Pectinesterases from the orange fruit - their purification, general characteristics and juice cloud destabilizing properties

Promotor: dr. W. Pilnik, hoogleraar in de levensmiddelenleer
Co-referent: dr. ir. F.M. Rombouts, wetenschappelijk hoofdmedewerker

C. Versteeg

Pectinesterases from the orange fruit - their purification, general characteristics and juice cloud destabilizing properties

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Abstract

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Twelve forms of pectinesterase were detected in citrus fruits. Two forms, representing over 90% of the total pectinesterase activity in Navel oranges, were purified. These pectinesterases, named Pectinesterase I and II have isoelectric points of 10.05 and > 11.0, respectively. Both pectinesterases have the same molecular weight of 36 200 but differ in amino acid composition. A third pectinesterase, with a higher molecular weight (about 54 000) was partly purified. The optimum pH of these three pectinesterases is about 7.5, but their pH-activity profiles differ and depend on the degree of esterification of the pectin. The high molecular weight pectinesterase is still active at pH 2.5. The K_m values of both purified pectinesterases decrease with decreasing degree of esterification of the pectin substrate and increase with decreasing pH. Pectinesterase II has a tenfold higher affinity for pectin than Pectinesterase I and is more strongly inhibited by pectate. The heat stabilities were determined: the D_{90} °C and Z values in orange juice are 0.00037 min and 6.5 °C for Pectinesterase I, 0.0015 min and 11 °C for Pectinesterase II and 0.375 min and 6.5 °C for the high molecular weight pectinesterase. The orange juice cloud destabilizing properties of the pectinesterases at 5 °C and 30 °C are remarkably different. The activity measurements were done, amongst other methods, by an improved gas chromatographic methanol assay. The literature on pectinesterase was reviewed.

Free descriptors: molecular weight, multiple forms, isolectric point, stability, heat stability, amino acid composition, K_m, K_i, V_{max}, mode of action, cloud stability, pH, literature review, methanol assay, citrus fruit,

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Stellingen

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1. De klaring van ondergepasteuriseerd sinaasappelsap door pectine-esterase wordt veroorzaakt door slechts enkele van de oorspronkelijk daarin aanwezige vormen van pectine-esterase.

Dit proefschrift, hoofdstuk 8.

2. Het uitdrukken van de Michaelis-Menten-constante van pectine-esterase in een methyl D-galactopyranosyluronaat concentratie, zoals aanbevolen door Rexová-Benková & Markovič, is af te raden.

L. Rexová-Benková & O. Markovič, 1976. Advances in Carbohydrate Chemistry and Biochemistry 33: 323-385.
Dit proefschrift, hoofdstuk 7.

3. De experimenten van Lee & Macmillan en Miller & Macmillan rechtvaardigen niet hun conclusie dat de helft van de enzymactiviteit van pectine-esterase van zowel de tomaat als ook van *Aspergillus niger* begint aan het reducerende einde van de hoogveresterde pectine.

M.Lee & J.D. Macmillan, 1970. Biochemistry 9: 1930-1934.
L. Miller & J.D. Macmillan, 1971. Biochemistry 10: 570-576.

4. De relatie tussen de Michaelis-Menten-constante van pectine-esterase van de sinaasappel en de veresteringsgraad van gedeeltelijk alkalisch voorverzeepte pectine zoals gevonden door Solms & Deuel is onjuist.

J. Solms & H. Deuel, 1955. Helv. Chim. Acta 38: 321-329.
Dit proefschrift, hoofdstuk 7.

5. Het glycoproteïne karakter van pectine-esterases van tomaten en pruimen, zoals gesuggereerd door Theron, De Villiers & Schmidt, is vooralsnog niet bewezen.

T. Theron, O.T. De Villiers & A.A. Schmidt, 1977. Agrochemophysica 9: 7-12.
T. Theron, O.T. De Villiers & A.A. Schmidt, 1977. Agrochemophysica 9: 93-96.

6. Het verdient aanbeveling om bij de bestudering van zowel fysiologische als technologische processen in relatie tot de activiteit van een enzym niet alleen de totale enzymactiviteit, maar ook de activiteit van eventueel aanwezige verschillende vormen van het enzym afzonderlijk in het onderzoek te betrekken.

7. De door Biran, Gilbert & Giacin gepubliceerde gegevens over de sorptie van vinylchloride in levensmiddelen bemoeilijken de toepassing van de methode van Van Lierop als routinebepaling van vinylchloride in levensmiddelen.

D. Biran, S.G. Gilbert & J.R. Giacin, 1979. J. Food Sci. 44: 56-58.
J.B.H. van Lierop, 1979. Determination of potentially carciogenic compounds in Food. Proefschrift Landbouwhogeschool, Wageningen.

8. De door Van der Stegen gegeven interpretatie van de door Viani & Horman aangegeven mogelijke afbraak van carboxy-5-hydroxytryptamides bij het branden van koffie als verklaring voor het niet verdragen van koffie door sommige mensen is niet gerechtvaardigd.

G.H.D. van der Stegen, 1979. Food Chem. 4: 23-29.
R. Viani & I. Horman, 1975. Proceedings of the 7th International Colloquium on the Chemistry of Coffee, p. 273-278.

9. Behalve bij kant-en-klaar-maaltijden, is het vermelden van voedingsstoffen en de energetische waarde op levensmiddelen slechts van beperkte waarde.

W. Pilnik & P. Folstar, 1979. Dtsch. Lebensm. Rundsch. In druk.

10. Onderzoek naar de toepassing van enzymen in afwasmachines dient gestimuleerd te worden, aangezien het gebruik van enzymen in afwasmachines zowel het energieverbruik van, als de fosfaatverontreiniging door deze machines zou kunnen verminderen.

11. Indien het oppervlak van het Nederlandse wegennet tevens gebruikt zou kunnen worden als zonne-energiecollector, zou jaarlijks een hoeveelheid energie opgevangen kunnen worden die het Nederlandse elektriciteitsverbruik benadert.

12. Witte wijn is niet wit.

13. De gebruikelijke opdracht voorin een proefschrift met de strekking van: "Aan mijn vrouw en kinderen" zou vaak volledigheidshalve moeten luiden: "Aan mijn vrouw en kinderen, ondanks wie dit proefschrift toch tot stand is gekomen".

Proefschrift van C. Versteeg
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Wageningen, 28 september 1979

Aan mijn ouders,
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Edwin

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Curriculum vitae

De auteur werd op 12 mei 1948 te Maarsseveen geboren. Na het behalen van zijn ULO-B diploma in 1964 werd de Rijks Hogere Tuinbouwschool te Utrecht bezocht en aldaar werd in 1968 het einddiploma behaald met als hoofdvak fruitteelt.

Daarop aansluitend werd de studie in de levensmiddelentechnologie gevolgd aan de Landbouwhogeschool te Wageningen. In 1975 werd het doctoraal diploma behaald met als hoofdvak de kennis van levensmiddelen en de bijvakken levensmiddelenmicrobiologie en proceskunde.

Een half jaar na zijn afstuderen kwam hij als promotie-assistent in dienst van de Landbouw Hogeschool op het laboratorium voor levensmiddelenchemie en -microbiologie. Sinds 1 maart 1979 verricht hij onderzoek voor Gist-Brocades.

Contents

1	*Introduction*		1
2	*Literature on pectinesterase*		
	2.1	Pectinesterase and other pectic enzymes	2
	2.2	Pectinesterase	3
	2.2.1	Occurrence	3
	2.2.2	Role in vivo	6
	2.2.3	Purification and properties	8
	2.2.4	Specifity and mode of action	13
	2.2.5	Inhibition and (in)activation	14
	2.2.6	Importance in food technology	16
	2.2.7	Assay and detection	18
	2.2.7.1	Changes in the substrate	18
	2.2.7.2	Methanol determination	19
3	*Materials and methods*		22
4	*Methanol determination*		28
	4.1	Experiments and results	28
	4.2	Discussion	39
5	*Purification and molecular properties of two citrus pectinesterases*		40
	5.1	Experiments and results	40
	5.2	Discussion	49
6	*Multiple forms of pectinesterase in Navel orange and other citrus fruit*		51
	6.1	Experiments and results	51
	6.1.1	Multiple forms of pectinesterase in Navel orange	51
	6.1.2	Multiple forms of pectinesterase in citrus fruit	54
	6.2	Discussion	57
7	*Kinetics and mode of action of Pectinesterase I and II*		58
	7.1	Experiments and results	58
	7.1.1	Initial reaction rates	58
	7.1.2	De-esterification limits	66
	7.2	Discussion	72

8 Stability of pectinesterases 76
8.1 Experiments and results 76
8.1.1 Stability during storage, assay and orange cloud stability tests 76
8.1.2 Heat stability 78
8.2 Discussion 83

9 Cloud loss of orange juice 86
9.1 Experiments and results 86
9.1.1 Pectinesterases and the cloud loss at 30 °C and 5 °C 86
9.1.2 Cloud stabilization by polygalacturonase 89
9.2 Discussion 91

10 General discussion and conclusions 93

Summary 95

Samenvatting 98

Literature 101

1 Introduction

Pectinesterase (pectin pectylhydrolase EC 3.1.1.11, older names: pectin methylesterase, pectin demethoxylase, pectin methoxylase, pectase) is present in large amounts in various citrus fruits (MacMillan & Sheiman, 1974; Rexová-Benková & Marković, 1976). The enzyme is firmly associated with the cell wall fractions and is situated mainly in the peel, the rag and juice sac tissue (MacDonnell et al., 1945; Rouse, 1953; Jansen et al., 1960b; Manabe, 1973a). Most of the enzyme present in the extracted juice is bound to pulp particles (Rouse, 1953).

In fresh citrus juices, pectinesterase de-esterifies the pectin, producing methanol and low methoxyl pectin. Subsequently, precipitation of the low methoxyl pectin with calcium ions from the juice results in cloud loss, a serious quality defect (Joslyn & Pilnik, 1961; Krop, 1974). In citrus juice technology pectinesterase is either inactivated by pasteurization or inhibited by frozen storage of juice concentrates at -20 ^{o}C or below (Joslyn & Pilnik, 1961).

Recently, several reports appeared on the stabilization of orange juice cloud, either by the addition of polygalacturonase to degrade the low methoxyl pectin (Baker & Bruemmer, 1972; Krop & Pilnik, 1974) or by adding oligogalacturonic acids to inhibit pectinesterase (Termote et al., 1977). In these studies no attention was paid to the possible occurrence of multiple forms of pectinesterase in citrus fruits, notably in orange. Multiple forms of pectinesterase have been found in tobacco pith (Glasziou & Inglis, 1958), banana (Hultin et al., 1966; Brady, 1976; Marković et al., 1975), tomato (Pressey & Avants, 1972; Marković & Kuniak, 1974; Rexová-Benková et al., 1977) and various other fruits and vegetables (Roeb & Stegeman, 1975). Multiple forms of pectinesterase may differ in kinetic properties and temperature stability (Hultin et al., 1966; Hultin & Levine, 1963) and therefore, if present in orange juice, may play a different role in cloud loss phenomena.

This work on orange pectinesterase was intended to be an initial step in the study of the mode of action of plant pectinesterase and also to obtain a more detailed picture of cloud loss phenomena in citrus juice. Extraction and extensive purification resulted in the isolation of two molecular forms of pectinesterase. In addition several other forms could be detected and one of them (in the impure state) was also incorporated in part of this study. Some of the molecular, kinetic and orange juice clarifying properties of the different forms were found to be remarkably different.

2 Literature on pectinesterase

2.1 PECTINESTERASE AND OTHER PECTIC ENZYMES

As this thesis is concerned with pectinesterase, the literature on pectinesterase is reviewed in this chapter, while other pectic enzymes are described only briefly. Several literature reviews dealing with pectic enzymes have been published recently (Voragen & Pilnik, 1970a; Rombouts & Pilnik, 1972; MacMillan & Sheiman, 1974; Rexová-Benková & Markovic, 1976). The importance of these enzymes for the fruit and vegetable juice technology was reviewed by Rombouts & Pilnik (1978) and also the literature on the production of these enzymes was discussed by the same authors (Rombouts & Pilnik, 1980, in press). Pectic enzymes degrade pectin, which is a major constituent of the plant middle lamella and primary cell wall. They are very generally produced by higher plants and micro-organisms. But also insects (Courtois et al., 1968), nematodes (Deubert & Rohde, 1971) and protozoa (Mah & Hungate, 1965) have been found to produce these enzymes.

Pectin is a (1→4)-polymer of partially methyl esterified α-D-galacturonic acid. However, it is not a homopolymer and contains various proportions of L-rhamnose in the main chain and arabinose, xylose, fucose, galactose and possibly also apiose in side chains (Voragen & Pilnik, 1970b; Fogarty & Ward, 1974; McNeil et al., in press). The secondary hydroxyl groups at C_2 and C_3 of the galacturonide monomer may partially be acetylated, depending on the source. Pectinesterase de-esterifies the methyl esters of the carboxyl groups. Polygalacturonases split the main chain in a hydrolytic way and the lyases (pectate lyase and pectin lyase) split the main chain by transelimination (Table 1; Fig. 1). Internal and terminal splitting of polygalacturonases and pectate lyases are known and all of these enzymes have a preference for substrates with a low content of methyl ester (Pilnik et al., 1973). Endo-pectin lyase is the only enzyme which preferentially attacks pectins with a high content of methyl ester. Many chain-splitting enzymes require free carboxyl groups for activity, and these enzymes can only degrade pectins with a high content of methyl ester when they operate in conjunction with pectinesterase.

Table 1. Chain-splitting pectic enzymes.

Name	EC number	Preferred substrate	Mechanism	Mode of attack
Endo-polygalacturonase	3.2.1.15	pectate	hydrolytic	internal
Exo-polygalacturonase	3.2.1.67	pectate	hydrolytic	terminal
Endo-pectate lyase	4.2.2.2	pectate	transeliminative	internal
Exo-pectate lyase	4.2.2.9	pectate	transeliminative	terminal
Endo-pectin lyase	4.2.2.10	pectin	transeliminative	internal

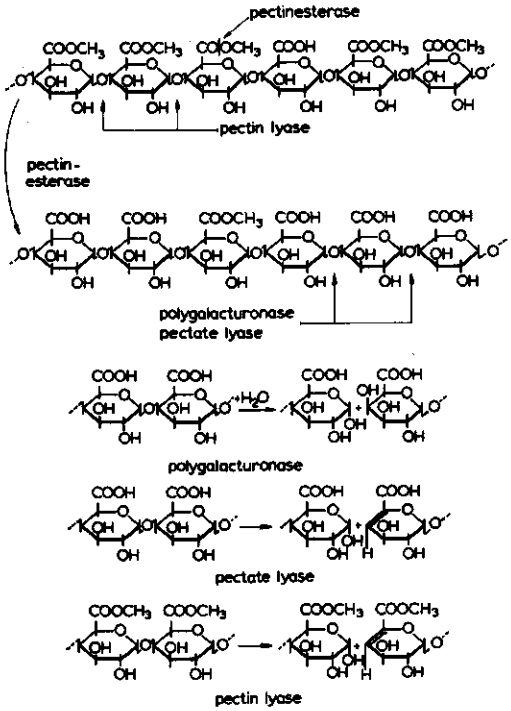


Fig. 1. Basic structure of galacturonan with points of attack and mode of attack of pectic enzymes.

From the heterogenous structure of pectin it is evident that also several other enzymes are needed for splitting all glycosidic linkages in the molecule. The basic structure of pectin together with places and mode of attack of some of the pectic enzymes are given in Fig. 1.

Because pectin is a structural element of the plant cell wall and a major constituent of the middle lamella, pectic enzymes play an important role in growth, ripening, tissue deterioration by parasites as well as by saprophitic micro-organisms and affect the texture and appearance of processed fruits and vegetables.

2.2 PECTINESTERASE

2.2.1 Occurrence

Pectinesterase was discovered in 1840 by Frémy (1840) in vegetable juice. Since then numerous higher plants and micro-organisms have been shown to contain or to be able to produce pectinesterase (Tables 2 and 3). In fact all higher plants seem to contain pectinesterase in all living tissues. For instance, pectinesterase was demonstrated in seeds, leaves, stems, petioles, flowers, green and ripe fruit of cucumbers (Bell et al., 1951). The level of activity varies considerably with plant species, variety, part of the plant and stage of growth. Also in fruits there are zones which are relatively rich and poor in pectinesterase activity, like in citrus (MacDonnell et al., 1945; Rouse, 1953; Rothschild et al., 1974; Tahir et al., 1975) and persimmon (Nakayama & Iwasaki, 1966).

Table 2. Occurrence of pectinesterase in higher plants.

Source	References
alfalfa	Lineweaver & Ballou (1945)
apple	Pollard & Kieser (1951); Lee & Wiley (1970); Miyairi et al. (1975)
apricot	Polacsek-Rački & Pozsár-Hajnal (1976)
artichoke	Glasziou & Inglis (1958)
avocado	Zauberman & Schiffmann-Nadel (1972)
banana	Hultin & Levine (1963); Marković et al. (1975); Brady (1976)
barley	Fuchs (1965)
bean (broad, french and snap)	Fuchs (1965); Buren et al. (1962)
cacao	Gamble (1973)
carrot	Marković (1978)
cauliflower	Hoogzand & Doesburg (1961)
cherry (sweet and Montmorency)	Schmid (1975); Al-Delaimy (1966)
cranberry	Arakji & Jang (1969)
cucumber	Bell et al. (1951)
gooseberry	Polacsek-Rácz & Pozsár-Hajnal (1976)
grape	Montedoro (1968); Datunashvili et al. (1976)
grapefruit	Rouse & Atkins (1953a); Eagerman & Rouse (1976)
guave	Shastri & Shastri (1975)
lilac	McColloch & Kertesz (1947)
lime	Evans & McHale (1978)
mandarin	Nath & Ranganna (1977a)
oat	Glasziou (1959)
onion	Vas et al. (1967)
orange	MacDonnell et al. (1945); Evans & McHale (1978); Versteeg et al. (1978)
papaya	Aung & Ross (1965)
paprika	Vas et al. (1967)
peach	Buescher & Furmanski (1978)
pea	Collins (1970)
pear	Vas et al. (1967); Weurman (1954)
persimmon	Nakayama & Iwasaki (1966)
plum	Theron et al. (1977a)
potato	Vas et al. (1967); Bartolome & Hoff (1972a)
radish	Polacsek-Rácz & Pozsár-Hajnal (1976)
raspberry	Polacsek-Rácz & Pozsár-Hajnal (1976)
rye	Fuchs (1965)
strawberry	Leuprecht & Schaller (1968)
tomato	Hills & Mottern (1947); Pressey & Avants (1972)
tobacco	Glasziou & Inglis (1958)
wheat	Fuchs (1965)

The activity changes in fruits and vegetables during growth and ripening do not follow a general trend. For instance the pectinesterase level increases during growth in orange (Rouse & Atkins, 1953b; Tahir et al., 1975), tomato (Sawamura et al., 1978; Poszár-Hajnal & Polacsek-Rácz, 1975), banana (Hultin & Levine, 1965), apple (Lee, 1969b), cacao (Gamble, 1973), guave (Shastri & Shastri, 1975) but decreases in cucumbers (Bell et al., 1951), pears (Weurman, 1954), peas (Collins, 1970) and avocado (Zauberman & Schiffmann-Nadel, 1972). In orange, tomato and guava there is a sudden drop at a stage during ripening. Brady (1976) suggested that the apparent low activity level in immature bananas is caused by inactivation through phenolic compounds.

Pectinesterase is situated in the free space between the cell walls as was shown for oat coleoptiles and tobacco pith (Glasziou, 1959; Bryan & Newcomb, 1954), and the main

Table 3. Pectinesterase-producing micro-organisms.

Micro-organisms	References
Bacteria	
Clostridium aurantibutyricum	Lund & Brocklehurst (1978)
Clostridium multifermentans	Miller & MacMillan (1970); Sheiman et al. (1976)
Corynebacterium (several species)	Lange (1970)
Erwinia (several species)	Lange (1970)
Pseudomonas solanacearum	Lange (1970)
Xanthomonas malvacearum	Abo-El-Dahab (1964)
Xanthomonas (several species)	Dye (1960); Lange (1970)
Fungi	
Acrocylindrium	Kimura et al. (1973)
Alternaria humicola	Abdel-Fattah & Mabrouk (1977)
Aspergillus carbonarius	Sreekantiah et al. (1975)
Aspergillus japonicus	Ishii et al. (1979)
Aspergillus niger	Rexová-Benková & Slezárik (1966); Baron (1978)
Aspergillus (several species)	Abdel-Fattah & Mabrouk (1977)
Botrytis cinerea	Verhoeff & Warren (1972)
Botryosphaeria ribis	Wallace et al. (1962)
Byssochlamys fulva	Reid (1952)
Cercosporella herpotrichoides	Hännsler et al. (1971)
Chaetomium globosum	Abdel-Fattah & Mabrouk (1977)
Colletotrichum trifolii	Hancock & Millar (1965)
Coniothyrium diplodiella	Endo (1964)
Corticium rolfsii	Yoshihara et al. (1977)
Curvularia lunata	Abdel-Fattah & Mabrouk (1977)
Diplodia gossypina	Wang & Pinckard (1971)
Epicoccum nigrum	Abdel-Fattah & Mabrouk (1977)
Fusarium oxysporum	Miller & MacMillan (1971)
Fusarium roseum	Perley & Page (1971)
Fusarium sp.	Abdel-Fattah & Mabrouk (1977)
Gibberella sp. 117	Abdel-Fattah & Mabrouk (1977)
Gilbertella persicaria	Mehrotra et al. (1971)
Glomerella cingulata	Wallace et al. (1962)
Macrosporium cladosporioides	Abdel-Fattah & Mabrouk (1977)
Monilia fructicola	Paynter (1975)
Nigrospora sphaerica	Agarwal et al. (1977)
Oospora sp.	Abdel-Fattah & Mabrouk (1977)
Ophiobolus graminis	Weste (1970)
Pellicularia filamentosa	Barker & Walker (1962)
Penicillium chrysogenum	Phaff (1947)
Penicillium (several species)	Abdel-Fattah & Mabrouk (1977)
Pleospora infectoria	Agarwal et al. (1977)
Plylasospora sp.	Wallace et al. (1962)
Physalospora obtusa	Wallace et al. (1962)
Phytophtora infestans	Clarke (1966)
Pseudoarachniotus roseus	Abdel-Fattah & Mabrouk (1977)
Rhizoctonia solani	Bateman (1963)
Sclerotina libertiana	Oi & Satomura (1965)
Sclerotina sclerotiorum	Astapovich (1976)
Sclerotium rolfsii	Yoshihara et al. (1977); Bateman & Beer (1965)
Stemphylium botryosum	Hancock & Millar (1965)
Trichoderma lignorum	Abdel-Fattah & Mabrouk (1977)
Trichothecium roseum	Abdel-Fattah & Mabrouk (1977)
Yeasts	
Kluyveromyces fragilus	Call & Emeis (1978)
Torulopsis candida	Call & Emeis (1978)

part is bound by ionic interaction. It can be liberated from the cell wall fraction by raising the pH above 7 and increasing the ionic strength, as was demonstrated, for example, in orange (MacDonnell et al., 1945; Jansen et al., 1960b), oat coleoptiles (Glasziou, 1959; Jansen et al., 1960a) and tomatoes (Pozsár-Hajnal & Polacsek-Rácz, 1975). In tomato cell walls and oat coleoptiles it was moreover demonstrated that there were binding sites specific for pectinesterase. The binding of pectinesterase was only partially or not at all affected by prior saturation with other proteins or vice versa (Nakagawa et al., 1971; Jansen et al., 1960a).

However, the situation is probably much more complicated. Glasziou & Inglis (1958) demonstrated three different fractions of pectinesterase from artichoke and two in tobacco pith. The pectinesterases appeared to differ with respect to binding properties to cell walls. One pectinesterase could increasingly be bound by addition of auxins, but not by calcium ions, the binding of another pectinesterase was neither affected by calcium ions nor by auxin and the binding of the third pectinesterase was only affected by calcium ions and not by auxin. Hultin & Levine (1963) demonstrated three different pectinesterases in bananas by differential extraction procedures. These enzymes appeared to be bound differently and possessed different properties with respect to pH-activity, activation or inhibition by sodium lauryl sulphate and temperature inactivation. Multiple forms and isoenzymes of pectinesterase are discussed further in Sections 2.2.2 and 2.2.3.

2.2.2 Role in vivo

The role of pectinesterase in growth of plants and in growth and ripening of fruits is not very well understood. As pectinesterase seems to be present in all living tissues of higher plants an essential function is likely.

Auxins like IAA (3-indolylacetic acid) promote cell enlargement. In tobacco pith this is accompanied by a rise in pectinesterase content (Bryan & Newcomb, 1954). IAA, depending on its concentration, stimulates or inhibits pectinesterase activity (Bryan & Newcomb, 1954; Nakagawa et al., 1970b). IAA and other auxins have been reported to affect the binding of pectinesterase to cell walls of tobacco pith (Glasziou, 1957; Glasziou & Inglis, 1958). It was suggested that immobilization of pectinesterase favours methylation of pectate of the cell wall and thus plasticity of cell wall increases. However, Jansen et al. (1960a) and Nakagawa et al. (1971) reported that there was no effect of IAA on pectinesterase binding to oat coleoptile and tomato cell walls, respectively, but it may be possible that they tested the wrong concentration ranges of IAA or irrelevant forms of pectinesterase. It was pointed out by Jansen et al. (1960a) that IAA did not change the degree of esterification of any pectic material in cell walls and that therefore elongation of cell walls mediated through an effect on pectinesterase was unlikely. Salt in the nutrient solution reduced the pectinesterase concentration in the leaves of *Aster tripolium* and promoted cell elongation (Binet, 1976); here an inverse relationship between cell size and pectinesterase concentration was found as by Bryan & Newcomb (1954) in tobacco pith.

