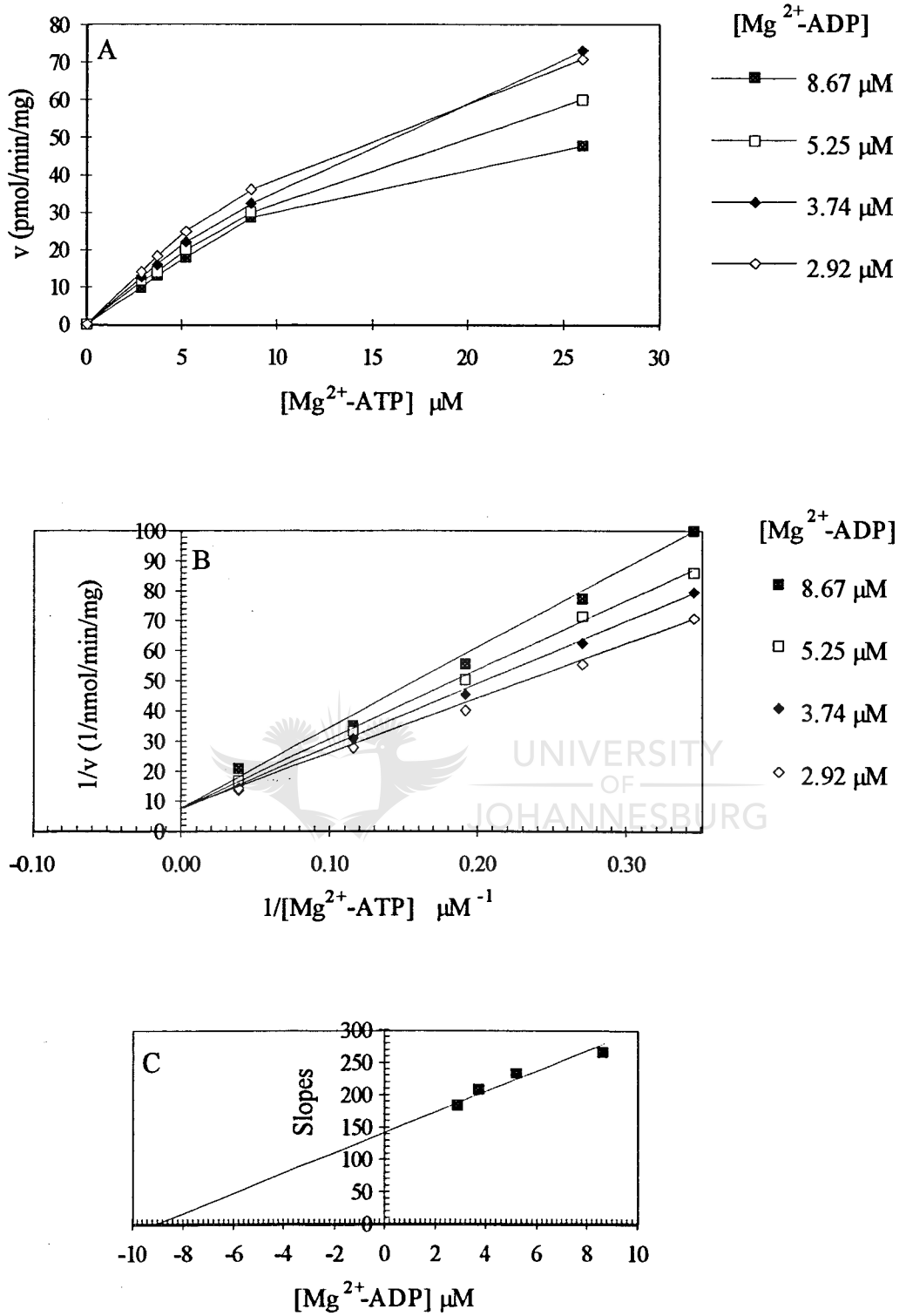



Figure 4.3.2.26 Product inhibition patterns of PK-I by ADP. A: Michaelis-Menten plot of v *versus* [$Mg^{2+}$-ATP] at 2 mg/ml casein with varying ADP concentration; B: Double reciprocal plots of 1/v *versus* 1/ [$Mg^{2+}$-ATP] at 2 mg/ml casein with varying ADP concentration; C: Secondary plot of slopes determined from plot B *versus* [$Mg^{2+}$-ADP]. Conditions are as described under Section 4.2.3.

## 4.3.3. Purification and Characterisation of Protein Kinase II (A Casein Kinase 2-Type Protein Kinase)

### 4.3.3.1 Purification and homogeneity of PK-II

The purification of PK-II from ripening mango fruit tissue (climacteric peak stage) is summarised in Table 4.3.3.1. The standard purification procedure as described under Section 4.2.11 and 4.2.13, allowed recovery of 1-5% of the total available kinase activity, and yielded approximately 100 µg of purified PK-II from 600 g of starting material (Table 4.3.3.1). The final specific activity, determined as described under Section 4.2.3 in the presence of 0.1 mM $Ca^{2+}$, was consistently 30-50 nmol/min/mg. PK-II was analysed by SDS-PAGE to assess its purity (Figure 4.3.3.5) and subunit structure. PK-II probably consists of a tetrameric $(\alpha\beta)_2$ structure (see discussion under Section 4.3.3.2.1). It was shown below that PK-II was not $Ca^{2+}$-dependent, but rather $Ca^{2+}$-stimulated and that it has casein kinase 2 (CK-2) characteristics.

#### 4.3.3.1.1 Chromatographic properties of PK-II

Four column chromatography steps and an ammonium sulphate precipitation step were used to purify PK-II over a period of a week. The elution profiles of the four chromatographic steps are shown in Figures 4.3.1.2, 4.3.3.2, 4.3.3.3, and 4.3.3.4. The calcium-stimulated casein phosphorylating activity was eluted from the DEAE-Sephacel column with a 0 to 0.4 M NaCl gradient. PK-II eluted at a NaCl concentration of 0.1 M NaCl (Figure 4.3.1.2).

The other protein kinases (not PK-III, but PK-I and PK-IV) bound tightly to Cibacron blue-Sepharose and could not be separated from PK-II which could only be eluted with 1 M NaSCN (Figures 4.3.3.1 and 4.3.3.2). Endogenous calmodulin was removed by this step.



The activity peak from Blue-Sepharose was concentrated by means of centrifugal-ultrafiltration through a 30 kDa cut off filter that could remove low molecular weight contaminants as well as the 30 kDa PK-I from the PK-II active pool (Figure 4.3.3.5, lane 5). The concentration of PK-II by centrifugal-ultrafiltration had a detrimental effect on the activity yield (only 4% - Table 4.3.3.1). Being highly hydrophobic, the pure protein kinase molecules tend to aggregate, forming an insoluble precipitate leading to high losses. As seen from SDS-PAGE studies (Figure 4.3.3.5, lane 3) an inactive 70 kDa enzyme species exists and certain experimental conditions might promote the formation of this inactive species.

Filtration chromatography on Toyopearl-HW55 (Figure 4.3.3.3) and Sephadex-G75 (Figure 4.3.3.4) resulted in a further 2-fold purification. The hydrophobic character of PK-II might lead to non-specific adsorption that lead to high activity losses (Table 4.3.3.1) although the possibility of the formation of an inactive enzyme form is higher. Casein kinases 2 (CK-2) is labile and readily undergo inactivation after gel-filtration, dialysis or dilution (Pinna 1990, Klimczak et al. 1992). This may also apply to PK-II.

#### 4.3.3.1.2 Homogeneity

SDS-PAGE was used to determine the homogeneity, molecular weight and subunit structure of the purified enzyme as described under Section 4.2.6 and the results discussed under Section 4.3.3.2.1. After the examination of SDS-PAGE (Figure 4.3.3.5), it could be assumed that PK-II was at least 90 % pure. Only two light contamination protein bands can be seen in lane 5, the Sephadex G-75 activity pool.

The hydrophobic character of PK-II could explain why native PAGE experiments (not shown) did not give satisfactory resolution. The tendency of aggregate formation was probably prevented in the crude extract by the higher protein concentration and lipids, that kept this protein kinase in suspension, at the same time stabilising it. High percentages of glycerol stabilised the enzyme to an extent. Concentration steps of the purification protocol made the enzyme especially vulnerable. It is suspected that PK-II is a membrane associated protein. The ripening process results in a natural breakdown in membranes and thus make membrane associated proteins more soluble. The high concentration EGTA (3 mM) in the extraction medium also attributed to the release of membrane associated proteins.

### 4.3.3.2 Physico-chemical properties of purified/isolated PK-II

#### 4.3.3.2.1 SDS-PAGE: Determination of the subunit structure and molecular weight

SDS-PAGE was used to determine the homogeneity, molecular weight and subunit structure of the purified enzyme as described under Section 4.2.6. The experimental information of the standardisation process and the determined results are given in Figure 4.3.3.6. Purified PK-II, which eluted as a seemingly single peak of activity during molecular filtration chromatography (Figures 4.3.3.3 and 4.3.3.4), resolved into four protein bands of molecular weight 34.6, 30, 24 kDa and an undetermined low molecular weight band on 10% SDS gels (Figure 4.3.3.5, lane 4). The molecular weight of 34.6 kDa agrees well with the size of 34 kDa determined by analytical gel chromatography (Section 4.3.3.2.2 and Figure 4.3.3.7). All efforts to remove the 24 and <21.5 kDa "contaminants" by means of centrifugal-ultrafiltration through a 30 k cut off membrane failed. The 30 kDa contamination band was removed by above mentioned treatment after which it is assumed that PK-II was at least 90% pure (Figure 4.3.3.5, lane 5).

The conclusion was that the 34 kDa species consisted of two subunits, i.e. a 24 kDa subunit and a <21.5 kDa (presumably a 10.6 kDa) subunit. Under the experimental conditions used to prepare the samples for SDS-PAGE analysis (incubate 2 min at 90°C), the 34 kDa enzyme species did not dissociate fully. In later studies the samples were incubated for 5 min at 55°C, and this seemed to dissociate the enzyme more completely. Using a new batch of mangoes, grown under other conditions and maybe not precisely at the same ripening stage, gave a lower yield of PK-II and more of PK-I (30 kDa protein - isolation discussed elsewhere). The results was never again as successful and photographs of better quality were not available.

Analysing the SDS-PAGE patterns of the Cibacron blue Sepharose and Toyopearl HW-55 a prominent 70 kDa band was detected. This molecular weight is twice that of the 34 kDa protein kinase species. The assumption that can be made is that this 70 kDa species consists of two 34 kDa protein species associated with each other. As discussed under Section 4.3.3.2.3, this 70 kDa species is slightly active. The results suggest that the holo-enzyme consists of a $\alpha\beta$ structure that can aggregate to a $(\alpha\beta)_2$ dimer with a $M_r$ of 70 kDa, were $\alpha$ = 24 kDa, $\beta$ = 10.6 and $\alpha\beta$ = 34 kDa. This kind of structure is typical of a casein kinase 2-type protein kinase (Pinna 1990, Klimczak et al. 1992).

Experience showed that the contaminants, in the Cibacron blue Sepharose activity pool, were mostly protein kinases with the same binding properties in respect to the Cibacron blue dye. The isolation of the 30 kDa protein kinase is discussed under Section 4.3.2. The 37 and 25 kDa contaminants turned out to be a similar higher molecular weight enzyme than PK-II. This enzyme was named PK-IV and co-eluted mostly with PK-III on the DEAE-Sephacel column as indicated in Figure 4.3.1.2.

4.3.3.2.2 IEF: Determination of the isoelectric point

Agar isoelectric focusing was used to separate the different species of PK-II according to their pI characteristics (see Section 4.2.7). The pI is the point at which the protein or its subunits are stationary and uncharged within an electric field and where its pI corresponds to a point in the appropriate pH gradient. The experimental information of the standardisation process and the determined results are given in Figures 4.3.2.8 and 4.3.2.9. Purified PK-II, resolved into three Coomassie stained bands with pI,s of 6.95, 7.5 and 9.9 (Figure 4.3.2.8, lane 2). The pI of 7.5 could represent the $\alpha\beta$ 34.6 kDa and/or the $(\alpha\beta)_2$ 70.8 kDa species of PK-II. The other two pI's, 6.95 and 9.9 could represent the 24 kDa and 10.6 kDa species, although one do not know which is which. The occurrence of three pI bands thus does not necessarily indicate that the preparation is not pure. The other contaminating protein kinase (PK-IV - see Figure 4.3.1.2 and Section 4.3.1.2) was also analysed and interesting results were obtained (Figure 4.3.2.8, lane 1). The obtained pI's show that two of the three bands had the same pI as that of PK-II and that only one, pI = 8.1, was different. This might be an indication that PK-II and PK-IV are isoenzymes. Further studies are necessary to make further conclusions, but it is known that the basal amounts of each enzyme differed in the different mango batches used. This could be as a result of different growing conditions, different areas where they were grown, temperature conditions after the fruit was picked and ripening stage (attempts were made to select fruit in the climacteric peak stage but accuracy is impossible).

4.3.3.2.3 Analytical gel chromatography: Determination of molecular weight

Analytical gel chromatography (Andrews 1970) on Toyopearl HW-55 (described under Section 4.2.8) was used to determine the molecular weight of PK-II. The experimental information of the standardisation process and the determined results were given in Figure 4.3.3.7. The apparent molecular weight of PK-II isolated from mango fruit tissue was approximately 34 kDa. The molecular weight of 34 kDa agrees well with the size of the one of the three bands determined in 10% SDS gels (70.8, 34 and 24 kDa; Figures 4.3.3.6). This was the most active form of the kinase that could be detected on the gel exclusion column.

A higher molecular weight for PK-II was not expected. However, the active protein pool from Toyopearl HW-55 resolved on Sephadex G-75SF in two approximately equal protein peaks; a lower molecular weight protein kinase active peak and a higher molecular weight (may be 70 kDa) low activity peak (Figure 4.3.3.4). At the time the last mentioned peak seemed to be a contaminant and only the high activity peak was pooled and analysed. By comparing the results from SDS-PAGE and IEF, one can assume that these two peaks are two different forms of the same enzyme, one the less active dimer of the other. The possibility exists that under certain experimental conditions, PK-II associated with itself forming more of the inactive species and less of the active lower molecular weight active enzyme species detected in the other isolation steps. Results from SDS-PAGE could support this theory (presence of 70 kDa species) and could also explain why the PK-II activity seemed to "vanish" on the gel exclusion chromatography columns giving a very low yield (4%).

### 4.3.3.3 Enzymatic properties of PK-II

4.3.3.3.1 Effect of pH on catalysis

Tests of the pH dependence of PK-II (Figure 4.3.3.8) showed that the optimum pH was in the vicinity of 9.6 under assay conditions used (Section 4.2.3). Relative to its activity at pH 9.6, its activity at pH 8.0 was approximately 50% and its activity at pH 6.5 was approximately 10%. The casein kinase-2 like kinase isolated by Li & Roux (1992) had a broad pH optimum near 8.

4.3.3.3.2 Effect of temperature on catalysis

The effect of temperature on PK-II enzyme activity was determined between 10 and 50 °C (Figure 4.3.3.9). The optimum reaction temperature under standard reaction conditions described under Section 4.2.3 with casein as exogenous substrate was 35 °C.

The activation energy of the catalytic reaction (E*), the free energy (ΔG*), enthalpy (ΔH*) and entropy of formation (ΔS*) of the transition state (activation complex) from the reactants, was determined as described under Section 4.2.16.2.

The value of 12.20 kcal/mole for E* was calculated from the slope of the Arrhenius-plot of log v *versus* 1/T (Figure 4.3.3.10) as described under Section 4.2.16.2. It was assumed that the E* value stays constant throughout the temperature range tested.

The values of 11.44 kcal/mole and -32.67 cal/mole/K for ΔH* and ΔS* were calculated from the slope and intercept of the modified Arrhenius-plot of log v/T *versus* 1/T (Figure 4.3.3.11) as described under Section 4.2.16.2. From these two known values ΔG* was be calculated as 21.18 kcal/mole.

4.3.3.3.3 Regulatory properties

- $Mg^{2+}$ and $Mn^{2+}$ dependency

The enzyme required $Mg^{2+}$ ($Mg^{2+}$-ATP) for its activity, and $Mn^{2+}$ could not substitute for $Mg^{2+}$ (Figure 4.3.3.12). In a plot of [$Mg^{2+}$] and [$Mn^{2+}$] *versus* kinase activity under standard assay conditions, except varying the $Mg^{2+}$- and $Mn^{2+}$ concentration from 0 to 20 mM, the optimal $Mg^{2+}$ concentration was reached at 8 to 10 mM (Figure 4.3.3.12). At a 20 mM $Mg^{2+}$ concentration inhibition of PK-II still did not occur. Kandror et al. (1990) reported an inhibiting $Mg^{2+}$ concentration for casein kinase 2 of 50 mM.



- $Ca^{2+}$ stimulation

Figure 4.3.3.13 shows the $Ca^{2+}$ stimulation of PK-II in the presence of casein at pH 8. Five to 50 μM $Ca^{2+}$ stimulated the activity of the purified PK-II to approximately 35%-40% higher than the basal EGTA activity. Although not significant, calmodulin did affect the activity of PK-II at the lower $Ca^{2+}$ concentration range (0 - 10 μM). The activation of the enzyme by $Ca^{2+}$ was not due to the presence of contaminating amounts of calmodulin. 2 mM $Ca^{2+}$ inhibits the activity of PK-II to approximately 50% of the basal $Ca^{2+}$-independent activity ($Ca^{2+}$ removal by EGTA chelation) activity (Figure 4.3.3.13).

- Effector and inhibitor studies

Using casein as an artificial substrate, the sensitivity of PK-II to various potential inhibitors and activators were tested (Table 4.3.3.2 and Figure 4.3.3.14). Kinase activity was insensitive to such inhibitors of $Ca^{2+}$/calmodulin-regulated protein kinases as chlorpromazine and calmidazolium (Figure 4.3.3.15). Calcium chelation by EGTA did not inhibit activity (Figure 4.3.3.13). $Ca^{2+}$ stimulated PK-II to approximately 35% higher than its basal $Ca^{2+}$-independent activity. The addition of 17 μM cAMP had no effect on its activity.

On the other hand PK-II was very sensitive to inhibition by heparin, as shown in Figure 4.3.3.14. Heparin (30 μg/ml) inhibited the phosphorylating activity of PK-II by approximately 70%. It is well known that heparin, as one of the negatively charged polysaccharides, has a diverse effect on the activity

of various protein kinases (Sakai et al. 1988) including casein kinase 2. They are of the most abundant serine-threonine kinases, inhibited by heparin (Hathaway et al. 1980, Yamamoto et al. 1979). The effect of heparin on PK-II is similar to those of CK-2 in which the inhibitory action by heparin is rather specific for the enzyme. Heparin is a glyco-aminoglycan that is structurally similar to $InsP_3$. It proved also to be a inhibitor of $InsP_3$-induced $Ca^{2+}$ efflux. However, inhibition by heparin of $InsP_3$ binding proteins (Brosnan & Sanders 1990) is not necessarily of significance in this study.

4.3.3.3. Substrate specificity

Under the assay conditions chosen, the PK-II favoured casein and phosvitin as artificial substrates, but it could also phosphorylate, with a much lower efficiency, histone III-S and BSA (Table 4.3.3.3 and Figure 4.3.3.16). Histone II-S and protamine sulphate were not stimulatory at 0.4 mg/ml. Protamine and phosvitin are relatively good substrates for protein kinase C (Kikkawa et al. 1982) and protein kinase A (Tao et al. 1980).

The substrate concentration dependency of histone II-S, histone III-S and casein phosphorylation by PK-II is shown in Figure 4.3.3.17. The phosphorylation of both the histone III-S and casein preparations showed non-linear kinetics with substrate inhibition at higher concentrations. The upper limit of enzyme activity for dephosphorylated casein was obtained at a concentration of 1 mg/ml. PK-II had a much lower affinity for histone III-S.

4.3.3.3.5 Endogenous substrates

Phosphorylation was carried out at the different purification stages with and without added purified PK-II and then subjected to SDS-PAGE analysis. Exogenous substrate was not added. Besides the $M_r$ 34.7 kDa band, the presence of more prominent phosphorylated protein bands could be detected in lane 2 where purified PK-II was added to the ammonium sulphate precipitation fraction (Figures 4.3.3.18 and 4.3.3.19). This indicated that PK-II had certain endogenous protein substrates in mango fruit tissue cells. The $M_r$'s of seven of these endogenous substrates were tabulated under Figure 4.3.3.19. The molecular weights of these phospho proteins were 22.9 kDa, 45.2 kDa, 47.9 kDa, 65.3 kDa, 80.4 kDa, 87.1 kDa and 104.7 kDa. The identities of the individual proteins are not known but it was determined that these proteins were either phosphorylated on a serine or threonine residue (see Section 4.3.3.3.8).

4.3.3.3.6 Autophosphorylation

Because the phosphorylation shown in Figure 4.3.3.18 lanes 6, 7 and 8 occurred with no exogenous substrate added, a possible interpretation of these results is that the 34.7 kDa species of PK-II the $Ca^{2+}$-stimulated protein kinase from ripening mango fruit tissue, is capable of autophosphorylation. Many protein kinases exhibit this activity, and this property may be an important way to regulate the enzyme activity (Lickteig et al. 1988, Guo & Roux 1990, Soderling 1993). Very limited information is known about this aspect of kinases especially in plant cells. It was reported that autophosphorylated CDPKs had increased activity (Bögre et al. 1988). Whether PK-II has a similar property remains to be tested. Details on the occurrence and function of autophosphorylation of plant protein kinases *in vivo* are not known. This autophosphorylation characteristic of PK-II confirmed the molecular weight determinations from SDS-PAGE and molecular sieve chromatography.

4.3.3.3.7 Kinetic properties

- Initial velocity studies

Initial velocity studies, in the absence of products, were performed over a wide range of ATP and casein concentrations (2.36 to 26 μM and 0.22 to 2 mg/ml, respectively), in order to adequately define both the kinetic mechanism and the kinetic constants of the reaction. In Figures 4.3.3.20 and 4.3.3.21 velocity is plotted *versus* $Mg^{2+}$-ATP or casein concentration respectively (Michaelis-Menten plot A), reciprocal velocity is plotted *versus* the reciprocal of $Mg^{2+}$-ATP or casein concentration respectively (Lineweaver-Burk plot B) and the slopes determined from the double-reciprocal plots is plotted *versus* the reciprocal of casein and $Mg^{2+}$-ATP concentrations respectively (Secondary plots C). The kinetic constants obtained from these plots are $K_m$ATP = 14 μM and $K_m$casein = 0.8 mg/ml. The $V_{max}$ for both was approximately 1.3 nmol/min/mg protein.

- Product inhibition studies

The results of initial velocity measurements made in the presence of the product (ADP) of the reaction are presented in Figures 4.3.3.22 and 4.3.3.23. As indicated, ADP was a competitive inhibitor *versus* ATP and a simple linear non-competitive inhibitor *versus* casein. Product inhibition by phospho-casein was not tested. The inhibition constant for ADP obtained from these data is $K_i$ADP = 3.2 μM.

- Enzyme mechanism

The initial velocity pattern studies of PK-II follows a sequential, rather than a Ping-Pong mechanistic pathway. That is, both of the substrates bind to the enzyme prior to product release.