As discussed in Section 2.2.1, there is no general relationship between ripening and pectinesterase level in fruits and vegetables. Also no relationship could be demonstrated

between available pectinesterase activity and firmness of tomato fruit (Hall & Dennison, 1960). It has often been suggested that tissue softening during ripening or plant pathogenesis is caused by pectinesterase activity followed by polygalacturonase action. However, pectinesterase action alone, and subsequent binding of pectate with bivalent cations increases tissue firmness and resistance against maceration (Brown, 1969). There are indications that this mechanism is involved in resistance against pathogens. In several cases it was demonstrated that host pectinesterase activity increased upon infection (Bateman, 1963; Langcake et al., 1973). In bean hypocotyls infected by *Rhizoctonia solani* calcium ions accumulate in the infected zone, pectinesterase activity increases, pectin is demethylated and insoluble calcium-pectate is formed, which cannot be degraded by the endo-polygalacturonase of the pathogen (Bateman & Millar, 1966). During pathogenesis *Sclerotium rolfsii* produces oxalic acid, which binds calcium ions and thus enables the polygalacturonase to degrade pectate. Also, the pH is lowered towards the optimum for polygalacturonase activity (Bateman & Beer, 1965). Pectinesterase of the pathogen is not always needed for virulence. Lange (1970) concluded that there was no relationship between virulence and pectinesterase activity of pathogenic bacteria. Paquin & Coulombe (1962) found that a virulent strain of *Fusarium oxysporum* produced 2 - 3 times more pectinesterase than an avirulent one. Cells of *Xanthomonas malvacearum* adapted to produce pectinesterase were more pathogenic than unadapted cells (Abo-El-Dahab, 1964). No relation was found between virulence of *Pellicularia filamentosa* and pectinesterase production (Barker & Walker, 1962).

The amount of pectinesterase is sometimes very high compared with the amount of available pectin. For instance, in citrus fruit there is sufficient pectinesterase (60 000 units (µmol/min)/kg) to de-esterify the quantity of pectin (30 g/kg) from high to low degree of esterification in 10 min at optimal pH or in less than 4 h at the pH of the juice. Possibly pectinesterase in vivo plays a role in the methylation of pectates. Glasziou (1959) suggested that pectinesterase might have a transmethylase function to methylate pectate to pectin in vivo. A transferase activity transferring methyl groups from *S*-adenosyl-L-methionine to the carboxyl groups of polygalacturonic acid was found in bean shoots (Kauss & Hassid, 1967). Kauss et al. (1969) found also that intact particles from the same source contained pectinesterase which could only de-esterify the plant's pectin if the lipid membranes protecting the pectic substances were destroyed by detergents or phospholipase. Although the authors did not mention this possibility, one might speculate that the transferase becomes an esterase after the lipid membranes are destroyed and water is introduced in the immediate environment of substrate and enzyme, and possibly also after some chemical or physical change of the enzyme. Then, after extraction and under the aqueous assay conditions, only pectinesterase activity would be measured. This theory is in agreement with the continuously increasing pectinesterase concentration during growth (see Section 2.2.1) as often found without simultaneous decrease in degree of esterification of the pectic substances. The degree of esterification of the pectic substances even increases in some fruits and no decrease is observed until the very ripe stage (Gee et al., 1959).

In several plants multiple forms of pectinesterase have been demonstrated, e.g. four to eight forms in tomatoes (Pressey & Avants, 1972; Delincée, 1976), two to six in

bananas (Hultin & Levine, 1963; Brady, 1976; Markovič et al., 1975), two in tobacco pith (Glasziou & Inglis, 1958), three in artichoke (Glasziou & Inglis, 1958) and two in orange (Evans & McHale, 1978; Versteeg et al., 1978). It is still not known whether these enzymes are derived from the same form and are merely breakdown products, still having pectinesterase activity, or whether they all have a different origin and function in the plant. Several explanations for the occurrence of multiple forms were given by Hultin et al. (1966) and Brady (1976). The fact that the two pectinesterases in citrus were found in different tissues (Evans & McHale, 1978) points to a functional difference. If so, the situation in vivo must be very complicated since eight forms were detected in tomato (Delincée, 1976) and even twelve forms in citrus, of which sometimes ten forms in the same fruit (this work; Nieuwenhuis, 1978). Multiple forms of pectinesterase always seem to differ in isoelectric point and usually differ in one or more of the following aspects: pH-activity profile, activation by cations, substrate affinity, inactivation by detergents, inhibition by sucrose, heat stability, molecular weight and binding properties to cell walls (Pressey & Avants, 1972; Hultin et al., 1966; Hultin & Levine, 1963; Brady, 1976; Glasziou & Inglis, 1958; Versteeg et al., 1978).

2.2.3 Purification and properties

Though in many plants and cultures of micro-organisms the presence of pectinesterase was established (Tables 2 and 3), the properties of these enzymes were often not determined and only in relatively few cases they were partially or completely purified. Especially pectinesterases from micro-organisms received little attention even though they differ more widely in properties than the plant pectinesterases seem to do.

The properties of pectinesterases which have been studied in more detail are listed in Tables 4a, 4b and 5. Comparison of most properties has only limited value because the assay conditions are not always the same. The data of the impure preparations usually represent the overall properties of a mixture of different forms of pectinesterase. The use of a mixture of different forms is sometimes indicated by irregularities reported such as pH curves with shoulders and irregular heat inactivation curves. Even in some of the pure preparations more than one single form of pectinesterase may be present. Thin-layer isoelectric focusing seems to be the most sensitive method to determine multiple forms or isoenzymes (Delincée, 1976).

Purification usually involves extraction (for plant pectinesterases), fractional ammonium sulphate precipitation or acetone precipitation and several types of column chromatography, such as gel filtration (separation based on molecular size), anion or cation exchange (separation based on charge and charge distribution). Sometimes other principles were employed as well, for instance affinity chromatography (Rexová-Benková et al., 1977; Versteeg et al., 1978), isoelectric focusing (Brady, 1976), calcium-phosphate adsorption chromatography (Dahodwala et al., 1974; Yoshihara et al., 1977). A rather detailed summary about purification methods used for pectinesterase is given by Rexová-Benková & Markovič (1976).

Except for one low molecular weight form from banana and one high molecular weight pectinesterase (complexed with exo-pectate lyase) from *Clostridium multifermentans*, all

pectinesterases have molecular weights in the range of 24 000 to 37 000. The molecular weights were not all determined in the same way and Delincée & Radola (1970) showed that the apparent molecular weight for the same tomato pectinesterase could range between 24 000 and 28 000, depending on the method employed. The isoelectric points of plant pectinesterases were always above pH 7 and the pH optimum ranged between 6 and 8.5. It should be noted that the pH-activity profile is broadened at the acid side by raising the concentration of cations (Lineweaver & Ballou, 1945). The pH optimum for microbial pectinesterases depends largely on the source of the enzyme. The Michaelis-Menten constants (K_m values) reported for the purified enzymes differ up to a factor 500. Between isoenzymes from the same source already a 20-fold difference was found (Versteeg et al., 1978). Rexová-Benková & Markovič (1976) suggested to express the K_m on the basis of methyl-D-galactopyranosyluronate residues. But this seems illogical because free carboxyl groups are regarded to be necessary for affinity (Solms & Deuel, 1955), see also Section 2.2.4.

Plant pectinesterases are competitively inhibited by pectate, one of their own reaction products (Termote et al., 1977). The effect of salts on alfalfa pectinesterase was thoroughly studied by Lineweaver & Ballou (1945). It appeared that cations and not anions stimulate pectinesterase at the acid side of its pH optimum. Also pectate inhibition is less at higher pH and at higher cation concentrations. It was concluded that cations do not stimulate the enzyme but liberate it from an inactive ionic complex with the product so that full activity can be obtained. Also MacDonnell et al. (1945) and Hills & Mottern (1947) found for orange and tomato pectinesterase, respectively, that a higher concentration of cations was required for activity at a lower pH. The optimum concentration of sodium chloride for plant pectinesterase is in the range of 0.1-0.25 mol/l and the optimum concentration of calcium chloride is about five times lower. This optimum salt concentration is usually determined near pH 7.0 and is higher at a lower pH. Though the optimum sodium chloride concentration for fungal pectinesterase is approximately the same, the stimulation is much less. Also for a fungal preparation it was found that more salt was required for optimal activity at a lower pH (Calesnick, 1950).

As can be seen from the tables in this chapter, the optimal temperature for plant pectinesterases is approximately 55 °C with a Q_{10} of 1.3-1.4 in the range 20-50 °C. The optimal temperature for microbial pectinesterases is about 10 °C lower, and usually they are also less heat stable. Lee (1969a) suggested that tomato pectinesterase was a lipoprotein. Markovic (1974) demonstrated that one of the multiple forms of tomato pectinesterase was neither a lipo-protein nor a glyco-protein. Theron et al. (1977a, 1977b) found indications for a glyco-protein character of plum and tomato pectinesterase. None of the six banana pectinesterases examined by Markovič et al. (1975) were lipo-proteins. Baron (1978) found indications for a lipo-protein character of *Aspergillus niger* pectinesterase.

Table 4a. Properties of purified plant pectinesterases.

Source	Molecular weight	$S_{20,w}$	Iso-electric point	Spec. act. (units/mg)	Optimal pH	K_m (mg/ml)	K_i pectate (mg/ml)	Optimal NaCl (mol/l)	Optimal $CaCl_2$ (mol/l)	Optimal temp. (°C)	Inactiv. temp. 5-15 min (°C)	References
Purified preparations												
apple	28 000	2.88 S		713	7.3			0.2	0.05	63	70	1
orange				2 200	8.0	2.3					80	2
orange I	36 200		10.05	694	7.6	0.083	0.416					3
II	36 200		> 11.0	762	8.0	0.0046	0.0016					3
tomato		3.17 S		724	8.0	2.4		0.1			80	4, 5
tomato	27 500	3.08 S		1 150	8.5	0.74	1.23	0.05	0.005			6, 7, 8
tomato	27 800[41]	3.25 S		704								9, 10
Partially purified preparations												
banana I	30 000		8.9	457	6.0			0.18				11
II	30 000		9.4	529	6.0			0.18				11
banana I	10 000											12
II	35 000											12
III	46 000											12
cacao				33	7.0							13
carrot	27 000			312	7.9			0.20			70	14
grape					5.5	3.5				50	90	15
grape			8.0		7.1							16
lime I				144		0.15						17
II				328		0.54						17
orange I				222		0.25						17
II				444		0.21						17
plum I		1.02 S		32								18
II				19								18
tomato	28 000	3.08 S		355								19
tomato I	35 500											20
II	27 000											20
III	23 700											20
IV	24 300											20
tomato I-VIII	22 000-32 000	3.30 S	7-9.3									21,22

Table 4b. Properties of crude plant pectinesterases.

Source	Optimal pH	K_m (mg/ml)	K_i pectate (mg/ml)	Optimal NaCl (mol/l)	Optimal $CaCl_2$ (mol/l)	Optimal temp. (°C)	Q_{10} about 20–50 °C	Inactiv. temp. 5–15 min (°C)	References
alfalfa	6.0–8.0	0.4		0.2	0.025				23
apple	7.0					55		80	24
apple	6.5							80	25
cranberry	7.5			0.15				100	26
cucumber	7.5			0.20			1.34	70	27
fruits (6)							1.31–1.53		28
oat				0.10					29
orange	7.5	0.8		0.1–0.2	0.025				30
papaya	7.5	0.3		0.2					31
papaya								85	32
peas	7.5–8.5			0.25		50	1.35		33
persimmon	6.5			0.15					34
snapbeans	8.2			0.2	0.05		1.40	90	35
strawberry	7.5					55	1.31		36
sweet cherries	7.5							80	37
tomato		0.55	0.24	0.15		60			38
tomato	7.5	0.41		0.05			1.44		39
tomato							1.47		40
vegetables (6)							0.81–1.43		28

Footnotes to table 4a and 4b.

1. Miyairi et al.(1975) 2. Manabe (1973a) 3. Versteeg et al. (1978) 4. Nakagawa et al. (1970a) 5. Nakagawa et al. (1970b) 6. Lee & MacMillan (1968) 7. Lee (1969a) 8. Miller & MacMillan (1971) 9. Markovič & Slezárik (1969) 10. Markovič (1974) 11. Brady (1976) 12. Markovič et al. (1975) 13. Gamble (1973) 14. Markovič (1978) 15. Montedoro (1968) 16. Datunashvili et al. (1976) 17. Evans & McHale (1978) 18. Theron et al. (1977a) 19. Theron et al. (1977b) 20. Pressey & Avants (1972) 21 Delincée & Radola (1970) 22. Delincée (1976) 23. Lineweaver & Ballou (1945) 24. Lee & Wiley (1970) 25. Pollard & Kieser (1951) 26. Arakji & Jang (1969) 27. Bell et al. (1951) 28. Vas et al. (1967) 29. Glasziou (1959) 30. MacDonnell et al. (1945) 31. Chang et al. (1965) 32. Aung & Ross (1965) 33. Collins (1970) 34 Nakayama & Iwasaki (1966) 35. Buren et al. (1962) 36. Leuprecht & Schaller (1968) 37. Schmid (1975) 38. Dahodwala et al. (1974) 39. Hills & Mottern (1947) 40. McColloch & Kertesz (1947) 41. One of its multiple forms.

Some of the original specific activities, K_m and K_i values were recalculated to uniform units to facilitate comparison.

Table 5. Properties of microbial pectinesterases.

Source	Molecular weight	$S_{20,w}$	Isoelectric point	Spec. act. (units/mg)	Optimal pH	K_m (mg/ml)	K_i pectate (mg/ml)	Optimal NaCl (mol/l)	Optimal $CaCl_2$ (mol/l)	Optimal temp. (°C)	Inactiv. temp. 5-15 min (°C)	References
Purified preparations												
Aspergillus japonicus	35 000		3.7	187	4.5					40		1, 2
Aspergillus niger				110								3
Clostridium multifermentans	400 000[18]			48	9.0	0.74		0.05		25-35	38	4, 5
Coniothyrium diplodiella I				300	4.8			0.25	0.035	45	55	6
II					4.8			0.25	0.035	45	55	6
Corticium rolfsii[17]	37 000			231	2.5-4.5					45		7
Fusarium oxysporum	35 000			205	7.0							15
Sclerotinia libertiana		4.41 S		16	5.0						90	8
Partially purified and crude preparations												
Acrocylindrium		5.4 S			7.5	0.7					70	9
Aspergillus niger					3-4	2.3	stimulation	no effect		40		10
Aspergillus niger	33 000		4.0									11
Aspergillus niger					4.5					40	45	12
Cercosporella herpotrichoides					7.5			0.085				13
Diplodia gossypina					6.5							14
Rhizoctonia solani					6-7			no effect				16

1. Ishii et al. (1978) 2. Ishii et al. (1979) 3. Rexová-Benková & Slezárik (1966) 4. Miller & MacMillan (1970) 5. Sheiman et al. (1976) 6. Endo (1964) 7. Yoshihara et al. (1977) 8. Oi & Satomura (1965) 9. Kimura et al. (1973) 10. Dahodwala et al. (1974) 11. Delincée (1978) 12. Baron (1978) 13. Hänssler et al. (1971) 14. Wang & Pinckard (1971) 15. Miller & MacMillan (1971) 16. 16. Bateman (1963) 17. One of its multiple forms 18. Complex with exo-pectate lyase.

Some of the original specific activities and K_m values were recalculated to uniform units to facilitate comparison.

2.2.4 Specificity and mode of action

Pectinesterase is highly specific for methyl esters of (1→4)-α-D-galacturonan chains. Both the D-galacturonan chain structure and the methyl ester group are needed for full activity. The methyl ester of alginic acid is not de-esterified by orange pectinesterase (MacDonnell et al., 1950). The methyl esters are not hydrolysed if the galacturonan chains are too short, for example trimers are not attacked but a degree of polymerization of 10 is sufficient for activity (McCready & Seegmiller, 1954). Also if part of the galacturonate ester units in the main chain are reduced to galactose units or part of the secondary hydroxyl groups are acetylated the reaction rate is much lower (Solms & Deuel, 1955). Pectinesterase has some activity on ethyl esters of D-galacturonan, approx. 10% of the rate on methyl esters at optimal pH (MacDonnell et al., 1950; Manabe, 1973b). The reaction rate on ethyl esters is much less affected by the pH than the rate on methyl esters and at pH 4 the rates are approximately equal (Manabe, 1973b). Some other esters (viz. propyl, allyl, propargyl) of α-D-galacturonan are attacked at a very low rate and the glycyl and glyceryl esters not at all (Deuel, 1947; Manabe, 1973b).

Crude orange pectinesterase preparations show activity on alkyl esters of several acids (MacDonnell et al., 1950). As these activities diminish with increasing purity of the pectinesterases, MacDonnell et al. (1950) concluded that the activity of crude pectinesterase on these substrates is due to contaminating carboxylesterases other than pectinesterase. The acetylesterase from citrus, which hydrolysis several carboxyl esters and the esters of acetic acid best, does not have pectinesterase activity (Jansen et al., 1947). It should be pointed out that the substrate specificity as described above pertains to plant pectinesterase (mainly citrus pectinesterase) and that the substrate specificity of microbial pectinesterases may appear to be different.

It is believed that the de-esterification of pectin by plant pectinesterases proceeds linearly along the pectin chain so that blocks of free carboxyl groups are produced. This conclusion was reached by comparing partially acid or alkali de-esterified pectins (which have randomly distributed free carboxyl groups) with partially enzymatically de-esterified pectin and pectic acid with respect to viscosity of solutions, calcium gel strengths, electrophoretic and chromatographic patterns and stability constants of calcium pectinates (Schultz et al., 1945; Hills et al., 1949; Heri et al., 1961; Kohn et al., 1968).

On the basis of the enzymatic activity on high methoxyl pectins, partially de-esterified by alkali, Solms & Deuel (1955) concluded that orange pectinesterase splits ester groups next to free carboxyl groups and that carboxyl groups are needed for the enzyme-substrate complex. Although carboxyl groups are necessary, they found a higher Michaelis-Menten constant (K_m) with an increasing number of free carboxyl groups in the substrate. Ishii et al. (1978; 1979) studied the elution patterns on DEAE cellulose and the gel strengths of pectins de-esterified with *Aspergillus japonicus* pectinesterase and they suggested this pectinesterase removes methoxyl groups at random.

For the complex of pectinesterase and exo-polygalacturonate lyase of *Clostridium multifermentans*, Sheiman et al. (1976) proved that both enzymes attacked highly esterified pectin (with a ^3H label at the reducing end) from the reducing end in a coordinated

manner. First the pectinesterase de-esterifies ester groups and without dissociation of the enzyme-substrate complex, the lyase splits off unsaturated dimers. From the equivalent release of the ^{3}H label and total product, and from the K_m and V_{max} of the lyase, it could be shown that no steady state concentration of the lyase substrate was available to account for the observed initial reaction rate. However, some doubt can be expressed about the conclusions of the same research group with respect to the mode of action of tomato pectinesterase and *Fusarium oxysporum* pectinesterase. Tomato pectinesterase and the exo-pectate lyase from *Clostridium multifermentans* (the pectinesterase of the pectinesterase-pectate lyase complex had been heat inactivated) were jointly incubated with highly esterified pectin (with an average degree of polymerization of 33). The activity of both enzymes was continuously monitored. The pectinesterase activity appeared to be rate limiting for the lyase activity and the lyase activity was always half that of the pectinesterase activity. It was concluded that half of the tomato pectinesterase activity was initiated at the reducing end of the pectin chain (Lee & MacMillan, 1970).

However, objections can be made to such a conclusion. Suppose the pectinesterase initiates its action somewhere in the pectin chain and moves to and reaches the reducing end of the pectin. With the turnover number of about 500 moles/mole.s of the pectinesterase, only 0.03 s are needed for a pectinesterase molecule to reach the reducing end of the pectin chain. In this way, every pectinesterase molecule can de-esterify 33 pectin chains per s. Then no lag period for lyase activity would be observed either. If all pectinesterase activity were initiated somewhere in the pectin chain and if half of the pectinesterase molecules were to move towards and reach the reducing end, the experimental results would have been the same. Therefore the conclusion that half of the pectinesterase activity is initiated at the reducing end of the pectin chain should not have been made.

A further point of criticism may be raised by comparing the observed initial activity of the lyase with the initial activity expected from the known Michaelis-Menten constant (K_m), maximal velocity (V_{max}) of the lyase (Sheiman et al., 1976) and the maximal quantity of substrate made available by the tomato pectinesterase. Even if all pectinesterase activity were initiated at the reducing end of the pectin chain, a considerable period (> 10 min) with lower lyase activity should have been observed.

Similar objections can be made to the conclusions on the mode of attack of *Fusarium oxysporum* pectinesterase (Miller & MacMillan, 1971).

2.2.5 Inhibition and (in)activation

The heat stability was discussed in Section 2.2.3. Pectinesterase is not inactivated by a dose of γ-radiation that can effectively reduce the microbial contamination of an industrial enzyme preparation (Delincée, 1978).

Table 6 shows the effects of a variety of agents on the activity of pectinesterases from different sources. Not all agents tested are listed, only those that are thought to give a good illustration of the array of effectors, e.g. the effect of potassium chloride is rather similar to that of sodium chloride whereas magnesium chloride behaves like calcium chloride. Also more sugars and phenols have been tested but the results were not

Table 6. Pectinesterase (in)activators and inhibitors. (++: strong stimulation; +: stimulation; o: no effect; -: inhibition/inactivation; =: strong inhibition/inactivation).

(In)activator/ inhibitor	Approx. conc. (mmol/l)	Higher plants									Micro-organisms				Cross references[22]
		apple	banana	carrot	grape	orange	papaya	tomato	7 plants	alfalfa	*Aspergillus niger*	*Coniothyrium diplodiella*	*Corticium rolfsii*	*Acrocylindrium*	
NaCl	100		+			++	++	++		++		+	+	+	1,2,3,4,5, 6,7,10
Li_2SO_4	100											+	+		3,5
$(NH_4)Cl$	100									++				-	7,6
$(NH_4)_2SO_4$	100											+	+	-	3,7,5
NaCN	100							o			o				8
NaN_3	100							o			-				8
$CaCl_2$	100					++		++		++		+	+	+	3,4,7,5,6, 16
$CuSO_4$	1							o			o		o		8,5
$ZnSO_4$	1											+			3
Hg acetate	1												=		5
$Hg\ Cl_2$	10							o			o			-	8,7
Pb acetate	1												o		5
$NiCl_2$	1				-										9
$ZnCl_2$	1				-										9
$Fe_2(SO_4)_3$	1												o		5
$AlCl_3$	1											-	o		3,5
$FeCl_3$	1				-							-			9,3
$SnCl_4$	1											-			3
glucose	1000		-				-								1,10
sucrose	1000	-	-				-								1,11,12,10, 13
glycerol	1000		-				-								1,10
ethanol	1000				-										9
tannic acid	3							=	=						14,15
(di)gallic acid	3				-			-	-						14,15,9
chlorogenic acid	3				-			-							14,9
caffeic acid	3				-			-							14,9
sodium lauryl sulphate	1		±					=			o				8,2,17
pectate						-		-		-					18,19,6,16
iodine	10			=				=			o				20,21,8
Cross references[22]		11 13	12 17 10	20	9	4 18 19	1	14 15 8 2 21 16	15	6	8	3	5	7	

1. Chang et al. (1965) 2. Pressey & Avants (1972) 3. Endo (1964) 4. MacDonnell et al. (1945) 5. Yoshihara et al. (1977) 6. Lineweaver & Ballou (1945) 7. Kimura et al. (1973) 8. McColloch & Kertesz (1947) 9. Montedoro (1968) 10. Brady (1976) 11. Lee (1969b) 12. Hultin et al. (1966) 13. Lee & Wiley (1970) 14. Hall (1966) 15. Fuchs (1965) 16. Lee & MacMillan (1968) 17. Hultin & Levine (1963) 18. Termote et al. (1977) 19. Versteeg et al. (1978) 20. Markovič (1978) 21. Markovič & Patočka (1977)
22. The references in the last vertical column refer to inhibitors used and the references at the bottom refer to the source of pectinesterase used. There is always at least one reference in common for a pectinesterase-inhibitor combination tested.

significantly different from those reported in the table. The principal effects of monovalent and bivalent cations on pectinesterase activity were also discussed in Section 2.2.3. Ammonium ions have an inhibitory effect on pectinesterase of *Acrocylindrium* but not on other microbial and plant pectinesterases. Mercury ions appear to be an effective inhibitor of some microbial pectinesterases. Some trivalent ions are inhibitory for both plant and fungal pectinesterases. Some of the cations may block the substrate instead of affecting the pectinesterase.

The inhibition by sugars and alcohols is ascribed to the reduction of the water activity and Chang et al. (1965) found an indication for the non-competitive character of the inhibition.

Tannic acid is the most effective phenolic inhibitor and cloud stabilization of orange juice by grape leave extracts (Kew & Veldhuis, 1961) was also ascribed to this compound (Hall, 1966).

The inactivatory effect of sodium lauryl sulphate differs somewhat depending on the molecular form or isoenzyme involved (Pressey & Avants, 1972; Hultin & Levine, 1963). At rather low concentrations it is an effective inactivator for plant pectinesterases.

A minimum chain length of 8 is required for the competitive inhibition of orange pectinesterase by oligomers from pectate. Pectate oligomers were successfully used to increase the cloud stability of orange juice (Termote et al., 1977).

The inhibition by iodine (irreversible and non-competitive) increased with the purity of tomato and carrot pectinesterase preparations (Markovič & Patočka, 1977; Markovič, 1978). On the basis of this inhibition it was assumed that tyrosine is present in the active centre of the enzyme (Markovič & Patočka, 1977).

2.2.6 Importance in food technology

Native pectinesterase in fruits and vegetables may produce desirable and undesirable effects before, during or after processing.

In fresh or underpasteurized citrus juices pectinesterase de-esterifies the pectin, producing methanol and low methoxyl pectin. Subsequently precipitation of the low methoxyl pectin by calcium ions results in cloud loss, a serious quality defect because of the appearance, reduction of taste and aroma components and the increased sensitivity to oxidation (Joslyn & Pilnik, 1961; Pilnik, 1958; Krop, 1974). Concentrates may gel. The drinks prepared by reconstitution have gel lumps, a curdy appearance and a rapidly setting pulp (Rouse & Atkins, 1952; Kew & Veldhuis, 1961). Gelating of papaya puree is also ascribed to pectinesterase activity (Chang et al., 1965). The pectinesterase in citrus juice is more heat stable than the micro-organisms which are involved in spoilage (about 20 ^{o}C) (Bisset et al., 1953) and than peroxidase which may give flavour changes (Nath & Ranganna, 1977b). Heat inactivation of pectinesterase brings the risk of a cooked off-flavour (Kew & Veldhuis, 1961).

Lemon juice on Sicily is clarified by storage in big tanks for months (Uhlig, 1978). The mechanism of clarification is not clearly understood since the pH of the juice is rather low (about pH 2.5) for native pectinesterase action. Nowadays the process can be speeded up by commercial fungal preparations, which contain several pectic enzymes

(Uhlig, 1978; Gonzalez et al., 1977).

In cider production clarification is desirable and calcium carbonate and sodium chloride are added deliberately to aid clarification (Pollard & Kieser, 1951).

In tomato juice production heat or acid must be applied quickly to produce a stable juice with a high viscosity (hot break process), whereas the combined action of pectinesterase and polygalacturonase simplifies the production of concentrated products (Fogarty & Ward, 1974; Wagner et al., 1969; Hobson & Davies, 1971; Paul, 1975).

By manipulating with blanching temperatures, holding times and sometimes with the aid of calcium ions or a pH shift, the texture of canned tomatoes (Hsu et al., 1965), apple slices (Wiley & Lee, 1970), cauliflower (Hoogzand & Doesburg, 1961), carrots (Lee et al., 1979) and potatoes (Bartolome & Hoff, 1972a) can be improved. Blanching conditions are chosen so as not to inactivate too much pectinesterase and to use its increased activity at the elevated temperature to reduce the degree of esterification of the pectic material in the tissues. Calcium bridges can then be formed which result in a better firmness retention after cooking. Presumably the heat applied for blanching is also needed to liberate pectinesterase or to render the substrate available for the enzyme since there is no direct correlation between pectinesterase level and firmness of fresh tomatoes (Hall & Dennison, 1960) or firmness retention of normal canned peaches (Shewfelt, 1965).

Citrus and tomato pectinesterase have been used to produce low methoxyl pectin, suitable to make gels with low sugar content or even without sugar (Mottern & Hills, 1946; Hills et al., 1942; Leo & Taylor, 1958; Leo & Taylor, 1962). The principal differences in low methoxyl pectins prepared by acid de-esterification and by plant pectinesterase are discussed by Hills et al. (1949). Low methoxyl pectin produced with pectinesterase from *Aspergillus japonicus* gives a high gel strength with calcium ions (Ishii et al., 1979).

Pectinesterases are usually an integral part of the commercially available pectolytic enzymes of fungal origin, which are used to increase press yields or pressability, improve extraction of pigments, reduce viscosity of juices, liquefy fruits and vegetables and clarify juices (Pilnik et al., 1975; Pilnik & Rombouts, 1978; Segal et al., 1966; Rombouts & Pilnik, 1978; Gonzalez et al., 1977; Delecourt, 1972).

A possible disadvantage is the methanol production, both by the native and by the added enzymes in fruit juice and wine technology (Segal et al., 1966; Dahodwala et al., 1974). Methanol concentrations can rise to 130 mg/l (Bock, 1966) or even 400 mg/l (Baumann & Gierschner, 1974) and concentrations of 50 mg/l are quite common. In fresh fruit the methanol concentration is usually 10-20 mg/l but can also be much higher and rise to nearly 200 mg/l (Segal et al., 1966; Al-Delaimy et al., 1966; Hultin & Proctor, 1961). Also this methanol is ascribed to pectinesterase action. In wine making, the heat inactivation of the native pectinesterase before treatment with the fungal enzyme preparation considerably reduces the final methanol concentration (Segal et al., 1966). The methanol in distilled spirits from fruits is caused by the activity of pectinesterase from the fruits during fermentation (Tanner, 1970).

2.2.7 Assay and detection

Several methods are available to determine pectinesterase activity. Some determine the changes in the substrate, others measure the methanol liberated.

2.2.7.1 Changes in the substrate

Titration of the carboxyl groups The activity of pectinesterase can be followed by titration of the liberated carboxyl groups with diluted (0.1-0.002 mol/l) alkali. If the pH is kept constant, the alkali used is a direct measure of the pectinesterase activity. The original manual titration with a pH indicator, used, for example, by Lineweaver & Ballou (1945), is nowadays usually done by automatic titration equipment. The pH is kept constant by the apparatus and the alkali consumption is monitored versus time. These titrations can be done in systems with a relatively low or no buffering capacity at the pH where the activity has to be determined. The uptake of carbon dioxide from the air must be corrected for by a blanc or prevented by saturating with nitrogen. Above pH 7 a correction must be made for the chemical de-esterification of the pectin. Also the pH should be well above the pK of the pectin (about 4.0). Correction for partial dissociation of the liberated carboxyl groups is difficult while the pK varies with the concentration of the pectin, the degree of esterification and the mode of de-esterification (Speiser et al., 1945). It is simpler to raise the pH quickly to 6 at the end of the reaction and to do the same for a blanc with inactivated enzyme (McColloch & Kertesz, 1947). However, this procedure may produce relatively large errors if the activity of the pectinesterase is much higher at pH 6 than at the pH at which the activity was measured.

Recording pH changes Somogyi & Romani (1964) proposed direct recording of the pH drop over a short pH interval in an unbuffered substrate solution. Within certain limits, the drop in pH per min appeared to be proportional to the amount of enzyme. A standard curve must be made to relate pH change to hydrogen-ion equivalents in a certain reaction mixture. The method is quick and little substrate is needed. Again, this method is unsuitable at a pH close to the pK-value of pectin. The method is suitable only to measure initial reaction rates and if the enzymes used do not differ in content of buffer salts.

Colour change of pH indicator A quantitative method in which the colour change of a pH indicator is measured with a spectrophotometer was used by Brady (1976). The method has much in common with the recording method for pH changes. A semi-quantitative method was described by Zimmerman (1978). A pH indicator is included in agar-pectin plates. Pectinesterase solutions are pipetted into wells and the area of the coloured zones after a certain incubation time is related logarithmically to the amount of enzyme. A suitable pH indicator (such as methyl red or bromothymol blue) with pectin in test tubes can be used as a sensitive qualitative screening method for pectinesterase activity (Delincée & Radola, 1970; Versteeg et al., 1978). Pectin and indicator impregnated paper can be used in printing techniques on gels after gel filtration or electrophoresis (Delincée & Radola, 1970).

Gelation and viscosity The oldest qualitative method is that of Frémy (1840) who discovered pectinesterase. After de-esterification pectin forms a gel with polivalent cations. Usually calcium ions are used. Pilnik & Rothschild (1960) developed a sensitive semi-quantitative variation to test under pasteurization of juices. The increase in viscosity of pectin solutions has also been used to measure pectinesterase quantitatively (Weurman, 1954). The method has only very limited possibilities for application and calcium also affects the rate of viscosity increase. In both methods the activity of chain-splitting enzymes may interfere.

Hydroxylamine and methylene blue Hydroxylamine reacts with the carbomethoxyl groups of the pectin. Subsequently addition of ferric ions leads to a brown complex of ferric-pectin hydroxamic acid. McComb & McCready (1958) used this as a semi-quantitative method to screen preparations on pectin-agar plates. Gee et al. (1959) used the reaction in histochemical studies to estimate the degree of esterification of pectic substances in fruits. Also recently Delincée (1976) used the principle for a print technique after isoelectric focusing of pectinesterases. The buffering capacity of the ampholites incorporated in the gel reduces the sensitivity of a print technique with a pH indicator. Another detection technique was used by Roeb & Stegeman (1975) in polyacrylamide gels. The pectin was included in the gel and after electrophoresis the gel was stained with methylene blue. Pectin is hardly coloured but the zones with de-esterified pectin strongly absorb the dye. In both staining techniques other pectic enzymes may interfere (Roeb & Stegeman, 1975; Delincée, 1978).

Non-pectin substrates Instead of pectin p-nitrophenylacetate has been used as a substrate for pectinesterase (Zimmerman, 1978). The pectinesterase activity was followed by a spectrophotometer at 400 nm. It would seem a simple procedure. However, from the work of MacDonnell et al. (1950) it can be presumed that not pectinesterase activity is measured, but some other esterase activity; see also Section 2.2.4.

Manometric assay Glasziou & Inglis (1958) used Warburg manometry to measure pectinesterase activity. Sodium hydrogen carbonate was added to the reaction mixture and the carbon dioxide development was measured. The sensitivity was higher than with a titrimetric procedure.

2.2.7.2 Methanol determination

Several colour reactions and gas chromatographic methods are available to determine methanol. Also, in some cases radioactive isotopes can be used.

Colour reactions and titrations The colour reactions require an oxidation step in which methanol is oxidized to formaldehyde. Several methods of oxidation were studied by Whright (1927) and the method with potassium permanganate was found to be the only satisfactory one. Whright (1927) found a modified Schiff's reagent to be the most suitable reagent to condense formaldehyde to a compound with a (violet) colour. Ethanol affects

the colour development and also glycerol and pectin interfere. Leaf & Zatman (1952) adjusted their test solutions to 15 g/l ethanol for optimal sensitivity and determined methanol concentrations down to 3 mg/l in body fluids. With chromotropic acid formaldehyde produces a violet colour which is measured at 580 nm and several other aldehydes do not interfere (Boos, 1948). There again the absorbance is affected by ethyl alcohol (Beyer, 1951). Interfering substances are described by Bricker & Johnson (1945). The method was not used under 20 mg/l methanol. Usually in organic material all these methods require distillation of the methanol before analysis can be carried out, which is the main disadvantage.

Wood & Siddiqui (1971) developed a sensitive method (down to 2 mg/l original methanol) in which pentane-2,4-dione was used to produce a colour with formaldehyde (absorbance peak at 412 nm). Because of the mild conditions, the method can directly be used in pectin solutions, but some other compounds interfere. The method was automated in an analyser by Vijayalakshmi et al. (1976) who found that polyphenols interfere. Segal et al. (1966) used a method in which formaldehyde is further oxidized to formic acid by hydrochloric acid and an excess of silver nitrate. The remaining silver nitrate is titrated and the methanol is calculated.

Methanol can also be determined by converting it to methyl iodite, after which it can be titrated (Vieböck & Schwappach, 1930; Clark, 1932; Hoffman & Wolfrom, 1947) or determined gravimetrically (Zeisel, 1885; Zeisel, 1886). The samples must first be distilled because methoxyl and other alkoxyl groups react also. The methods are labourous, insensitive and not specific for methyl alcohol.

Gas chromatography Several gas chromatographic methods for analysing lower alcohols have been published. Many of them were developed for analysis in wines and spirits and were not used for concentrations lower than 10-20 mg/l (Dyer, 1971; Venturella et al., 1974; Klaushofer & Bandion, 1968). Nowadays usually porous polymers without liquid phase are used as column material (Porapak, Chromosorb, Tenax). In several applications gas chromatography was used to determine methanol in concentrations of about 1 mg/l (Baker et al., 1969; Berger et al., 1974; Majchrowicz & Mendelson, 1971; Krop, 1974). However, the sensitivity of the detector can be affected by the water in the sample (Lucero, 1972) and low concentrations (< 10 mg/l) of methanol in aqueous samples appeared to give irregular response in routine analysis (Krop, 1974). For biological materials usually a precolumn or inlet is needed which must regularly be replaced to prevent rapid column deterioration (Baker et al., 1969; Berger et al., 1974). Also samples can be distilled or pretreated before injection (Baumann & Gierschner, 1974; Majchrowicz & Mendelson, 1971). Lee & Wiley (1970) used a dialysis cell to separate the methanol from the reaction mixture. Demethylation of carboxyl groups is thus prevented, however, the diffusion process limits the possibility to measure initial reaction rates. Usually headspace techniques are only qualitatively used (Wolford et al., 1963; Norman, 1970). Davis & Chase (1969) measured quantitatively the alcohols of citrus juices by a headspace technique, but the coefficient of variation determining concentrations of lower than 40 mg/l was not given.

Alcohols can also be determined gas chromatographically after derivatization to

alkyl iodites (after distillation because esters and ethers such as pectin, hesperidin and naringin also react). In contrast to the volumetric and gravimetric determinations of alkyl iodites, the method is specific for various alcohols because the alkyl iodites are separated on the column (Kratzl & Gruber, 1958; Vertalier & Martin, 1958). Derivatization to alkyl iodites is advantageous if an electron capture detector is used, which has a sensitivity of about 50 times that of a flame ionization detector (Dickes & Nicholas, 1978).

Another approach is derivatization of alcohols to nitrite esters, which are much more volatile than their alcohols. The headspace is sampled and injected and concentrations of about 1 mg/l can be determined quantitatively (Gessner, 1970; Litchman & Upton, 1972). If the method is properly used, pectin is not de-esterified under the derivatization conditions and pectin solutions and plant tissue homogenates can be assayed without prior distillation (Bartolome & Hoff, 1972b). Recently Baron et al. (1978) described a very sensitive and reproducable variation of this principle. At a methanol concentration of about 1 mg/l the coefficient of variation was only 1.2%, better than any of the gas chromatographic or colorimetric methods described. Generally, the gas chromatographic methods are not very time consuming (4-15 determinations/h).

Radioisotopes A sensitive method of another nature is the use of pectin labelled with ^{14}[C] methyl as a substrate. The substrate can be prepared biosynthetically (Kauss et al., 1969) or by esterification with ^{14}[C] diazomethane (Milner & Avigad, 1973). After the enzymatic reaction the substrate is separated from the liberated methanol by precipitation with alcohol or by extraction. The radioactivity of the methanol is then counted in a liquid scintillation counter. The sensitivity of the method depends on the specific activity of the label, but a sensitivity of 0.03 mg/l methanol is easily achieved (Milner & Avigad, 1973).

The method is of little value if natural substrates are to be used (e.g. natural pectic substances and fruit juices), but can be advantageous in studies of working mechanisms and in routine assays of enzymes.

Generally a sensitive methanol analysis offers many more possibilities than titration e.g. in buffered systems, at low reaction rates, at pH values below 5 and if a limited amount of substrate is available. But where it can be applied, automatic titration is the most simple method, also because the activity is continuously monitored and deviations from linearity in time are easily recognized.

3 Materials and methods

Citrus fruit Citrus fruits were purchased as single lots from local dealers in the Netherlands. Navel oranges (*Citrus sinensis*) from Spain, Salustiana oranges (*Citrus sinensis*) from Spain, Shamouti oranges (*Citrus sinensis*) from Israel, Valencia oranges (*Citrus sinensis*) from the USA, White Marsh seedless grapefruits (*Citrus paradisi*) from Israel, Clementine mandarins (*Citrus reticulata*) from Spain and lemons (*Citrus limon*) from the USA.

Substrates and inhibitors Green ribbon apple pectin(62% esterified; anhydrogalacturonic acid content 64%,degree of polymerization 368)and brown ribbon apple pectin(72% esterified; anhydrogalacturonic acid content 63%, degree of polymerization 423) were obtained from Messrs. Obipectin Ltd., Bischofszell, Switzerland. Pectins with different degrees of esterification were prepared by esterification of the same brown ribbon apple pectin to 95.6% and subsequent alkali de-esterification as described by van Deventer-Schriemer & Pilnik (1976). The characteristics of the preparations are given in Table 7. Various other substrates were obtained from commercial suppliers and from the laboratory collection as indicated in Table 16 (page 46). Polygalacturonic acid (anhydrogalacturonic acid content 88%) used as pectinesterase inhibitor was obtained from ICN Pharmaceuticals, Inc., Cleveland, Ohio, USA. Pasteurized frozen, pectinesterase inactive, Valencia orange concentrate of 65 ^{o}Brix from Letaba (South Africa) was supplied by the Coca-Cola Company and had the characteristics as listed by Krop (1974).

Fractionation of pectins Pectins were fractionated according to their degree of esterification by chromatography on a DEAE cellulose (Whatman DE22) column. Five ml of a 10 g/l pectin solution was applied to a column of 2.5x12.5 cm, equilibrated with starting buffer (0.05 mol/l sodium phosphate, pH 4.8) and eluted with a linear gradient

Table 7. Characteristics of pectin preparations.

Degree of esterification (%)	Anhydrogalacturonic acid content (g/100 g)	Degree of polymerization (monomers/chain)
95.6	75.0	76
88.0	73.4	84
78.2	73.4	81
67.8	74.6	81
52.6	80.7	77
32.3	82.2	79

from 0.05 to 1 mol/l sodium phosphate buffer pH 4.8 in 3 l. The remaining material was washed from the column with 250 ml 0.1 mol/l sodium hydroxide. Fractions of 15 ml were collected and analysed for uronic acids. This method is a modification of the method described by van Deventer-Schriemer & Pilnik (1976).

Degree of esterification of pectins The degree of esterification was determined titrimetrically. The pectin (300 mg) was dissolved in 100 ml water and passed through a (2x18 cm) Dowex 50 W and an (2x18 cm) Amberlite 45 IR ion exchange column, placed in series. The columns were washed with 200 ml distilled water and the total volume collected was titrated with 0.1 mol/l sodium hydroxide with phenolphthalein as an indicator (= x ml). Fifty ml of 0.1 mol/l sodium hydroxide was added and the mixture was left in a glass stoppered flask for one hour at room temperature. Then 50 ml 0.1 mol/l sulphuric acid was added and the solution was slowly titrated under nitrogen with 0.1 mol/l sodium hydroxide (= y ml).
Formula:

$$\text{Degree of esterification} = \frac{y}{x+y} \cdot 100\%$$

$$\text{Anhydrogalacturonic acid content} = \frac{\frac{(x+y)}{10} \cdot 176}{300} \cdot 100\%$$

The ion exchange columns were regenerated separately after about 4 g of pectin had been treated.

Degree of polymerization of pectins The degree of polymerization of pectins was estimated viscosimetrically. The viscosity of 4 pectin concentrations (2.0, 1.5, 1.0 and 0.5 g/l) in 0.1 mol/l tris-succinate buffer pH 6 with 0.2 mol/l sodium chloride was measured in an Ubbelohde viscosimeter No. 1. The intrinsic viscosity $[\eta]$ was determined by plotting the ratio $(\eta_r-1)/c$ against c and by extrapolating to zero concentration (c = pectin concentration, η_r = viscosity relative to solvent). The formula $[\eta] = 1.4\times10^{-6} \cdot M^{1.34}$ was used to calculate the average molecular weight (Owens et al., 1946). Dividing the molecular weight of the polymer by the average molecular weight of the monomers (176-190, depending on the degree of esterification) gave the degree of polymerization of the pectins.

Cloud stability of orange juice For measuring the effect of pectinesterase on the cloud stability of orange juice, reconstituted Valencia orange concentrate was used. The cloud was determined by optical density of the supernatant at 660 nm after centrifuging for 10 min at 360xg. Krop (1974) gave detailed information about the turbidity measurements. However, instead of 10 ml samples, only 3 ml samples were removed from the glass stoppered bottles and samples were not returned to the bottles but used for methanol analysis.

Sugar analysis The neutral sugar composition of proteins and pectins was analysed gas chromatographically according to the method of Jones & Albersheim (1972). Proteins (about 2 mg) were dialysed salt free and lyophilized in a glass tube. The sample was dissolved in 1 ml of distilled water. To this solution 0.5 ml of 2 mol/l tri fluoro-acetic acid containing 100 µg of inositol as internal standard was added. The tube was closed by melting and heated in an oil bath at 121 °C for one hour. The tube was opened and the hydrolysate was dried at room temperature by a soft stream of air. After addition of 0.25 ml of 1 mol/l ammonium hydroxide with 10 g/l sodium borhydride the samples were left for one hour at ambient temperature. Next 0.15 ml of distilled concentrated acetic acid was added in 3 portions. After addition of 0.5 ml of 100 g/l acetic acid in methanol the sample was dried in an air stream and this procedure was repeated three times and the same was done with 4 successive additions of 0.5 ml methanol. Thereupon 0.1 ml of acetic anhydride was added and the tube resealed by melting and placed for three hours in an oil bath at 121 °C. The tubes were opened and dried at room temperature by a soft stream of air. To the residu 0.1 ml of chloroform and 0.1 ml of water were added and after dissolving, the samples were transferred to a 1 ml test tube. The aqueous layer was removed with a syringe and the remaining chloroform solution was dried in a stream of air. After dissolving the residue in 50 µl of chloroform, 10 µl was injected in a gas chromatograph (Hewlett Packard 5750 G with dual flame ionization detector) equipped with a dual column system of two glass columns of 190 cmx4 mm (I.D.), packed with 3 g/kg O.V.275+4 g/kg X.F.1150 on Gas Chrom Q 100-120 mesh (Darvill et al., 1975). Column oven temperature programme: 3 min at 140 °C, 140-190 °C with 1 °C/min, 50 min at 190 °C (to elute amino sugars). Peak areas were measured by an Infotronics CRS 304 computing integrator.

The neutral sugars of pectins (about 2 mg) were determined by virtually the same procedure, but uronic acids were removed by a Dowex 1x8 treatment after hydrolysis (batchwise 300 mg Dowex 1x8, one hour stirring, supernatant with 200 mg Dowex 1x8, one hour stirring, collected sediments washed with 3 x 2.5 ml distilled water, supernatants and washing water used for further procedure). The holding of the gas chromatograph at the upper temperature limit was omitted.

Uronic acids were determined by the carbazole test in an automated analyser as described by van Deventer-Schriemer & Pilnik (1976).

Amino acid analysis About 0.1 mg protein was dialysed salt free against distilled water and lyophilized together with 3 µg norleucine internal standard. The samples were hydrolysed under nitrogen for 22 h at 110 °C with 1 ml 6 mol/l hydrochloric acid. The hydrochloric acid was evaporated at 40 °C under vacuum and samples were analysed by either an amino acid analyser or a gas chromatographic procedure.

For the analysis on the amino acid analyser, the dried samples were redissolved in 0.3 ml of 0.2 mol/l lithium citrate buffer (pH 2.20). The amino acid analyser used was a Biotronik LC-2000 and the amino acids were separated on a single column (Durrum DC-6A) by stepwise elution with lithium citrate buffers. Amino acids were detected with ninhydrin at 570 nm and 440 nm (for proline). Peak areas were integrated with a Spectra Physics Autolab System I computing integrator.

Amino acids were also analysed gas chromatographically with the procedure as described by Mackintosh et al. (1977) with the derivatization of amino acids to N(O)-heptafluorobutyryl-iso-amyl esters.

Tryptophan was determined by two methods. The colour reaction of Opieńska-Blauth et al. (1963) was used without prior enzymatic hydrolysis and the absorbance of standards and samples was measured at 450 nm instead of 545 nm. Also the fluorescence method of Pajot (1976) in 6 mol/l guanidine hydrochloric acid pH 6.8 was used to determine tryptophan.

Methanol analyses Methanol was determined after the general procedure of Litchman & Upton (1972), which includes conversion of alcohols to their volatile nitrite esters and subsequent analysis by gas chromatography after headspace sampling. In this work much attention was paid to the derivatization conditions, which are given in Chapter 4. The gas chromatography was carried out on a Hewlett Packard 5750 G research gas chromatograph with flame ionization detector. Column: 90 cmx4 mm (I.D.) glass column packed with 1 mol/l potassium hydroxide washed textured glass beads 80-100 mesh (Chrompack, Middelburg, the Netherlands) over a length of 15 cm starting from the injection port end and packed with Porapak R 120-150 mesh (Waters Associates, Milford, Mass.) over the rest of the column length. Column oven temperature: 150 °C, temperature of injection port and detector: 250 °C. Carrier gas (nitrogen) 80 ml/min. New columns were conditioned at 260 °C for 20 h. Peak areas or peak heights were measured with an Infotronics CRS 304 computing integrator.

The mass spectra of alcohols and their reaction products were determined with a combined gas chromatograph (Pye 204) mass spectrometer (VG Micromass 7070F). The gas chromatographic conditions were the same as for the methanol analysis described above, except that helium instead of nitrogen was used as carrier gas. Spectra were recorded with the aid of a connected VG Datasystem 2040.

Pectic enzymes Polygalacturonase was obtained from the yeast *Kluyveromyces fragilis* (Jörgenson) v.d. Walt strain CBS 397. Endo-polygalacturonase is the only pectic enzyme produced by this organism (Phaff, 1966). The enzyme was prepared as described by Krop (1974) but further purified on a cross-linked alginate column (6x1.5 cm). The cross-linked alginate was prepared as described by Rombouts et al. (1979). After dialysis against 0.1 mol/l sodium acetate buffer pH 4.2 the culture filtrate (4000 ml) was passed through the cross-linked alginate column and the column was washed with starting buffer until the eluate was free of glucose. The polygalacturonase was washed from the column with 0.1 mol/l sodium acetate pH 6 with 1 mol/l sodium chloride. Specific activity 100 units/mg. Endo-pectate lyase of *Pseudomonas fluorescens* GK-5, purified as described by Rombouts et al. (1978), was obtained from the laboratory collection. Specific activity 956 units/mg. Endo-pectin lyase was purified from Ultrazym 20 (an *Aspergillus niger* preparation from CIBA-Geigy AG, Basel, Switzerland) as described by van Houdenhoven (1975). The enzyme preparation used here only contained isoenzyme Type II, specific activity 10 units/mg. All enzyme activity units are in µmol/min at optimal conditions and 30 °C.

Enzyme assay and detection One unit of pectinesterase is defined as the amount of enzyme which liberates one micromole of carboxyl groups per min at standard assay conditions. In standard assays for pectinesterase activity, 20 ml of 5 g/l green ribbon pectin in 0.15 mol/l sodium chloride, pH 7.0 was pipetted into a glass beaker. After equilibration at 30 °C 0.01 to 1 ml of pectinesterase solution was added. While stirring with a magnetic stirrer and purging with nitrogen, the initial reaction velocity was measured by automatic titration of the liberated carboxyl groups with 0.02 mol/l sodium hydroxide, in a Combi recording pH-stat (E 300 B - E 473 - E 425) from Metrohm Ltd., Herisau, Switzerland. For kinetic measurements and pectinesterase activities in juices other techniques were used as well. Activities below pH 5.0 were determined by incubating 10 ml of buffered substrate with enzyme in a 10 ml glass stoppered flask with 25 mg/l of an alcohol (ethanol or propanol-2 or propanol-1) in a water bath of 30 °C. At time intervals samples of 1 ml were taken and the methanol concentrations were determined gas chromatographically, for details see Chapter 4. In titrimetric assays above pH 7 the blanc values for chemical de-esterification were substracted. If initial reaction rates were too short because of shortage of substrate or product inhibition, reaction volumes were increased and more diluted alkali (0.002 mol/l) was used. Also, in this case, the pH of the reaction mixture was continuously recorded on a recorder with extended scale (1 pH unit/full scale). For more sensitive measurement the pH drop method of Somogyi & Romani (1964) was used. The pH shift - alkali equivalents were determined with 0.002 mol/l potassium hydroxide. The drop in pH per minute without enzyme in the reaction mixture was substracted. Pectinesterase detection on thin-layer gels after isoelectric focusing or pH-gradient electrophoresis was done with the hydroxylamine-ferric chloride print technique of Delincée (1976).