The product inhibition patterns observed are entirely consistent with a steady state ordered Bi-Bi kinetic mechanism with the binding first. This mechanism is in concurrence with the findings of Gronot et al. (1981) and Whitehouse et al. (1983) who have shown binding of nucleotide to enzyme in the absence of peptide substrate and concluded that if the reaction mechanism is ordered, ATP must be the first substrate to bind. The kinetic scheme can be represented by the following:

$$\begin{array}{ccccccc} & A & & B & & P & Q \\ & k_1 \downarrow k_{-1} & & k_2 \downarrow k_{-2} & & k_{-3} \uparrow k_3 & k_{-4} \uparrow k_4 \\ E & \text{———} & EA & \text{———} & (EAB \Leftrightarrow EPQ) & \text{———} & EQ \text{———} E \end{array}$$

where A and B represent $Mg^{2+}$-ATP and casein, and P and Q the products, phospho-casein and $Mg^{2+}$-ADP, respectively. The competitive inhibition by $Mg^{2+}$-ADP, when $Mg^{2+}$-ATP was the varied substrate, supports the assignment of $Mg^{2+}$-ATP as the first substrate to bind, and assuming that there are no unusual rate constants, suggests that the mechanism is not random. The product inhibition patterns also exclude the possibility that the reaction mechanism might be non-rapid equilibrium random sequential since, if it was, both products should have been competitive inhibitors with their substrate counterparts (Segel 1985).

4.3.3.3.8 Identification of amino acids phosphorylated by PK-II

Protein recognition anti-P-Tyr antibody studies (Section 4.2.9.2 and Figures 4.3.2.21 and 4.3.2.22) showed that PK-II did not phosphorylate Tyr residues. It is known that casein phosphorylation is a confirmation of phosphorylation on Ser and Thr residues (Hirayama & Oka 1992). PK-II could serve as an endogenous substrate for PK-I (see discussion in Section 4.3.2.8). It is reported that phospho-Tyr

kinases could control the activity of Ser and Thr protein kinases (Krebs et al. 1993). See further discussions under Section 4.4.

#### 4.3.3.4 Conclusion

The first report of highly purified protein kinases from higher plant nuclei were by Murray et al. (1987 (a,b)). Although these appeared to be CKs, one of them could not use GTP as a phosphoryl donor (Murray et al. 1978a) and the other did not bind to DEAE under relatively low salt conditions (Murray et al. 1978b); thus both were likely to be CK-1-type kinases. Later, Erdmann et al. (1982) reported the isolation of NI (= CK-1) and NII (= CK-2) protein kinases from the nuclei of cultured tobacco cells. However, these kinase preparations were not pure enough to characterise very thoroughly. The same is true for the casein kinase 2-type kinase preparation partially purified by Datta et al. from pea nuclei (1987). Yan and Tao (1982) have also purified and characterised a CK from a higher plant source, wheat germ, but the sub-cellular locale of this kinase was not tested, and spermine inhibited rather than stimulated this kinase except at below 2 mM $Mg^{2+}$ concentrations.

The pea casein kinase 2-type kinase preparation described by Li and Roux (1992) was highly purified, and was suitable for detailed biochemical characterisation and comparison with purified animal casein kinase 2 kinases. According to the criteria reviewed by Pinna (1990), the pea nuclear protein kinase is casein kinase 2-like in several respects: (a) native and subunit molecular weights; (b) ability to be stimulated by polycations, such as polyamines and high sensitivity to inhibition by polyanions, such as heparin; (c) ability to use ATP or GTP as phosphoryl donor; (d) preference for casein or phosvitin and ability to use RRREEETEEE (Kuenzel & Krebs 1985) as artificial substrates; (e) selectivity for serine and threonine as the side chains phosphorylated; (f) binding properties to phosphocellulose; and (g) kinetic (Km, Vmax) properties.



Further evidence that the pea nuclear kinase has a structure highly similar to that of animal casein kinase 2-type kinases, was that it specifically bound anti-*Drosophila* casein kinase 2 kinase antibodies in a western blot analysis. The anti-*Drosophila* casein kinase 2 kinase antibodies will also recognise calf thymus and yeast casein kinase 2 kinases but not a CK-1 kinase tested (Dahmus et al. 1984). Sequences that were highly conserved among widely divergent species in the $\alpha$ and $\beta$ subunits of casein kinase 2 kinases have been identified (Pinna 1990) and may be among those recognised in the pea casein kinase 2-like kinase by the anti-*Drosophila* antibodies.

PK-II isolated from ripening mango fruit tissue was highly purified as well, and suitable for biochemical characterisation and comparison with purified animal casein kinase 2 kinases. Although all the criteria were not tested, e.g. ability to use GTP as phosphoryl donor, there are still enough similarities to classify PK-II as a casein kinase 2-type protein kinase. The mango protein kinase is casein kinase 2-like in the following respects: (a) native and subunit molecular weights (Section 4.3.3.2.1); (b) high sensitivity to inhibition by polyanions, such as heparin (Section 4.3.3.3.3); (c) preference for $Mg^{2+}$ above $Mn^{2+}$ (Section 4.3.3.3.3); (d) preference for acidic artificial substrates such as casein or phosvitin (Section 4.3.3.3.4); (e) selectivity for serine and threonine as the side chains phosphorylated (Section 4.3.3.3.8); (f) the ability to autophosphorylate (Section 4.3.3.3.6) and (g) kinetic ($K_m$, $V_{max}$) properties - it was in the range observed for identified physiological substrates of casein kinase 2 (Section 4.3.3.3.7).

At this stage PK-II can not be identified as originating from the nuclear fractions of the fruit, because the natural ripening process leads to the breakdown of sub-cellular structures. The function of casein kinase 2 is not yet fully understood (see Chapter 2 for further discussion).

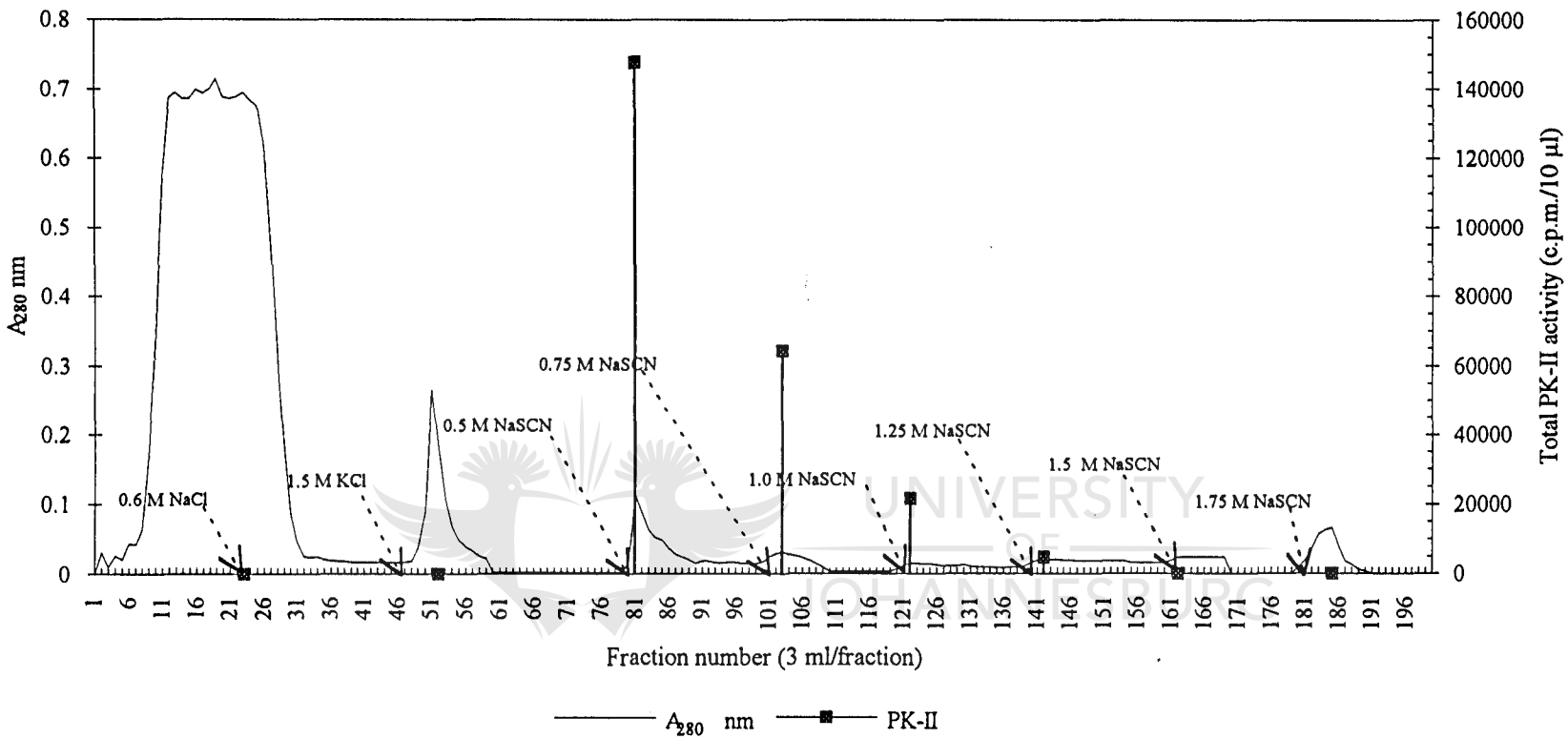



Figure 4.3.3.1 Step-wise elution of PK-II by means of affinity chromatography on a Cibacron-Sepharose column. The optimum NaSCN concentration needed to elute PK-II was determined to be 1 M.

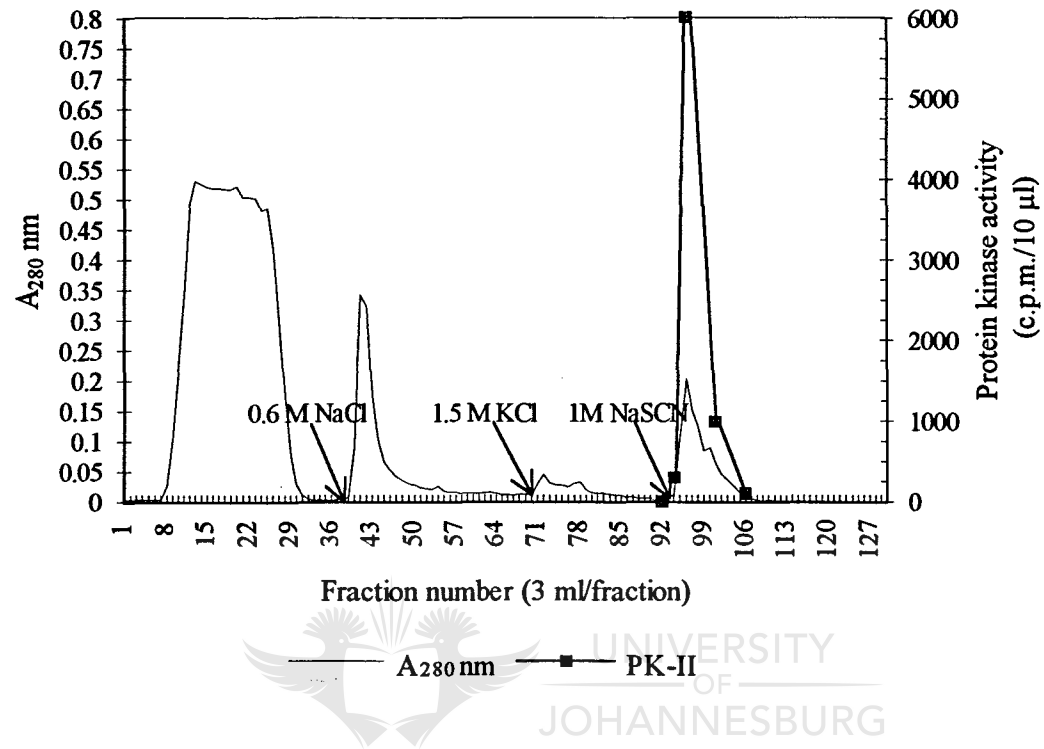





Figure 4.3.3.2 Elution profile of PK-II from a Cibacron Blue-Sepharose column after affinity chromatography. The experimental conditions are described under Section 4.2.13.2. Protein content of eluted fractions was determined by absorption at 280 nm. Activity of PK-II was measured, using dephosphorylated casein as substrate in the presence of 0.1 mM $CaCl_2$.

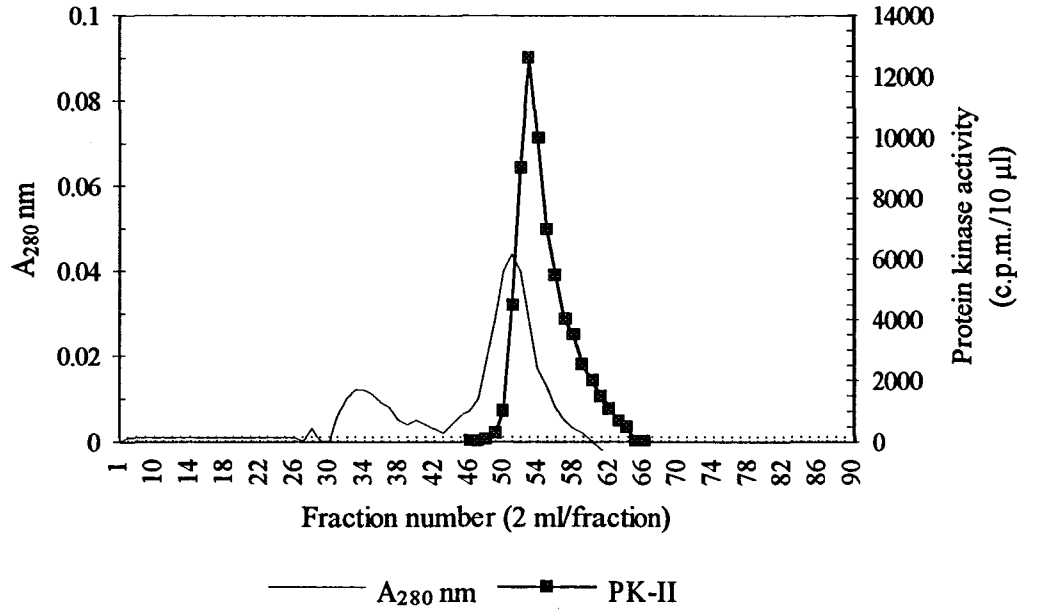



Figure 4.3.3.3 Purification of PK-II by means of exclusion chromatography on a Toyopearl HW-55F column. The experimental conditions are described under Section 4.2.13.3. Protein content of eluted fractions was determined by absorption at 280 nm. Activity of PK-II was measured, using dephosphorylated casein as substrate in the presence of 0.1 mM $CaCl_2$



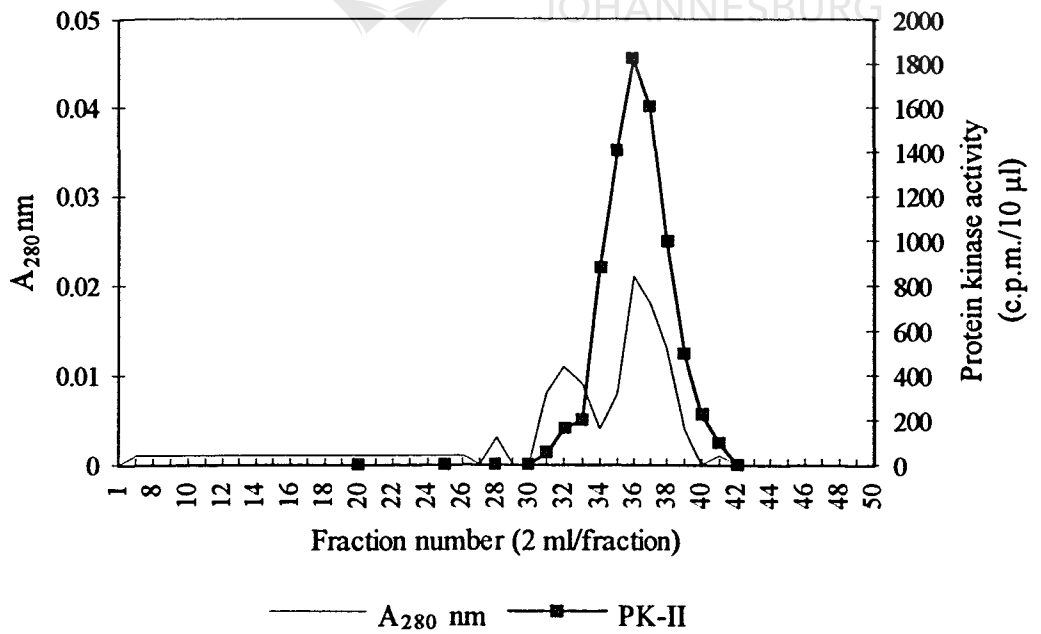



Figure 4.3.3.4 Purification of PK-II by means of exclusion chromatography on a Sephadex G-75SF column. The experimental conditions are described under Section 4.2.13.3. Protein content of eluted fractions was determined by absorption at 280 nm. Activity of PK-II was measured, using dephosphorylated casein as substrate in the presence of 0.1 mM $CaCl_2$.

Table 4.3.3.1 Summary of purification of PK-II from ripening mango fruit tissue

| Purification step | Volume (ml) | [Protein] mg/ml | Total protein (mg) | Activity (pmol/min/ml) | Total activity (pmol/min) | Specific activity (pmol/min/mg) | Yield (%) | Purification (x-fold) |
|---|---|---|---|---|---|---|---|---|
| 1. Crude homogenate | 1290 | 1,00 | 1290 | 48 | 61920 | 48 | 100 | 1 |
| 2. Ammonium sulphate precipitation (30-70%) | 100 | 2,68 | 268 | 627 | 62700 | 234 | 101 | 5 |
| 3. DEAE-Sephacel | 65 | 0,75 | 49 | 1441 | 93665 | 1921 | 151 * | 40 |
| 4. Blue-Sepharose | 4 | 0,80 | 3 | 8971 | 35884 | 11214 | 58 | 234 |
| 5. Toyopearl-HW55 | 3 | 0,10 | 0,3 | 4874 | 14622 | 48740 | 24 | 1015 |
| 6. Sephadex G75 | 5 | 0,015 | 0,08 | 452 | 2260 | 30133 | 3.6 | 628 |

Substrate used was casein

*The removal of protein kinase inhibiting substances by the previous purification steps result in a higher apparent yield of PK-II.

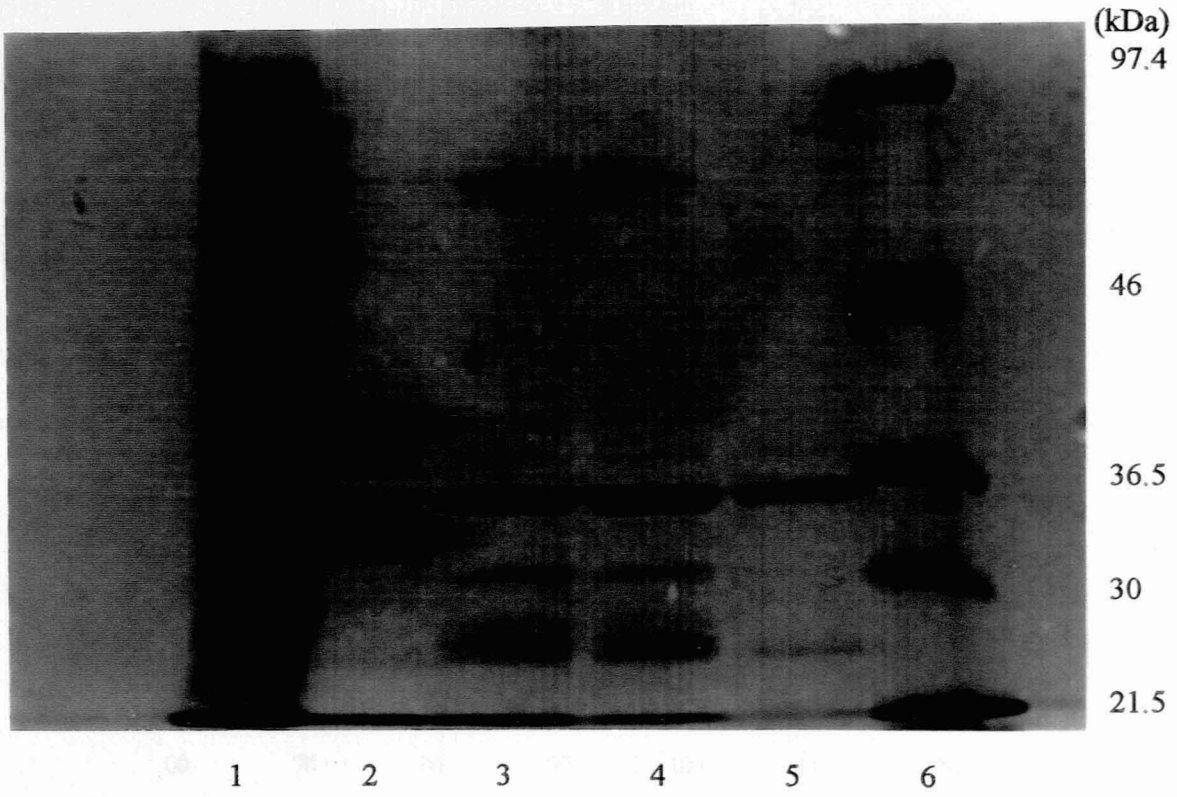



Figure 4.3.3.5 SDS-Polyacrylamide gel electrophoretic analysis of proteins from each stage of purification of PK-II. Aliquots from each stage of purification were treated as described in Section 4.2.6 and resolved in a 10% polyacrylamide gel in the presence of SDS and stained with Coomassie blue. Lane 6 contained the following molecular weight markers (in kDa): phosphorylase b, 97.4; ovalbumin, 46; lactate dehydrogenase, 36.5; carbonic anhydrase, 30; and trypsin inhibitor, 21.5. Other lanes show the following fractions from each step of the purification procedure: lane 1, ammonium sulphate precipitation pool; lane 2, DEAE pool; lane 3, Cibacron blue-Sepharose pool; lane 4, HW-55 pool; and lane 5, G75 pool.

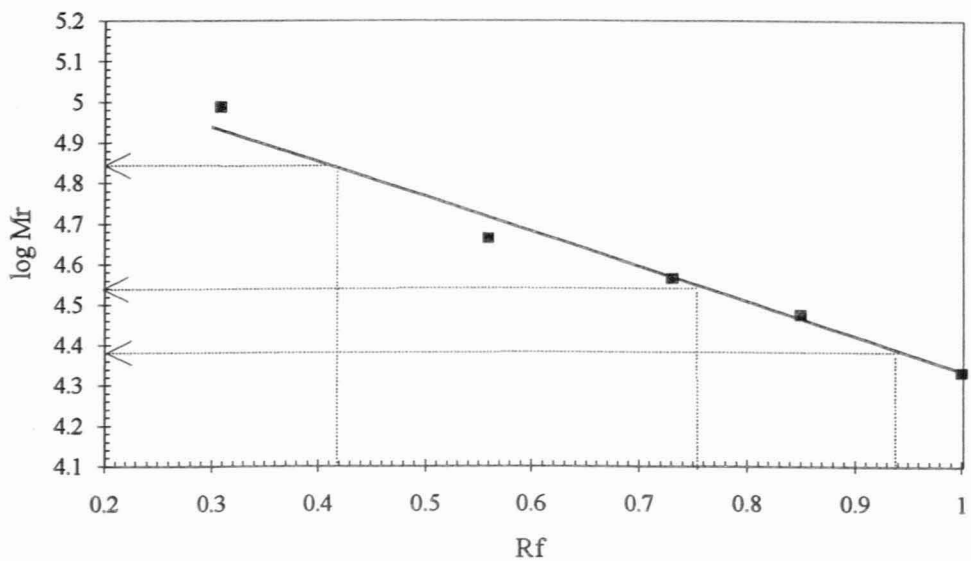



Figure 4.3.3.6 Plot of $R_f$ (Electrophoretic mobility) *versus* log $M_r$ (Molecular weight) for the determination of the $M_r$'s of the "subunits" PK-II. The determined weights in kDa were 70.0, 34.6 and 24. See Section 4.3.3.2.1. for the elucidation of the results.