Non-esterase activities of pectinesterase preparations on various substrates were measured in reaction mixtures (0.5 ml) containing 4 mg per ml of substrate, 0.04 mol/l sodium succinate buffer, pH 5.0 and 3 units of pectinesterase. After incubation at 30 °C for 20 h, the increase in reducing groups was determined by the Nelson-Somogyi method (Spiro, 1966). Phosphatase (Arnold, 1972), carboxylesterase and protease (Cabib & Ulane, 1973) activities were determined using p-nitrophenylphosphate, p-nitrophenylmyristate and azocoll, respectively.

Polygalacturonase activity was determined by the Nelson-Somogyi method (Spiro, 1966) or by a viscometric assay (Krop, 1974). Pectin and pectate lyase were measured by the increase in absorbance at 235 nm (Voragen, 1972; Rombouts et al., 1978) or by the Nelson-Somogyi method (Spiro, 1966).

Chromatography of pectinesterase and analysis of fractions Column chromatography was done with Bio-Gel P-100 (100-200 mesh), CM Bio-Gel A (100-200 mesh) and Bio-Gel HTP (hydroxylapatite) obtained from, and used according to the specifications of Bio-Rad Laboratories, Richmond, California, USA. Cross-linked pectate was a gift from Dr L.Rexová-Benková, Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Czechoslovakia. From the bed volume of this preparation (3.7 ml/g) it was estimated that it contained 3.5 uronic acid units per one cross-link (Kohn, 1975). The protein concentration in eluted fractions was determined by using Folin reagent (Lowry et al., 1951), with bovine

serum albumin (A-4378, Sigma Chemical Company, St.Louis, Missouri, USA) as standard. Pectinesterase activity in fractions was assayed with a methyl-red indicator test. Aliquots of 10 - 25 µl of fractions were added to small test tubes containing 1 ml of a solution (pH 7.0) composed of 5 g/l green ribbon pectin, 0.15 mol/l sodium chloride, 0.1 g/l sodium azide as preserving agent and a few drops of a 10 g/l ethanolic methyl-red solution per 25 ml. Fractions giving a positive methyl-red test (red colour) within one hour were also assayed titrimetrically.

Electrophoresis and isoelectric focusing Enzyme purity in various stages of the purification procedure was checked by electrophoresis on thin slabs of polyacrylamide gel in the presence of sodium dodecyl sulphate (Ames, 1974), with the discontinuous buffer system of Laemmli (1970). The separating gels contained a final acrylamide concentration of 120 g/l. Staining of slab gels was done with Coomassie Brilliant Blue (Fairbanks et al., 1971). The molecular weight of the purified pectinesterases was determined with the same electrophoresis system, using the following protein standards: bovine serum albumin (Boehringer, Mannheim, mol.wt. 67 000), beef liver catalase (Boehringer, mol.wt. 60 000), ovalbumin (Boehringer, mol.wt. 45 000), chymotrypsinogen A (Worthington, mol.wt. 25 700) and trypsinogen (Boehringer, mol.wt. 23 500).

Isoelectric focusing was done in a 110 ml column (Type 8100-1, LKB-Produkter AB, Bromma, Sweden), following the instructions of Winter & Karlsson (1976). While mixing the sorbitol density gradient, enzyme samples were introduced as a band at about one fourth of the height of the column. A constant power supply (Type 2103, LKB) was used for electrofocusing at max. 4 W and max. 1400 V during approximately 40 h at 6 °C. While continuously recording the absorbance at 280 nm, the column was emptied from the bottom, at a rate of 2 ml per min, collecting fractions of 1, 2 or 4 ml. The pH of the fractions, kept at 6 °C, was determined within 5 min with an accurate and stable pH meter previously calibrated with freshly prepared standard buffers (Electronic Instruments, Ltd., Chertsey, England) of pH 7.08 and 9.41 at 6 °C.

pH Gradient electrophoresis (a separation method developed in this work) was applied if isoelectric focusing was impossible because of inavailability of commercial ampholites in the pH range needed. Thin-layer gels 11x24 cm of Ultrodex 100 (a dextran gel from LKB) with ampholines for the pH range 7-10 were prepared according to the instructions of Winter et al. (1975) for the LKB 2117 multiphor flat bed electrophoresis apparatus. Enzymes were introduced 1 cm from the anode side and power was applied for about 4.5 h. Temperature 6 °C, power settings: maximal 8 W, 20 mA and 1400 V over the length of the gel. Pectinesterase activity was detected with the printing technique. Then the gel was segmented with a fractionating grid. The pH of the segments was measured with a pH-surface electrode and for quantitative pectinesterase determinations, segmented zones in which pectinesterase activity was detected were scraped off and transferred to small filters (LKB 2117-502 PEGG elution columns). The pectinesterase was eluted into tubes with 0.1 mol/l potassium chloride and 0.1 g/l sodium azide solutions and activity in the eluates was measured under standard assay conditions.

4 Methanol determination

Because of the need to measure pectinesterase activities under conditions where no titrimetric assay could be used, much attention was paid to the gas chromatographic analysis of methanol. Thereby results were obtained which are also useful for the gas chromatography of other lower alcohols. The method described below was based on the method of Litchman & Upton (1972). In this method alcohols are esterified to volatile nitrites and determined gas chromatographically after headspace sampling.

4.1 EXPERIMENTS AND RESULTS

Reaction conditions 0.2 g of urea was weighed into 25 ml aluminium painted flasks (Pierce, Rockford, Illinois, Reacti-flasks No. 13320). The flasks were purged with nitrogen for about 5 min and stoppered with a rubber stopper. Then 1 ml of a saturated oxalic acid solution was added and the flask was swirled. Next 1 ml of sample was added (t=0) and the flask was swirled again. The rubber stopper was replaced by an open screw cap (Pierce, No. 13218) with a silicone rubber septum (Pierce, No. 10155) and the flask was swirled until t=30 sec. At t=45 sec 0.5 ml of a sodium nitrite solution was injected through the septum and the flask was swirled until t=70 sec. Next the flask was held at a certain temperature-time combination for reaction. Thereupon, 1 ml headspace was withdrawn with a gas syringe and analysed by a gas chromatograph under the condition as described in Chapter 3. Several combinations of reaction time, reaction temperature, type of gas syringe and sodium nitrite concentration were tested. Litchman & Upton (1972) prepared the esters at room temperature and Bartolome & Hoff (1972b) at 0 °C. Using a saturated sodium nitrite solution and injection with a 2.5 ml Gastight Hamilton syringe (# 1002, Hamilton Co., Reno, Nevada) it appeared that holding the Reacti-flask in ice from t=70 sec on, was better than holding it at room temperature. Fig. 2 shows the peak areas of methanol against different reaction time whereas Fig. 3 shows the ratio of the areas of ethanol to methanol against the reaction time. The peaks are named after their respective alcohols (methanol, ethanol, propanol-1 and propanol-2) and not after their nitrite esters for reasons which are explained later in this Section. The nitrite esters were more stable and the sensitivity of the method was higher at 0 °C (Fig. 2). But from Fig. 3 it can be seen that with the use of ethanol as internal standard, reaction temperature and time become less critical.

With the reaction at room temperature an overpressure was built up (the reaction is exotherm), and some gas escaped from the syringe after withdrawal from the vial. The premature escape of the gas from the syringe was effectively prevented by the use of a 2 ml Pressure-Lok syringe (Precision Sampling Corp., Baton Rouge, Louisiana) fitted with a valve to close the needle. This doubled the sensitivity of the method at room

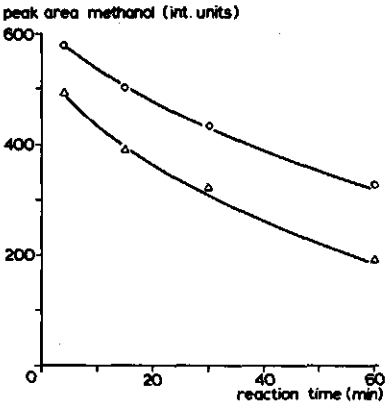


Fig. 2. Peak areas of methanol as a function of reaction time with nitrous acid. A solution of 25 mg/l methanol was used for the reactions. Reaction flasks and reagents were at room temperature. After the reagents were combined in the flasks, the flasks were incubated at room temperature (Δ) or in ice (o) till injection of 1 ml of headspace with a Gastight syringe in the gas chromatograph.

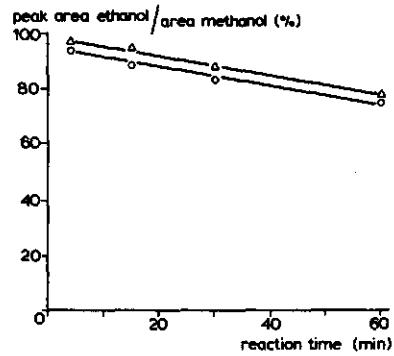


Fig. 3. Peak areas of ethanol relative to methanol as a function of reaction time with nitrous acid. The solution used for the reaction contained a mixture of 25 mg/l ethanol and 25 mg/l methanol. Reaction and conditions as in Fig. 2. The reaction flasks were incubated at room temperature (Δ) or in ice (o).

temperature but hardly affected the method at 0 °C.

In order to prevent the regular blocking of the injection needle of the syringe used to inject the sodium nitrite solution into the reaction vial, the saturated sodium nitrite solution was replaced by a 250 g/l sodium nitrite solution.

Fig. 4 shows the peak areas of 4 alcohols with different reaction times when the reagents and vial were prechilled at 0 °C and the whole reaction was done in ice. Fig. 5 shows the areas relative to each other under the same conditions. Similarly Figs 6 and 7 show the values when the reagents and reaction were kept at room temperature. Peak areas of different figures (Figs 2, 4, 6, 8 and 9) are not comparable because sometimes different settings of integrator and gas chromatograph were used. From Figs 4 and 6 it can be concluded that at 0 °C the reaction products are more stable. However, at 0 °C a longer reaction time is required for optimal sensitivity. In spite of the rapid reduction of the sensitivity with extended reaction times with the method at room temperature, the relative areas are less susceptible to variations in reaction time (Figs 6 and 7).

Table 8 shows the absolute and relative sensitivity of several methods for a mixture of 4 alcohols. The data were presented both absolutely and relatively to values obtained by direct injection of 5 µl liquid under the same gas chromatographic conditions. Table 9 shows the variation coefficients of some of these methods. From Tables 8 and 9 it can be concluded that the method at room temperature, using a Pressure-Lok syringe (Table 8, method No. 8) is the most sensitive. The method is also very reproducible if accurately

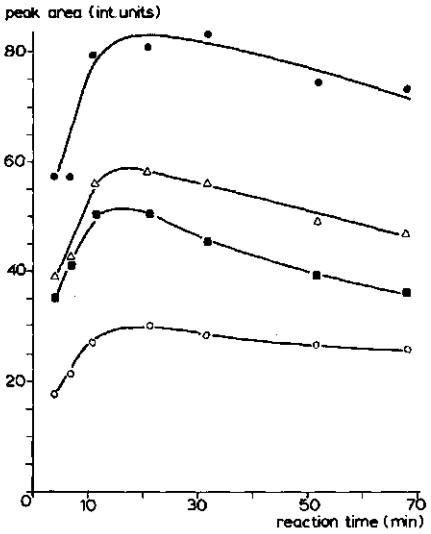


Fig. 4. Peak areas of four alcohols as a function of reaction time with nitrous acid at 0 °C. The solution used for the reaction contained a mixture of four alcohols, 25 mg/l of each. Prechilled reagents and reaction flasks were used. One ml of headspace was injected in the gas chromatograph with a Pressure-Lok syringe. Alcohols: Methanol (●); ethanol (Δ); propanol-2 (o) and propanol-1 (■).

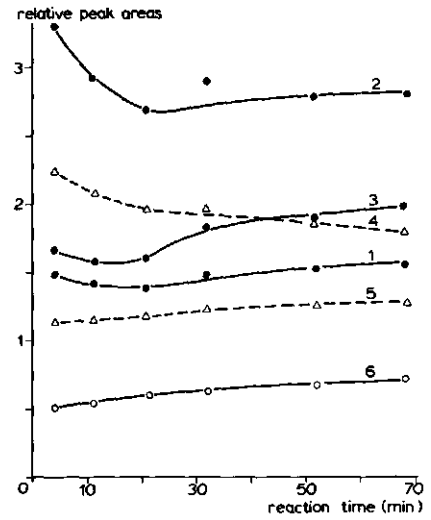


Fig. 5. Relative peak areas of four alcohols as a function of reaction time with nitrous acid at 0 °C. Reaction and conditions as in Fig. 4. Relative peak areas: methanol/ethanol (1); methanol/propanol-2 (2); methanol/propanol-1 (3) ethanol/propanol-2 (4); ethanol/propanol-1 (5) and propanol-2/propanol-1 (6).

timed (Table 9, method No. 8). The short reaction time is convenient too. This procedure which was finally used in the experiments for methanol determination is summarized in Table 10.

Typical retention times on a 90 cmx4 mm (I.D.) column with the gas chromatographic conditions as described in Chapter 3 are 43 s for methanol, 82 s for ethanol, 136 s for propanol-2 and 192 s for propanol-1. Depending on the internal standard used, 10-15 methanol determinations, derivatization included, could be done in an hour.

Linearity and reproducibility The correlation between methanol concentration and peak area was tested for aqueous methanol solutions, single strength orange juice, pectin concentrations up to 10 g/l and human blood serum. Fig. 8 shows the correlation for

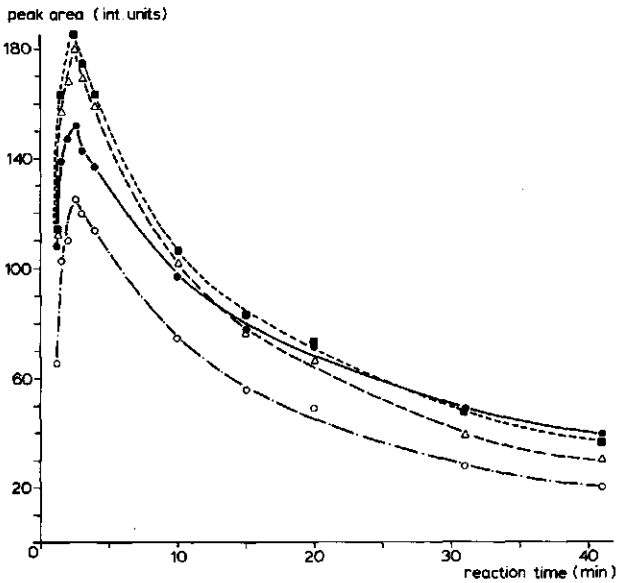


Fig. 6. Peak areas of four alcohols as a function of reaction time with nitrous acid at room temperature. The solution used for the reaction contained a mixture of four alcohols, 25 mg/l of each. One ml of headspace was injected in the gas chromatograph with a Pressure-Lok syringe. Alcohols: methanol (●); ethanol (Δ); propanol-2 (o) and propanol-1 (■).

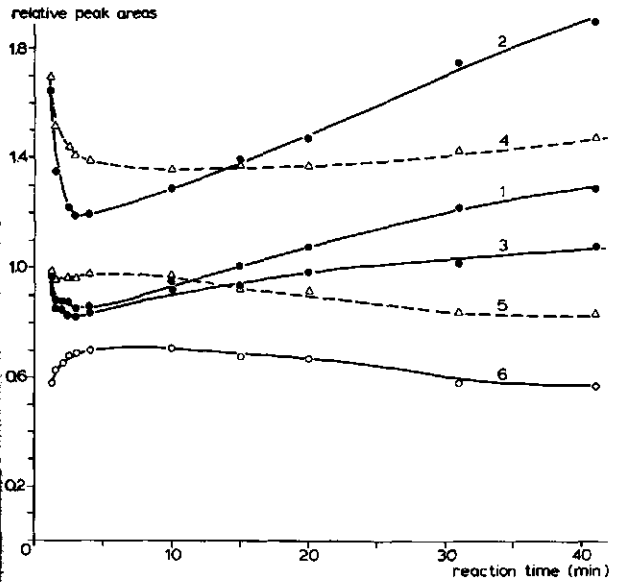


Fig. 7. Relative peak areas of four alcohols as a function of reaction time with nitrous acid at room temperature. Reaction and conditions as in Fig. 6. Relative peak areas: methanol/ethanol (1); methanol/propanol-2 (2); methanol/propanol-1 (3); ethanol/propanol-2 (4); ethanol/propanol-1 (5); propanol-2/propanol-1 (6).

Table 8. Absolute and relative peak areas obtained by different gas chromatographic procedures for a mixture of four alcohols, 25 mg/l of each.

No.	Method	Methanol		Ethanol		Propanol-2		Propanol-1	
		area (int.units)[7]	rel.area	area (int.units)[7]	rel.area	area (int.units)[7]	rel.area	area (int.units)[7]	rel.area
No derivatization									
1	5 µl liquid	11.1	1.00	19.4	1.00	22.6	1.00	22.1	1.00
2	1 ml headspace[1,2] (1 ml in 30 ml vial)	1.2	0.11	1.8	0.09	2.4	0.11	2.9	0.13
Nitrite esters									
3	sat.$NaNO_2$, 4 min[2,3]	82.8	7.46	101.1	5.21	78.5	3.47	69.2	3.13
4	250 g/l $NaNO_2$,60 min[2,4]	53.7	4.84	44.3	2.28	25.3	1.12	35.2	1.59
5	250 g/l $NaNO_2$, 5 min[2,3]	66.2	5.96	75.1	3.87	54.6	2.42	72.3	3.27
6	250 g/l $NaNO_2$,10 min[2,3]	56.1	5.05	58.5	3.02	41.6	1.84	52.6	2.38
7	250 g/l $NaNO_2$,2.5 min[2,5]	67.9	6.12	82.8	4.27	63.3	2.80	83.7	3.79
8	250 g/l $NaNO_2$,2.5 min[5,6]	145.6	13.12	182.1	9.39	140.1	6.20	196.5	8.89
9	sat.$NaNO_2$,2.5 min[5,6]	159.5	14.37	210.9	10.87	176.3	7.80	220.1	9.96

1. Room temperature.
2. Gastight syringe.
3. Reagents and vial at room temperature, reaction on ice.
4. Reagents, vial and reaction on ice.
5. Reagents, vial and reaction at room temperature.
6. Pressure-Lok syringe.
7. Integrator units.

Table 9. Variation coefficients (%) of a ten-fold analysis of a mixture of four alcohols, 25 mg/l of each, with and without the use of one of them as internal standard.

Method No.[1]	Methanol				Ethanol			Propanol-2		Propanol-1
	area	relative to			area	relative to		area	relative to	area
		ethanol	propanol-2	propanol-1		propanol-2	propanol-1		propanol-1	
3	4.27	1.61	2.93	3.10	4.84	1.56	1.91	5.91	1.14	6.29
4	5.69	2.16	2.57	3.29	4.82	0.98	2.02	4.18	2.12	4.97
5	4.34	1.20	2.70	2.34	4.90	1.58	1.28	6.30	1.12	5.81
6	1.84	1.88	3.79	4.95	1.96	2.23	3.42	3.90	3.03	3.95
7	3.35	1.10	2.60	1.67	3.01	1.79	1.02	3.50	1.49	3.70
8	2.44	0.75	1.56	1.14	2.43	1.16	0.62	3.00	1.14	2.49

1. Numbers refer to the methods listed in Table 8.

Table 10. Modified procedure for the gas chromatographic methanol determination with reagents and reaction at room temperature.

- Weigh 0.2 g of urea into a 25 ml aluminium painted vial.
- Purge at least 4 minutes with nitrogen.
- Close vial with rubber stopper and start purging of the next vial.
- Add 1 ml of a saturated oxalic acid solution and swirl vial.
- Add 1 ml of the sample (start timing : $t = 0$).
- Swirl vial, recap with open screw cap with silicone rubber septum.
- Swirl vial till $t = 30$ s.
- Inject 0.5 ml of a 250 g/l sodium nitrite solution through septum at $t = 45$ s.
- Swirl vial till $t = 70$ s.
- Withdraw with Pressure-Lok syringe 1 ml of headspace at $t = 150$ s., insert needle in injection port, precompress the sample,open valve of the syringe and inject.
- Gas chromatographic analysis as described in Chapter 3.

aqueous methanol solutions and Fig. 9 for human blood serum. The human blood serum contained 1.1 mg/l of methanol. There was always a good linear relationship between the peak areas and peak areas relative to an internal standard and the methanol concentration. On a column of 180 cmx4 mm (I.D.) the linear range extended at least from 2-400 mg/l methanol and on a 90 cmx4 mm (I.D.) column at least from 1-200 mg/l methanol in the sample. In human blood serum only the concentrations between 1 and 15 mg/l methanol were tested.

The peak areas, the relative areas and the variation coefficients of these areas at fixed alcohol concentrations of 25 mg/l but at different pectin concentrations are given in Table 11. The pectin concentration slightly affected the peak area of the methanol. However, with the use of area ratios, with one of the other alcohols as internal standard, the pectin concentration did not affect the response factor. Thereby, ethanol and propanol-1 were slightly better internal standards than propanol-2. Addition of 0.2 g/l of sodium azide or 300 g/l of sugars (100 g/l sucrose + 100 g/l glucose + 100 g/l fructose) to the alcohol solution did not affect the response factor. However, blancs must be substracted as pectin contains some free methanol which is released during dissolving and neutralization. Pectin may also contain considerable amounts of alcohol used for precipitation during pectin manufacture. Several mg/l of ethanol are frequently found in sugar solutions.

Table 12 shows the variation coefficients at 3 methanol concentrations with ethanol as internal standard. At lower methanol concentrations, the variation coefficient rises but is still less than 3% at 1 mg/l. For the calculations of the variation coefficients the methanol concentration in the blancs were substracted. In this case the methanol concentration in the blancs was 0.5 mg/l. The total variation coefficient at a concentration of 1 mg/l methanol was less than 2%.

Applications The action of pectinesterases can be followed by sampling from the reaction mixture. Pectinesterase from *Aspergillus niger* (Pectin methylesterase, rein, Röhm, Darmstadt, Fed. Rep. Germ.) was incubated with buffered substrate in glass stoppered flasks and sampled at time intervals. The results are shown in Fig. 10. It can be seen that this fungal pectinesterase is effectively stopped by the oxalic acid and also that

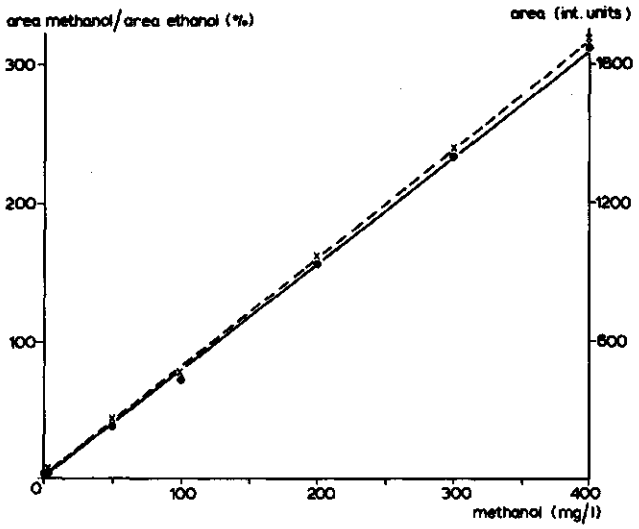


Fig. 8. Correlation between methanol concentration and peak area after reaction of methanol in a aqueous solution with nitrous acid. Peak area of methanol (•); peak area of methanol relative to peak area of 100 mg/l ethanol in the same aqueous solution (×).

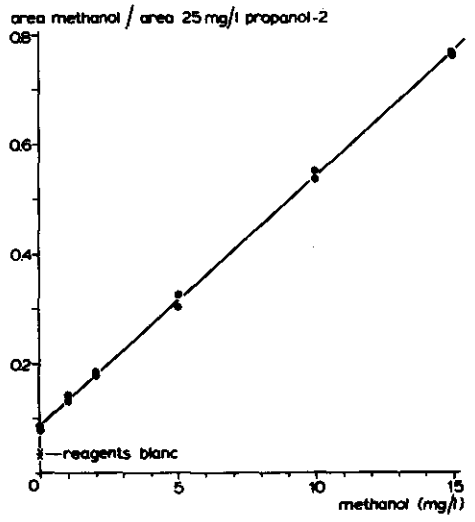


Fig. 9. Correlation between methanol concentration and peak area after reaction of methanol in human blood serum with nitrous acid. Peak area of methanol relative to peak area of 25 mg/l propanol-2 added to the same blood serum (•).

Table 11. Average peak areas, relative peak areas and their variation coefficients (%) of a ten-fold gas chromatographic analysis of a mixture of four alcohols, 25 mg/l of each at different pectin concentrations.

Pectin conc.		Methanol				Ethanol			Propanol-2		Propanol-1
		area (int.units)	relative to ethanol	relative to propanol-2	relative to propanol-1	area (int.units)	relative to propanol-2	relative to propanol-1	area (int.units)	relative to propanol-1	area (int.units)
10 g/l	average	172	0.835	1.020	0.726	206	1.221	0.867	169	0.712	234
	var.coeff.	4.81	1.27	1.78	1.71	5.00	1.25	0.62	5.1	1.30	5.40
5 g/l	average	172	0.834	1.026	0.735	206	1.231	0.875	168	0.710	236
	var.coeff.	4.01	1.54	2.84	2.10	4.80	1.80	1.30	5.60	1.65	5.90
1 g/l	average	170	0.835	1.074	0.739	201	1.282	0.873	155	0.677	230
	var.coeff.	4.34	2.62	2.20	1.25	5.10	1.62	0.51	6.1	1.36	5.61
0.5 g/l	average	178	0.834	1.084	0.739	213	1.297	0.885	164	0.682	240
	var.coeff.	5.21	1.17	2.53	0.97	4.80	1.75	1.16	4.0	2.04	5.22
0.0 g/l	average	181	0.833	1.092	0.739	218	1.311	0.888	168	0.676	246
	var.coeff.	2.71	1.16	2.37	1.52	3.50	1.43	0.73	4.1	1.44	3.62

Table 12. Variation coefficients of a 10-fold gas chromatographic analysis of methanol in distilled water with ethanol as internal standard.

Methanol (mg/l)	Variation coefficient (%)
25	1.2
5	1.7
1	2.9

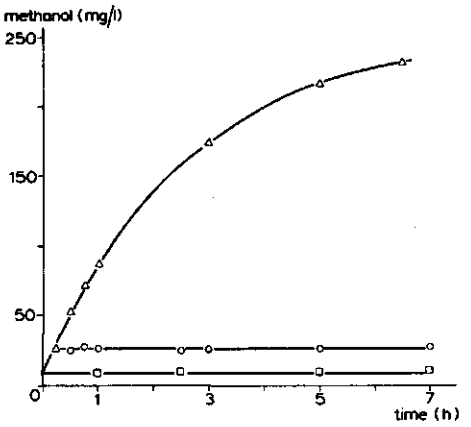


Fig. 10. Effect of saturated oxalic acid on pectinesterase activity and pectin stability upon incubation at 30 °C. Brown ribbon pectin (5 g/l) in 0.1 mol/l sodium succinate buffer pH 4.5 and 0.1 g/l sodium azide was incubated with a fungal pectinesterase (Δ). In parallel experiments, 1:1 of a saturated oxalic acid solution was added to a pectin solution without pectinesterase (□) and to a pectin solution after 15 min pectinesterase activity (o). At time intervals samples were taken for the reaction with nitrous acid and subsequent gas chromatographic assay.

the de-esterification of the pectin by the acid was negligible. Similar results were obtained with crude citrus pectinesterase.

The method may also be used to determine the degree of esterification of small amounts of pectic substances. This possibility was checked by dissolving 2 mg of different pectin preparations in water (sodium chloride as spacing agent and heating with vigorous stirring). After cooling, propanol-1 was added to give a final concentration of 50 mg/l and the volume was adjusted to 10 ml. The solutions were divided into 2 portions of 5 ml in 10 ml glass stoppered flasks. To one flask of each set 0.2 ml of 1 mol/l potassium hydroxide was added, these flasks were swirled and left for 1 hour at room temperature. Then 0.2 ml of 1 mol/l hydrochloric acid was added to these flasks. The contents of all flasks were analysed in triplicate for methanol and from the untreated flasks samples were taken for a triplicate uronic acid analysis with the automated carbazole test. Dividing the differences in μmoles methanol by the μmoles uronic acid gave the degree of esterification. The results are compared with a duplicate titrimetric assay of the same preparations in Table 13. The results are in good agreement. The advantage of the method is the small pectin quantity needed: 30-100 times less than for a single titrimetric assay. The method proved its usefulness in the analysis of cell wall fractions.

Identity of the detected compounds Gessner (1970) and Litchman & Upton (1972) noted that alcohols had the same retention time in the gas chromatograph as their reaction products with nitrous acid. The same observation was made in this gas chromatographic work. In the

Table 13. Comparison of titrimetric assay and methanol + uronic acids assay for the degree of esterification of pectins.

Degree of esterification (%)	
Titrimetric method	Gas chromatographic method
94.8	93.4
78.2	78.2
47.3	46.7
28.0	27.9

combined gas chromatograph-mass spectrometer analysis the mass spectra of the compounds eluting after injection of methyl nitrite or 2-propyl nitrite, were (computor) corrected for the background spectrum due to the Porapak R bleed. The resulting spectra did not correspond with the spectra of methyl nitrite, or 2-propyl nitrite (Mass Spectrometry Data Centre, 1974) but appeared to be identical to the mass spectra of directly injected corresponding alcohols. Apparently the alkyl nitrites are labile and are decomposed upon injection in the gas chromatograph (250 °C). If the reaction was done in orange juice, an extra peak was found with a shorter retention time than methanol in the gas chromatogram (Fig. 11). This peak was not found when the headspace of untreated orange juice was sampled under the same conditions. To avoid the temperature effect of the exotherm reaction, in both cases the vials were held in ice. The gas chromatographic-mass spectrometric analysis revealed that this peak was propene. The origin of this product is unknown and it was not found in other products like apple juice, pectin, saccharose, glucose and fructose solutions and blood serum.

Problems and sources of errors Formaldehyde and acetaldehyde have nearly the same retention times as methanol on Porapak R. These were confirmed in this work. The acetone peak (103 s) appeared in between that of ethanol (82 s) and propanol-2 (136 s). Low concentrations (25 mg/l) of these compounds did not interfere with the methanol determination. The acetone and acetaldehyde peaks did not increase upon treatment with nitrous acid. The

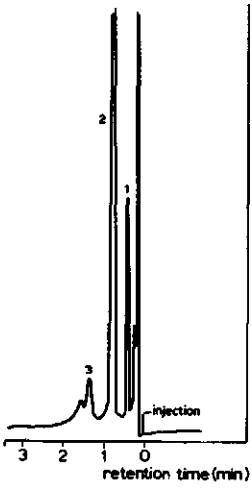


Fig. 11. Gas chromatographic separation of compounds in reconstituted Valencia orange concentrate after reaction with nitrous acid. Compounds: Unknown (*1*); methanol (2) and ethanol (3).

effect of nitrous acid on the formaldehyde peak was not verified because the available commercial 350 g/l formaldehyde solution appeared to contain about 100 g/l methanol as a stabilizer. Blanc values of methanol could be reduced by the use of distilled water, which was boiled to about half the volume, before being used in making up solutions and reagents. It appeared to be necessary to inject several blancs (up to 15 for relatively new columns and about 4 for old columns) to eliminate a false methanol peak which appeared when the column had been out of use for a certain time. The size of this peak could be equivalent to one representing 10 mg/l of methanol in the sample but could easily be reduced to one equivalent to 0.3-0.5 mg/l of methanol by these preinjections. The samples for conditioning the column could be taken from the same reaction flask with the blanc reagents, held in ice. Preconditioning a new column at 260 °C instead of 240 °C (Litchman & Upton, 1972) reduced this false peak considerably. Columns could be used for over a thousand injections before a decrease in performance became apparent.

The type of gas syringe and the batch of silicone rubber septa for the vials affected the minimum blanc value. Relatively soft septa could give rise to a peak comparable with that obtained with about 3 mg/l methanol with a retention time only a few seconds longer than methanol. The minimum blanc peak with the Gastight syringes was about twice as high as with the Pressure-Lok syringes. After repeated use, the plunger of the syringes could leak under the pressure of the carrier gas in the gas chromatograph. The silicone rubber septa for the vials could be used repeatedly, but if a very low concentration of methanol was analysed with the same septum after a high one there was some carryover. Also special attention had then to be paid to the air washing of the gas syringe. At low concentrations, it appeared to be advantageous to measure peak heights instead of peak areas.

4.2 DISCUSSION

If properly used, the gas chromatographic alcohol analysis, after conversion of alcohols to nitrite esters and with headspace sampling, appears to be very sensitive (<1 µg/ml) and reproducible (variation coeff. about 1.5%). The method, as described here, gives a better reproducibility than the methods described by Litchman & Upton (1972) and Bartolome & Hoff (1972b). Baron (1978) reported an even better reproducibility but worked with a five-fold larger sample and an inconvenient reaction time of 45 min. Presumably the reproducibility can be further improved by thermostating the reaction vials in a water bath. Reproducibility at concentrations of lower than 1 mg/l would be improved if the blanc methanol peak could be eliminated.

Under the gas chromatographic conditions used in this work (150-250 °C), the alkyl nitrites are unstable. Baron (1978) used a lower temperature in the gas chromatograph (50 °C). The alkyl nitrites are stable then (Baron, personal communication).

The method is very convenient, because it can be applied directly with pectin solutions, sugar solutions, fruit juices, blood serum and presumably with many other systems.

The details of the procedure are not very important, as long as they are carefully standardized. Most time and temperature combinations are suitable, but precise timing and a standardized procedure are requirements. If low concentrations are to be assayed, the method described in Table 10 will give the best results.

5 Purification and molecular properties of two citrus pectinesterases

5.1 EXPERIMENTS AND RESULTS

Extraction of pectinesterase from oranges Oranges (5 kg) were cut in halves and pressed on a rotary kitchen citrus press. The juice was drained through cheesecloth and the pulp was pressed in a galenic press (H. Fischer & Co., Norf, West Germany). The press cake, together with the peel segments were frozen at -40 °C and stored at -20 °C, till further use.

After thawing at 4 °C, pectinesterase was extracted from the preparation of peel and pulp by a borate buffer according to MacDonnell et al. (1945) and Krop (1974) with extra 15 g sodium chloride per liter added, to improve extraction. Hundred gram portions of the preparation were homogenized with 200 ml buffer for 5 min in a Braun Multimix homogenizer. The total homogenized preparation (9 670 ml) was stirred for one hour at room temperature, then packed in cheesecloth and pressed. The press liquid (7 500 ml) was rapidly cooled to 5 °C. The press cake was homogenized with buffer and pressed one more time to yield a second batch of press liquid (6 500 ml). To both batches cooled to 5 °C, 17 g per 100 ml of solid ammonium sulphate was added (to 30% saturation; Dixon & Webb (1964)). All ensuing operations were done at 2 to 5 °C. After two hours the two batches of press liquid were centrifuged at 10 000xg for 20 min. The pellet and an oil layer floating at the top were discarded. To the supernatants 31 g per 100 ml of solid ammonium sulphate (to 75% saturation) was added. After standing overnight the precipitates were collected by centrifugation at 10 000xg for 20 min. The precipitates of the two batches were redissolved in distilled water with the aid of an ultrasonic bath (Sonicor, SC-100-22H, Farmingdale, N.Y., USA). Undissolved particles (which contained no enzyme activity) were removed by centrifugation at 10 000xg for 20 min. The enzyme solutions were pooled and dialysed against four changes of 10 volumes of 0.01 mol/l sodium maleate buffer pH 7.0 in 0.1 mol/l sodium chloride. Dialysis bags were always previously cleaned by boiling in 100 g/l acetic acid and rinsing with distilled water and buffer, since appreciable losses of enzyme activity were noticed during dialysis in untreated bags, especially at low ionic strengths. The dialysed enzyme solution was freeze-dried and stored as a powder (5 410 mg of protein) at - 20 °C. Table 14 presents a summary of the steps used in the extraction of pectinesterase.

Purification of Pectinesterase I and II A portion of the freeze-dried powder (715 mg of protein) was dissolved in 20 ml of distilled water. After centrifugation at 20 000xg for 20 min, the clear enzyme solution was applied to a Bio-Gel P-100 column for gel filtration. The elution pattern is shown in Fig.12. No more pectinesterase was detected beyond fraction 37. Fractions 30 to 33 were pooled and dialysed against three changes of 1 l of 0.02 mol/l sodium succinate buffer, pH 6.0, containing 0.1 g/l of sodium azide as

Table 14. Extraction of pectinesterase from peels and pulp of 5 kg oranges.

Fraction	Volume (ml)	Pectinesterase (units)	Yield (%)
Homogenized preparation	9 670	312 700	100
First extraction			
Press liquid	7 500	206 250	65.9
Ammonium sulphate precipitation	365	165 510	52.9
Second extraction			
Press liquid	6 500	43 560	13.9
Ammonium sulphate precipitation	201	35 880	11.5
Pool of dissolved ammonium sulphate precipitates	566	201 390	64.4

preserving agent. Then the enzyme preparation was applied to a column of cross-linked pectate and eluted with a sodium chloride gradient (Fig.13). Fractions 6 to 16 (designated as Pectinesterase I) and fractions 39 to 44 (Pectinesterase II) were pooled separately. It was established by rechromatography and also by isoelectric focusing of some of the material of Pectinesterase I and of the pectinesterase peak of fractions 28 to 32 that the latter pectinesterase peak was identical to Pectinesterase I. Both Pectinesterase I and II were dialysed against 0.005 mol/l sodium phosphate buffer pH 7.5 with 0.1 g/l sodium azide and applied to a CM Bio-Gel A column. The elution patterns are shown in Fig.14 (Pectinesterase I) and Fig.15 (Pectinesterase II). Apparently the binding of

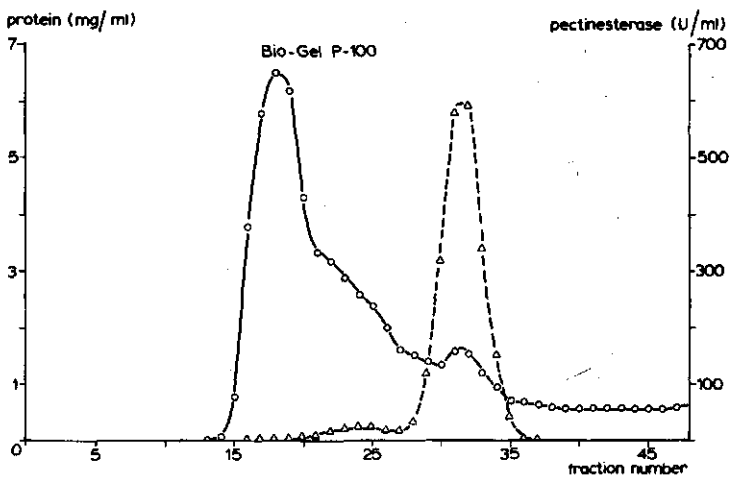


Fig. 12. Bio-Gel P-100 chromatography of crude pectinesterase. 21 ml concentrated crude enzyme was applied to a column (85x3.2 cm) of Bio-Gel P-100 equilibrated with a solution of 0.01 mol/l sodium maleate buffer pH 7.0, 0.5 mol/l sodium chloride and 0.1 g/l sodium azide. Elution was done with the same solution at a flow rate of 8 ml per h and fractions of 8 ml were collected. Protein (o); pectinesterase activity (Δ).

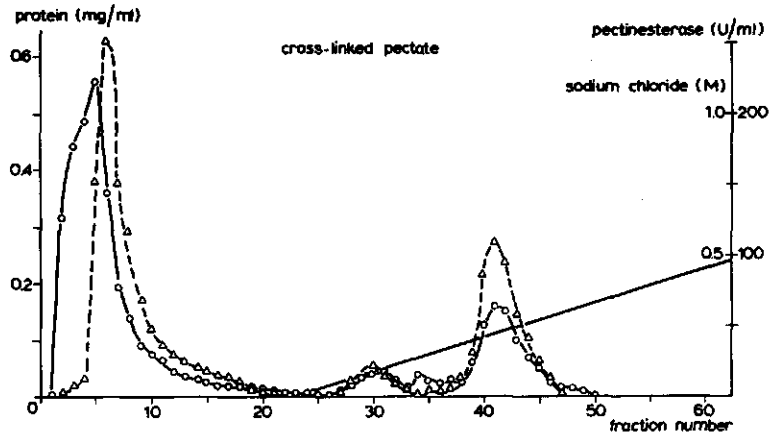


Fig. 13. Cross-linked pectate chromatography of the pectinesterase peak from Bio-Gel P-100. 32 ml pectinesterase from Bio-Gel P-100 were dialysed against 0.02 mol/l sodium succinate buffer pH 6 with 0.1 g/l sodium azide and applied to a cross-linked pectate column (10.0x2.0 cm) equilibrated with the same buffer. The column was eluted with equilibration buffer up to fraction 22 and then with a linear gradient of sodium chloride in the same buffer. The flow rate was 25 ml per h and fractions of 10 ml were collected. Protein (o); pectinesterase activity (Δ) and sodium chloride concentration (————).

Pectinesterase I to CM Bio-Gel A is not very strong since prolonged washing of the column with equilibration buffer (without sodium chloride) causes the enzyme to elute as a tailing peak (result not shown). The purification procedure of Pectinesterase I and II is summarized in Table 15. The total yield of purified pectinesterases was 26.3%, and Pectinesterase I and II appeared to occur approximately in a two to one ratio in terms of units in the crude pectinesterase preparation.

The specific activity of the two enzymes could not be increased by rechromatography or application of other chromatography steps such as hydroxylapatite adsorption chromatography. It may be of interest to report that an eight-fold purification of the crude enzyme preparation could be obtained by chromatography on a hydroxylapatite column, equilibrated with 0.01 mol/l sodium phosphate buffer pH 6.8 and elution with a linear gradient up to 0.2 mol/l of the same buffer.

Electrophoretic properties, molecular weights and isoelectric points Samples of up to 5 µg of purified Pectinesterase I and Pectinesterase II were applied to the wells of a slab gel for sodium dodecyl sulphate electrophoresis. Only a single band of protein was observed with both pectinesterase preparations. The detection limit of this technique is 0.2 µg of protein in a single band. Both enzymes had the same mobility, which corresponded to a molecular weight of 36 200 (Fig.16).

The isoelectric point of the two pectinesterases was determined by isoelectric focusing in a column (Fig.17). For Pectinesterase I an isoelectric point of 10.05±0.05 at 6 °C was found. That of Pectinesterase II was at or above 11.0, a value too high to be determined accurately with the commercially available carrier ampholytes. Only 48% of the

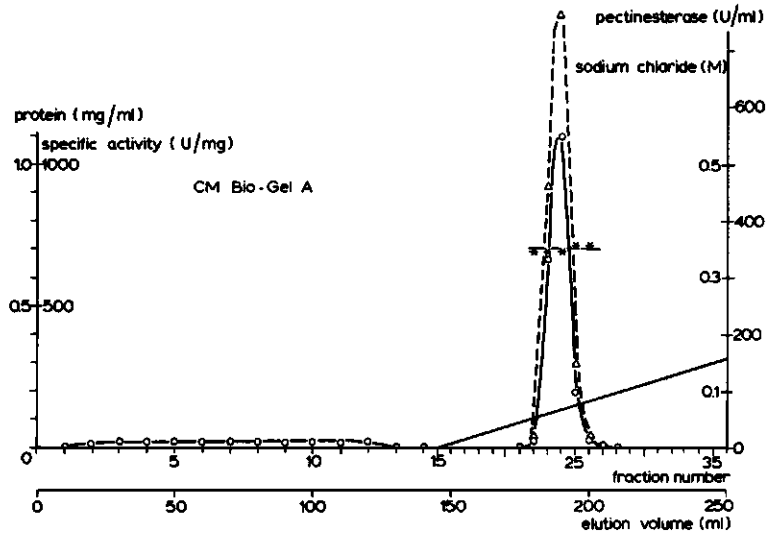


Fig. 14. CM Bio-Gel A chromatography of Pectinesterase I. Fractions 6 to 16 (110 ml) from the cross-linked pectate column were dialysed against 0.005 mol/l sodium phosphate buffer pH 7.5 with 0.1 g/l sodium azide and applied to a CM Bio-Gel A column (18x1.1 cm) equilibrated with the same buffer. The column was washed with equilibration buffer (40 ml) and then eluted with a linear sodium chloride gradient in the same buffer. The activity peak was collected in 5 ml fractions. Protein (o); pectinesterase activity (Δ); sodium chloride concentration (————); specific activity (*).

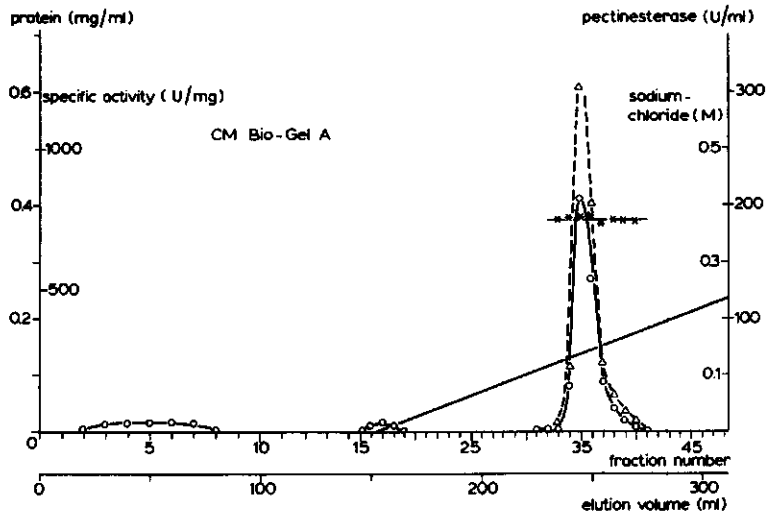


Fig. 15. CM Bio-Gel A chromatography of Pectinesterase II. Fractions 39 to 44 (60 ml) from the cross-linked pectate column were dialysed against 0.005 mol/l sodium phosphate buffer pH 7.5 with 0.1 g/l sodium azide and applied to a CM Bio-Gel A column (18x1.1 cm) equilibrated with the same buffer. The column was washed with equilibration buffer (90 ml) and then eluted with a linear sodium chloride gradient in the same buffer. The activity peak was collected in 5 ml fractions. Protein (o); pectinesterase activity (Δ); sodium chloride concentration (————); specific activity (*).

Table 15. Purification of orange Pectinesterase I and II.

Step of purification	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg)	Purification factor	Yield (%)
Homogenized preparation	1 278	40 900	6 100[1]	7	1	100
Ammonium sulphate precipitation	71	26 350	951	28	4	64.4
Dialysis and freeze drying[2]	21	20 180	715	28	4	49.3
Chromatography on Bio-Gel P-100	33.5	15 360	48	319	48	37.7
Pectinesterase I						
Chromatography on cross-linked pectate	110	8 060	13.6	594	89	19.7
Chromatography on CM Bio-Gel A	15	7 160	10.3	694	104	17.5
Pectinesterase II						
Chromatography on cross-linked pectate	60	4 300	8.1	527	79	10.5
Chromatography on CM Bio-Gel A	20	3 600	4.7	762	114	8.8

1. Kjeldahl nitrogen determination x 6.25.
2. This freeze-dried preparation is designated in the text as crude pectinesterase preparation.

activity of Pectinesterase II was recovered in this focusing experiment. Apparently enzyme inactivation occurred in the alkaline electrode solution. In a focusing experiment which was terminated prematurely, the greater part of the enzyme remained below pH 11 and over 90% of activity was recovered. Both pectinesterases migrated as single peaks in the focusing experiments. Moreover, no other protein peaks were monitored at 280 nm (Fig.17). Compared with pectate lyase (Rombouts et al., 1978) the pectinesterases moved extreme-

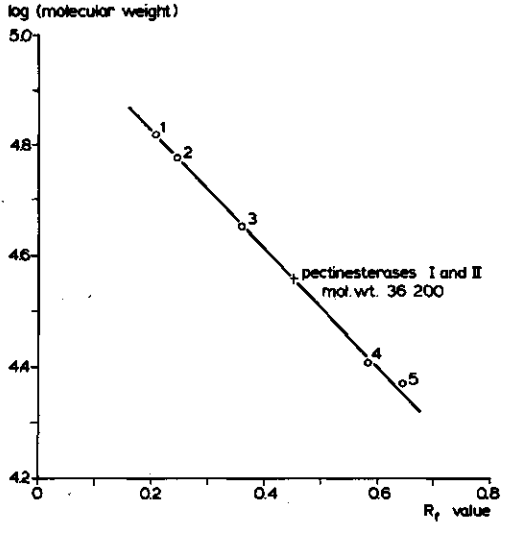


Fig. 16. Molecular weight determination of Pectinesterase I and II. Sodium dodecyl sulphate electrophoresis of enzyme and standard proteins was done in a slab gel with a final acrylamide concentration of 120 g/l. Standards: bovine serum albumin (1) beef liver catalase (2); ovalbumin (3); chymotrypsinogen (4) and trypsinogen (5).

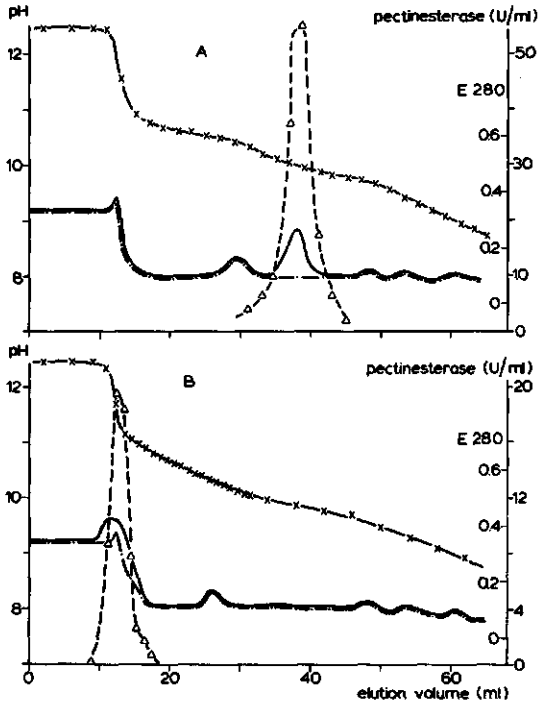


Fig. 17. Isoelectric focusing of Pectinesterase I and II. The enzymes (300 units) were focused as described in Materials and Methods (Chapter 3). A: Pectinesterase I, 85% of activity recovered. B: Pectinesterase II, 48% of activity recovered. pH (×); pectinesterase activity (Δ); absorbance at 280 nm (————); absorbance at 280 nm in an identical run without enzyme (— · — · —).

ly slowly at pH values close to their isoelectric points. Apparently the net charge of these molecules near their isoelectric points is very low.

Occurrence of other enzyme activities In order to assess the state of purity in terms of absence of other enzyme activities, various substrates were incubated with 3 units of pectinesterase for 20 h at 30 °C (Table 16). In addition to purified Pectinesterase I and II the preparation, obtained by ammonium sulphate precipitation (crude enzyme, Table 15), was tested. The crude preparation contained a variety of other enzymes, some of which may be involved in cloud loss phenomena: β-(1→3)-glucanase, β-(1→6)-glucanase, amylase, β-(1→4)-xylanase, β-(1→3)-galactanase, arabanase, α and β glucosidase, α and β galactosidase, fosfatase and carboxylesterase. None of these enzyme activities could be detected in the purified enzyme preparations.