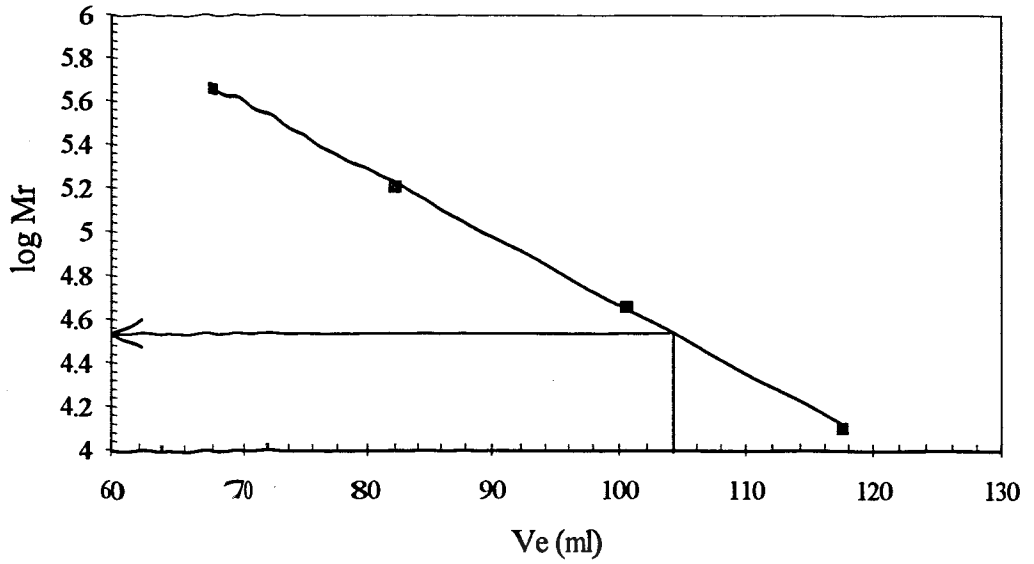



Figure 4.3.3.7 Plot of elution volume ($V_e$) *versus* molecular weight ($M_r$) for the determination of the molecular weight of PK-II by means of analytical molecular exclusion chromatography on Toyopearl HW-55. The experimental conditions are described under Section 4.2.8. The bed volume of the column was 157 ml, the flow rate was 26 ml/h and 3 ml fractions were collected. Standards used were blue dextran, 1000 kDa; ferritin, 450 kDa; aldolase 158 kDa; ovalbumin, 45 kDa; cytochrome c, 12.5 kDa; and β-ME, 0.087 kDa. The $M_r$ of PK-II is ca. 34.7 kDa.

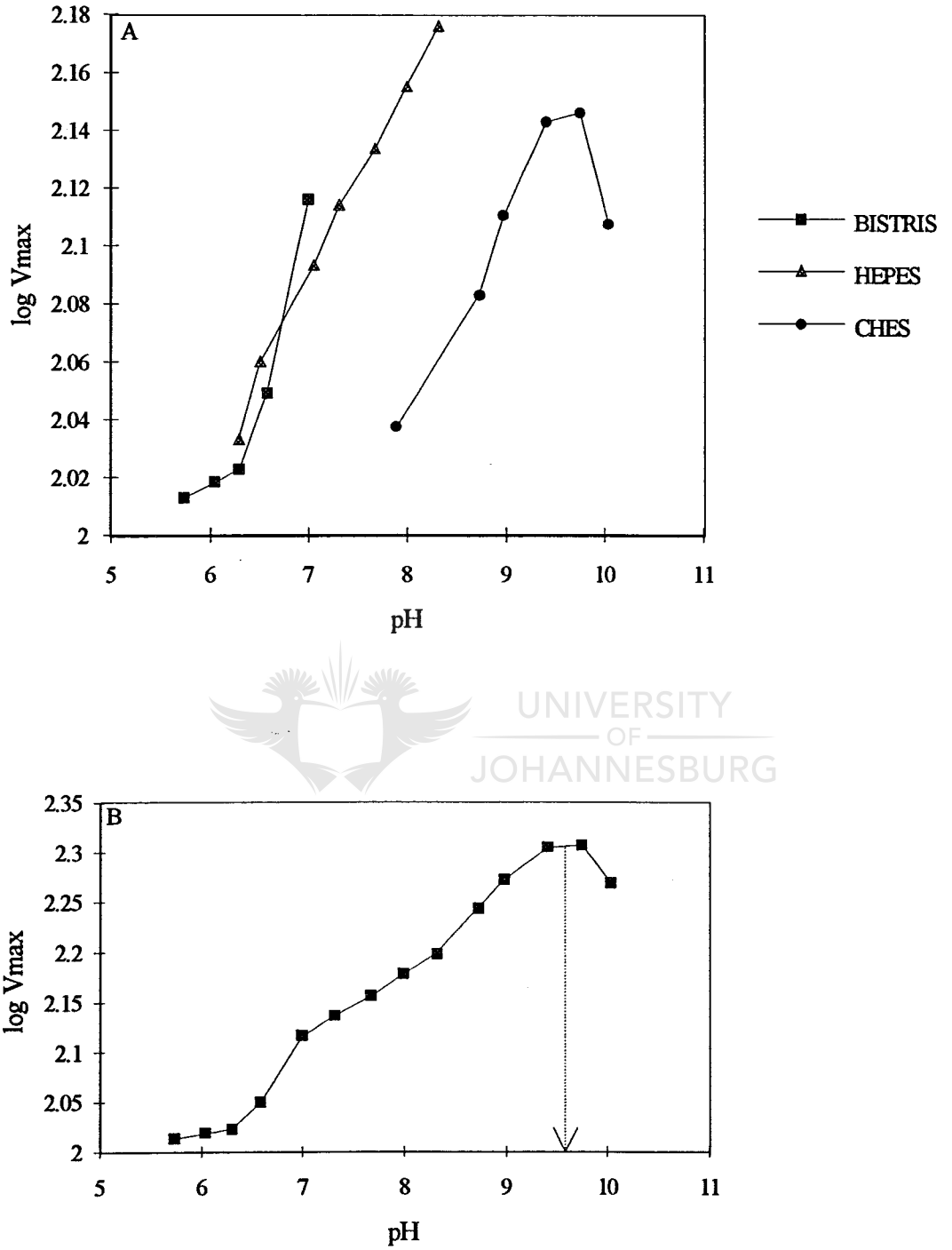





Figure 4.3.3.8 pH dependence of PK-II. Plots of log $V_{max}$ *versus* pH. For reaction conditions see Section 4.2.3 and 4.2.16.1. The value of each point is the average of two determinations in duplicate. The pH optimum = ~9.6. A: Activities of PK-II were determined in three different buffers namely, 100 mM BISTRIS (pKa = 6.5), 100 mM HEPES (pKa = 7.33) and 100 mM CHES (pKa = 9.5). B: The pH-activity curves determined in the different buffers were extrapolated to give one curve.

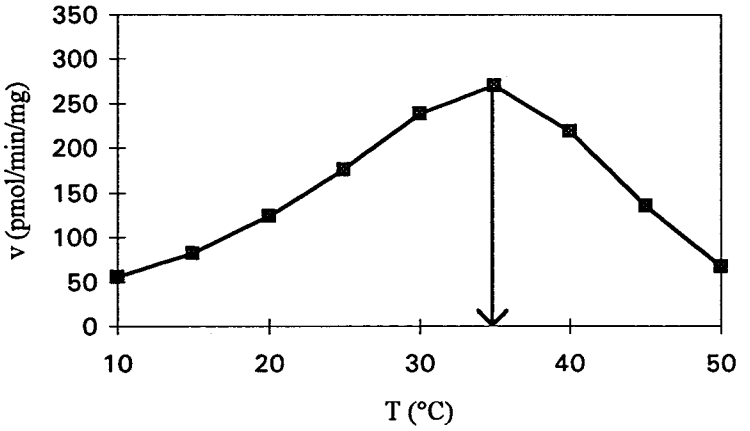



Figure 4.3.3.9 Effect of temperature on PK-II activity. The optimum reaction temperature at the standard reaction conditions as described under Section 4.2.3 was ca. 35°C. Casein was used as exogenous substrate. v = Initial reaction rate.

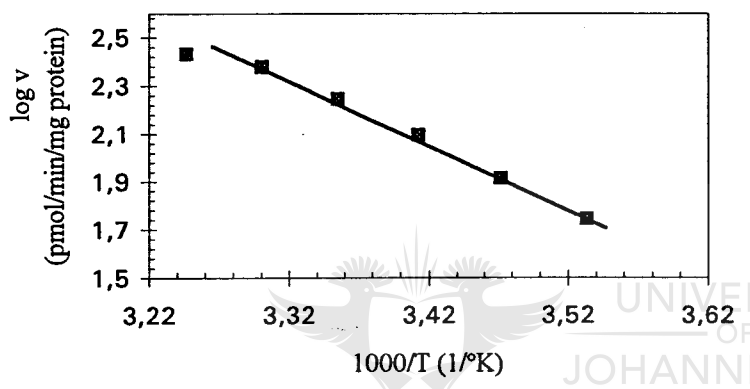



Figure 4.3.3.10 Arrhenius-curve for the determination of the activation energy of PK-II in HEPES buffer (100mM, pH 7.3) at saturated levels of ATP (26 µM) and casein (0.8 mg/ml). v = Initial reaction rate. Slope = -2667 and calculated activation energy (E*) = 12.2 kcal/mole.



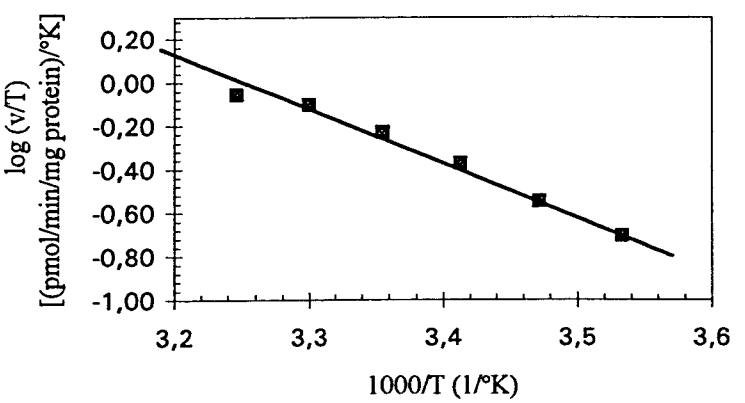



Figure 4.3.3.11 Modified Arrhenius-plot for the determination of the free energy, enthalpy and entropy of formation of the transition state of the PK-II reaction in HEPES buffer (100mM, pH 7.3) at saturated levels of ATP (26 µM) and casein (0.8 mg/ml). v = Initial reaction rate. Slope = -2500 and intercept = 0.16. Calculated $\Delta G$ = 21.18 kcal/mole, $\Delta H$ = 11.44 kcal/mole, and $\Delta S$ = -32.67 cal/mole/K.

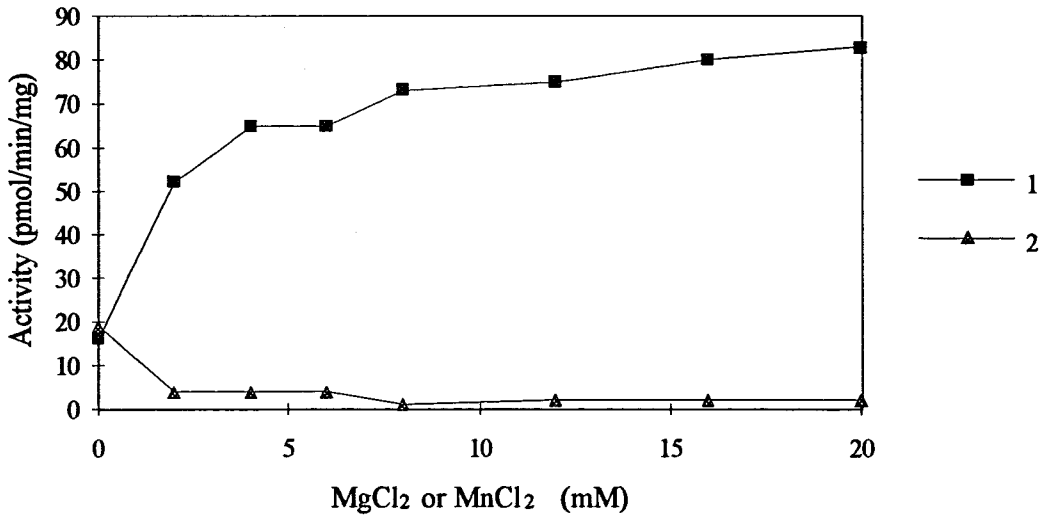



Figure 4.3.3.12 Effect of 1) $MgCl_2$ and 2) $MnCl_2$ on PK-II activity. PK-II assays were performed as described under Section 4.2.3 at pH 8 with 2 mg/ml casein as exogenous substrate and 0.1 mM $CaCl_2$. The value of each point is the average of two determinations in duplicate.

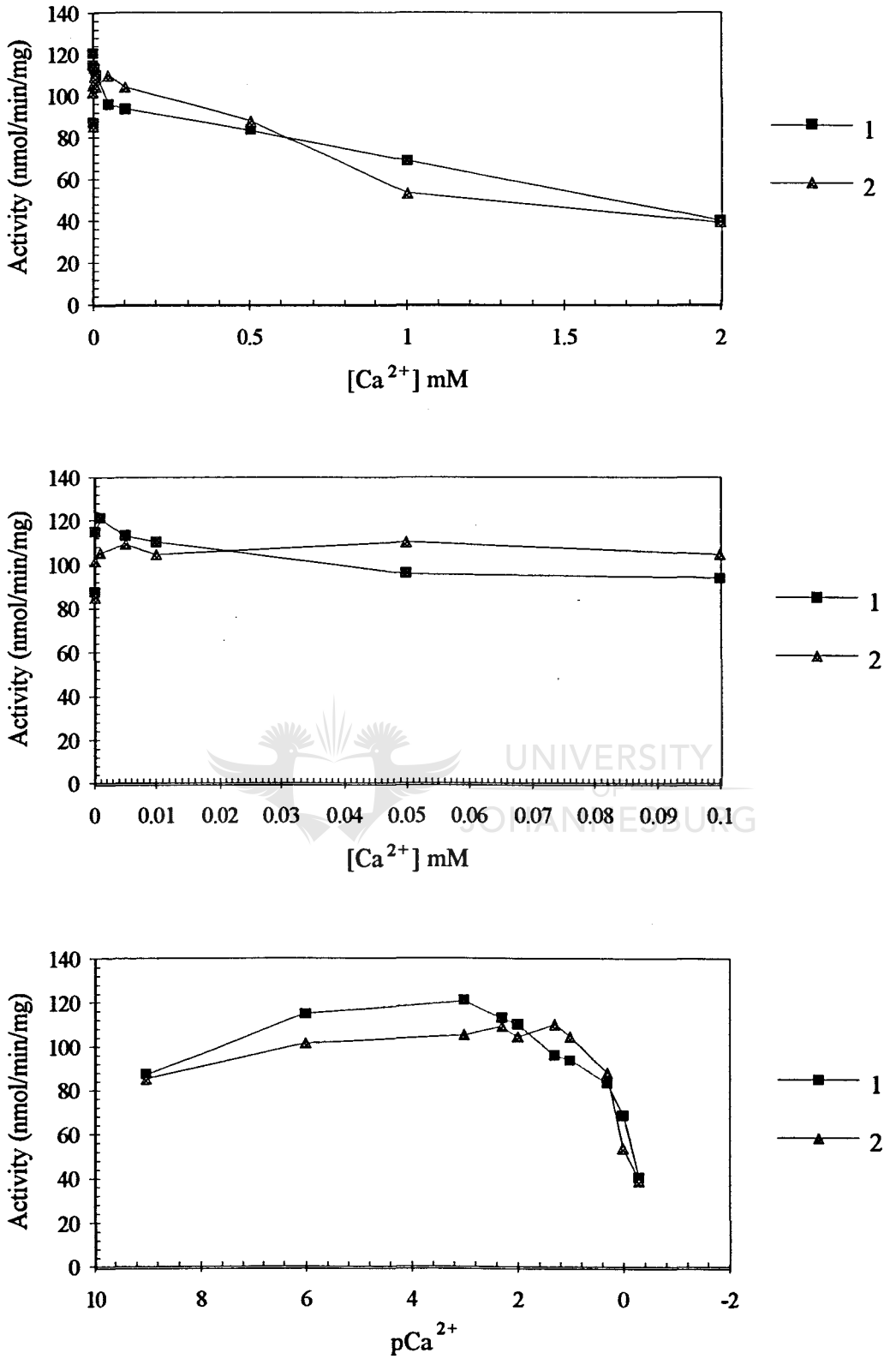



Figure 4.3.3.13 Effect of calcium on PK-II activity. Assays were performed as outlined under Section 4.2.3 in a calcium/EGTA buffer. Values are averages of duplicates. A: Plot of activity *vs.* $[Ca^{2+}]$, 0 - 2 mM range. Casein was used as exogenous substrate and 8 mM $MgCl_2$ was present in the assay mixture at pH 8. B: Plot of activity *vs.* $[Ca^{2+}]$, 0 - 0.1 mM range. C: Plot of activity *vs.* $pCa^{2+}$. The different curves 1 and 2 represents the presence of 1) 1 µM exogenous calmodulin and 2) no exogenous calmodulin added.

Table 4.3.3.2 Effects of various agents on PK-II activity. Values are the means of duplicate determinations. The enzyme activity was determined as described under Section 4.2.3 in the presence of 0.1 mM $CaCl_2$ under standard assay conditions with 2 mg/ml casein as exogenous substrate.

| Agent | Concentration | Maximal activity (%) |
|---|---|---|
| Heparin | 30 µg/ml | 30 |
| Calmidazolium | 250 µM | 105 |
| Chlorpromazine | 112 µM | 97 |
| cAMP | 17 µM | 100 |
| Calmodulin | 1 µM | 100 |
| DMSO | 16% | 98 |
| Control | | 100 |

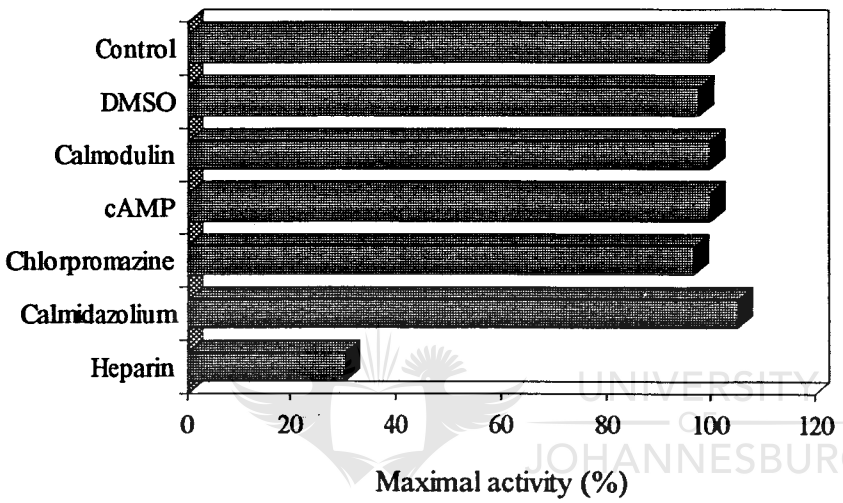



Figure 4.3.3.14 Graphical representation of the effects of the various effectors and inhibitors tabulated in Table 4.3.3.2 on PK-II activity.

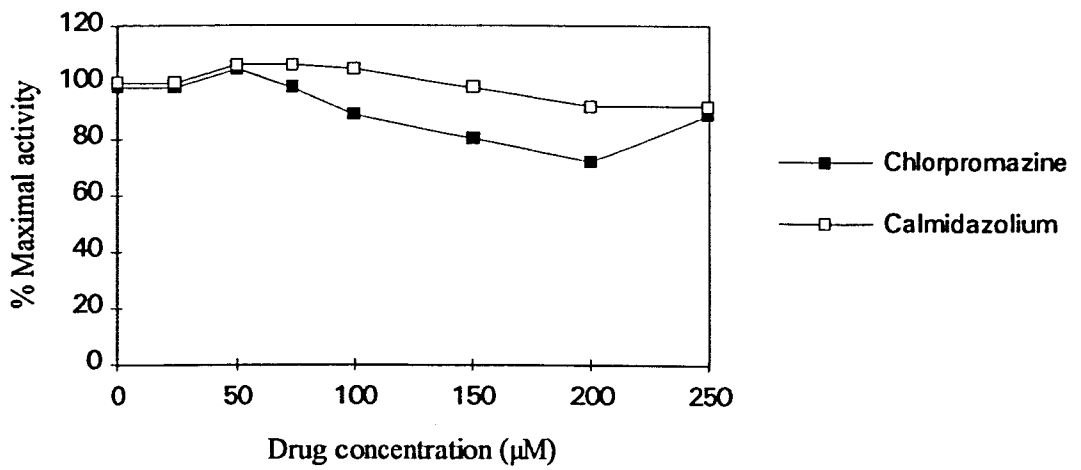



Figure 4.3.3.15 Effect of chlorpromazine and calmidazolium on PK-II activity. Values are the means of duplicate determinations. The enzyme activity was determined as described under Section 4.2.3 in the presence of 8 mM $MgCl_2$, 5 µM $CaCl_2$ and 2 mg/ml casein as exogenous substrate.

Table 4.3.3.3 Substrate specificity of PK-II from ripening mango fruit tissue. Values are the means of duplicate determinations. The enzyme activity was determined as described under Section 4.2.3 in the presence of 0.1 mM $CaCl_2$ with 0.4 mg/ml of each of the exogenous substrates tested and 36 µg PK-II. 100% activity was 103 nmol/min/mg.

| Protein | Activity (nmol/min/mg) | Relative activity (%) |
|---|---|---|
| Casein | 48 | 46.6 |
| BSA | 4 | 3.88 |
| Phosvitin | 103 | 100 |
| Protamine | 2 | 1.94 |
| Histone type II-S | 1 | 0.97 |
| Histone type III-S | 8 | 7.77 |
| No substrate | 1 | 0.97 |

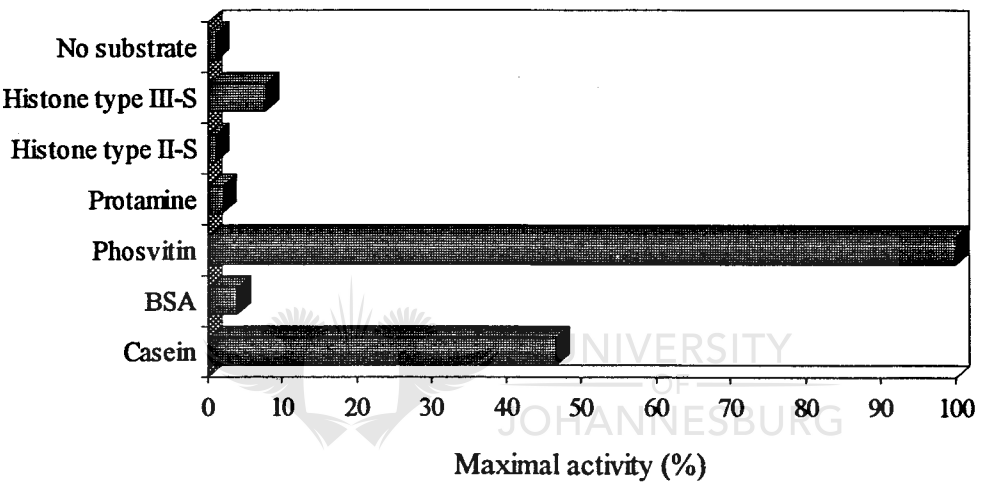



Figure 4.3.3.16 Graphical representation of the substrate specificity of PK-II tabulated in Table 4.3.3.3.