Protease activity, not detected at pH 5.0 with azocoll as substrate, could be demonstrated in the crude preparation at pH 7.5 with strips of exposed and developed photographic negative film, a technique described by Krop (1974). No polygalacturonase was detected in the crude preparation. Riov (1975) reported the presence of extremely low

Table 16. Enzymatic activity of crude and purified pectinesterase preparations on various substrates.

Supplier[1]	Substrate	Types of monomers and linkages	Activity in preparations[2]		
			crude	I	II
Dow	methyl cellulose	β-1,4 (glucose)	-	-	-
Difco	cellobiose	β-1,4 (glucose)	-	-	-
ICN	laminarin	β-1,3;β-1,6 (glucose)	+	-	-
Lab	pachyman	β-1,3 (glucose)	+	-	-
Calbiochem	pustulan	β-1,6 (glucose)	+	-	-
Lab	yeast glucan	β-1,3;β-1,6 (glucose)	+	-	-
K & L	lichenan	β-1,4;β-1,3 (glucose)	+	-	-
F.C.& R.	chitosan	β-1,4 (glucosamine)	-	-	-
F.C.& R.	chitin	β-1,4 (N-acetylglucosamine)	-	-	-
Difco	soluble starch	α-1,4;α-1,6 (glucose)	+	-	-
K & L	nigeran	α-1,4;α-1,3 (glucose)	-	-	-
ICN	dextran	α-1,6;α-1,3;α-1,4 (glucose)	-	-	-
Merck	inulin	β-1,2 (fructose;α-glucose)	+	-	-
K & L	xylan	β-1,4 (xylose)	+	-	-
K & L	galactan	β-1,3 (galactose)	+	-	-
K & L	araban	α-1,5;α-1,3 (arabinose)	+	-	-
K & L	mannan	α-1,6;α-1,3;α-1,2 (mannose)	-	-	-
ICN	polygalacturonate	α-1,4 (galacturonic acid)	-	-	-
K & L	ρ-nitrophenyl-β-D-glucoside		+	-	-
K & L	ρ-nitrophenyl-α-D-glucoside		+	-	-
K & L	ρ-nitrophenyl-β-D-galactoside		+	-	-
K & L	ρ-nitrophenyl-α-D-galactoside		+	-	-
K & L	ρ-nitrophenylphosphate		+	-	-
K & L	ρ-nitrophenylmyristate		+	-	-
Calbiochem	azocoll		-	-	-

1. Suppliers: Dow, Dow Chemical International S.A., Brussels; Difco, Difco Laboratories, Detroit; ICN, ICN Pharmaceuticals, Inc., Cleveland, Ohio; Lab, this laboratory's collection; Calbiochem, San Diego, Calif.; K & L, Koch and Light Laboratories, Ltd., Colnbrook, Bucks, England; F.C. & R., Food Chemical and Research Lab., Inc., Seattle, USA; Merck, E.Merck, Darmstadt, West Germany.
2. Activity detected (+). no activity detected (-).

activities of polygalacturonase in peel and pulp from orange. This enzyme activity, however, could only be measured when precautions were taken to inhibit uronic acid oxidase, also present in orange.

Amino acid composition About 100 µg of the enzyme were hydrolysed (in duplicate) after lyophilization. In a parallel experiment, the same was done for solutions containing 100 µg bovine serum albumin (Sigma A 4378) and a mixture of common L-amino acids, about 3 µg each. These preparations were analysed on an amino acid analyser together with standard mixtures of amino acids which had not been subjected to hydrolysis conditions, to determine the response factors. Both in the amino acid standard which had been subjected to hydrolysis conditions and in the bovine serum albumin, cystine and cysteine were completely oxidized to cysteic acid. Using the recovery factors from the amino acids which had been subjected to hydrolysis conditions, the cysteine contents of the bovine serum albumin and the enzymes were calculated. Methionine was nearly completely lost in

all the hydrolysed proteins and in the amino acids which had been subjected to hydrolysis conditions. The experiments were repeated with better precautions to exclude oxygen during hydrolysis and the amino acids were analysed by gas chromatography (Chapter 3). Now the recovery of methionine was nearly complete and these values were used in the data presented.

The tryptophan determination by the method of Pajot (1976) yielded higher values than the method of Opieńska-Blauth et al. (1963), presumably because the proteins were not enzymatically hydrolysed before the reaction as described in the latter method. Besides, in this method the tryptophan standards and the samples appeared to have hardly any absorbance at the indicated wavelength of 545 nm but had an absorbance maximum near 450 nm. Therefore this latter wavelength was used.

The amino acid composition of bovine serum albumin found by the amino acid analyser was very similar to the literature values (Reeck, 1970). All values were within 2% from the literature values, except phenylalanine (5% deviation), arginine (10% deviation) and methionine (75% deviation). In Table 17 the amino acid composition of the orange pectinesterases are listed, using the values of the amino acid analyser together with the gas-chromatographic values for methionine and the spectrometric values for tryptophan.

The recovery of bovine serum albumin in the amino acid analyser was 98% (value corrected for tryptophan and methionine). Assuming the same recovery for the pectinesterases, the correlation factor between E 280 nm and the protein concentration for Pectinesterase

Table 17. Amino acid composition of purified orange pectinesterases.

Amino acid	Pectinesterase I		Pectinesterase II	
	mol %	mol/mol[1]	mol %	mol/mol[1]
Cys[2]	3.13	9	1.79	5
Asp[3]	11.06	32	9.69	28
Thr	8.62	25	7.94	23
Ser	6.37	19	7.28	21
Glu[4]	6.77	20	7.34	21
Pro	3.69	11	3.57	10
Gly	8.87	26	11.05	32
Ala	11.55	34	10.80	31
Val	8.00	23	7.87	23
Met[5]	1.55	5	1.56	5
Ile	5.33	16	5.31	15
Leu	5.31	15	4.64	14
Tyr	2.12	6	1.36	4
Phe	4.91	14	5.60	16
Trp[6]	2.28	7 (4)[7]	1.87	6 (4)[7]
Lys	3.17	9	4.66	14
His	1.00	3	2.35	7
Arg	6.30	18	5.29	15

1. Nearest integer.
2. Determined as cysteic acid.
3. Asparagine + aspartic acid.
4. Glutamine + glutamic acid.
5. Methionine gas chromatographically.
6. Tryptophan with method of Pajot (1976).
7. Tryptophan with method of Opieńska-Blauth et al. (1963).

I is 0.569 (0.569 x E 280 nm = mg protein/ml) and for Pectinesterase II is 0.626. For Pectinesterase I $E^{1\%}_{1\,cm}$ can be estimated as 17.6, and for Pectinesterase II as 16.0. The available quantities of pure enzymes were insufficient to determine these values more accurately by weighing.

Fig.18 shows the absorbance spectra of the pectinesterases in neutral environment and in 0.1 mol/l potassium hydroxide. Using the formula of Beaven & Holiday (1952):

$$\frac{MTyr}{MTrp} = \frac{0.592.E294.4 - 0.263.E280.0}{0.263.E280.0 - 0.170.E294.4}$$

for the spectra in 0.1 mol/l potassium hydroxide gave much lower tryptophan values than listed in Table 17. But as stated by Leggett Baily (1967), the absorbance spectra of tyrosine and tryptophan in proteins may shift 1-3 nm to higher wavelengths.

Glycoprotein character About 2 mg of both pectinesterases and ovalbumin (Sigma, A-2512, Grade VI) were gas chromatographically analysed for sugars as described in materials and methods (Chapter 3). Standards contained rhamnose, arabinose, xylose, mannose, glucose, inositol (internal standard), glucose amine, galactose amine and mannose amine. After correction for the trace of glucose, contaminated from the dialysis bags, it appeared that none of both pectinesterases contained any of the sugars mentioned. In the parallel experiment with ovalbumin 1.7% mannose and 1.1% glucose amine was found. The literature values are 2% and 1.2% respectively (Winzler, 1970). The method was sensitive enough to determine 1 mol of a sugar/1 mol of enzyme.

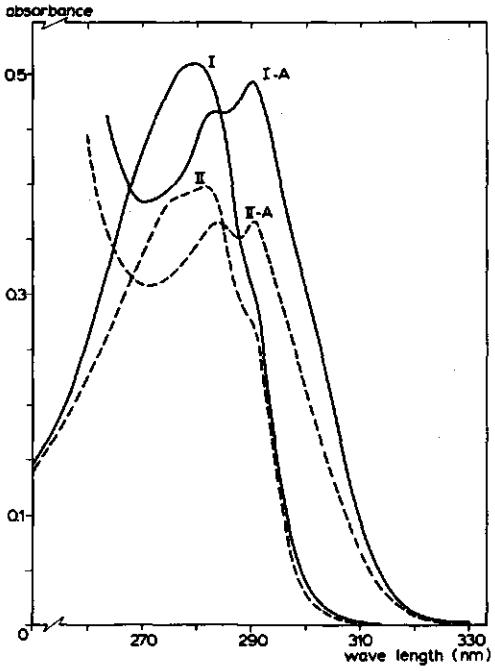


Fig. 18. Ultraviolet absorbance spectra of purified pectinesterases. Enzyme concentrations: 460 units/ml for Pectinesterase I, a 320 units/ml for Pectinesterase II. In 0.1 mol/l potassium hydroxide the enzyme concentrations were 10% lower. I. Pectinestera I in neutral environment. II. Pectinesteras II in neutral environment. I-A and II-A the same in 0.1 mol/l potassium hydroxide.

5.2 DISCUSSION

Extraction and purification of the two pectinesterases The first step in the preparation of orange pectinesterase was the separation of juice from peel and pulp. The juice was discarded since it contained only traces of pectinesterase (<1% of total activity) presumably mainly bound to small pulp particles. The enzyme could not be extracted from the peel and pulp fraction in one step: the occurrence of equilibria between bound and solubilized pectinesterase during repeated extractions has already been described by Jansen et al. (1960b). Solubilization of almost all of the pectinesterase by repeated extraction was considered important to minimize the chance that selectively one of the molecular forms would not be extracted.

The two forms of pectinesterase could be separated by chromatography on cross-linked pectate. However, Rexová-Benková et al. (1977) who, by electrophoresis, demonstrated five molecular forms of pectinesterase in tomato, were unable to separate these on cross-linked pectate. Rombouts et al. (1979) described how the separation of orange pectinesterases is affected by the degree of cross-linking of the pectate. Multiple forms of tomato pectinesterase could, however, be separated on DEAE-Sephadex A 50 (Pressey & Avants, 1972), and three forms of banana pectinesterase were obtained by differential extraction (Hultin & Levine, 1963; Manabe, 1973a), who thoroughly purified pectinesterase from the orange *Citrus natsudaidai* found no evidence for the existence of more than a single pectinesterase in that fruit.

The two forms of orange pectinesterase could eventually be obtained as homogeneous proteins by chromatography on CM Bio-Gel A (Figs 14 and 15). The enzymes could be obtained in relatively favourable yields by maintaining a high ionic strength in the early steps of the purification scheme and by working at pH 7.5 in the last purification step. Also, cleaning of dialysis bags with boiling acetic acid solution was necessary to prevent big losses of Pectinesterase II during dialysis probably by adsorption to the dialysis membrane. The purity of the enzymes was confirmed by sodium dodecyl sulphate electrophoresis, by isoelectric focusing (Fig. 17) and by the absence of a great variety of enzyme activities, which were found to be present in the crude enzyme extracts (Table 16).

Properties of the enzymes On the basis of their mobility in sodium dodecyl sulphate electrophoresis, both enzymes appeared to have the same molecular weight of 36 200. This molecular weight was also indicated by gel filtration on Bio-Gel P-100 in 0.5 mol/l sodium chloride. Apparently both enzymes are composed of a single peptide chain. It is therefore unlikely that one of the forms is derived from the other, e.g. by dissociation of subunits or by limited proteolysis. As suggested by their different isoelectric points (Fig.17), the two enzymes will have genetically determined differences in primary structure, and could therefore be called 'isoenzymes' or 'isozymes' (CBN, 1971). This was confirmed by the amino acid analysis.

Two pectinesterase isoenzymes of molecular weight 30 000 were recently found in banana (Brady, 1976). These isoenzymes were separated by isoelectric focusing and their isoelectric points were 8.8 and 9.3, well below those of the orange isoenzymes (10.05 and

>11,0). The multiple forms of tomato pectinesterase have different molecular weights ranging from 24 300 to 35 500 (Pressey & Avants, 1972). Isoelectric points of 7-9.3 have been reported for tomato pectinesterases (Delincée & Radola, 1970; Delincée, 1976).

The amino acid composition of the enzymes differed considerably on cystine + cysteine, asparagine + aspartic acid, glycine, tyrosine, lysine and hystidine. The isoelectric points cannot be explained without also knowing asparagine, aspartic acid, glutamine and glutamic acid separately. Comparing the amino acid compositions with the amino acid composition of tomato (Markovič & Sajgó, 1977; Nakagawa et al., 1970b) or *Citrus natsudaidai* orange pectinesterase (Manabe, 1973a) reveals that not one of the pectinesterases has the same or nearly the same amino acid composition. In literature suggestions have been made about the glycoprotein character of tomato and plum pectinesterases (Theron et al., 1977a; 1977b). These were contradicted by Markovič (1974) for one form of tomato pectinesterase and by Markovič et al. (1975) for 6 forms of banana pectinesterase. The two pectinesterases purified in this work could be demonstrated not to be glycoproteins.

6 Multiple forms of pectinesterase in navel orange and other citrus fruit

In Chapter 5 only two isoenzymes are purified and described, whereas in some other fruit six to eight forms of pectinesterase have been detected (Delincée, 1976; Markovič et al., 1975). Differences in properties of pectinesterases from different citrus fruits are known to exist, such as heat stability (Rouse & Atkins, 1952; Rouse & Atkins, 1953a) and juice clarifying properties (Krop, 1974). For these reasons a search for more multiple forms of pectinesterase in Navel oranges and other citrus fruits was initiated.

6.1 EXPERIMENTS AND RESULTS

6.1.1 Multiple forms of pectinesterase in Navel orange

Search for molecular forms of pectinesterase Crude pectinesterase, obtained by ammonium sulphate precipitation of the extract from peel and pulp was subjected to isoelectric focusing. The column was loaded with a sample of 10 ml of this preparation (2 500 pectinesterase units, 85 mg protein), and carrier ampholytes were used to obtain a pH gradient from pH 4 to 11. Large pectinesterase peaks were found at pH 10 (Pectinesterase I) and just above 11.0 (Pectinesterase II). In addition four or more small activity peaks were found in the pH range of 6.0-9.6. However, the combined activity of these small peaks accounted for less than 4% of the total activity of the sample. Moreover, contaminating material precipitated in heavy bands throughout the column during focusing. It is likely that small amounts of pectinesterase were thus prevented from migrating to their isoelectric point. Further, the minor peak of pectinesterase in fractions 20 to 27 from Bio-Gel P-100 (Fig. 12) was also subjected to isoelectric focusing and gave a major activity peak with an isoelectric point of 10.2 and a trace of activity with an isoelectric point >11.

When the heat stabilities of the pure and crude enzyme preparations were determined, there was a small heat stable fraction (about 6% of the total activity) in the crude enzyme preparation, which could not be ascribed to Pectinesterase I or II. It appeared that most of the pectinesterase in the fractions 20 to 27 from Bio-Gel P-100 (Fig. 12) was more heat stable than either Pectinesterase I and II. Rechromatography of these fractions on Bio-Gel P-100 resulted in the same elution volume as before. Because of the highly impure state of the enzyme (specific activity 9 units/mg protein) the molecular weight could not be determined with sodium dodecyl sulphate electrophoresis. Rechromatography of 10 ml containing 120 units of pectinesterase was done on Bio-Gel P-100 under the same experimental conditions as described before (Fig.12) in a mixture with 10 mg blue dextran 2 000 (Pharmacia, Uppsala, Sweden, mol.wt. 2 000 000), 10 mg bovine serum albumin (Boehringer, Mannheim, mol.wt. 67 000), and 300 units Pectinesterase I + II

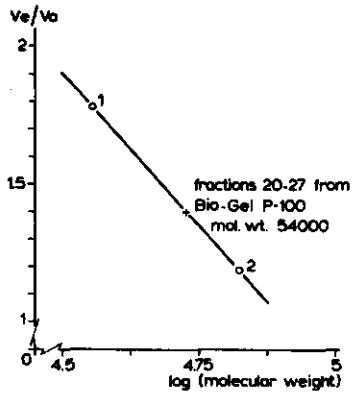


Fig. 19. Molecular weight estimation of fractions 20-27 from Bio-Gel P-100. A sample of fractions 20-27 from Bio-Gel P-100 (Fig. 12) was re-applied to the column together with standard proteins under the same column conditions as described before (Fig. 12). Standards: Pectinesterase I + II (1); bovine serum albumin (2).

(mol.wt. 36 200) to estimate the molecular weight of this small pectinesterase peak. Proteins were measured by absorbance at 280 nm and fractions were assayed for pectinesterase activity. The log molecular weight was plotted against V_e/V_0 (V_e=elution volume protein, V_0=elution volume blue dextran)(Fig. 19). The molecular weight was roughly estimated to be 54 000 and this pectinesterase will be called high molecular weight pectinesterase.

For crude enzyme preparations column isoelectric focusing was unsuitable to detect with certainty other molecular forms of pectinesterase because of the flocculation of contaminating proteins. Also thin layer or gel isoelectric focusing could not be used because the highest obtainable pH in these gels is about 10 (Winter et al., 1975) and several pectinesterases were expected to have isoelectric points above pH 10. In preliminary gel isoelectric focusing experiments, Pectinesterase II migrated considerably faster towards the cathode than Pectinesterase I, but eventually both enzymes disappeared in the cathode strip. This observation resulted in the development of a preparative flat bed pH gradient electrophoresis in a granulated gel as described in materials and methods. Crude Navel orange pectinesterase (50 units in 0.3 ml) was introduced as a narrow band over the full width of the gel and the power was switched off before Pectinesterase II, the fastest migrating pectinesterase, reached the cathode end. Fig. 20 shows the photograph of a strip used for pectinesterase detection with the print technique and Fig. 21 shows the activity recovered from the scraped off gel zones with a drawing from the print. By using pure Pectinesterase I and II and the high molecular weight pectinesterase as references, 3 of the 5 pectinesterase active zones could be identified as indicated in Fig. 20. In pH gradient electrophoresis most of the high molecular weight pectinesterase migrated slightly faster than Pectinesterase I, a trace as fast as Pectinesterase II and a trace slightly slower than Pectinesterase I (results not shown). Because some of the bands are rather close to each other, the quantitative analysis of the scraped off zones showed a poor resolution (Fig. 21).

Homogenization and pressing in the extraction procedure of pectinesterase was normally done at room temperature (Chapter 5). In a new purification procedure, these initial steps were done at 4 ^{o}C. After gel filtration of the crude enzyme on Bio-Gel P-100 (as in Fig. 12), the fraction of high molecular weight pectinesterase represented exactly as

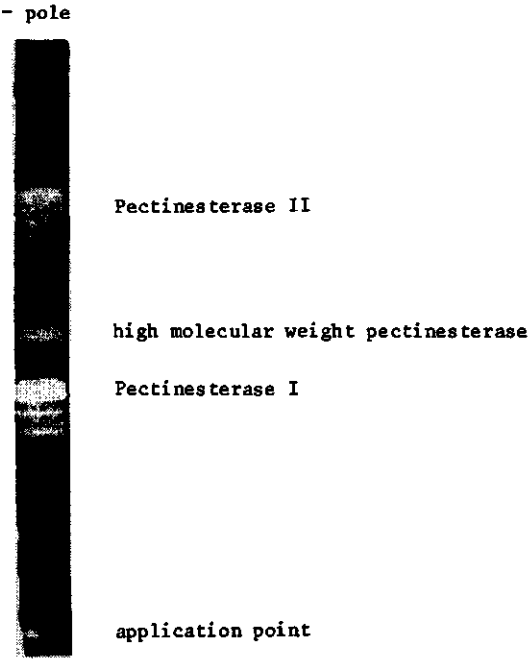


Fig. 20. pH Gradient electrophoresis of crude Navel orange pectinesterase. Photograph of print for pectinesterase detection, light zones are pectinesterase active (for method see Chapter 3).

much enzyme as in the normal procedure (6% of the total recovered activity). Therefore it seems unlikely that Pectinesterase I or II are derived from the high molecular weight pectinesterase due to some kind of degradation during extraction.

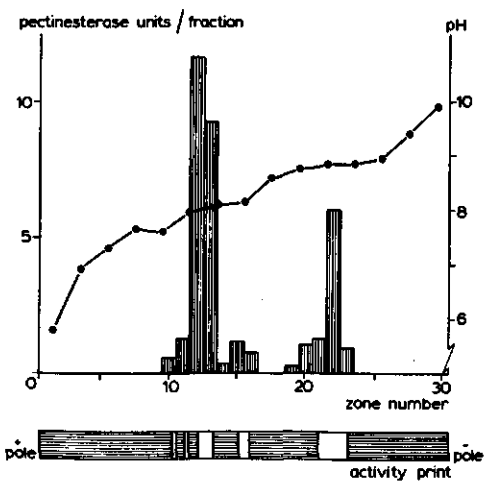


Fig. 21. pH Gradient electrophoresis of crude Navel orange pectinesterase. After taking the print for pectinesterase detection, the gel was segmented and the pH of the segments measured. The segments were extracted and assayed for pectinesterase activity. pH (●——●).

Composition of the high molecular weight pectinesterase The high molecular weight pectinesterase was chromatographed on cross-linked pectate under the conditions as used in Section 5.1 for the separation of Pectinesterase I from Pectinesterase II. Fig. 22 shows the results of this experiment. The high molecular weight pectinesterase was separated into 3 peaks of which the first peak in the salt gradient (HM II) was dominant (85% of the total activity). This peak seemed to be a mixture of three or perhaps more forms, but even after repeated chromatography of this material it was not possible to establish the number of forms involved. The enzymes in three different fractions of this peak (fractions 36, 39 and 41) migrated equally fast in pH gradient electrophoresis runs; slightly faster than Pectinesterase I. The activity of Pectinesterase HM I was lost after pH gradient electrophoresis experiments and Pectinesterase HM III migrated as fast as Pectinesterase II. It can be seen in Fig. 22 that Pectinesterases HM II and HM III are purified to a good extent on the cross-linked pectate column (about 20 and 10 fold, respectively).

6.1.2 Multiple forms of pectinesterase in citrus fruit

Five kg portions of several citrus fruits were pressed and mixed well with the pressed juice filtrate as described in Section 5.1 and frozen in portions of 200 g. The pectinesterase was extracted from a 200 g portion and precipitated with ammonium sulphate as described in Section 5.1. The 30-75% ammonium sulphate precipitate was redissolved in a minimum amount of distilled water and dialysed against 10 g/l glycine sodium hydroxide

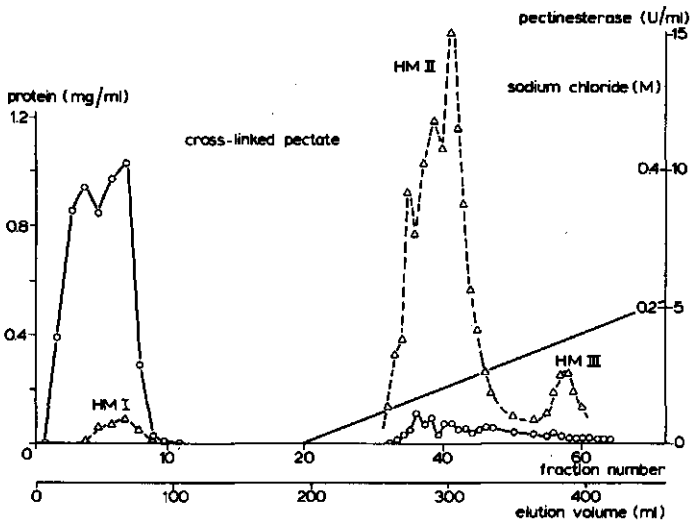


Fig. 22. Cross-linked pectate chromatography of the high molecular weight pectinesterase. 700 Units of pectinesterase (9 units/mg protein) from fractions 20-27 of Bio-Gel P-100 (Fig. 12) were applied to a cross-linked pectate column as described in Fig. 13. Protein (o); pectinesterase activity (Δ) and sodium chloride concentration (————).

buffer pH 7.0. The dialysis bags were previously treated with acetic acid and rinsed in water and buffer. Sometimes the ammonium sulphate precipitates were hard to redissolve. Then 1 unit/ml of yeast endo-polygalacturonase was mixed through the gel-like precipitate and left overnight before being treated further. This step then increased greatly the pectinesterase recovery.

Approximately 1 500 units of the crude pectinesterases were used for column isoelectric focusing in the pH range 9-11 and approximately 75 units were used for the pH gradient electrophoresis. The extraction was repeated for each citrus fruit and approximately 75 units were used for a second pH gradient electrophoresis. In all citrus fruits Pectinesterases I and II were identified by their isoelectric points (10.05 and >11 respectively) in column isoelectric focusing experiments. Pectinesterase I and II were the major pectinesterase enzymes in all citrus fruits and no other major pectinesterase peaks were found. Frequently a shoulder could be observed at the high pH side (pH 10.2) of the Pectinesterase I peak. In those instances (lemon, grapefruit and mandarin), pH gradient electrophoresis demonstrated the high molecular weight pectinesterase to be present in a quantity of more than 20% of that of pectinesterase I. The result of the grapefruit pectinesterase column isoelectric focusing is presented in Fig. 23. In all fruits, except in grapefruit, a small peak could be observed as a shoulder of Pectinesterase I at pH 9.6. The recovery in column isoelectric focusing experiments amounted to about 75% of the total activity.

Drawings of the prints for pectinesterase activity in the pH gradient electrophoresis experiments are given in Fig. 24. Compared with column isoelectric focusing, thin-layer pH gradient electrophoresis gave much better separation of Pectinesterase I from the high molecular weight pectinesterase and also allowed detection of more molecular forms. In

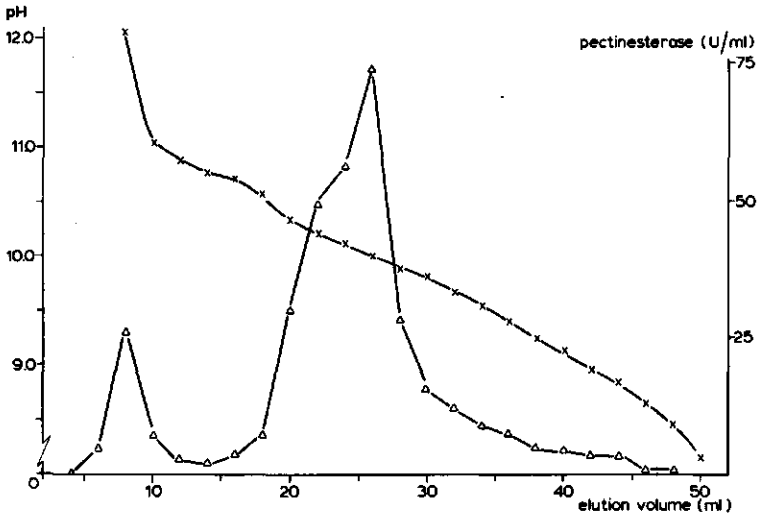


Fig. 23. Isoelectric focusing of crude grapefruit pectinesterase. The enzymes (1000 units) were focused as described in Chapter 3, activity recovery 75%. pH (×); pectinesterase activity (Δ).

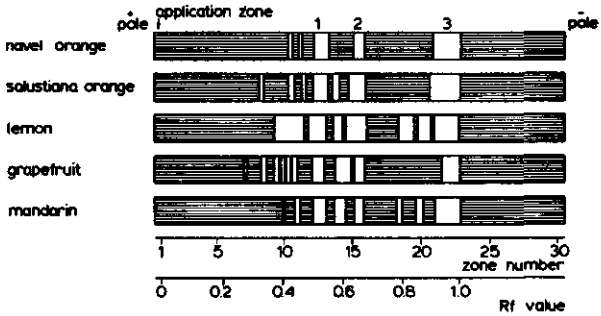


Fig. 24. pH Gradient electrophoresis patterns of crude pectinesterases from different citrus fruits. The pH electrophoresis experiments were done as described in Chapter 3. Drawings were made from the prints for pectinesterase detection. Light zones are pectinesterase active. Identified zones: Pectinesterase I (1); high molecular weight pectinesterase (2) and Pectinesterase II (3).

spite of the detection of activities as separated zones in the prints, the zones did not always show distinct activity peaks upon collection. Therefore the activity measurements of the collected bands are compiled in 4 groups in Table 18. The values are the averages of two pH gradient electrophoresis experiments. The group with a migration rate between Pectinesterase I and II represents mainly the high molecular weight pectinesterase in recovered activity (90-100%), except for the mandarins (about 50%). The migration distance of Pectinesterase II and the relative migrations (R_f values) of Pectinesterase I (R_f =0.55) and the high molecular weight pectinesterase (R_f =0.68) were reproducible. The activity recovery of all pectinesterases together was rather low (about 60%). The print technique allowed detection of zones to about 2% of the total activity (= 1 unit pectinesterase/ zone). This sensitivity could be influenced by the contact time of gel and printpaper. But a too long contact (more than 1 min) resulted in fading of dark zones between two close bands with a higher activity.

Table 18. Relative quantities of pectinesterase forms in citrus fruits.

Fruit	Migration rates			
	R_f<0.55	R_f=0.55	0.55<R_f<1.00	R_f=1.00
		Pectin-esterase I		Pectin-esterase II
Navel orange	5.1	62.0	5.7	27.2
Salustiana orange	3.1	58.2	11.3	27.4
Shamouti orange	10.3	58.4	6.8	24.5
Lemon	10.7	45.6	9.1	33.6
Grapefruit	7.3	45.8	33.2	13.7
Mandarin	14.2	44.2	10.2	31.4

6.2 DISCUSSION

Thin-layer pH gradient electrophoresis combined with the print technique of Delincée (1976) gave better resolution of the already known pectinesterases and allowed detection of several others. The high molecular weight pectinesterase was not detected in column isoelectric focusing experiments because its isoelectric point differs only very little from Pectinesterase I. The small peak of high molecular weight pectinesterase disappeared under the Pectinesterase I peak. Flocculation in the upper part of the isoelectric focusing column made detection of forms with isoelectric points lower than Pectinesterase I difficult.

The molecular weight of the high molecular weight pectinesterase was roughly estimated to be 54 000. This is much less than the double of the molecular weight of Pectinesterase I and II (2x36 200 = 72 400). If the high molecular weight pectinesterase is related to Pectinesterase I or II, a simple dissociation of a double molecule is unlikely to be the cause. The complexity of the situation is illustrated by Fig. 22. The major form of the high molecular weight pectinesterase (peak HM II, Fig. 22) is contaminated with other forms, which have the same molecular weight but differ with respect to affinity to cross-linked pectate and charge properties. The major form of high molecular weight pectinesterase may consist out of several more forms. In pH gradient electrophoresis, the three pectinesterases with known isoelectric points migrate in order of their isoelectric points. This is not necessarily the case for all pectinesterases.

In citrus fruits twelve forms of pectinesterase could be recognized (Fig. 24). Maximally ten forms of these were present in one citrus fruit (grapefruit). Pectinesterase I and II together always represented more than 60% of the total activity (Table 8) and in oranges always more than 80%. In oranges, the concentration quotient of pectinesterase I to pectinesterase II was 2.1-2.4. In grapefruit there was more Pectinesterase I and in lemons and mandarins there was more Pectinesterase II. The high molecular weight pectinesterase is strongly represented in grapefruit. Navel oranges contained the least of this form.

Whether the differences found can be ascribed to orange cultivar and citrus species or to other factors like ripeness and growth conditions remains unresolved. Also the relative quantities in the component parts of each fruit may differ considerably as was demonstrated by Evans & McHale (1978) in orange for two forms of pectinesterase. One of these forms was found in the juice sacs and membrane covers (OPE II) and the other form was found in the peel (OPE I). It may be presumed from the elution order of OPE I and OPE II on DEAE Sephadex at pH 7.0 (Evans & McHale, 1978) that Pectinesterase I is identical to OPE II and that Pectinesterase II is identical to OPE I of these authors.

7 Kinetics and mode of action of Pectinesteras I and II

The kinetic studies of orange pectinesterase reported in literature were done with preparations of undefined pectinesterase composition. It is very likely that mixtures of different forms were used. Often pectinesterases were not or only partially purified. It is important to know the properties of the single forms of pectinesterase to be able to explain phenomena which are observed in processing of fruits and vegetables and to adapt processing conditions.

Further, some of the experiments give information about the mode of action of the enzymes. Knowledge about the mode of action will contribute to elucidate the structure of pectic substances.

In the experiments to be described purified Pectinesterase I and II were used. In some cases the crude Navel orange pectinesterase and the partially purified high molecular weight pectinesterase containing all the forms, Pectinesterase HMI, HMII and HMIII were included.

7.1 EXPERIMENTS AND RESULTS

7.1.1 Initial reaction rates

pH For most pectinesterases the pH-activity curves were determined both on brown ribbon and green ribbon pectin. At pH 5 and above, initial activities were measured by automatic titration and at pH 5 and below initial activities were determined by the gas chromatographic methanol assay. This assay was described in Chapter 4. For the gas chromatographic assay, pectin solutions were buffered with 0.04 mol/l citric acid, adjusted to the desired pH with potassium hydroxide. The ionic strength was adjusted at 0.1 by adding potassium chloride. At low pH values with some enzymes, reaction rates decreased rapidly with time. The reaction rate decreased sometimes before 1% of the available substrate was de-esterified. In such cases tangents were drawn to the curves to determine the initial reaction rate. The high molecular weight pectinesterase was the least susceptible and Pectinesterase II the most susceptible to this phenomenon. Fig. 25 shows the methanol formation at and below pH 5 by the high molecular weight pectinesterase on green ribbon pectin. The reaction rate, decreasing with decreasing pH, increased again below pH 3.5. Figs 26 to 29 show the pH-activity curves for the different pectinesterases on both green and brown ribbon pectin. The activities determined at pH 5 with recorded titration did not always coincide with the activities at pH 5 determined by the gas chromatographic methanol assay. The potassium citrate buffered solutions for the gas chromatographic methanol assay were adjusted to an ionic strength of 0.1 with potassium chloride. The potassium ion concentrations were then 0.073 mol/l at pH 5, 0.086 mol/l

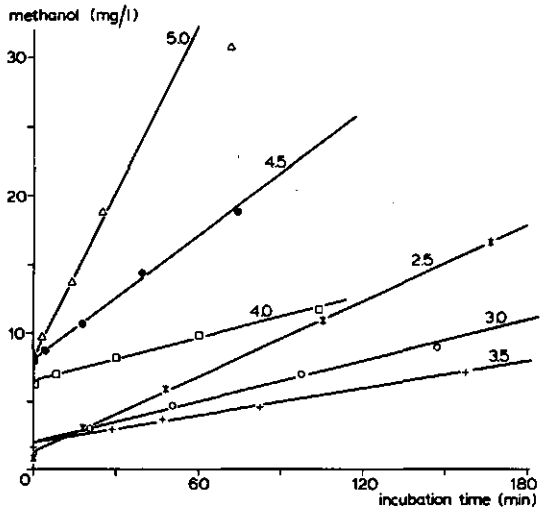


Fig. 25. Activity of the high molecular weight pectinesterase at low pH values. Reaction mixtures (10 ml) contained 5 g/l green ribbon pectin, 0.1 g/l sodium azide, 0.04 mol/l potassium citrate, potassium chloride to an ionic strength of 0.1 and 0.1 units per ml of pectinesterase. Incubation at 30 °C. Samples of 1 ml were taken for gas chromatographic methanol assay. The figures indicate the pH values of the reaction mixtures.

at pH 4.5, 0.095 mol/l at pH 4.0 and 0.1 mol/l at the lower pH values. In recorded titration experiments there were always 0.1 mol/l potassium ions. The potassium ions introduced during the neutralization of the pectin were neglected. The poor fit of several curves at pH 5 can probably be ascribed to this difference in potassium ion concentrations, as at pH 5 in the potassium citrate buffered solutions there was a lower concentration of potassium ions and also the activity of the pectinesterases was lower (for the influence of cations see Section 2.2.3).

On brown ribbon pectin, Pectinesterase I has its optimum at pH 7.6 and Pectinesterase II at pH 8.0. Further, Pectinesterase I is relatively more active at low pH values, which is particularly evident at pH 5.0. As anticipated, the curve for the crude enzyme preparation runs between the curves for the two purified enzymes. The two purified enzymes account for 90% of the total activity in crude Navel orange pectinesterase (see Section 6.1.2). On green ribbon pectin, the curves are broadened at the acid side, approximately, over a full pH unit. The difference in the curves between green ribbon and brown ribbon pectin cannot be caused by the different quantities of potassium hydroxide needed for the neutralization of the pectins. The potassium ion concentration was about 0.106 mol/l in the brown ribbon pectin and about 0.108 mol/l in the green ribbon pectin solutions.

None of the pectinesterases, except the high molecular weight pectinesterase had any activity at pH 3.0 or below. The crude enzyme must have some activity at pH 2.5 but the concentration of the high molecular weight pectinesterase in this preparation was too low to measure activity within a few hours.

The three enzyme peaks of the high molecular weight pectinesterase after separation

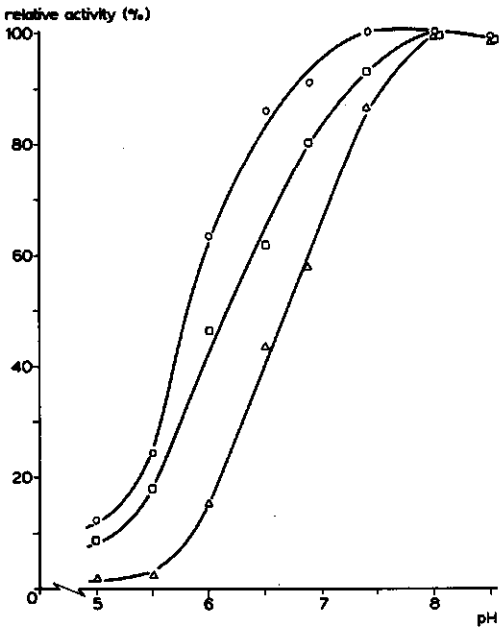


Fig. 26. pH Activity curve of pectinesterase on brown ribbon pectin (degree of esterification 72%). Reaction mixtures (25 ml) contained 5 g/l brown ribbon pectin; 0.1 g/l sodium azide; 0.1 mol/l potassium chloride and 0.2 units per ml of pectinesterase. Initial activities were measured at 30 °C by recorded titration. Crude pectinesterase (□); Pectinesterase I (o); Pectinesterase II (Δ).

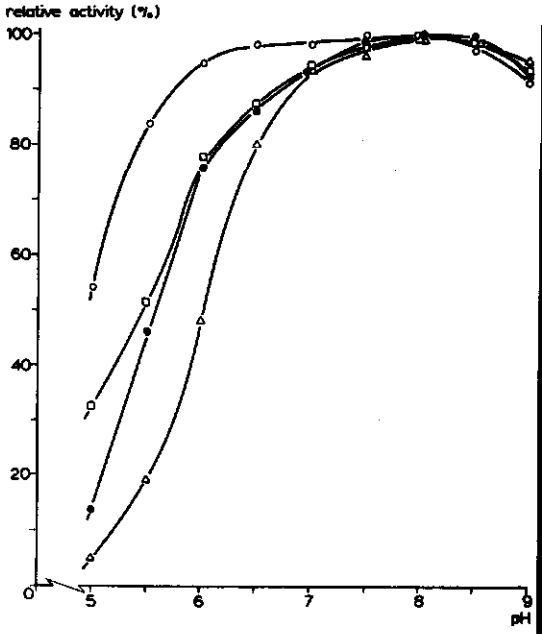


Fig. 27. pH Activity curve of pectinesterase on green ribbon pectin (degree of esterification 62%). Reaction mixtures and conditions as in Fig. 26, except that 5 g/l green ribbon pectin instead of brown ribbon pectin was used. Crude pectinesterase (□); Pectinesterase I (o); Pectinesterase II (Δ); high molecular weight pectinesterase (●).

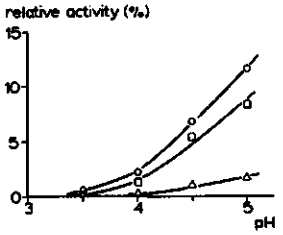


Fig. 28. pH Activity curve of pectinesterase on brown ribbon pectin (degree of esterification 72%). Reaction mixtures (10 ml) contained 5g/l brown ribbon pectin, 0.1 g/l sodium azide, 0.04 mol/l potassium citrate, potassium chloride to an ionic strength of 0.1 and 0.1 units per ml pectinesterase. Incubation at 30 °C. Samples of 1 ml were taken for the gas chromatographic methanol assay. Crude pectinesterase (□); Pectinesterase I (o); Pectinesterase II (Δ).

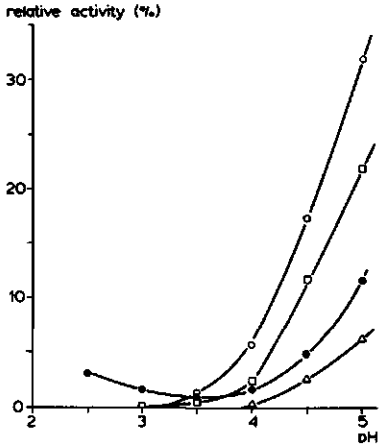


Fig. 29. pH Activity curve of pectinesterase on green ribbon pectin (degree of esterification . 62%). Reaction mixtures and conditions as in Fig. 28, except that 5 g/l of green ribbon pectin instead of brown ribbon pectin was used. Crude pectinesterase (□); Pectinesterase I (o); Pectinesterase II (Δ); high molecular weight pectinesterase (●).

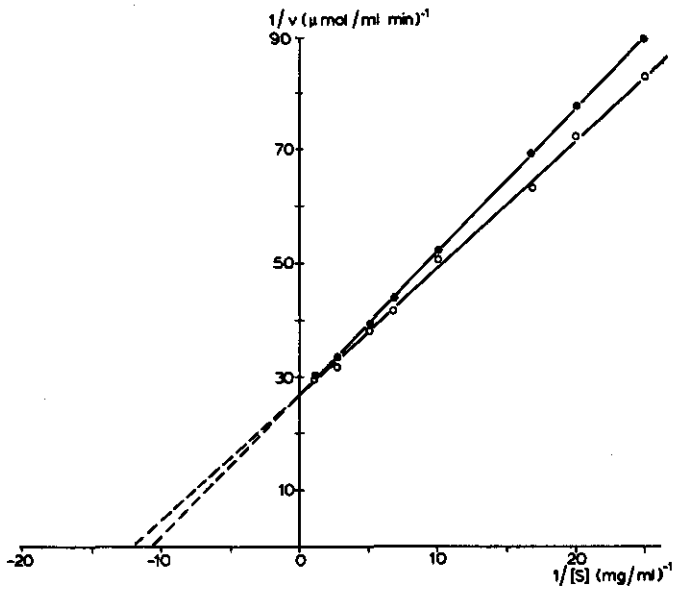


Fig. 30. Lineweaver-Burk plots for purified Pectinesterase I with and without inhibitor. Reaction mixtures (30 ml, pH 7.0) contained 0.04 to 1 mg per ml of green ribbon pectin, 0 or 0.05 mg per ml of polygalacturonic acid inhibitor, 0.1 mol/l of potassium chloride and 0.05 units per ml of pectinesterase. While purging with nitrogen, initial activities were measured at 30 °C by recorded titration with 0.01 mol/l potassium hydroxide. Without inhibitor (o); with inhibitor (●).

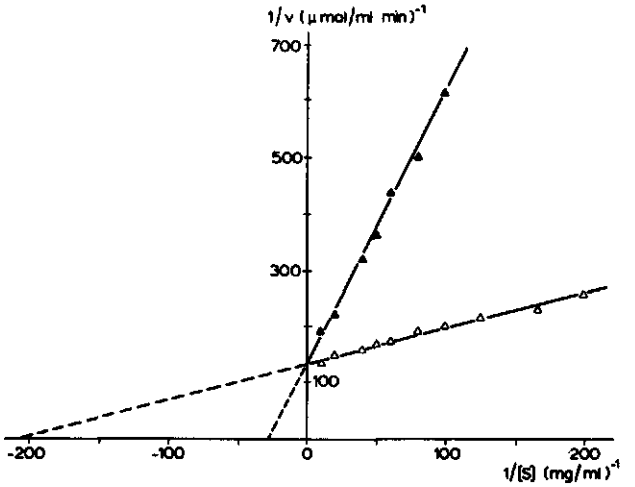


Fig. 31. Lineweaver-Burk plots for purified Pectinesterase II with and without inhibitor. Reaction mixtures (160 ml, pH 7.0) contained 0.005 to 0.1 mg per ml of green ribbon pectin, 0 or 0.01 mg per ml of polygalacturonic acid inhibitor, 0.1 mol/l potassium chloride and 0.0095 units per ml of Pectinesterase II. While purging with nitrogen, initial activities were measured at 30 °C by recorded titration with 0.01 mol/l potassium hydroxide. Without inhibitor (Δ); with inhibitor (▲).

on cross-linked pectate (Section 6.1.1.; Fig. 22) were assayed separately at pH 2.5 on green ribbon pectin. All three peaks had activity at pH 2.5 but the relative activities differed. The relative activities (the activities relatively to the activity at standard assay conditions) were 4.8% for Pectinesterase HM I, 3.5% for Pectinesterase HM II and 0.4% for Pectinesterase HM III. Different fractions (fractions 36, 39 and 41) of the broad Pectinesterase HM II peak differ not or very little from each other in relative activity at pH 2.5.

K_m, V_{max} and K_i Figs 30 and 31 show initial enzyme activities measured at different concentrations of green ribbon pectin in the absence and presence of polygalacturonic acid, a competitive inhibitor. Various kinetic parameters calculated from these figures are given in Table 19. The values for V_{max} and turnover number (molecular activity) of both enzymes are virtually equal. However, the K_m values for green ribbon pectin and also the K_i values for polygalacturonate inhibitor differ widely. Pectinesterase I has a relatively low affinity for its pectin substrate and is rather weakly inhibited by polygalacturonate, whereas Pectinesterase II has a high affinity for its substrate and is strongly inhibited by polygalacturonate. Pectinesterase II is much more active at low substrate concentrations, as may be judged from the largely different saturation curves for the two enzymes, shown in Fig. 32. Activities measured for both purified pectinesterases in the presence of various substrate concentrations (data from Figs 30 and 31) were used in log [S] versus log $V/(V_{max}-V)$ plots. Straight lines were obtained from which K_m values were calculated which were the same as those given in Table 19. The slopes of the lines were 1 $\pm$ 0.02, which is typical of enzymes following Michaelis-Menten kinetics (Whitaker, 1972).

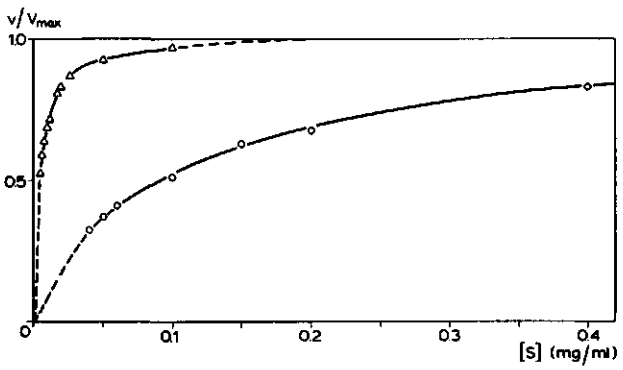


Fig. 32. Substrate saturation curves for Pectinesterase I and II. The ratio of initial reaction velocity and maximum initial reaction velocity (V/V_{max}) is plotted against substrate concentration. Reaction conditions are specified in Figs 30 and 31. V_{max} values were taken from Figs 30 and 31.
Pectinesterase I (o); Pectinesterase II (Δ).

Table 19. Kinetic data of Pectinesterase I and II at pH 7.0 on green ribbon pectin. For reaction conditions see Figs 30 and 31.

Enzyme	V_{max} (μmole/min.unit)	Turnover number (mole/mole.sec)	K_m (mg/ml)[1]	K_i (mg/ml)[2]
Pectinesterase I	0.81	340	0.083	0.416
Pectinesterase II	0.74	338	0.0046	0.0016

1. Expressed as mg of green ribbon pectin per ml of reaction mixture.
2. Expressed as mg of polygalacturonic acid (ICN) per ml of reaction mixture.

Relationship of K_m and degree of esterification of substrate Lineweaver-Burk plots were made with pectins, partially de-esterified by alkali, with different degrees of esterification as listed in Chapter 3. For Pectinesterase I initial reaction rates were determined with recorded titration. However, for Pectinesterase II initial reaction rates decreased too rapidly for the titrimetric assay. The pH drop method as described in Chapter 3 appeared to be more sensitive and was used for all Pectinesterase II assays. For this method conical flasks were used, filled with substrate, and topped with 1 cm petroleum ether (b.p. 60-80 °C) to exclude carbon dioxide. Figs 33 and 34 show the Lineweaver-Burk plots for Pectinesterase I and II, respectively on all substrates. Activity measurements on some pectins are only partly shown in these figures. However, the kinetic data listed in Table 20 were obtained from plots (not shown) in which the scales were chosen in agreement with the points of measurements on each particular substrate. Both pectinesterase I and II have a strongly increased affinity to substrates with a lower degree of esterification. As found for green ribbon pectin, the affinity for the substrate is much higher for Pectinesterase II than for Pectinesterase I. The K_m values (Table 19) found on green ribbon pectin (degree of esterification 62%) fit fairly well in Table 20. However, the V_{max} values on the green ribbon substrate were lower. The V_{max} (Table 20) appears to be rather independant from the degree of esterification of the substrate, only at the highest and lowest degree of esterification tested, there was some deviation. The slopes of the log [S] versus log $V/(V_{max}-V)$ plots were 1.00 ($\pm$ 0.05), except for Pectinesterase II at the highest and lowest degree of esterification of the substrate (slopes 1.21 and 1.25, respectively). It appeared that log $(1/K_m)$ was linearly related to log (% free carboxyl groups in the substrate) for both Pectinesterase I and II, with a slope of 2.08 for Pectinesterase I and a slope of 2.03 for Pectinesterase II (Fig. 35).

Another observation was made during the measurements for these Lineweaver-Burk plots. On highly esterified pectin, initial reaction rates increased for Pectinesterase I after some of the substrate was de-esterified. The reaction rate was maximal after about 2% of the substrate was de-esterified, as is illustrated in Fig. 36 by Lineweaver-Burk plots. Thus affinity increased and the V_{max} was not affected. This phenomenon was not observed at degrees of esterification of the substrate of 78% and lower.

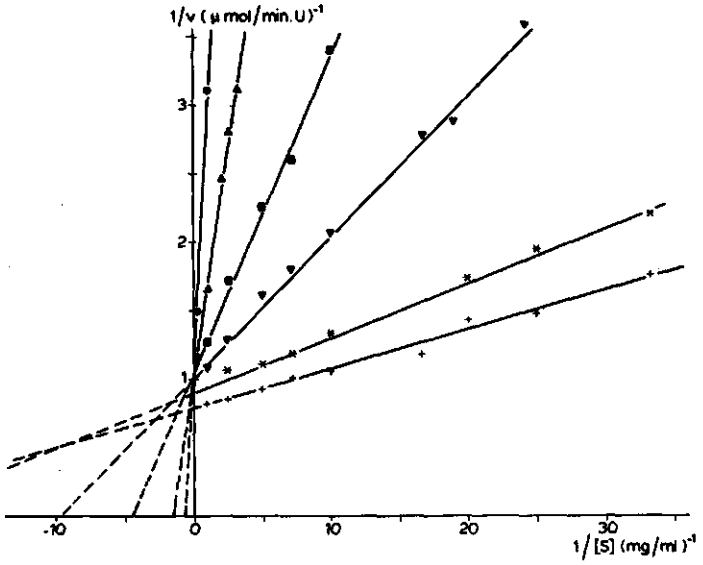


Fig. 33. Lineweaver-Burk plots for purified Pectinesterase I on pectins with different degrees of esterification. Reaction mixtures (100 ml, pH 7.0) contained various pectins, 0.1 mol/l potassium chloride, 0.1 g/l sodium azide and 0.02 units per ml of pectinesterase. Initial activities were measured at 30 °C by recorded titration with 0.01 mol/l potassium hydroxide. Degrees of esterification of pectins: 95.6% (●); 88.0% (▲); 78.2% (■); 68.3% (▼); 52.5% (✳) and 32.3% (+).