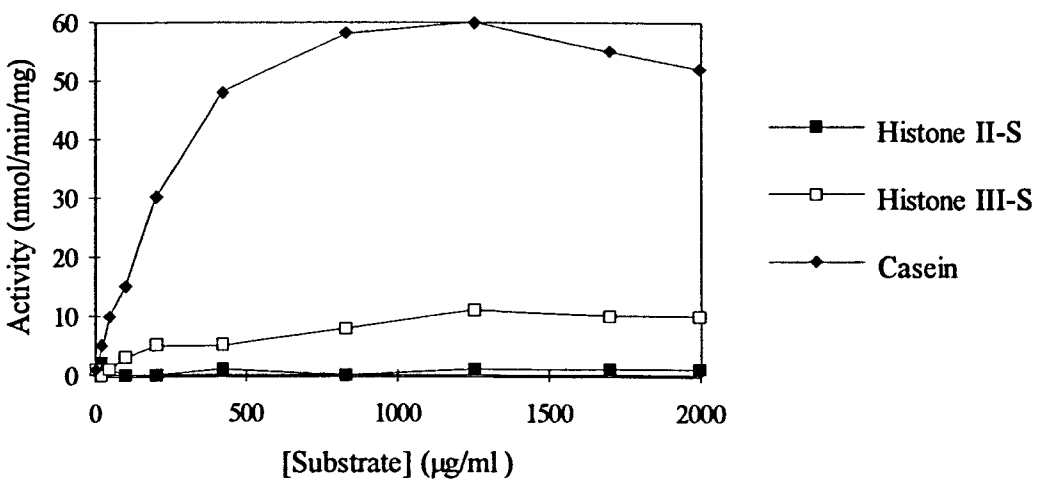



Figure 4.3.3.17 Substrate concentration dependency of histone II-S, histone III-S and casein phosphorylation by PK-II isolated from ripening mango fruit tissue. Histone II-S, histone III-S and casein were incubated with enzyme fractions in the standard incubation media in the presence of 0.1 mM $CaCl_2$ and 8 mM $MgCl_2$. Values are the means of duplicate determinations.

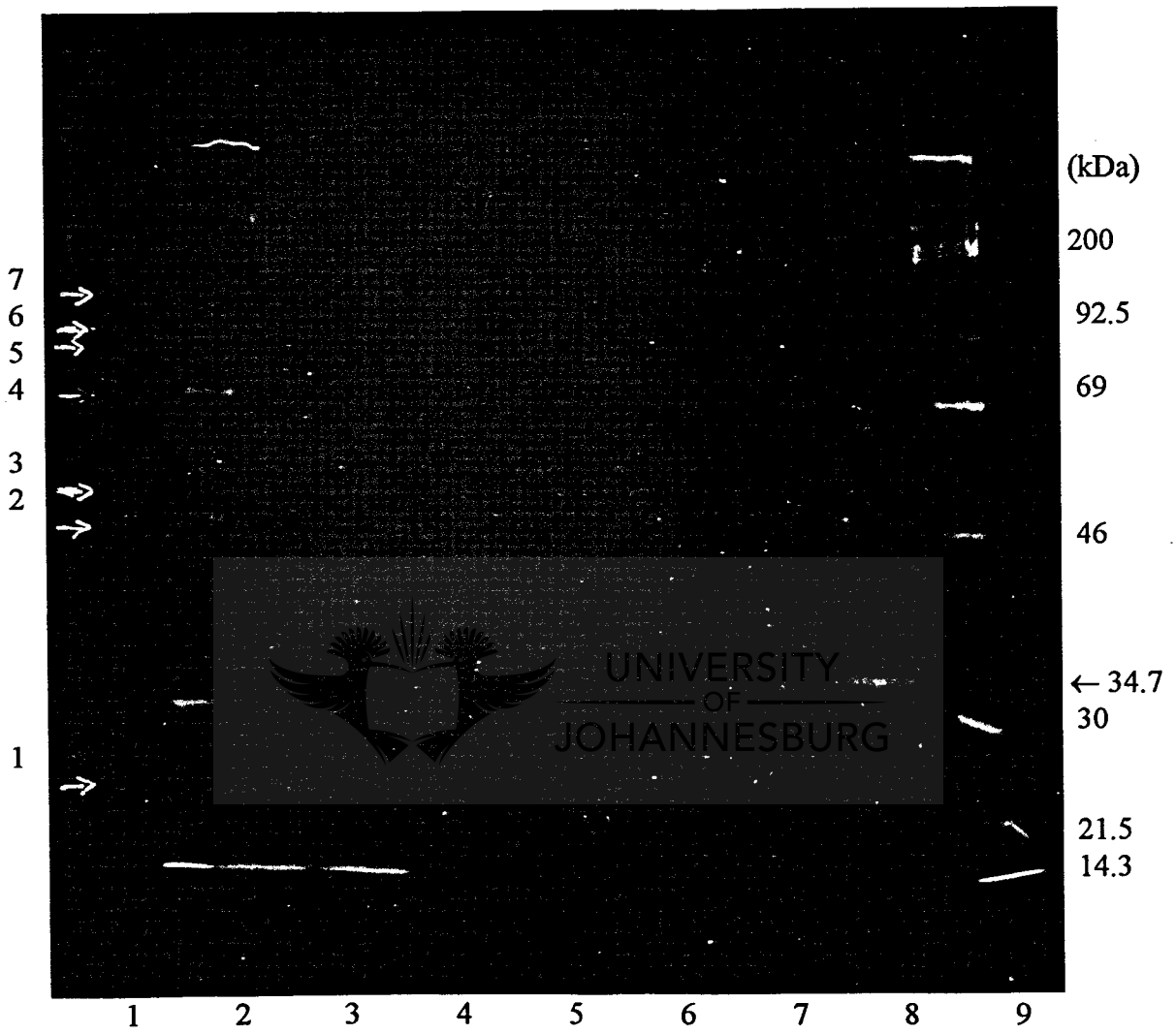





Figure 4.3.3.18 Autoradiogram showing the autophosphorylation of the 34.6 kDa PK-II species and some endogenous substrates in the ammonium sulphate precipitation fraction separated by SDS-polyacrylamide electrophoresis. Lane 2, ammonium sulphate precipitation fraction plus purified PK-II from Cibacron blue Sepharose pool; lane 3, ammonium sulphate precipitation fraction alone; lane 4, DEAE-Sephacel PK-II pool; lane 5, DEAE-Sephacel PK-II pool plus purified PK-II from Cibacron blue Sepharose pool; lane 6, purified PK-II from Cibacron blue Sepharose pool; lane 7, purified PK-II from Sephadex G75 pool; and lane 8, purified PK-II from Cibacron blue Sepharose plus Sephadex G75 pools. Lanes 1 and 9 contained $^{14}C$-labelled protein molecular weight markers tabulated under Figure 4.3.3.19. The phosphorylated proteins were also tabulated under Figure 4.3.3.19. Experimental conditions were described under Section 4.2.6. The results were described under Sections 4.3.3.3.5 and 4.3.3.3.6.

Table 4.3.3.5 Tabulation of the determined weights of the phosphorylated proteins separated in Figure 4.3.3.18.

| Standards | $R_f$ | $M_r$ (Da) | log $M_r$ | Relative intensity of bands |
|---|---|---|---|---|
| Myosin | 0.096 | 200000 | 5.301 | |
| Phosphorylase b | 0.235 | 92500 | 4.966 | |
| BSA | 0.348 | 69000 | 4.839 | |
| Ovalbumin | 0.52 | 46000 | 4.663 | |
| Carbonic anhydrase | 0.773 | 30000 | 4.477 | |
| Trypsin inhibitor | 0.92 | 21500 | 4.332 | |
| Lysozyme | 1 | 14300 | 4.155 | |
| PK-II (autophosphorylated) | 0.7 | 34 700 | 4.54 | |
| Endogenous substrates: | | | | |
| 1. | 0.9 | 22 900 | 4.36 | +++ |
| 2. | 0.545 | 45 200 | 4.655 | ++ |
| 3. | 0.505 | 47 900 | 4.68 | + |
| 4. | 0.354 | 65 300 | 4.815 | + |
| 5. | 0.283 | 80 400 | 4.905 | + |
| 6. | 0.263 | 87 100 | 4.94 | + |
| 7. | 0.212 | 104 700 | 5.02 | ++ |



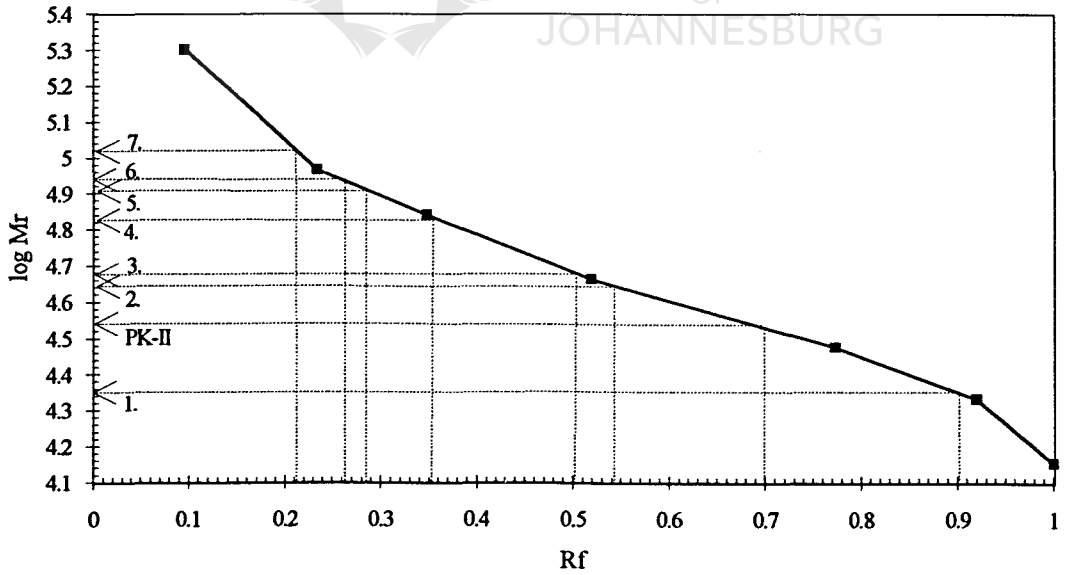



Figure 4.3.3.19 Plot of $R_f$ *versus* log $M_r$ for the determination of the $M_r$'s of the phosphorylated proteins including the autophosphorylated species of PK-II. The determined weights in Da are tabulated above.

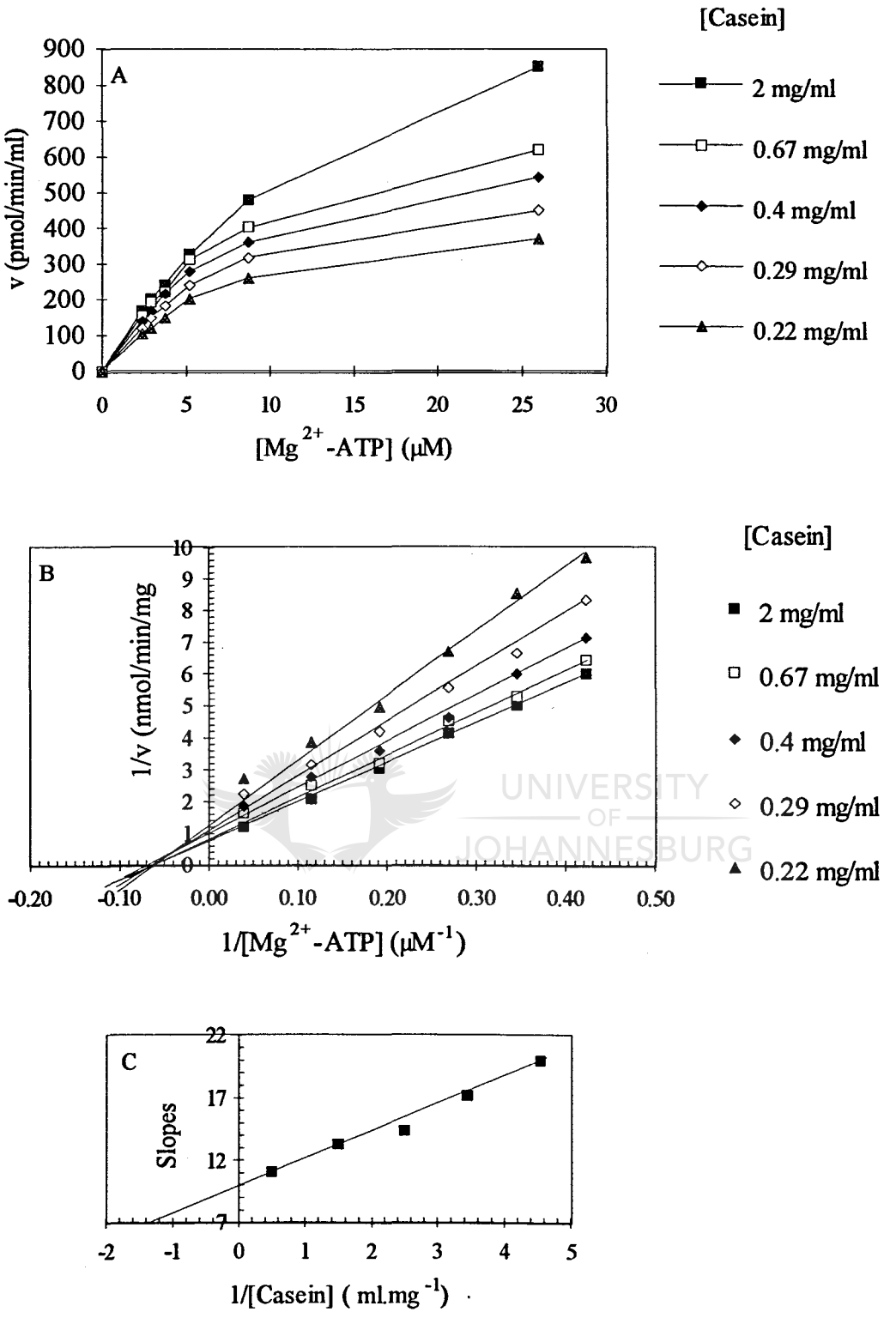



Figure 4.3.3.20 Initial velocity patterns for PK-II with varying ATP and casein concentrations. A: Michaelis-Menten plot of v *versus* [$Mg^{2+}$-ATP] at different fixed concentrations of casein. B: Double reciprocal plots of 1/v *versus* 1/[$Mg^{2+}$-ATP] at different fixed concentrations of casein. C: Secondary plot of slopes determined from plot B *versus* 1/[casein]. Conditions are as described under Section 4.2.3.

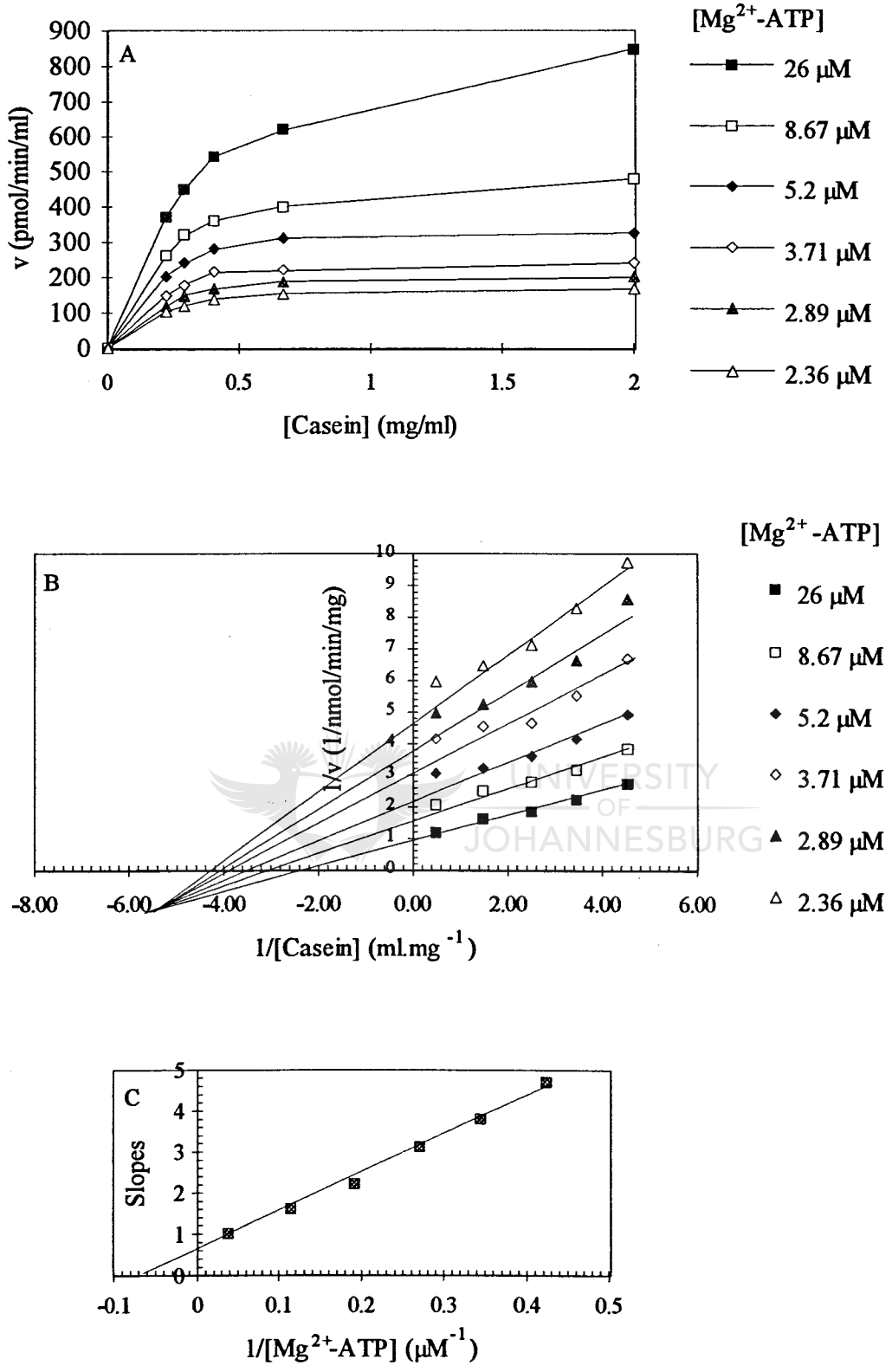



Figure 4.3.3.21 Initial velocity patterns for PK-II with varying ATP and casein concentrations. A: Michaelis-Menten plot of v *versus* casein at different fixed concentrations of [$Mg^{2+}$-ATP]. B: Double reciprocal plots of 1/v *versus* 1/[casein] at different fixed concentrations of [$Mg^{2+}$-ATP]. C: Secondary plot of slopes determined from plot B *versus* 1/[$Mg^{2+}$-ATP]. Conditions are as described under Section 4.2.3.

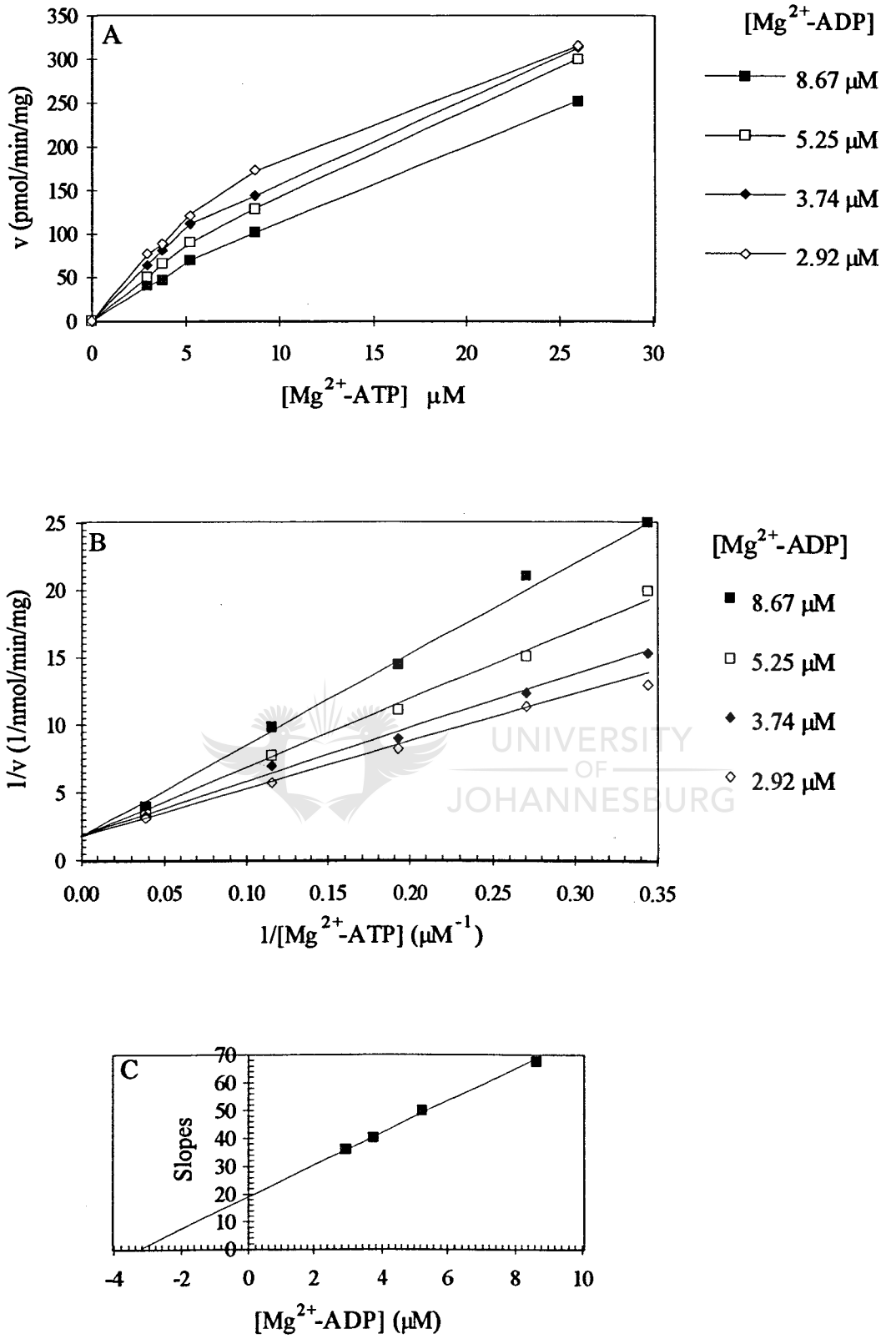



Figure 4.3.3.22 Product inhibition patterns of PK-II by ADP. A: Michaelis-Menten plot of v *versus* [$Mg^{2+}$-ATP] at 2 mg/ml casein with varying ADP concentration; B: Double reciprocal plots of 1/v *versus* 1/ [$Mg^{2+}$-ATP] at 2 mg/ml casein with varying ADP concentration; C: Secondary plot of slopes determined from plot B *versus* [$Mg^{2+}$-ADP]. Conditions are as described under Section 4.2.3.

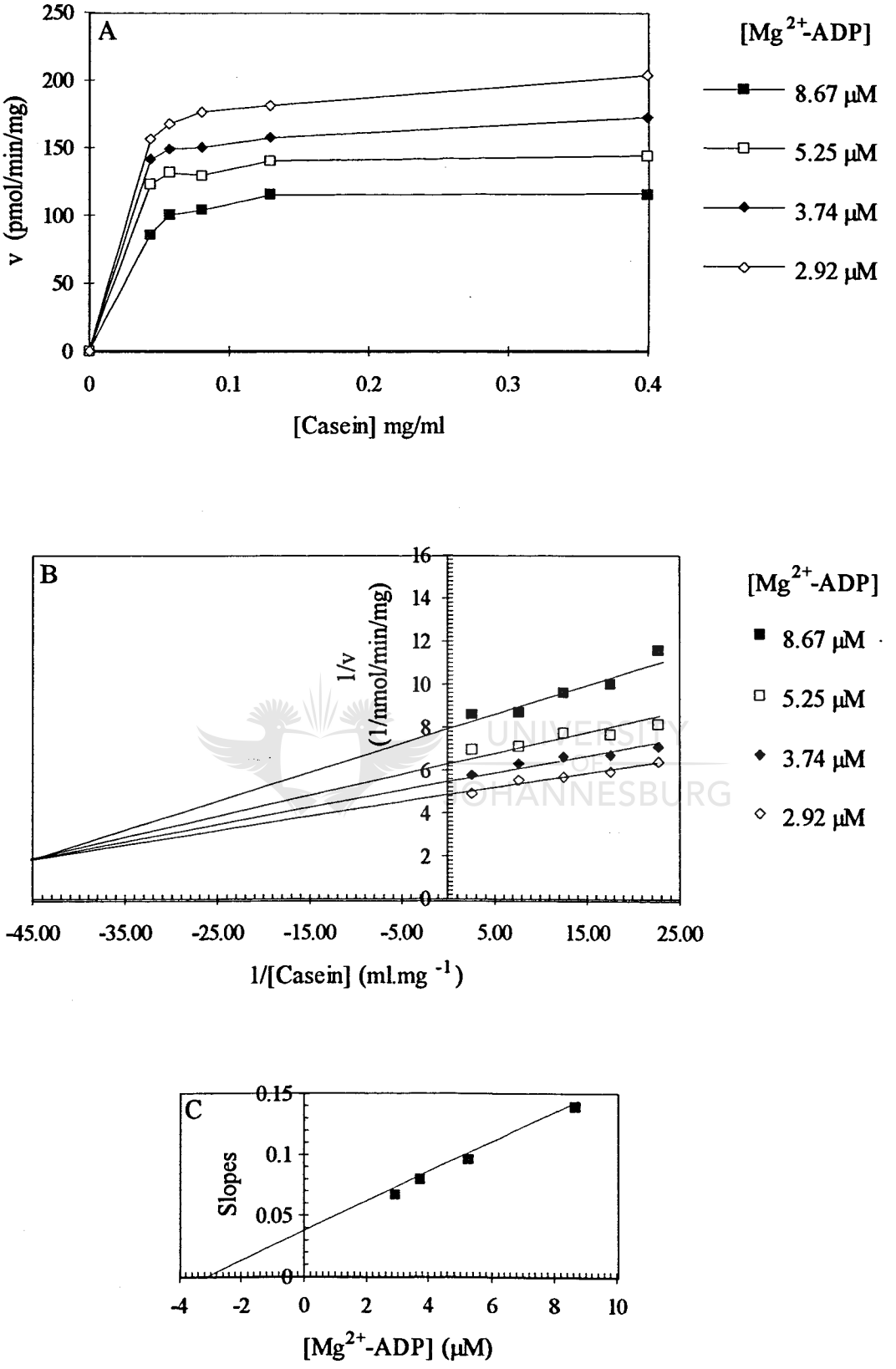



Figure 4.3.3.23 Product inhibition patterns of PK-II by ADP. A: Michaelis-Menten plot of v *versus* [Casein]at 26 µM ATP with varying ADP concentration; B: Double reciprocal plots of 1/v *versus* 1/[Casein] at 26 µM ATP with varying ADP concentration; C: Secondary plot of slopes determined from plot B *versus* [$Mg^{2+}$-ADP]. Conditions are as described under Section 4.2.3

## 4.3.4 Purification and Characterisation of Protein Kinase III (A $Ca^{2+}$-Dependent/Calmodulin-Independent Protein Kinase)

### 4.3.4.1 Purification and homogeneity of PK-III

The purification of PK-III from ripening mango fruit tissue is summarized in Table 4.3.4.1. PK-III, a protein kinase which catalyses the phosphorylation of the artificial substrate histone III-S and casein was purified 70-fold from mango fruit tissue as described under Section 4.2.11 and 4.2.14. PK-III was analysed by SDS-PAGE to assess its purity (Figure 4.3.4.5). Two protein-stained bands centred at $M_r$ 48 kDa and $M_r$ 50 kDa in lanes 5 and 6 were possibly a doublet. As discussed below, it was shown that these bands possess calcium-dependent protein kinase activity. The $V_{max}$ was 70-fold higher with calcium than without. Autophosphorylation was also stimulated by the addition of free calcium (Figures 4.3.4.18 and 4.3.4.19).

#### 4.3.4.1.1 Chromatographic properties of PK-III

Five column chromatography steps and an ammonium sulphate precipitation step were used to purify PK-III. The elution profiles of the five chromatographic steps are shown in Figures 4.3.1.2, 4.3.4.1, 4.3.4.2, 4.3.4.3 and 4.3.4.4. Only one peak of calcium-dependent casein phosphorylating activity was eluted from the DEAE-Sephacel with a 0 to 0.4 M NaCl gradient. PK-III activity could not be detected with histone HI (type III-S) as substrate at this stage because phosphorylation of histone HI (type III-S) was inhibited by the presence of increasing concentrations of NaCl (0.3 M). This could be a result of the salt disrupting ionic interactions between the positively charged histone and a negatively charged site(s) on the enzyme (Putnam-Evans et al. 1990).

Many $Ca^{2+}$ binding proteins undergo a conformational change in the presence of $Ca^{2+}$ such that a hydrophobic site is exposed (Moore & Dedman 1982, Putnam-Evans et al. 1990), allowing the protein to bind to hydrophobic resins such as phenyl-Sepharose. In this case it was not necessary to add $Ca^{2+}$ because the enzyme bound tightly to the hydrophobic resin and could only be eluted with a high percentage ethylene glycol (30-50%). PK-III is thus highly hydrophobic. Attempts to use the $Ca^{2+}$-dependent hydrophobic properties to elute PK-III failed completely. A large purification (38-fold) resulted from chromatography on phenyl-Sepharose (Figure 4.3.4.1). This highly hydrophobic nature of PK-III point towards the fact that it probably is a membrane associated protein.

The purification factor decreased after the use of the Cibacron blue-Sepharose chromatography step. The other protein kinases being purified bound tightly with Cibacron blue-Sepharose and could be separated from PK-III which could be eluted with 0.6 M NaCl (Figure 4.3.4.2). Endogenous calmodulin was also removed by this step.

Calmodulin ($M_r$ 16.7 kDa) co-purified with PK-III through the DEAE-Sephacel and phenyl-Sepharose chromatographic steps. A lightly Coomassie-stained low molecular weight band could be seen in the DEAE-Sephacel and Blue-Sepharose elution pools (Figure 4.3.4.5, lanes 2 and 3). Calmodulin was absent from the purified PK-III pool after the Cibacron blue-Sepharose step (Figure 4.3.4.5 lane 4). According to Putnam-Evans (1990) calmodulin does not bind to Blue-Sepharose. The activity peak from Blue-Sepharose was concentrated by means of centrifugal-ultrafiltration through a 30 k filter that could also remove calmodulin from the PK-III active pool. The concentration of PK-III by centrifugal-ultrafiltration with a 30 k cut off filter did not affect the activity of PK-III. Although precipitate formed, the total activity stayed the same. This means that this step did in effect serve as a purification step.

Molecular filtration chromatography on Toyopearl-HW55 and Sephadex-G75 resulted in a further 2-fold purification. The hydrophobic nature of PK-III possibly lead to non-specific adsorption

accompanied by high activity losses (Table 4.3.4.1). Since the activities of purified PK-III, like PK-I, are almost completely lost upon gel filtration, this step proved ineffective for improving purification. Appreciable recoveries however can be obtained if gel filtration is performed with more crude preparations, after the DEAE-Sephacel step. At this stage PK-III eluted from Sephadex G200 as a single peak with a molecular weight of approximately 50 kDa (Figure 4.3.4.7).

4.3.4.1.2 Homogeneity and stability

Purified PK-III, which eluted as a single peak of activity during gel filtration column chromatography (Figures 4.3.4.3 and 4.3.4.4), resolved into two protein bands of molecular weight 44 and 50 kDa on 10% SDS gels (Figures 4.3.4.5 and 4.3.4.6). The two proteins appear to be related and may have resulted from proteolysis. According to Harmon et al. (1987) and Putnam-Evans et al. (1990) the following observations may be in support of this possibility:

- several CDPK preparations have contained two bands whose molecular weights are smaller than 52 and 55 kDa;
- the relative proportions of the Coomassie stained bands in SDS gels vary among preparations;
- preliminary data from one-dimensional maps of $^{32}P$-labelled peptides resulted from digestion with V-8 protease were consistent with the bands generated by proteolysis;
- monoclonal antibodies to CDPK (calcium-dependent protein kinase) recognised both bands.

Although PK-III studies did not include protein mapping of protease digested polypeptides and monoclonal antibody studies on CDPKs, the first two events described above were also observed.

The hydrophobic nature of PK-III could explain why native PAGE experiments were not successful. High protein concentration and lipids in crude extracts kept this protein kinase in suspension and the same time stabilising it. High percentages of glycerol stabilised the enzyme to an extent. It is suspected that PK-III is a membrane associated protein. The ripening process results in a natural breakdown in membranes and thus make membrane associated proteins more soluble. The high concentration EGTA (3 mM) in the extraction medium also attributed to the release of membrane associated proteins (see discussion under Section 4.3.1.1).

### 4.3.4.2 Physico-chemical properties of purified / isolated PK-III

4.3.4.2.1 SDS-PAGE: Determination of the subunit structure and molecular weight

SDS-PAGE was used to determine the homogeneity, molecular weight and subunit structure of the purified enzyme as described under Section 4.2.6. Purified PK-III, which eluted as a single peak of activity during molecular sieve chromatography (Figures 4.3.4.3 and 4.3.4.4), resolved into two protein bands of molecular weight 50 and 44 kDa on 10% SDS gels (Figure 4.3.4.5). The molecular weight of 50 and 44 kDa agrees well with the size of 50 kDa determined by analytical gel chromatography (Section 4.3.4.2.2 and Figure 4.3.4.7). This indicated that the active form of the PK-III kinase was a monomer.

4.3.4.2.2 Analytical gel chromatography: Determination of the molecular weight

Analytical gel chromatography (Andrews 1970) on Sephadex G200 (described under Section 4.2.8) was used to determine the molecular weight of PK-III. The experimental information of the standardization process and the determined results are given in Figure 4.3.4.7. The molecular weight of PK-III isolated from mango fruit tissue was approximately 50 kDa.

The molecular weight of 50 kDa agrees well with the size of the two bands determined in 10% SDS gels (44 and 50 kDa; Figures 4.3.4.6). This confirmed that the active form of the kinase is a monomer.

### 4.3.4.3 Enzymatic properties of PK-III

#### 4.3.4.3.1 Effect of pH on catalysis

The enzyme displayed a broad pH optimum, with maximal activity occurring between pH 6.6 and 9.5 (Figure 4.3.4.8). A similar pH profile was obtained for the $Ca^{2+}$-dependent protein kinase (CDPK) purified by Yuasa & Muto (1992) from green alga *Dunaliella tertiolecta* and for the CDPK purified by Putnam-Evans (1990) from soybean. This pH optimum seems to be characteristic of CDPKs.

#### 4.3.4.3.2 Effect of temperature on catalysis

The effect of temperature on PK-III enzyme activity was determined between 10 and 50 °C (Figure 4.3.4.9). The optimum reaction temperature under standard reaction conditions described under Section 4.2.3 with histone III-S as exogenous substrate was 35 °C. The activation energy of the catalytic reaction (E*), the free energy (ΔG*), enthalpy (ΔH*) and entropy of formation (ΔS*) of the transition state (activation complex) from the reactants, was determined as described under Section 4.2.16.2.

The value of 41.18 kcal/mole for E* was calculated from the slope of the Arrhenius-plot of log v *versus* 1/T (Figure 4.3.4.10) as described under Section 4.2.16.2. It was assumed that the E* value stays constant throughout the temperature range tested.

The values of 20.02 kcal/mole and -25.84 cal/mole/K for ΔH* and ΔS* were calculated from the slope and intercept of the modified Arrhenius-plot of log v/T versus 1/T (Figure 4.3.4.11) as described under Section 4.2.16.2. From these two known values ΔG* was be calculated as 33.54 kcal/mole.



#### 4.3.4.3.3 Regulatory properties

- $Mg^{2+}$ and $Mn^{2+}$ dependency

PK-III requires $Mg^{2+}$ or $Mn^{2+}$ for its activity in addition to $Ca^{2+}$. The $Mg^{2+}$ optimum of the kinase was 5-10 mM, with inhibition of the enzyme activity observed above 10 mM $Mg^{2+}$ (Figure 4.3.4.12). In contrast the $Mn^{2+}$ optimum of PK-III was 1-3 mM, with inhibition of the enzyme observed above 5 mM $Mn^{2+}$. Under conditions tested, it seemed as if PK-III had a two fold increase in activity in the presence of a lower concentration $Mn^{2+}$ compared to the usual 8 mM $Mg^{2+}$.

- $Ca^{2+}$ dependency

Figure 4.3.4.13 shows the determination of $Ca^{2+}$ dependency of PK-III in the presence of casein at pH 7.3 (A) and histone HI type III-S at pH 8 (B and C).

The purified PK-III was activated 3-fold by $Ca^{2+}$ alone (Figure 4.3.4.13). The activation on the enzyme by $Ca^{2+}$ was not due to the presence of contaminating amounts of calmodulin. The concentration of $Ca^{2+}$ required for 50% activation of histone phosphorylation (for 80% homogenous PK-III) at pH 8 was 1 μM. Maximal activation occurred at 10 μM free $Ca^{2+}$ at pH 8. The phosphorylation of histone III-S by PK-III was stimulated 20-fold by the addition of > 10 μM-free calcium (Figure 4.3.4.13). At pH 7.3 and dephosphorylated casein as substrate, 0.5 mM $Ca^{2+}$ was required for 50% activation and 1.0 mM free $Ca^{2+}$ for maximal activation.

- Effector and inhibitor studies

To investigate the effects of calmodulin on PK-III activity, several experiments were carried out. The exogenous addition of calmodulin (1 μM) had no obvious effect on enzyme activity and did not change the $Ca^{2+}$ sensitivity of the enzyme in the presence of 8 mM $Mg^{2+}$ at pH 8 (Figure 4.3.4.12). In the presence of 2 mM $Mn^{2+}$ calmodulin had a slightly stimulating effect of 10% at $Ca^{2+}$ concentrations higher than 0.01 mM (Figure 4.3.4.13).

It was important to separate calmodulin from the kinase to determine whether or not it was required for the calcium-stimulated activity. From Figure 4.3.4.5 it was evident that calmodulin co-eluted with PK-III through the DEAE-Sephacel column as well as the phenyl-Sepharose column. Calmodulin only separated to a large extent on the blue-Sepharose column and the molecular exclusion chromatography steps. In an additional step calmodulin was removed by centrifugal-ultrafiltration by means of a 20 k filter in the presence of a buffer containing 3 mM EGTA. After the separation from calmodulin, both histone kinase activity and phosphorylation of endogenous proteins still showed stimulation by $Ca^{2+}$, indicating that this stimulation was calmodulin independent (Table 4.3.4.2).

On the other hand this protein kinase was inhibited by the calmodulin antagonists, chlorpromazine and calmidazolium in a dose-dependent manner over a range of 0 to 100 μM (Figure 4.3.4.14 and 4.3.4.15). This result does not counter the conclusion that the enzyme is calmodulin independent, since many other calmodulin-independent protein kinases that are regulated by $Ca^{2+}$, are inhibited by these drugs (Battey & Venis 1988, Bögre et al. 1988, Harmon et al. 1987). It was recently discovered that the amino-terminal catalytic domain of some plant CDPKs is similar to that of the calcium/calmodulin-dependent protein kinases, while the carboxy-terminal domain is similar to calmodulin: these regions are separated by a junction domain (Ma 1993, Harper et al. 1991). This phenomenon might offer an explanation why calmodulin antagonists inhibit PK-III activity while no external calmodulin is necessary for the activation of the enzyme.



There is some measure of concern among plant scientists regarding the use of calmodulin antagonists. All the evidence obtained from studies with calmodulin antagonists for the involvement of calmodulin should be considered suggestive because of their reported non-specific effects (Nishizuka 1984, Hepler & Wayne 1985, Raghothama et al. 1985).

4.3.4.3.4 Substrate specificity

PK-III displayed a somewhat narrow protein substrate specificity (Figure 4.3.4.16 and Table 4.3.4.3). PK-III did not phosphorylate histone II-S (0-4% incorporation of $^{32}P$ compared to 100% incorporation into 0.4 mg/ml histone HI type III-S), protamine sulphate and phosvitin. PK-III did however, phosphorylate casein and BSA (15-20%) to a much lesser extent than histone III-S (100%).

PK-III, utilising the positively charged histone III-S as substrate, might have a negatively charged site which is necessary for the interaction with the histone. Protamine, a polycation might interact with such a site and thus inhibit the activity of PK-III (Putnam-Evans et al. 1990). Polycations have been documented to affect the activity of a number protein kinases (Ahmed et al. 1985).

The substrate concentration dependency of histone II-S, histone III-S and casein phosphorylation by PK-III is shown in Figure 4.3.4.17. The phosphorylation of both the histone III-S and casein preparations showed non-linear progression curves with substrate inhibition at higher concentrations. The upper limit of enzyme activity for histone was obtained in a lower concentration (1 mg/ml) than that for casein (1.75 mg/ml). At a calcium concentration of 1 mM, histone III-S phosphorylation was higher in a reaction mixture containing 8 mM $MgCl_2$ than in a reaction mixture containing 2 mM $MnCl_2$.

Bovine histone HI (Sigma type III-S) in animals is known as a common substrate for a group of different protein kinases including animal protein kinase C, which need basic amino acids as specificity determinants. In the histone gene family the HI is the most variable, showing tissue and species specificities. Contrary to these diversities all histones have some homologous sequences that are conserved during evolution (Gantt & Key 1987). The first primary structure of a plant histone HI has been published. It revealed that the sequence surrounding the phenylalanine residue found near to the carboxyl terminus of the globular domain is extremely well conserved (Allan et al. 1980, Gantt & Key 1987).

Protein kinases are known to recognise the primary sequence of their substrate proteins around the phosphorylated residue. According to Olah et al. (1989) histone HI contains a specific serine residue that is recognised by both animal PK-C and plant CDPK. Although it is difficult to determine the extent of homology of animal $Ca^{2+}$/phospholipid-dependent (Ashendel 1985, Hunter 1987, Nishizuka 1984) and plant $Ca^{2+}$-dependent protein kinases (Harmon et al. 1987, Hetherington & Trewavas 1982, Schäfer et al. 1985), it is clear that the two enzymes show significant similarities in substrate specificity and phosphorylation sites (Olah et al. 1989). The substrate specificity of the plant and animal $Ca^{2+}$-dependent protein kinases presumably relies on the amino acid residues surrounding the phosphorylatable serine and/or on the similarity of substrate binding sites.

#### 4.3.4.3.5 Endogenous substrates

When phosphorylation was carried out with preparations from the different purification steps without the addition of exogenous substrate and then subjected to SDS-PAGE analysis, the presence of phosphorylated bands at positions in addition to that of $M_r$ 49 kDa and 44.7 kDa, could be detected (Figures 4.3.4.18 and 4.3.4.19). This indicated that the kinase could phosphorylate some of the endogenous protein substrates that co-purify with it. The $M_r$'s of seven of these endogenous substrates were tabulated under Table 4.3.4.4. The molecular weights of these phosphorylated proteins were 21.4 kDa, 36.3 kDa, 38 kDa, 46.8 kDa, 57.5 kDa, 70.8 kDa, and 85.1 kDa. The identities of the individual proteins are not known although it is known that these proteins were either phosphorylated on a serine or threonine (see Section 4.3.4.3.8).

#### 4.3.4.3.6 Autophosphorylation

Because the phosphorylation shown in Figure 4.3.4.18 lane 6 occurred with no exogenous substrate added, a possible interpretation of these results is that the 49 kDa and 44.7 kDa $Ca^{2+}$-dependent protein kinase(s) from ripening mango fruit tissue is capable of autophosphorylation. Many protein kinases are capable of autophosphorylation, and this property may be an important way to regulate the enzyme activity (Lickteig et al. 1988, Guo & Roux 1990, Soderling 1993). Very limited information is known about this aspect of kinases especially in plant cells. It was found that autophosphorylated CDPKs had increased activity (Bögre et al. 1988). Whether PK-III has a similar property remains to be tested. Details on the occurrence and function of autophosphorylation of plant protein kinases *in vivo* are not known. This autophosphorylation characteristic of PK-III confirmed the molecular weight determinations from SDS-PAGE and molecular filtration chromatography.

#### 4.3.4.3.7 Kinetic properties

- Initial velocity studies

Initial velocity studies, in the absence of products, were performed over a wide range of ATP and casein concentrations (2.36 to 26 μM and 0.22 to 2 mg/ml, respectively), in order to adequately define both the

kinetic mechanism and the kinetic constants of the reaction. In Figures 4.3.4.20 and 4.3.4.21 velocity is plotted *versus* $Mg^{2+}$-ATP or casein concentration respectively (Michaelis-Menten plot A), reciprocal velocity is plotted *versus* the reciprocal of $Mg^{2+}$-ATP or casein concentration respectively (Lineweaver-Burk plot B) and the slopes determined from the double-reciprocal plots is plotted *versus* the reciprocal of casein and $Mg^{2+}$-ATP concentrations respectively (Secondary plots C). The kinetic constants obtained from these plots are $K_m$ATP = 17 μM and $K_m$casein = 1 mg/ml. The $V_{max}$ for both was approximately 4.17 nmol/min/mg protein.

- Product inhibition studies

The results of initial velocity measurements made in the presence of the product (ADP) of the reaction did not show product-inhibition.

- Enzyme mechanism

The initial velocity pattern studies of PK-III follows a sequential, rather than a Ping-Pong mechanistic pathway. That is, both of the substrates bind to the enzyme prior to product release.

Being a $Ca^{2+}$-stimulated protein kinase, the kinetic studies on this enzyme proved to be much more complicated than that of PK-I and PK-II (kinetics being done without the addition of effectors like $Ca^{2+}$). Kinetic studies done with histone III-S as exogenous substrate proved to be non-linear and can not be interpreted. To avoid complications due to autophosphorylation and ligand-induced instability of the holoenzyme, others (Kwiatkowski et al. 1990, Whitehouse et al. 1983) have used the autonomous kinase domain for their study of the kinetic mechanism of calmodulin kinase II- and cAMP-dependent protein kinases.



4.3.4.3.8 Identification of amino acids phosphorylated by PK-III

It was clear from protein recognition anti-P-Tyr antibody studies (Section 4.2.9.2 and Figures 4.3.2.22 and 4.3.2.23) that PK-III showed no phospho-Tyr activity. Although it is known that histone III-S could also serve as a tyrosine substrate (Hirayama & Oka 1992), PK-III only phosphorylated this exogenous substrate on a Ser or Thr residue. On the other hand PK-III could serve as a endogenous substrate for PK-I (see discussion in Section 4.3.2.8). It is reported that phospho-Tyr kinases could control the activity of Ser and Thr protein kinases (Krebs et al. 1993).

### 4.3.4.4 Conclusion

The protein kinase described here is highly $Ca^{2+}$-sensitive, with activation at free $Ca^{2+}$ concentrations in the micro molar range, depending on the assay conditions. As such it has the potential to serve as a stimulus-response coupler in $Ca^{2+}$-regulated cell processes in ripening mango fruit tissue. Specifically, it could be an important participant in the ripening process, because these responses are known to involve the increased turnover of membrane inositol phospholipids (Paliyath & Poovaiah 1984, Leshem et al. 1984, Poovaiah & Reddy 1987), which, in turn, is often coupled to increases in intracellular $Ca^{2+}$. The increased cytoplasmic $Ca^{2+}$ concentration consequently may activate PK-III in the ripening fruit cells. The activated membrane protein kinase can then phosphorylate target membrane proteins. Schaller & Sussman (1988) have reported that oat root plasma membrane $H^+$-ATPase is activated or stimulated by $Ca^{2+}$-stimulated protein kinase.

The partially purified mango fruit calcium-dependent protein kinase (PK-III) showed several common characteristics with the soybean CDPK (Harmon et al. 1987, Putnam-Evans et al. 1990) and alfalfa CDPK (Bögre et al. 1988, Olah et al. 1989), such as the $M_r$'s, that are 46 to 51 kD and 50 to 65 kD, respectively, autophosphorylation properties and substrate specificity. These enzymes did not require externally added calmodulin for their activity.

The purified enzyme consisted of two related polypeptides of molecular weight 43.7 and 50 kDa in 10% SDS-polyacrylamide gels (Figures 4.3.4.5 and 4.3.4.6). These did not represent two different subunits since PK-III eluted from a gel filtration column with a molecular weight of 50 kDa (Figure 4.3.4.7), and several lines of evidence indicate these bands were generated by proteolysis.

The *in vitro* protein substrates identified for PK-III were histone H-I type III-S and to a much lesser extent dephosphorylated casein and BSA. According to Putnam-Evans et al. (1990) histone is probably not an *in vivo* substrate, since its phosphorylation by soybean CDPK is inhibited in the presence of physiological concentrations of NaCl. Similar observations were made for PK-III. See discussion under Section 4.3.4.1.

The phosphorylation of a number of endogenous proteins by PK-III was observed, but the identity of the individual proteins (Figure 4.3.4.18) is not yet known. Determination of the amino acid substrates of the enzyme showed that PK-III is a serine/threonine protein kinase (Section 4.3.4.3.8).

Antibody and sequence data would allow for a more rigorous evaluation of the relationship of PK-III from ripening mango fruit tissue to the CDPKs that have been purified from other plant material and from animal sources.

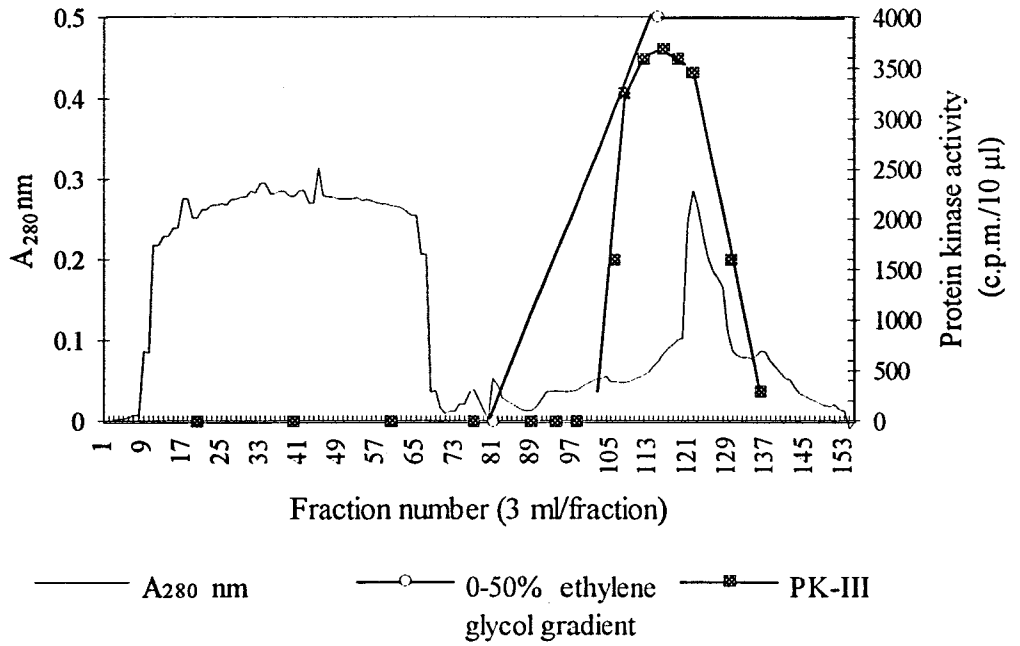



Figure 4.3.4.1 Elution profile of PK-III from mango extracts by means of hydrophobic chromatography on a phenyl-Sepharose column. The experimental conditions are described under Section 4.2.14.2. Protein content of eluted fractions was determined by absorption at 280 nm. Activity of PK-III was measured, using dephosphorylated casein as substrate in the presence of 1 mM $CaCl_2$.



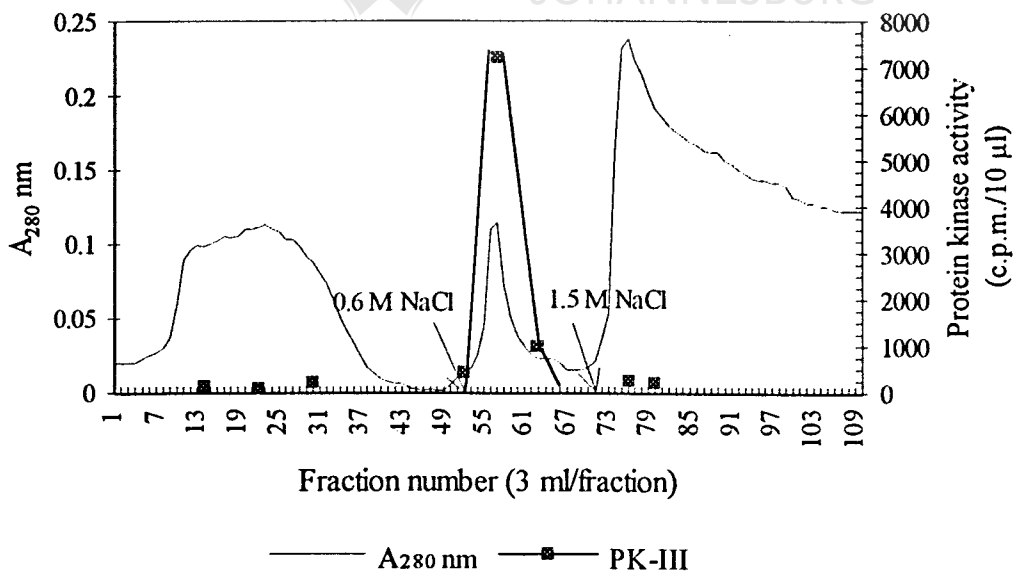



Figure 4.3.4.2 Elution profile of PK-III from a Cibacron Blue-Sepharose column after affinity chromatography. The experimental conditions are described under Section 4.2.14.3. Protein content of eluted fractions was determined by absorption at 280 nm. Activity of PK-III was measured, using dephosphorylated casein as substrate in the presence of 1 mM $CaCl_2$.

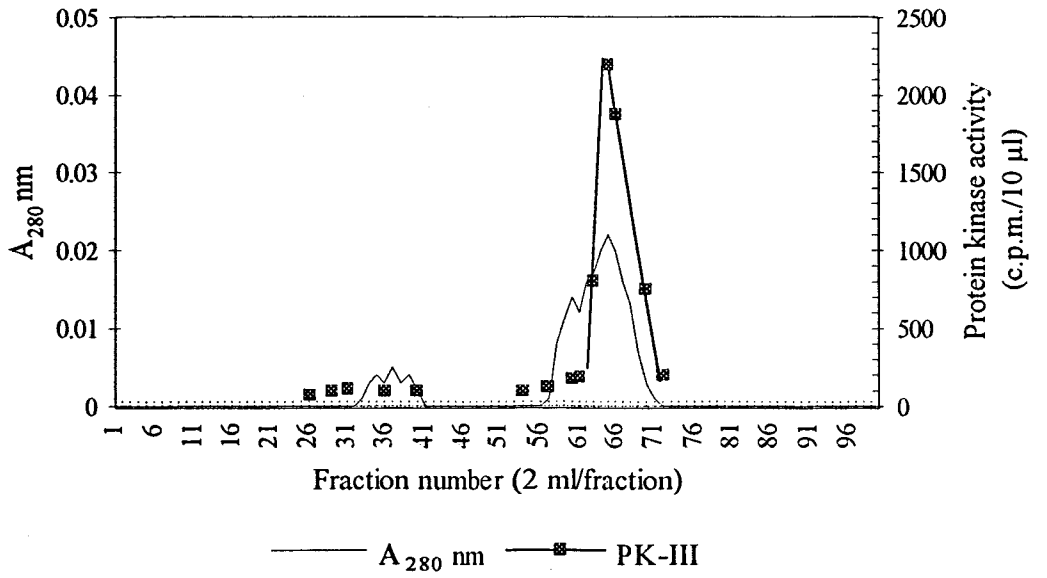



Figure 4.3.4.3 Purification of PK-III by means of exclusion chromatography on a Toyopearl HW-55F column. The experimental conditions are described under Section 4.2.14.4. Protein content of eluted fractions was determined by absorption at 280 nm. Activity of PK-III was measured, using dephosphorylated casein as substrate in the presence of 1 mM $CaCl_2$.



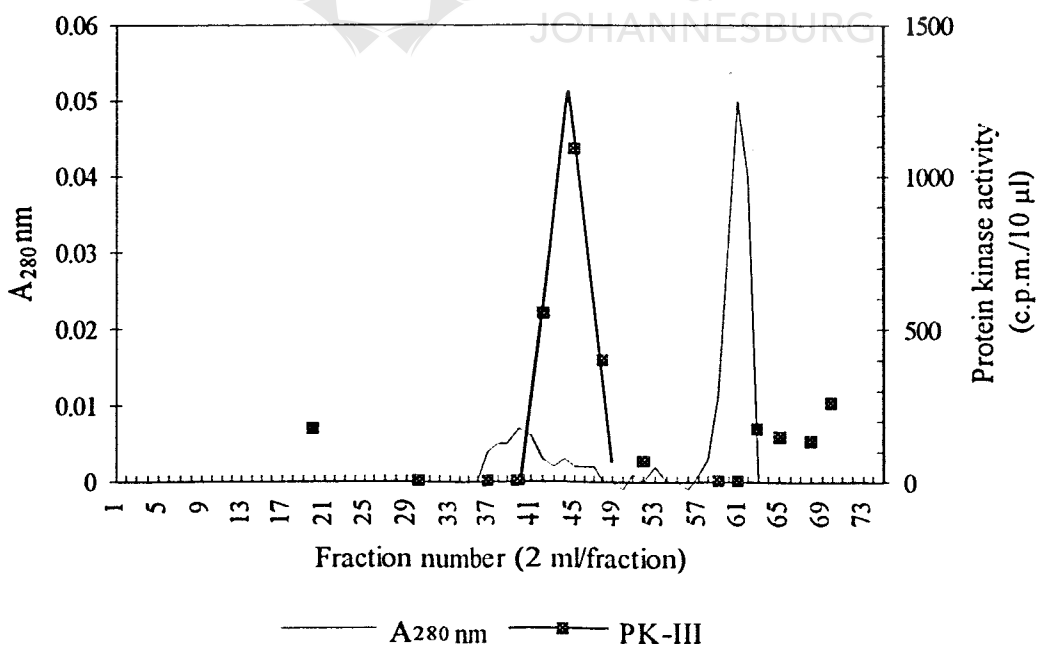



Figure 4.3.4.4 Purification of PK-III by means of exclusion chromatography on a Sephadex G-75SF column. The experimental conditions are described under Section 4.2.14.4. Protein content of eluted fractions was determined by absorption at 280 nm. Activity of PK-III was measured, using dephosphorylated casein as substrate in the presence of 1 mM $CaCl_2$.

Table 4.3.4.1 Summary of purification of PK-III from ripening mango fruit tissue

| Purification step | Volume (ml) | [Protein] (mg/ml) | Protein (mg) | Activity (pmol/min/ml) | Total activity (pmol/min) | Specific activity (pmol/min/mg) | Yield (%) | Purification (x-fold) |
|---|---|---|---|---|---|---|---|---|
| 1. Crude homogenate | 2580 | 0,125 | 322,5 | 16 | 41280 | 128 | 100 | 1 |
| 2. Ammonium sulphate precipitation (30-70%) | 137 | 2,68 | 367,16 | 627 | 85899 | 234 | 208* | 2 |
| 3. DEAE-Sephacel | 170 | 1 | 170 | 341 | 57970 | 341 | 140 | 3 |
| 4. Phenyl-Sepharose | 102 | 0,035 | 3,57 | 314 | 32028 | 8971 | 77,59 | 70 |
| 5. Blue-Sepharose | 4 | 0,541 | 2,164 | 752 | 3008 | 1390 | 7,29 | 11 |
| 6. Toyopearl-HW55 | 2 | 0,23 | 0,46 | 451 | 902 | 1961 | 2,19 | 15 |
| 7. Sephadex G75 | 10 | 0,004 | 0,04 | 37 | 365 | 9125 | 0,88 | 71 |

Substrate used was casein.

*The removal of protein kinase inhibiting substances by the previous purification steps result in a higher apparent yield of PK-III.

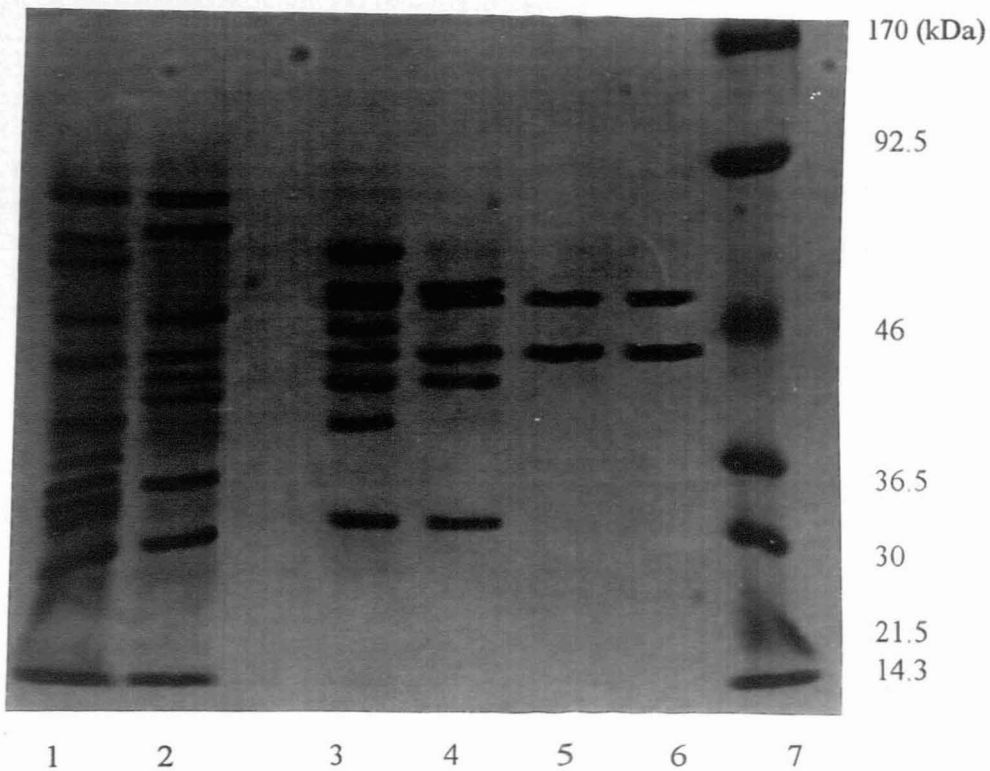



Figure 4.3.4.5 SDS-Polyacrylamide gel electrophoretic analysis of proteins from each stage of purification of PK-III. Aliquots from each stage of purification were treated as described in Section 4.2.6 and resolved in a 10% polyacrylamide gel in the presence of SDS and stained with Coomassie blue. Lane 7 contained the following molecular weight markers (in kDa): macroglobulin, 170; phosphorylase b, 97.4; ovalbumin, 46; lactate dehydrogenase, 36.5; carbonic anhydrase, 30; trypsin inhibitor, 21.5. and lysozyme, 14.3. Other lanes show the following fractions from each step of the purification procedure: lane 1, ammonium sulphate precipitation pool; lane 2, DEAE pool; lane 3, Blue-Sepharose pool; lane 4, Phenyl-Sepharose pool; lane 5, HW-55 pool; and lane 6, G75 pool

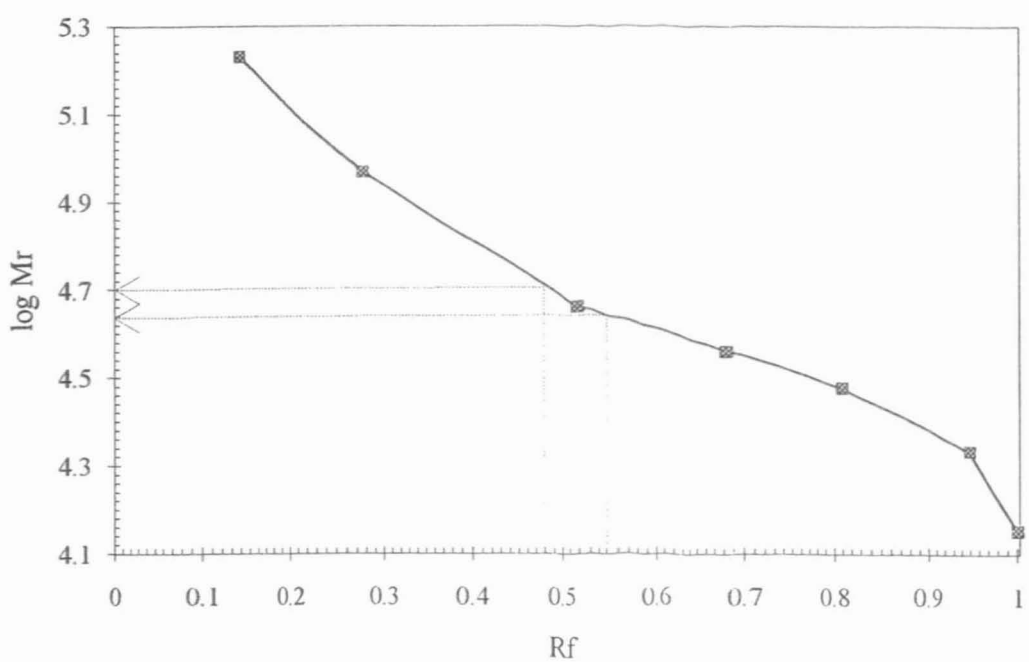



Figure 4.3.4.6 Plot of log $M_r$ versus $R_f$ for the determination of the $M_r$'s of PK-III. The determined weights in kDa were 50 and 43.7.

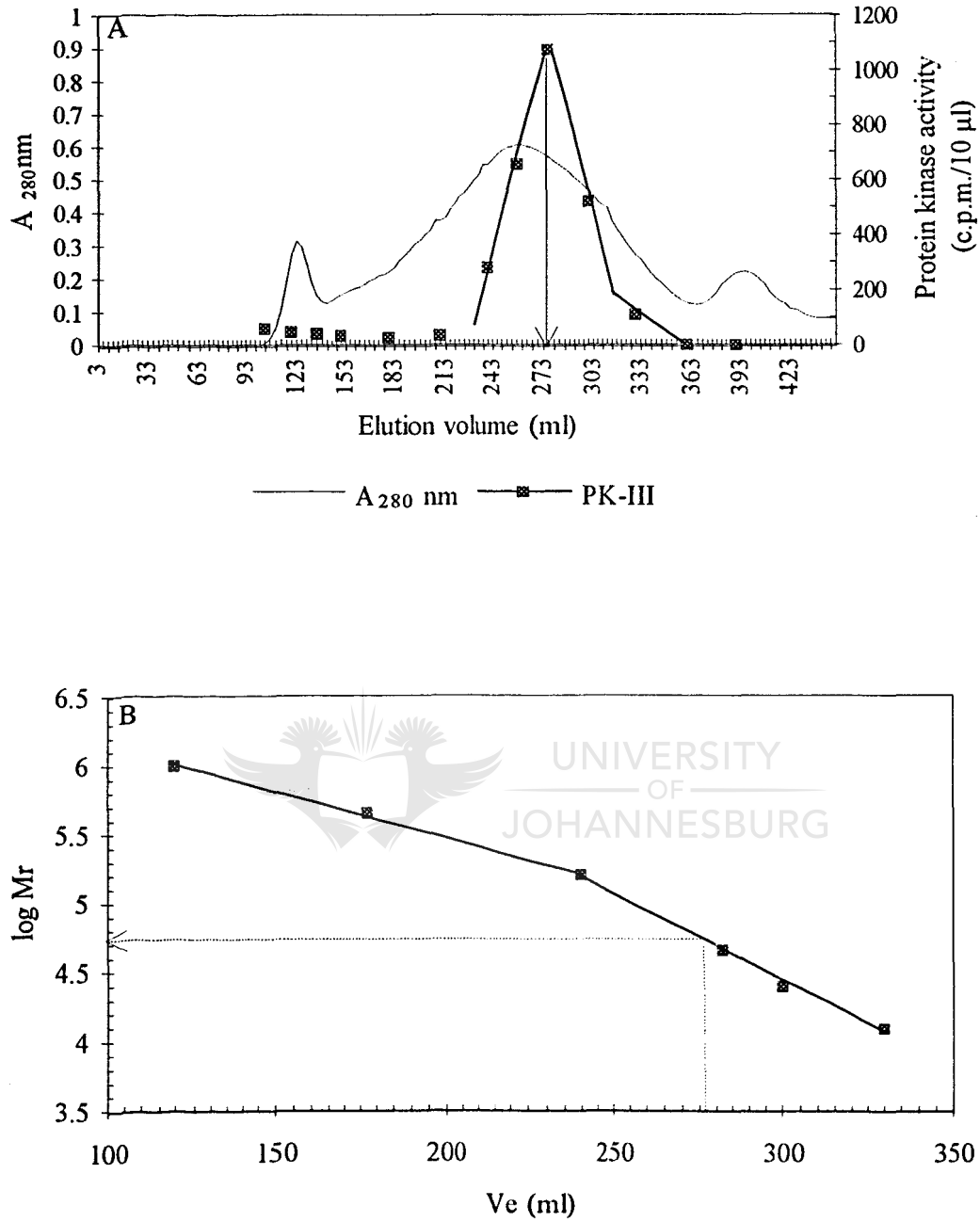



Figure 4.3.4.7 A: Separation of PK-III by means of gel filtration chromatography on a Sephadex G200 column. The experimental conditions are described under Section 4.2.8. The bed volume of the column was 373 ml, the flow rate was 8 ml/h and 3 ml fractions were collected. B: Plot of elution volume ($V_e$) versus molecular weight ($M_r$) for the determination of the molecular weight of PK-III by means of analytical gel chromatography on Sephadex G-200. Standards used (in kDa) were blue dextrin, 1000; ferritin, 450; aldolase, 158; ovalbumin, 45 kDa; chymotrypsinogen A, 25 kDa; lysozyme, 14.4 and β-ME, 0.078 kDa. The $M_r$ of PK-III is ca. 50 kDa.

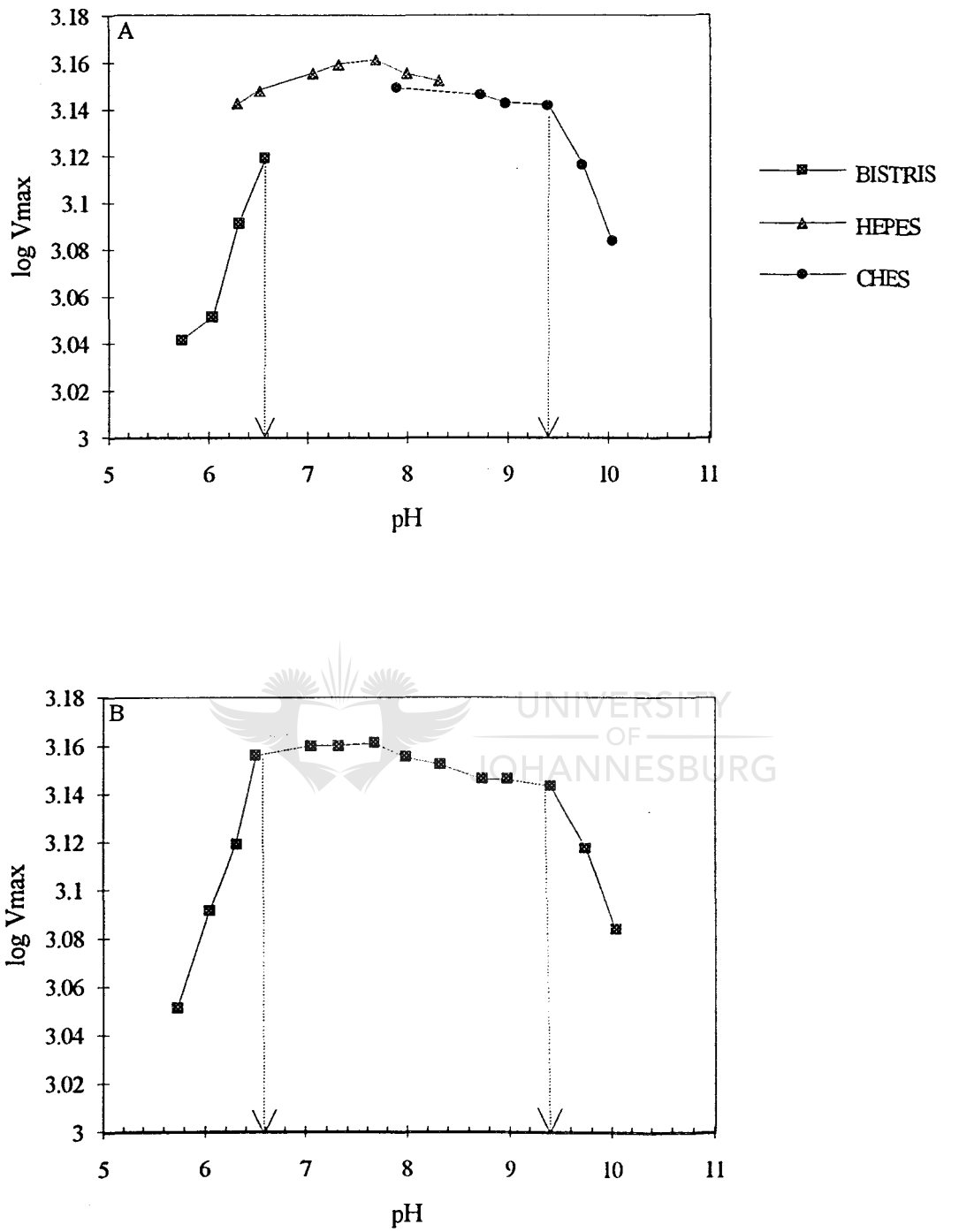





Figure 4.3.4.8 pH dependence of PK-III. Plots of log $V_{max}$ *versus* pH. For reaction conditions see Section 4.2.3 and 4.2.16.1. The value of each point is the average of two determinations in duplicate. PK-III has a broad pH optimum between pH 6.6 and 9.5. A: Activities of PK-III were determined in three different buffers namely, 100 mM BISTRIS (pKa = 6.5), 100 mM HEPES (pKa = 7.33) and 100 mM CHES (pKa = 9.5). B: The pH-activity curves determined in the different buffers were extrapolated to give one curve.

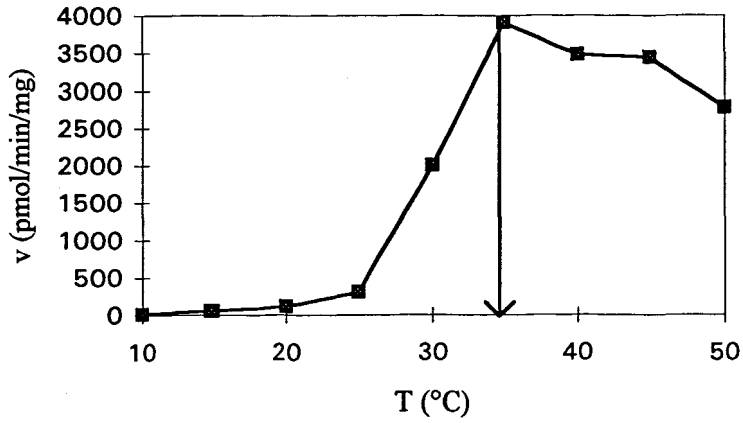



Figure 4.3.4.9 Effect of temperature on PK-III activity. The optimum reaction temperature at the standard reaction conditions as described under Section 4.2.3 was ca. 35°C. Histone IIIS was used as exogenous substrate. v = Initial reaction rate.

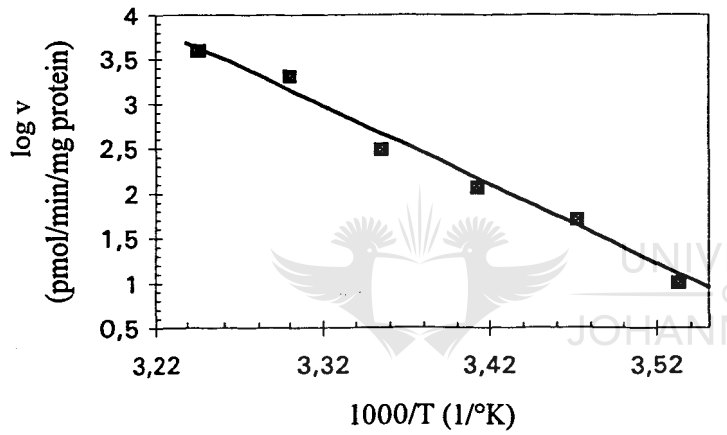



Figure 4.3.4.10 Arrhenius-plot for the determination of the activation energy of PK-III in HEPES buffer (100mM, pH 7.3) at saturated levels of ATP (26 µM) and histone IIIS (0.8 mg/ml). v = Initial reaction rate. Slope = 9000 and calculated activation energy (E*) = 41.18 kcal /mole.



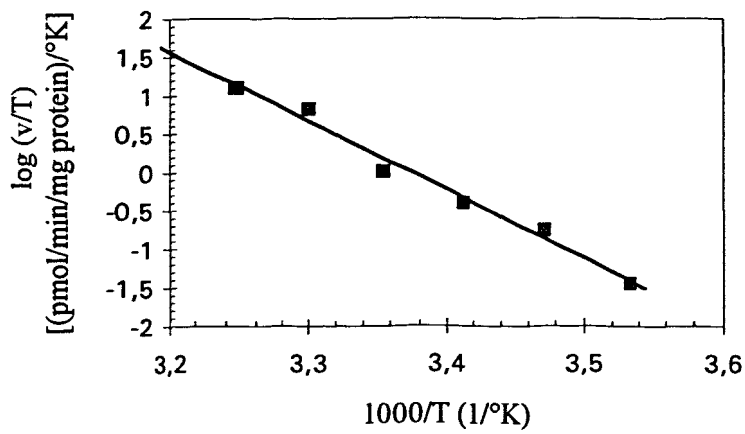



Figure 4.3.4.11 Modified Arrhenius-plot for the determination of the free energy, enthalpy and entropy of formation of the transition state of the PK-III reaction in HEPES buffer (100mM, pH 7.3) at saturated levels of ATP (26 µM) and histone IIIS (0.8 mg/ml). v = Initial reaction rate. Slope = -4375 and intercept = 1.65. Calculated $\Delta G$ = 33.54 kcal /mole, $\Delta H$ = 20.02 kcal/mole, and $\Delta S$ = -25.84 cal/mole/K.

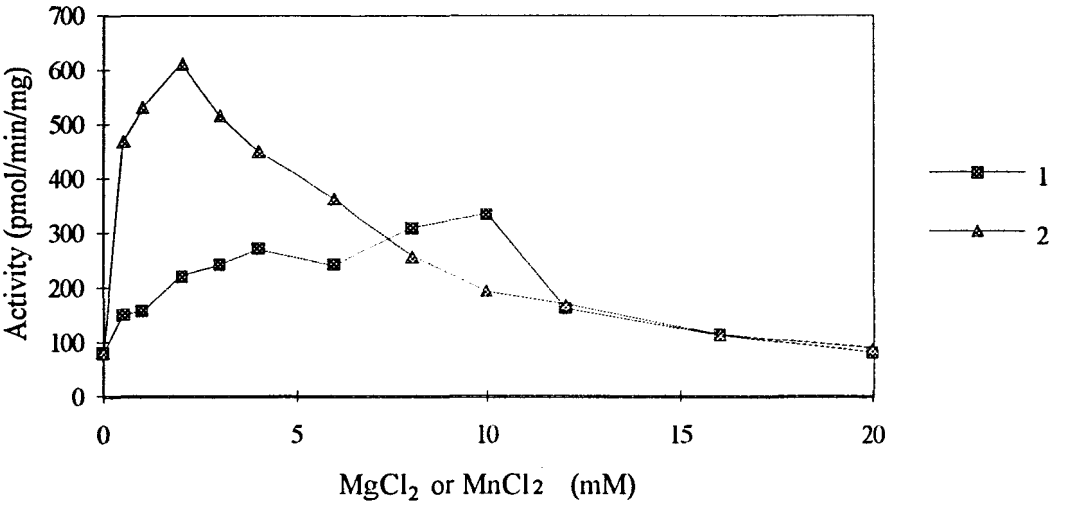
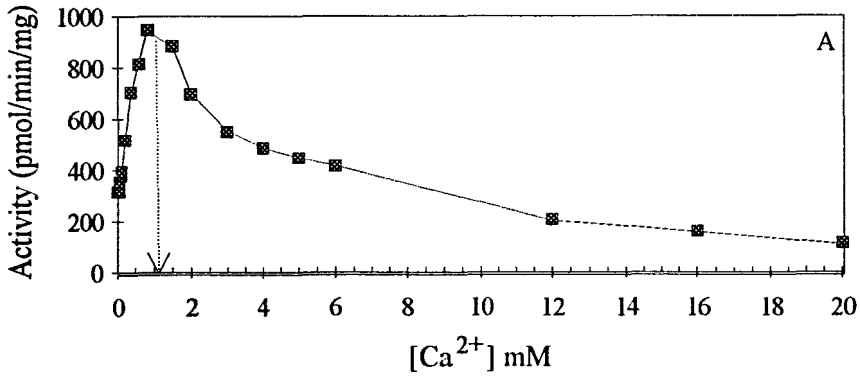
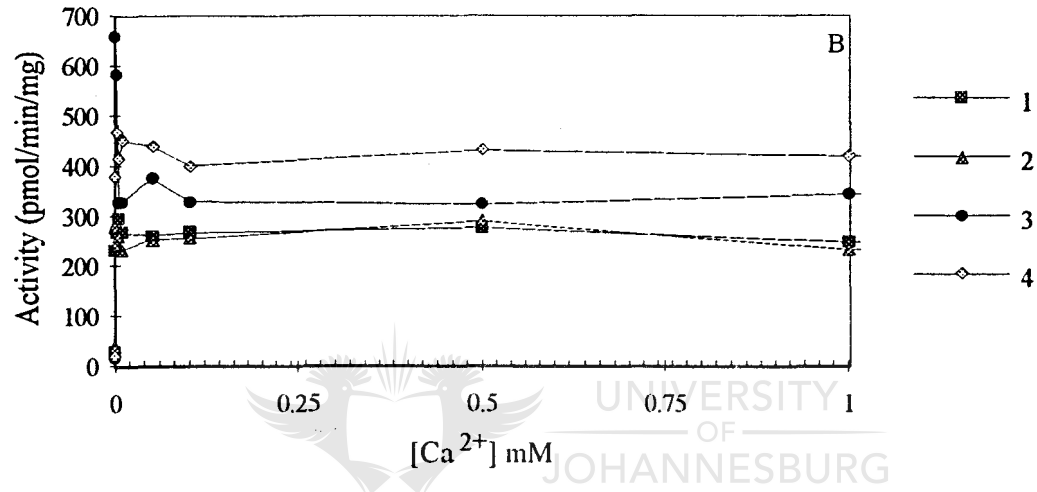



Figure 4.3.4.12 Effect of 1) $MgCl_2$ and 2) $MnCl_2$ on PK-III activity. PK-III assays were performed as described under Section 4.2.3 at pH 8 with 1.3 mg/ml histone III-S as exogenous substrate and 1 mM $CaCl_2$. The value of each point is the average of two determinations in duplicate.



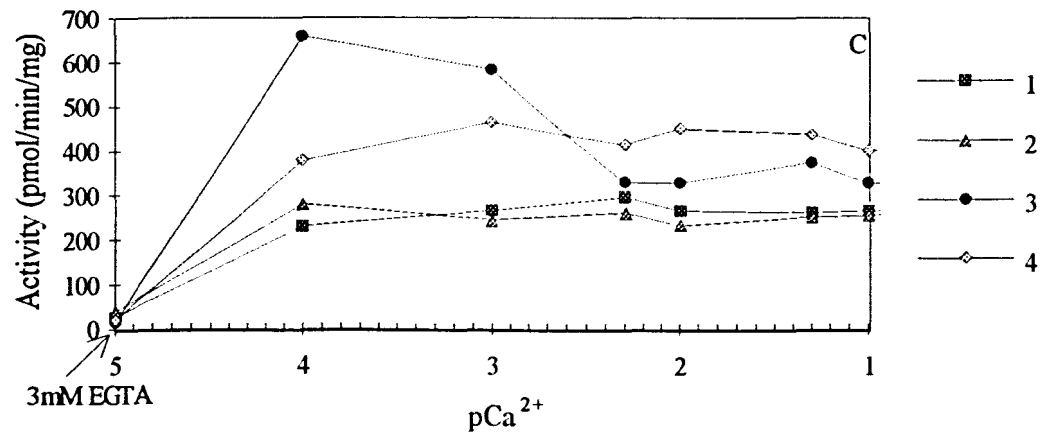




Figure 4.3.4.13 Effect of calcium on PK-III activity. Assays were performed as outlined under Section 4.2.3 in a calcium/EGTA buffer. Values are averages of duplicates. A: Plot of activity *versus* $[Ca^{2+}]$, 0 - 20 mM range. Casein was used as exogenous substrate and 8 mM $MgCl_2$ was present in the assay mixture at pH 7.3. B: Plot of activity *versus* $[Ca^{2+}]$, 0 - 1 mM range. C: Plot of activity *versus* $pCa^{2+}$. The different curves in B and C represents the presence of 1) 8 mM $MgCl_2$, 2) 8 mM $MgCl_2$ + 1 mM exogenous calmodulin; 3) 2 mM $MnCl_2$; 4) 2 mM $MnCl_2$ + 1 mM exogenous calmodulin. The results in plots B and C were obtained from assays at pH 8 with histone III-S as exogenous substrate.

Table 4.3.4.2 Effects of various agents on PK-III activity. Values are the means of duplicate determinations. The enzyme activity was determined as described under Section 4.2.3 in the presence of 1 mM $CaCl_2$ under standard assay conditions with 1.3 mg/ml histone III-S as exogenous substrate.

| Agent | Concentration | Maximal activity (%) |
|---|---|---|
| Heparin | 30 µg/ml | 116 |
| Calmidazolium | 250 µM | 18 |
| Chlorpromazine | 112 µM | 58 |
| cAMP | 17 µM | 88 |
| Calmodulin | 1 µM | 69 |
| DMSO | 16% | 97 |
| Control | | 100 |

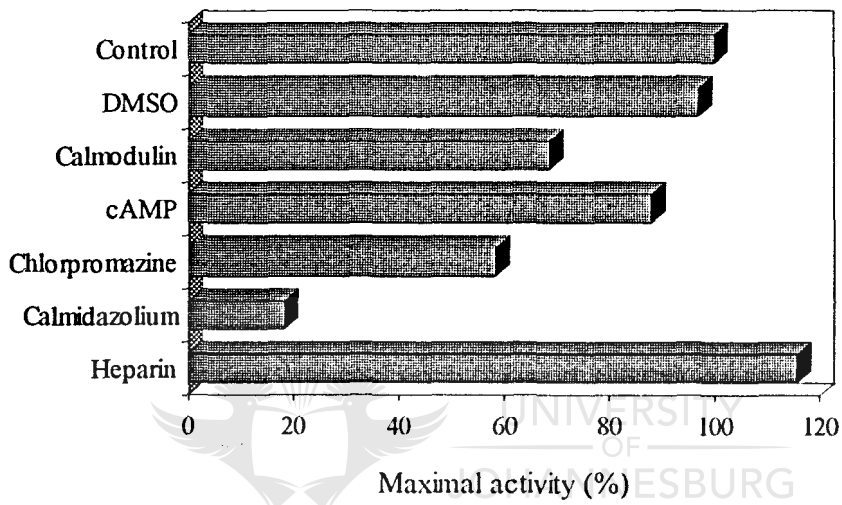



Figure 4.3.4.14 Graphical representation of the effects of the various effectors and inhibitors tabulated in Table 4.3.4.2 on PK-III activity.

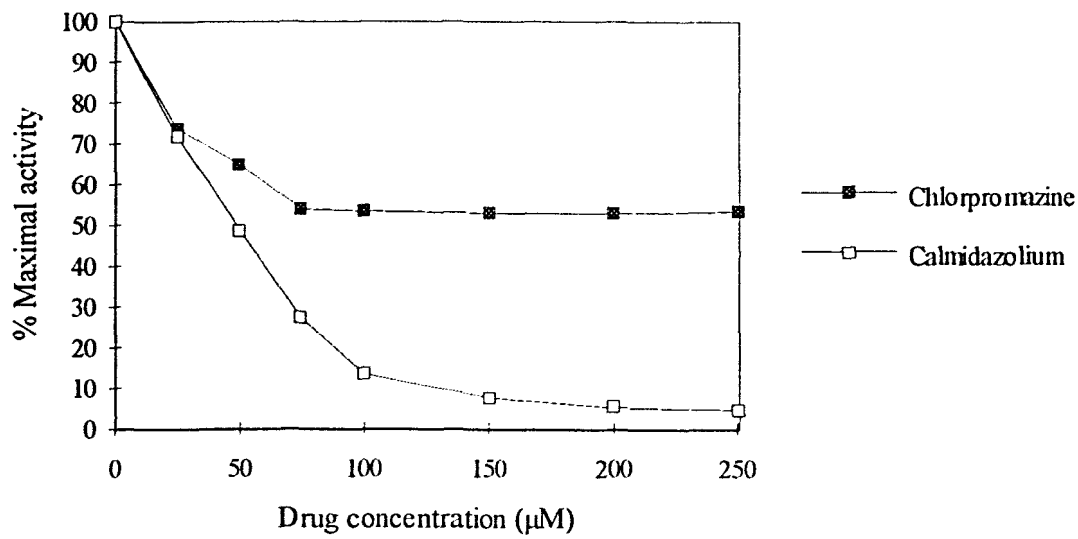



Figure 4.3.4.15 Effect of chlorpromazine and calmidazolium on PK-III activity. Values are the means of duplicate determinations. The enzyme activity was determined as described under Section 4.2.3 in the presence of 2 mM $MnCl_2$, 1 mM $CaCl_2$ and 1.3 mg/ml histone III-S as exogenous substrate.

Table 4.3.4.3 Substrate specificity of PK-III from ripening mango fruit tissue. Values are the means of duplicate determinations. The enzyme activity was determined as described under Section 4.2.3 in the presence of 1 mM $CaCl_2$ with 0.4 mg/ml of each of the exogenous substrates tested and 21 mg PK-III. 100% activity was 893 nmol/min/mg.

| Protein | Activity (nmol/min/mg) | Relative activity (%) |
|---|---|---|
| Casein | 161 | 18 |
| BSA | 121 | 14 |
| Phosvitin | 27 | 3 |
| Protamine | 27 | 3 |
| Histone type II-S | 2 | 0 |
| Histone type III-S | 893 | 100 |
| No substrate | 35 | 4 |



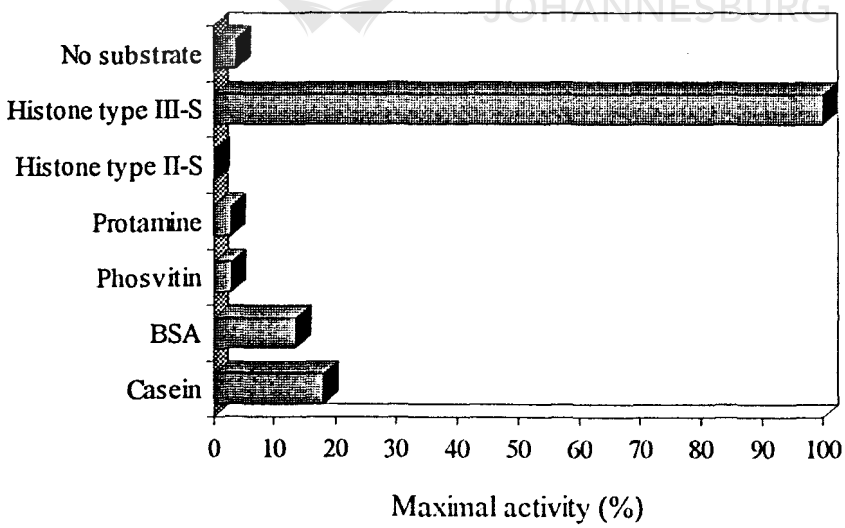



Figure 4.3.4.16 Graphical representation of the substrate specificity of PK-III tabulated in Table 4.3.4.3.

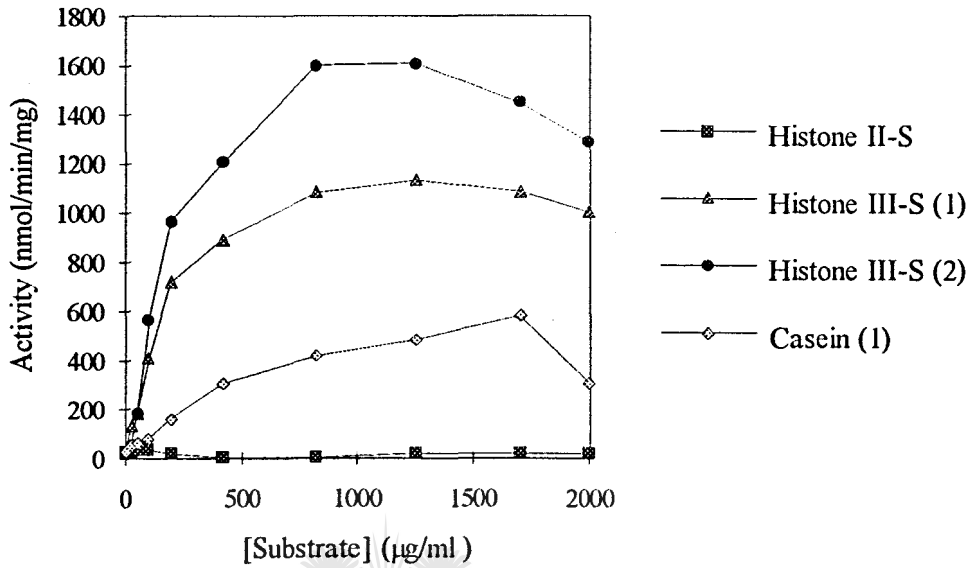





Figure 4.3.4.17 Substrate concentration dependency of histone II-S, histone III-S and casein phosphorylation by PK-III isolated from ripening mango fruit tissue. Histone II-S and histone III-S(1) were incubated with enzyme fractions in the standard incubation media in the presence of 1 mM $CaCl_2$ and 2 mM $MnCl_2$. Histone III-S(2) and casein were incubated with enzyme fractions in the standard incubation media (Section 4.2.3) in the presence of 1 mM $CaCl_2$ and 8 mM $MgCl_2$. Values are the means of duplicate determinations.

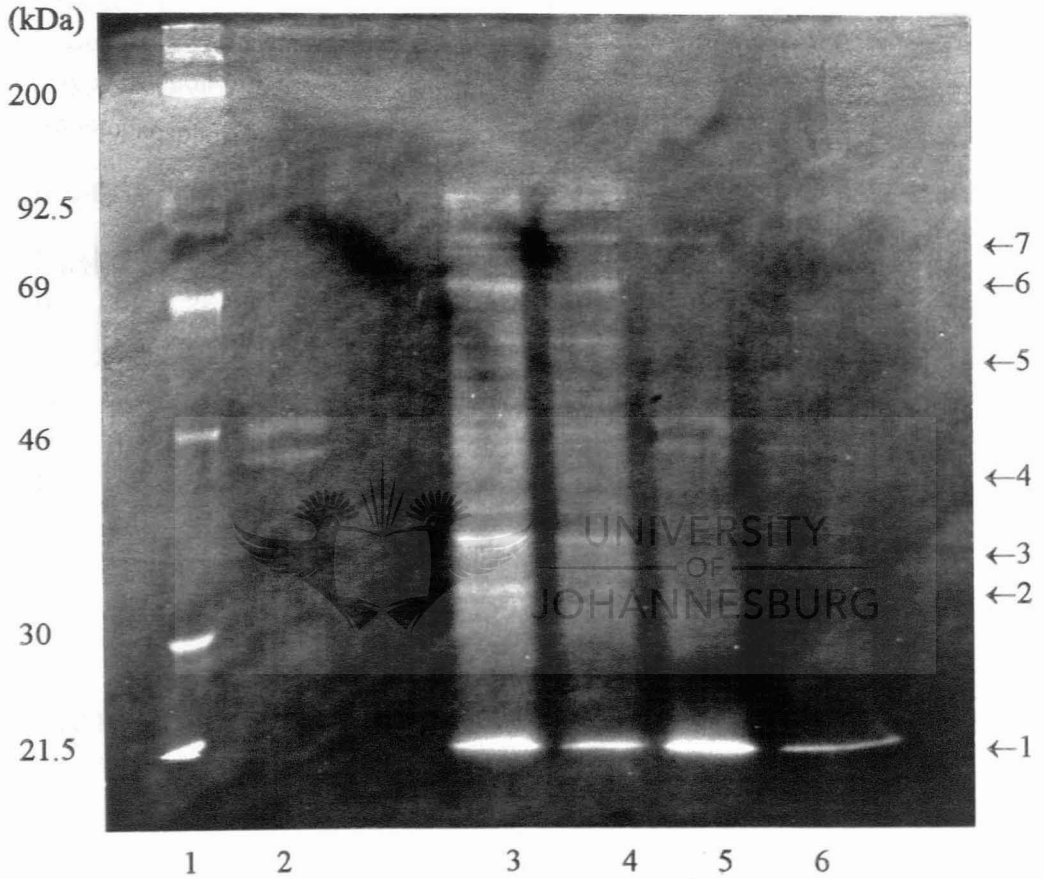





Figure 4.3.4.18 Autoradiogram showing the autophosphorylation of the two PK-III bands, 45 kDa and 49 kDa, as well as some endogenous substrates in the ammonium sulphate precipitation and DEAE fractions separated by SDS-polyacrylamide electrophoresis. Lane 2, purified PK-III from Sephadex G75 pool; lane 3, ammonium sulphate precipitation fraction; lane 4, DEAE-Sephacel PK-III pool; lane 5, phenyl Sepharose PK-III pool; and lane 6, Cibacron blue Sepharose pool. Lane 1 contained $^{14}C$-labelled protein molecular weight markers tabulated under Table 4.3.4.4. The phosphorylated proteins were also tabulated in Table 4.3.4.4. Experimental conditions were described under Section 4.2.6. The results were described under Sections 4.3.4.3.5 and 4.3.4.3.6.

Table 4.3.4.4 Tabulation of the molecular weights of phosphorylated proteins determined from the autoradiogram showed in Figure 3.4.3.18

| Standards | $R_f$ | $M_r$ (Da) | log $M_r$ | Relative intensity of bands |
|---|---|---|---|---|
| Myosin | 0.1031 | 200000 | 5.30 | |
| Phosphorylase b | 0.2783 | 92500 | 4.96 | |
| BSA | 0.381 | 69000 | 4.83 | |
| Ovalbumin | 0.562 | 46000 | 4.66 | |
| Carbonic anhydrase | 0.8454 | 30000 | 4.47 | |
| Trypsin inhibitor | 0.99 | 21500 | 4.33 | |
| Lysozyme | 1 | 14300 | 4.15 | |
| PK-III (autophosphorylated) | 0.541 | 49 000 | 4.69 | +++ |
| | 0.577 | 44 700 | 4.65 | +++ |
| Endogenous substrates: | | | | |
| 1. | 1 | 21 400 | 4.33 | +++ |
| 2. | 0.715 | 36 300 | 4.56 | + |
| 3. | 0.673 | 38 000 | 4.58 | +++ |
| 4. | 0.56 | 46 800 | 4.67 | ++ |
| 5. | 0.451 | 57 500 | 4.76 | ++ |
| 6. | 0.363 | 70 800 | 4.85 | +++ |
| 7. | 0.301 | 85 100 | 4.93 | ++ |



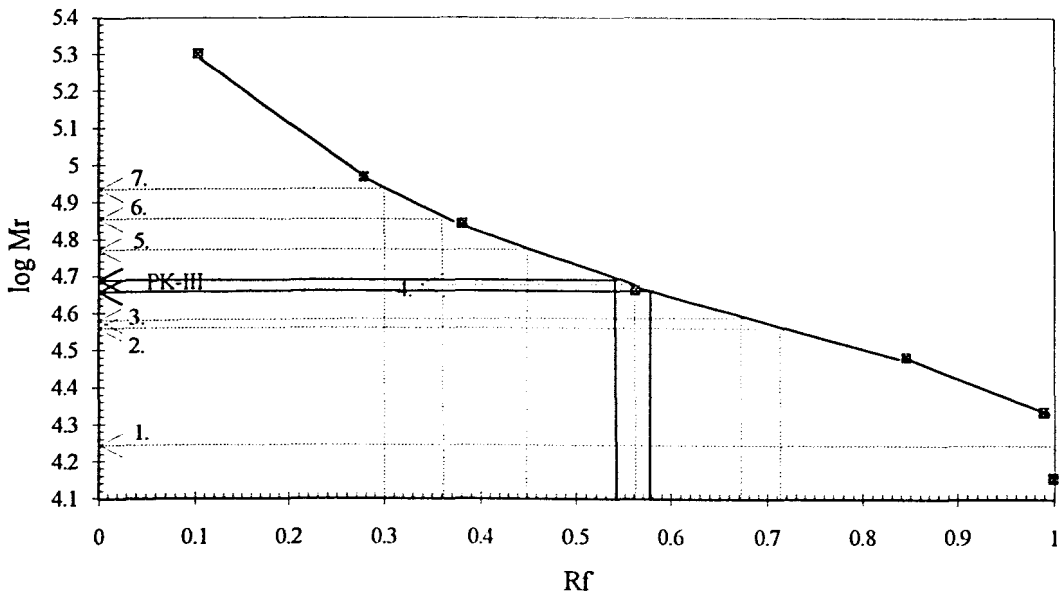



Figure 4.3.4.19 Plot of log $M_r$ *versus* $R_f$ for the determination of the $M_r$'s of the endogenous phosphorylated proteins including the autophosphorylated PK-III. The determined weights in Da were tabulated in Table 4.3.4.4.

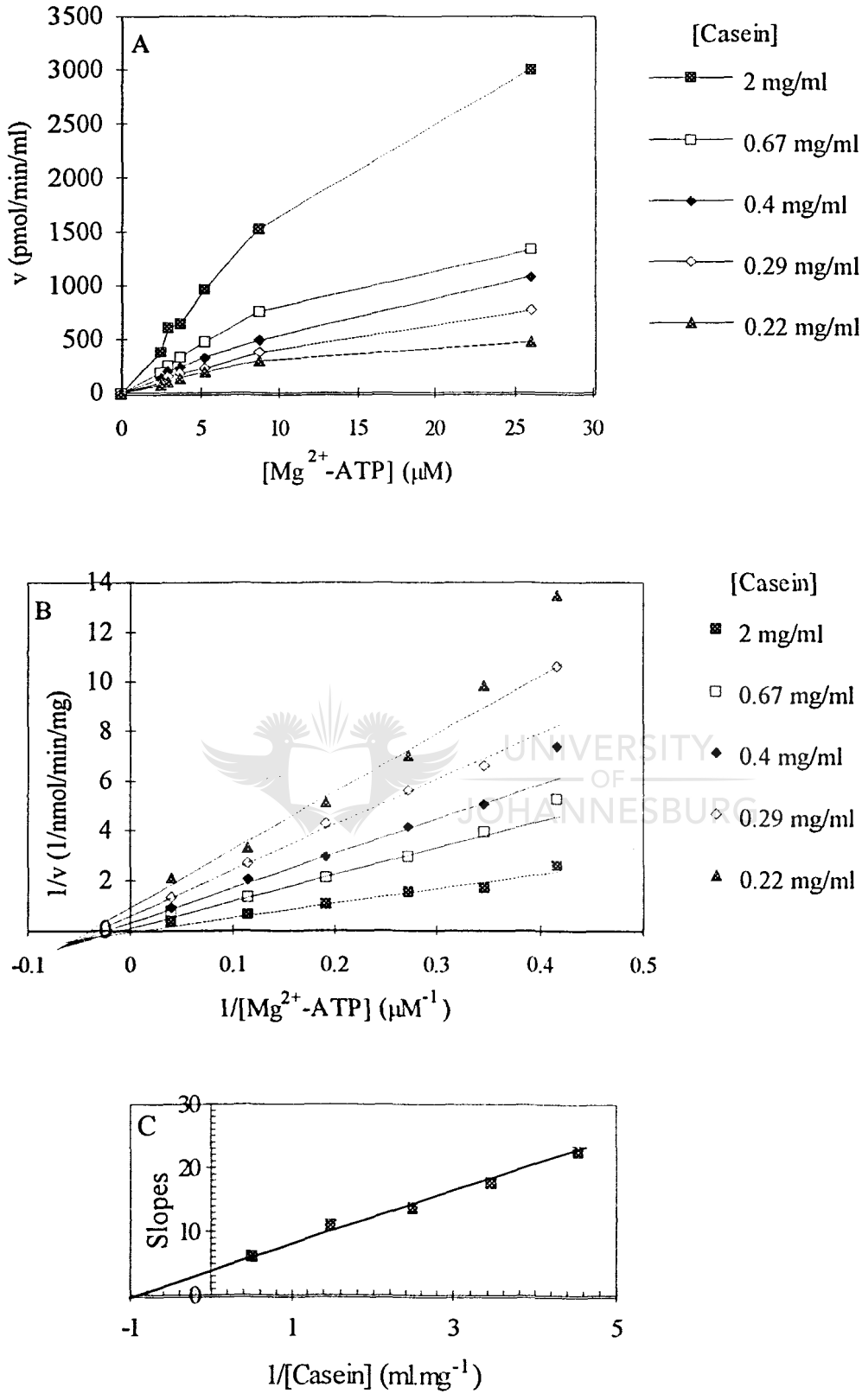



Figure 4.3.4.20 Initial velocity patterns for PK-III with varying ATP and casein concentrations. A: Michaelis-Menten plot of v *versus* [$Mg^{2+}$-ATP] at different fixed concentrations of casein. B: Double reciprocal plots of 1/v *versus* 1/[$Mg^{2+}$-ATP] at different fixed concentrations of casein. C: Secondary plot of slopes determined from plot B *versus* 1/[casein]. Conditions are as described under Section 4.2.3.

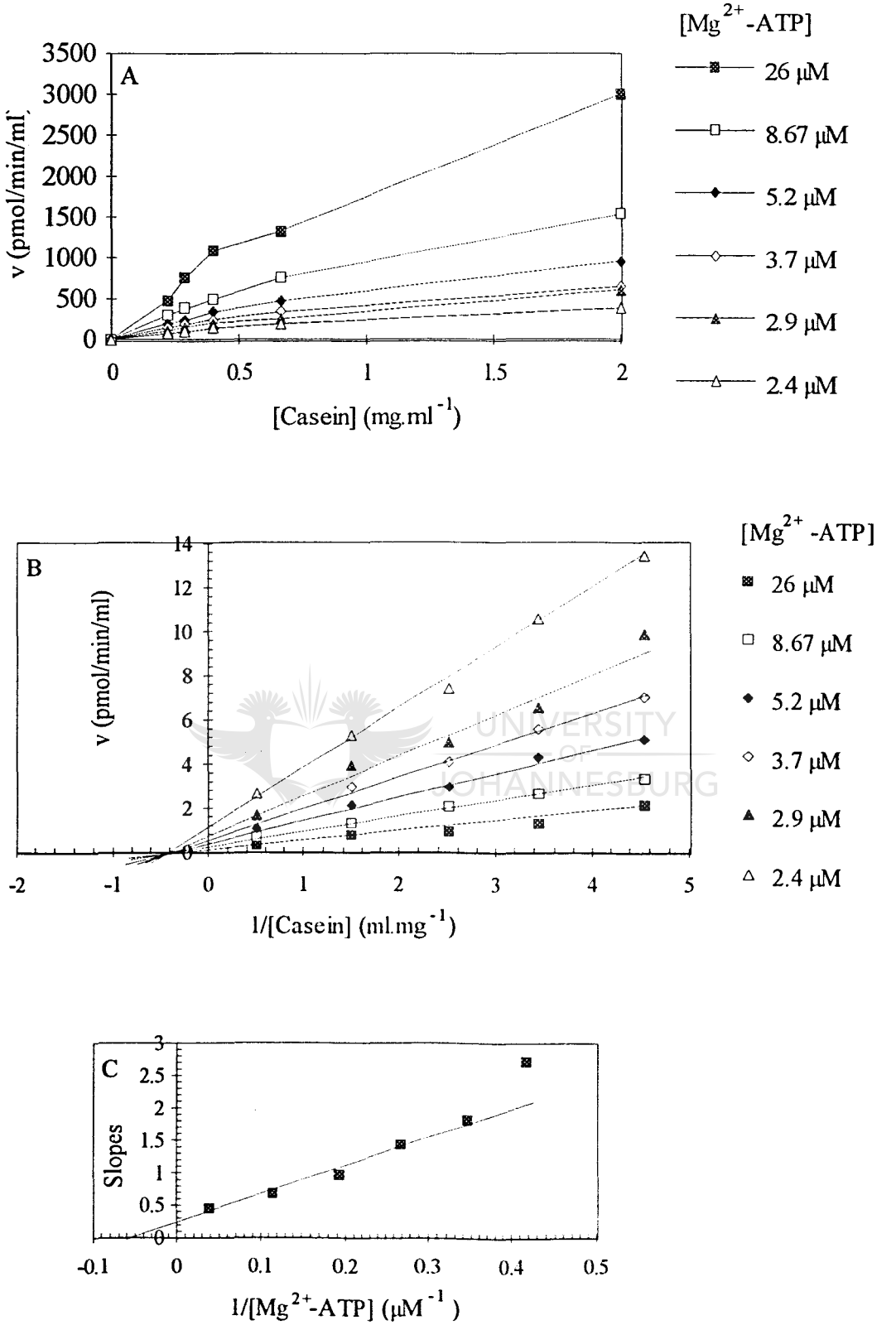



Figure 4.3.4.21 Initial velocity patterns for PK-III with varying ATP and casein concentrations. A: Michaelis-Menten plot of v *versus* casein at different fixed concentrations of [$Mg^{2+}$-ATP]. B: Double reciprocal plots of 1/v *versus* 1/[casein] at different fixed concentrations of [$Mg^{2+}$-ATP]. C: Secondary plot of slopes determined from plot B *versus* 1/[$Mg^{2+}$-ATP]. Conditions are as described under Section 4.2.3.

## 4.4. General Discussion and Conclusions

### 4.4.1 Comparison of the characteristics of the isolated protein kinases, PK-I, PK-II, and PK-III.

The known characteristics of the three protein kinases isolated from ripening mango fruit tissue are summarised and compared in Table 4.4.1. The diverse properties of each enzyme emphasise the fact that they are three distinct enzymes with their own specific role to play in the control of the physiology of the mango fruit.

Table 4.4.1 Comparison of the characteristics of the isolated protein kinases; PK-I, PK-II and PK-III.

| Characteristic | PK-I | PK-II | PK-III |
|---|---|---|---|
| Classification of protein kinase | Dual-specificity | Casein kinase 2 | $Ca^{2+}$-dependent |
| Physico-chemical properties: | | | |
| Molecular weight | 30 kDa | 34 kDa | 50 kDa |
| Subunit structure | monomeric | active αβ dimer | monomeric |
| Isoelectric point | 5.1 | 6.95, 7.5, 9.9 | ND |
| Hydrophobic nature | Hydrophobic | Hydrophobic | Hydrophobic |
| Enzymatic properties: | | | |
| Optimum pH | 6.5 | 8 | 6.6 - 9.5 |
| Optimum temperature | 35°C | 35°C | 35°C |
| Thermodynamic properties: | | | |
| E* (kcal/mole) | 8.07 | 12.2 | 41.2 |
| ΔH* (kcal/mole) | 8.5 | 11.44 | 20.02 |
| ΔS* (cal/mole/K) | -36.28 | -32.67 | -25.84 |
| ΔG* (kcal/mole) | 19.32 | 21.18 | 33.54 |
| Regulatory properties: | | | |
| $Mg^{2+}$ and $Mn^{2+}$ dependency | 8 - 12 mM $Mg^{2+}$ | 8 - 10 mM $Mg^{2+}$ | 5 - 10 mM $Mg^{2+}$ or 1 - 3 mM $Mn^{2+}$ |
| $Ca^{2+}$ stimulation/dependency | 50% stimulation | 40% stimulation | 100% dependency |
| Effect of calmodulin | None | None | 30% inhibition |
| Effect of calmodulin antagonists | None | None | 40 to 80% inhibition |
| Effect of cAMP | 40% inhibition | None | 10% inhibition |
| Effect of heparin | 30% inhibition | 70% inhibition | None |
| Substrate specificity | Casein, phosvitin | Casein, phosvitin | Histone III-S, casein |
| Autophosphorylation properties | Autophosphorylate | Autophosphorylate | Autophosphorylate |
| Amino acid specificity | Ser, Thr or Tyr | Ser or Thr | Ser or Thr |
| Kinetic properties: | | | |
| $K_m$ATP | 20 μM | 14 μM | 17 μM |
| $K_m$Casein | 0.2 mg/ml | 0.8 mg/ml | 1.0 mg/ml |
| $K_i$ADP | 9 μM | 3.2 μM | ND |
| Enzyme mechanism | Ordered Bi-Bi | Ordered Bi-Bi | Sequential |

One of the most interesting differences between PK-I and the other two enzymes is the low optimum temperature and the low activation energy (E*) of the catalytic reaction of PK-I compared to that of PK-

II and PK-III. It is possible that this property could correlate with the observation that PK-I might phosphorylate PK-II and PK-III and thus control their activity. Being easily activated/catalytically more active, it might be the biochemical trigger to phosphorylate other protein kinases involved in ripening in response to the presence of appropriate signal products.

## 4.4.2 Concluding Remarks

It was not a coincidence that three different protein kinases, each with its own unique properties and presumably important role in the control of the ripening process of mango fruit, were isolated. With more perseverance even more protein kinases could have been isolated. Knowledge exists already of two additional protein kinases (Section 4.3.1, Figure 4.3.1.2). With three enzymes having such different characteristics, there is no evidence that any of these enzymes is in fact an artefact. There might be isoenzymes present; experience showed that PK-IV (Figure 4.3.1.2 and 4.3.2.8) could be similar to PK-II. The enzyme's structural features could change along with ripening, as more proteases are released from their compartments when the membranes start to disintegrate during senescence. There is evidence of drastic changes in the protein kinase profiles during all the stages of ripening. Profile changes occur even as a result of different growing conditions, treatment and storage conditions after picking (compare Figures 4.3.1.2 and 4.3.1.3). Further complications could occur when the fruit are under stress as a result of temperature treatment or gamma-irradiation.

$Ca^{2+}$-dependent- (Putnam-Evans et al. 1990, Ma 1993) and casein kinase 2-type (Klimczak et al. 1992, Vasiliev et al. 1992, Li & Roux 1992) protein kinases have been isolated from plant sources, but, as far as could be ascertained, a dual-specificity enzyme has not yet been described.

From the results obtained in this study, it can be postulated that PK-I, the dual-specificity protein kinase, phosphorylate the PK-II, the casein kinase 2 type enzyme and PK-III, the $Ca^{2+}$-dependent protein kinase. Due to the seasonal availability of the fruit tissue, it was necessary to store the tissue in a frozen condition. The fragility of the membranes at the later stages of ripening and the rigidity of the cell walls during the earlier stages of ripening complicated the situation further. It was thus necessary to isolate these protein kinases from crude extracts, making it impossible to determine their location within the cell. However all three these enzymes had a strong hydrophobic nature (see their isolation characteristics) and one can assume that they could all be membrane associated. Casein kinases 2 and dual specificity enzymes isolated from other sources, indicate that they are usually situated in the nucleus, and that they play important roles in proliferation, mitosis and DNA transcription processes (Klimczak et al. 1992, Vasiliev et al. 1992, Li & Roux 1992). It is reported that a dual-specificity protein kinase isolated from *Schizosaccharomyces pombe* (Rowley et al. 1992, Lindberg et al. 1992), inhibits mitosis after DNA-damage occurred as a result of gamma-irradiation. The postulation was that mitosis was delayed so that DNA-repair could first take place (Anderson 1993). It was in turn reported that casein kinase 2 play a role in DNA-repair. Fruit, and more specifically mango fruit, are in particular prone to stress conditions, whether it is high temperature exposure, pathological infections or the more unusual gamma-irradiation treatment, and thus DNA-damage. Although cell division probably does not feature at this stage of the fruit physiology, other appropriate processes could be delayed - a possible control point where the normal ripening process is temporarily halted, to allow for DNA-damage repair, before the completion of the ripening process.

The effect of stress in the form of DNA-damage is also applicable in gamma-irradiated mango fruit - a process that leads to the delay of fruit ripening and at very high dosages even to the prevention of ripening all together. From previous studies (Dubery et al. 1984, Frylinck et al. 1987) it was already evident that mango fruit has the ability to overcome the damage done by low dosages (<1 kGy) of gamma-irradiation. The biochemical processes involved in fruit ripening and senescence are evidently an intricate, network of metabolic activities that cannot be underestimated.

# CHAPTER 5

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