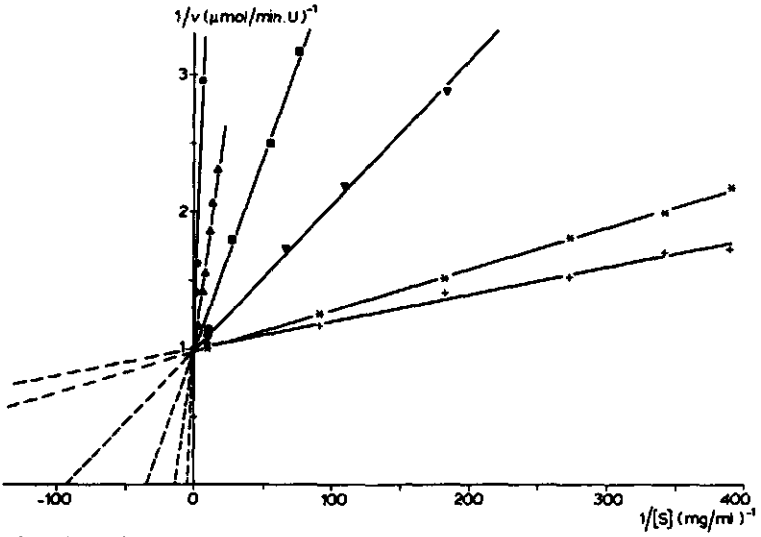


Fig. 34. Lineweaver-Burk plots for purified Pectinesterase II on pectins with different degrees of esterification. Reaction mixtures (500 ml) contained various pectins, 0.1 mol/l potassium chloride, 0.1 g/l sodium azide and 0.001 units per ml of pectinesterase. Initial activities were measured by the pH drop per min between pH 7.02 and 6.97. Degrees of esterification of pectins: 95.6% (●); 88.0% (▲); 78.2% (■); 68.3% (▼); 52.5% (✳) and 32.3% (+).

Table 20. Kinetic data of Pectinesterase I and II at pH 7.0 on pectins with different degrees of esterification. For reaction conditions see Figs 33 and 34.

Degree of esterification of pectin (%)	Pectinesterase I		Pectinesterase II	
	K_m (mg/ml)[1]	V_{max} (µmole/min.unit)	K_m (mg/ml)[1]	V_{max} (µmole/min.unit)
95.6	1.63	0.87	0.238	0.94
88.0	0.724	1.02	0.074	1.00
78.2	0.231	0.98	0.023	1.00
68.3	0.102	0.98	0.0108	1.02
52.5	0.042	1.06	0.0031	1.04
32.3	0.038	1.30	0.0019	0.96

1. Expressed as mg pectin per ml of reaction mixture.

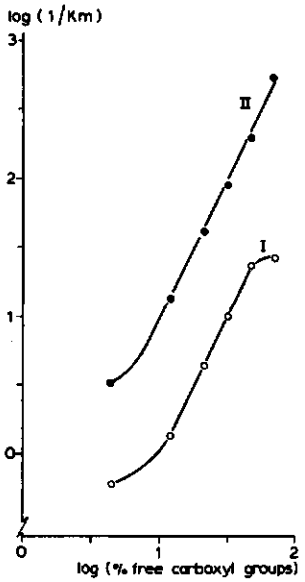


Fig. 35. Log ($1/K_m$) versus log (% free carboxyl groups in substrate). Data calculated from Table 20, reaction conditions as in Figs 33 and 34. Pectinesterase I (o); Pectinesterase II (•).

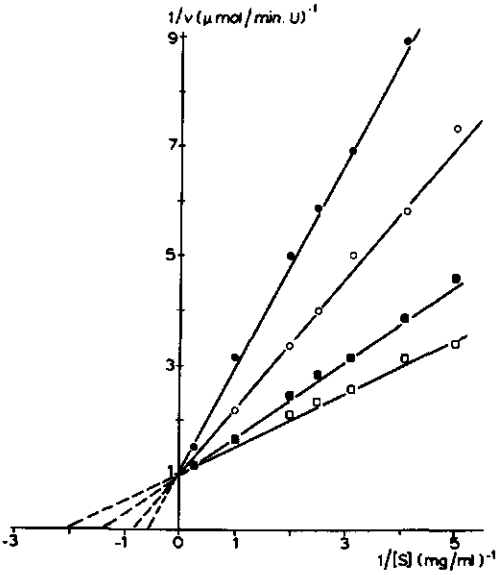


Fig. 36. Lineweaver-Burk plots for purified Pectinesterase I with increasing initial velocities. Reaction conditions and concentrations as in Fig. 33. Initial velocity on pectin 95.6% esterified (•); velocity after 2% enzymatic de-esterification on the same substrate (o); initial velocity on pectin 88.0% esterified (■); velocity after 2% enzymatic de-esterification on the same substrate (□).

Table 21. Kinetic data of Pectinesterase I and the high molecular weight pectinesterase on green ribbon pectin at different pH values. For reaction conditions see text.

pH	Pectinesterase I			High molecular weight pectinesterase		
	V_{max} (μmole/min.unit)	K_m (mg/ml)[1]	K_i (mg/ml)[2]	V_{max} (μmole/min.unit)	K_m (mg/ml)[1]	K_i (mg/ml)[2]
7.0	0.81	0.083	0.416	0.83	0.041	0.010
4.0	0.47	1.19	0.002	0.071	1.35	0.002
2.5				0.85[3]	50[3]	0.002

1. Expressed as mg of green ribbon pectin per ml of reaction mixture.
2. Expressed as mg of polygalacturonic acid (ICN) per ml of reaction mixture.
3. Approximate values because substrate concentrations which could be used were much lower than the K_m.

K_m, K_i and V_{max} Dependence on pH Lineweaver-Burk plots were made with green ribbon pectin as substrate, in the presence or absence of polygalacturonic acid as inhibitor. This was done at different pH values for both Pectinesterase I and the high molecular weight pectinesterase. Determinations at pH 7.0 were done with recorded titration under the conditions as in Fig. 30. Measurements at pH 4.0 and 2.5 were made with the gas chromatographic methanol assay. The reaction mixtures (10 ml) contained green ribbon pectin, 0.1 g/l sodium azide, 0.1 mol/l potassium chloride, 25 mg/l propanol-1 and 0.01 units pectinesterase/ml. During the initial reaction stage, no significant pH drop (< 0.05 pH units) was observed in these unbuffered substrates. The K_m, K_i and V_{max} values from these plots (not shown) are presented in Table 21. The polygalacturonic acid exhibited competitive inhibition. Table 21 clearly shows the reduction of the affinity to the substrate with decreasing pH for both pectinesterases. The pectate inhibition increases, but the K_i is a parameter which depends on the K_m. It can be seen that whereas Pectinesterase I is hardly inhibited at pH 7, it is strongly inhibited at pH 4 by polygalacturonic acid. The V_{max} is reduced at pH 4 for both enzymes, but remarkably the V_{max} of the high molecular weight pectinesterase increases to about the value of that at pH 7 if the pH is reduced to 2.5.

7.1.2 De-esterification limits

Pectinesterase on pectins partially de-esterified by alkali Pectinesterases were incubated with alkali de-esterified pectins with different degrees of esterification (pectins listed in Chapter 3). The reactions were followed by recorded titration with 0.02 mol/l potassium hydroxide. When the reaction rate had decreased to nearly zero, the amount of enzyme in the reaction mixture was doubled and when the reaction rate was virtually zero again another form of pectinesterase was added. Figs 37 and 38 show the decrease in degree of esterification of the substrate with time for Pectinesterase I and II. Pectinesterase I was able to de-esterify the highly esterified pectins better than Pectinesterase II and a lower degree of esterification could be reached. The lower the initial degree of esterification the lower the ultimate degree of esterification. It was

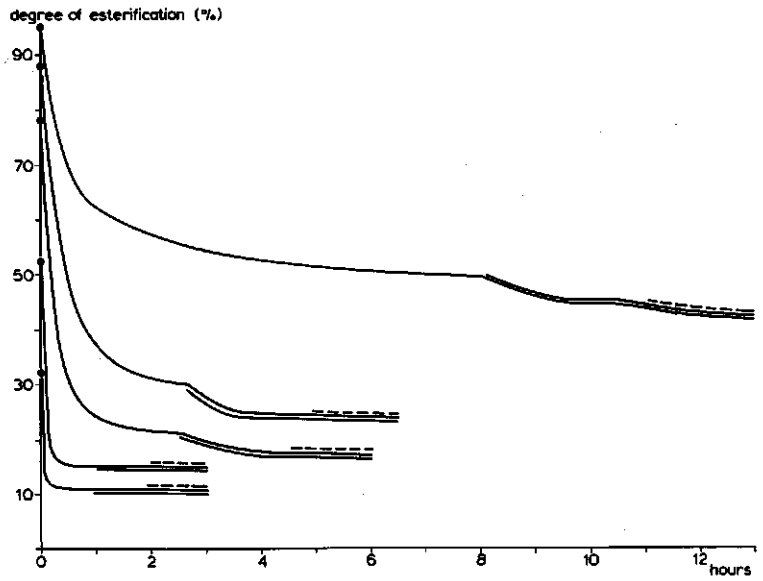


Fig. 37. Action of purified Pectinesterase I on pectin, partially de-esterified to different degrees of esterification by alkali. Reaction mixtures (25 ml, pH 7.0) contained 1 mg per ml of pectin, 0.1 mol/l potassium chloride, 0.1 g/l sodium azide. While purging with nitrogen the reactions were followed by recorded titration with 0.02 mol/l potassium hydroxide at 30 °C. Pectinesterase I 0.2 units per ml (———); Pectinesterase I 0.4 units per ml (═══); Pectinesterase I 0.4 units per ml + Pectinesterase II 0.2 units per ml (═ ═ ═).

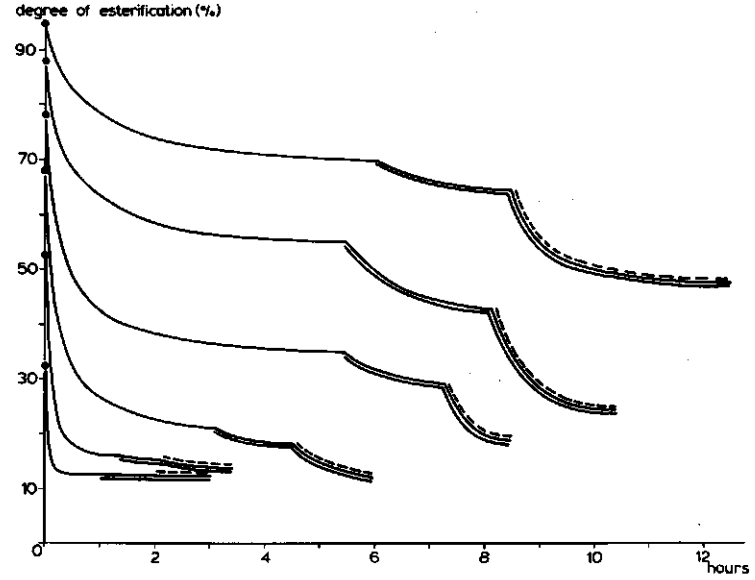


Fig. 38. Action of purified Pectinesterase II on pectin, partially de-esterified to different degrees of esterification by alkali. Reaction conditions and concentrations as in Fig 37. Pectinesterase II 0.2 units per ml (———); Pectinesterase II 0.4 units per ml (═══); Pectinesterase II 0.4 units per ml + Pectinesterase I 0.2 units per ml (═ ═ ═).

impossible to reach a degree of esterification of lower than 11%. At that point addition of more enzyme had no effect. It is remarkable that when the amount of enzyme is doubled during the reaction (especially with Pectinesterase II), the reaction velocity is more than doubled. The original enzyme was not inactivated since addition of green ribbon pectin and sodium chloride to the level of standard assay conditions restored the activity to the level of the added units (for stability of the enzymes see also Chapter 8). When Pectinesterase II is added after Pectinesterase I is not longer active, no more carboxyl groups are liberated (Fig. 37). When Pectinesterase I is added after Pectinesterase II is not longer active, the degree of esterification is rapidly lowered to the value that Pectinesterase I can reach alone (Figs 37 and 38). The curves (not shown) for crude pectinesterase and a 1:1 mixture of Pectinesterase I and II resemble very much the figure for Pectinesterase I (Fig. 37).

Separation of reaction products Twenty-five units of different pectinesterases were incubated with 100 ml of 5 g/l highly esterified pectin (degree of esterification 95.6%) in 0.1 ml/l potassium chloride. The pH was held at 7.0 by recorded titration with 0.1 mol/l potassium hydroxide. When the amount of alkali equivalent to the lowering of the degree of esterification from 95.6% to 60% was used, the reaction was stopped by the addition of concentrated hydrochloric acid to pH 2.0. The pectinesterases were inactivated by heating for 15 min at 100 ^{o}C and after cooling the pectin was precipitated with 2 volumes of ethanol 96%. The precipitate was washed chloride free with ethanol 70%, then washed with acetone and air dried. Part of the pectin preparations were fractionated on a DEAE-cellulose column as described in Chapter 3. Figs 39A - 39E show the elution patterns of several pectin preparations. The elution pattern of pectin treated with Pectinesterase II and crude pectinesterase were very similar to the elution pattern of pectin treated with Pectinesterase I (Fig. 39B). It can be seen from the figure that highly esterified pectin de-esterified by pectinesterase was separated in a large fraction apparently not de-esterified and fractions de-esterified to several rather different degrees of esterification (Figs 39B and C). Whereas alkali de-esterified pectins eluted as rather sharp peaks (Figs 39D and E) and were much more uniform with respect to degree of esterification.

Sugar analysis of separated reaction products The highly esterified pectin and fractions from the DEAE-cellulose column of pectin de-esterified by Pectinesterase I were analysed for neutral sugars. Approximately 2.5 mg of the original highly esterified pectin, 2.5 mg of the fraction which was not de-esterified (eluted with 0.05 mol/l sodium phosphate, Fig. 39B) and 2.5 mg of the de-esterified fraction (peak fraction eluted with 0.45 mol/l sodium phosphate, Fig. 39B) were dialysed against distilled water. Samples were taken for uronic acid analysis, the solutions were freeze dried and analyzed gas chromatographically for neutral sugars as described in Chapter 3. There were considerable differences in galactose and glucose contents and only slight and not very reproducible differences in other neutral sugars. Table 22 lists the results for galactose and glucose. The fraction not de-esterified was rich in galactose and glucose. It is difficult to draw conclusions from the differences in glucose content since the preparation contained

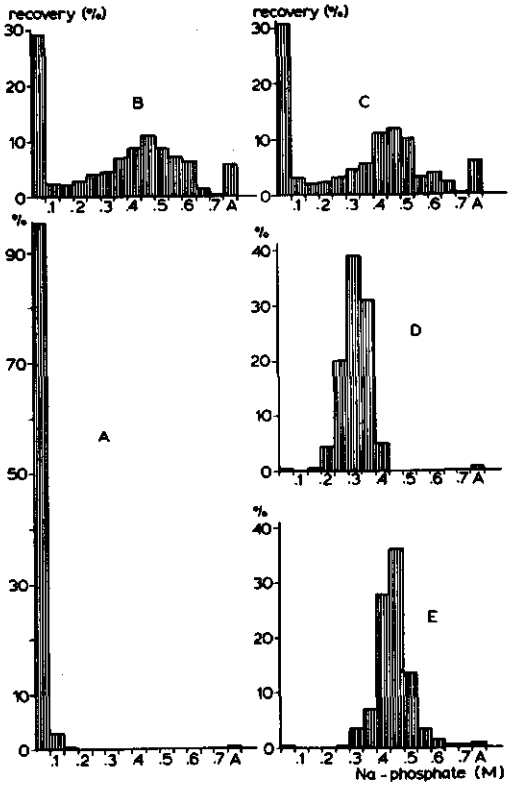


Fig. 39. Chromatography of pectins on DEAE-cellulose. Five ml of 10 g/l pectin solutions were applied to a DEAE-cellulose column and eluted with a linear gradient of sodium phosphate buffer, pH 4.8 as described in Chapter 3. Then the column was washed with 0.1 mol/l sodium hydroxide (A). A. Highly esterified pectin (degree of esterification 95.6%). B. Pectin de-esterified with Pectinesterase I (degree of esterification 60.4%). C. Pectin de-esterified with high molecular weight pectinesterase (degree of esterification 60.8%). D. Alkali de-esterified pectin (degree of esterification 52.6%). E. Alkali de-esterified pectin (degree of esterification 32.3%).

Table 22. Neutral sugar contents of the highly esterified pectin, the not de-esterified and the de-esterified fractions of the highly esterified pectin treated with Pectinesterase I.

Neutral sugar	Pectinesterase treated pectin			Original pectin
	not de-esterified	de-esterified	weighed sum[1]	
galactose (mg/g)[2]	71.6	6.0	27.8	29.0
glucose (mg/g)[2]	91.4	14.6	40.2	38.8

1. In this preparation 1/3 was not de-esterified.
2. mg/g (uronic acid + neutral sugars).

some starch which also elutes with 0.05 mol/l sodium phosphate from the DEAE-cellulose column. Highly purified apple pectin (the purification of which includes a copper precipitation step) never contains less than 25 mg/g galactose (Voragen, Department of Food Chemistry, Agricultural University, Wageningen; personal communication). This galactose is part of the side chains in pectin polymers (Rombouts, 1972). Apparently the highly esterified pectin contains a fraction relatively rich in side chains which is more difficult to be de-esterified by the pectinesterase than the rest of the preparation with less side chains.

The first fraction from the DEAE-cellulose column and the original highly esterified pectin were incubated with crude pectinesterase under the same conditions (0.3 mg pectin/ml, 0.1 mol/l potassium chloride, 0.1 g/l sodium azide and 0.2 units pectinesterase/ml at 30 ^{o}C and pH 7.0). The reactions were followed by recorded titration. Both the initial reaction rate and the de-esterification limit using the first fraction of the column as a substrate were half the value on the original highly esterified pectin (figures not shown).

Effects of other pectic enzymes Purified Pectinesterase I and II were incubated with highly esterified pectin at pH 6.0. The reactions were followed by recorded titration. When the reaction rate had decreased considerably, other purified pectic enzymes were added. The reaction pH of 6.0 was chosen to allow activity of all added pectic enzymes: pectin lyase has its optimum at about pH 6.0 (Houdenhoven, 1975), polygalacturonase at about pH 4.0 (Phaff, 1966) and pectate lyase at pH 9.4 (Rombouts et al., 1978). The amounts of pectic enzymes added were chosen to obtain comparable rates of chain splitting of the pectin, which was determined in preliminary experiments. Table 23 lists the amounts of pectic enzymes added and the average number of splits per pectin chain after one hour of activity in the reaction mixture together with the pectinesterases. The degree of polymerization gradually dropped to values corresponding to these numbers of splits per pectin chain (about 80 monomers each, see Chapter 3). Fig. 40 shows the action of Pectinesterase I and II on highly esterified pectin before and after the addition of other pectic enzymes at some stage during the reaction.

Because calcium ions are an absolute requirement for pectate lyase activity, 0.25 mmol/l calcium chloride was present in the reaction mixtures where pectate lyase was

Table 23. Quantities of pectic enzymes added to reaction mixtures with highly esterified pectin, partially de-esterified by pectinesterase and the number of splits per pectin chain after one hour of simultaneous action.

Pectic enzyme	With Pectinesterase I		With Pectinesterase II	
	units added[1]	splits/chain[2]	units added[1]	splits/chain[2]
polygalacturonase	1.76	7.5	8.8	7.5
pectin lyase	0.076	2.5	0.076	2.5
pectate lyase	15.0	2.5	50.0	2.5

1. Per reaction mixture of about 25 ml.
2. Average number of splits per pectin chain with a degree of polymerization of 80.

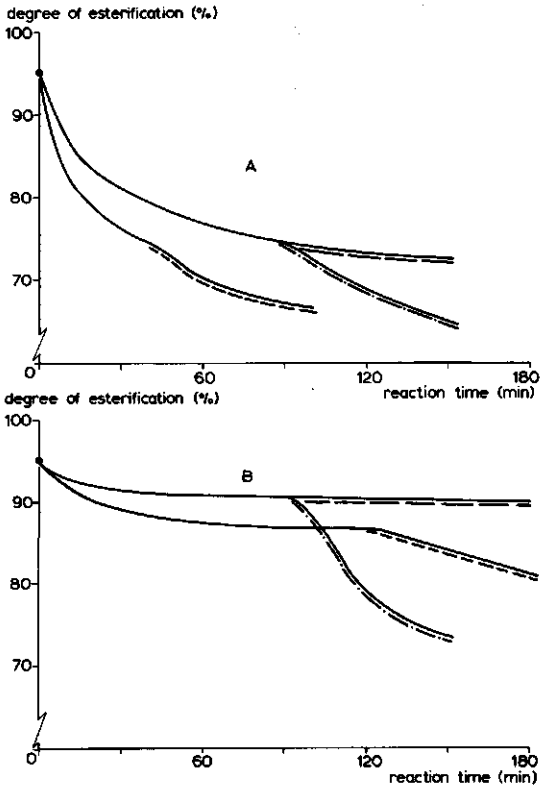


Fig. 40. Stimulation of pectinesterase by other pectic enzymes. Reaction mixtures (25 ml, pH 6) contained 1 g/l highly esterified pectin (degree of esterification 95.6%), 0.1 g/l sodium azide and 0.2 units Pectinesterase I (A) or 0.2 units Pectinesterase II (B) per ml. In the reaction mixtures where pectate lyase was added also 0.25 mmol/l calcium chloride was present. While purging with nitrogen, the reactions were followed by recorded titration at 30 °C. Pectinesterase (———); pectinesterase + pectin lyase (═══); pectinesterase + polygalacturonase (═·═·═); pectinesterase + pectate lyase (═ ═ ═).

used. This addition markedly stimulated both Pectinesterase I and II (Fig. 40). Calcium ions were assumed to be only 5-10 fold as effective in stimulating pectinesterase as potassium or sodium ions (see Chapter 2, Table 4). In the presence of 0.1 mol/l potassium chloride only a maximum stimulation in reaction rate of about 2.5% was expected. Pectin lyase had no effect but polygalacturonase highly stimulated the pectinesterases, especially Pectinesterase II (Fig. 40). Also pectate lyase stimulated the pectinesterases, but to a smaller extent. It can be seen from Table 23 that the pectate lyase was about 3-fold less effective in degrading the polymer.

7.2 DISCUSSION

The two pectinesterase isoenzymes described here differ markedly in their K_m value for green ribbon pectin and K_i value for pectic acid inhibitor (Table 19). Pectinesterase II with its isoelectric point of > 11 is much more strongly inhibited by pectic acid than Pectinesterase I with an isoelectric point of 10.05. Lineweaver & Ballou (1945) developed a theory about the inhibition of plant (alfalfa) pectinesterase by pectic acid (end product inhibition). Without knowing the isoelectric point of their enzyme, they reasoned that because of its positive charge below its pH optimum for activity it would form an inactive ionic complex with pectate. They carefully studied the stimulatory effect of various salts on pectinesterase activity and concluded that cations, by liberating the enzyme from its inactive ionic complex, stimulate enzyme activity by removal of inhibition rather than by specific activation. This theory is supported by the decreased inhibition of orange pectinesterase by pectic acid hydrolysates with increasing salt concentration, and also at increased pH values (Termote et al., 1977). In the light of this theory it is quite comprehensible that Pectinesterase II, with its relatively high isoelectric point, has less activity at low pH, is much stronger inhibited by polygalacturonate and binds to cross-linked pectate.

Through alkali de-esterification pectins with a statistical distribution of carboxyl groups are produced (Kohn & Furda, 1967). On alkali pre de-esterified pectins the K_m values are largely reduced but the V_{max} values are hardly affected (Table 20). The affinity ($1/K_m$) is increased much more than would be expected if only one carboxyl group is involved in the enzyme-substrate complex formation. Doubling the number of free carboxyl groups in the substrate gives a four fold increase of $1/K_m$ of both Pectinesterase I and II. From Fig. 35 the following relationship can be deduced for a wide range of degrees of esterification of the substrate:

$$\log (1/K_m) = \log a + 2 \log (\% \text{ free carboxyl groups})$$

$$\text{or } 1/K_m = a\ (\% \text{ free carboxyl groups})^2$$

where a is approximately 10 times as large for Pectinesterase II as for Pectinesterase I.

For pectins with different degrees of esterification and the free carboxyl groups randomly distributed over the polymer chain some calculations were made. Consider a pectin with a *DE* (degree of esterification) of 95% and an indefinite chain length. This pectin contains (100-*DE*) = 5% free carboxyl groups. The chance that at one side of a free carboxyl group another free carboxyl group occurs at some fixed distance (e.g. 4 monomer units) is 0.05. The pectin contains (100-*DE*) x 0.05 = 0.25% units of 2 free carboxyl groups at some fixed distance. For 3 carboxyl groups in some fixed arrangement this is (100-*DE*) x 0.05 x 0.05 = 0.0025%. These calculations were made for several degrees of esterification of pectins. The relationship between log (% units of n carboxyl groups in some fixed arrangement) and log (% free carboxyl groups in the pectin) is shown in Fig. 41, with n = 1, 2 and 3. The slope for log (% units of 2 carboxyl groups) in Fig. 41 is

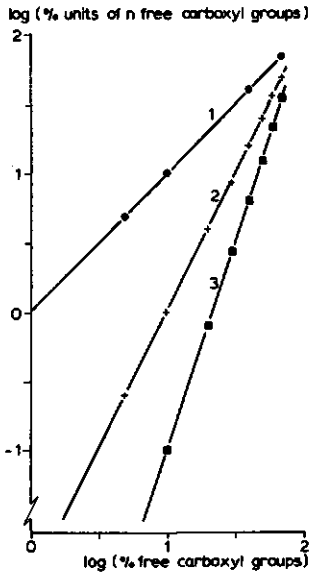


Fig. 41. Units of n carboxyl groups at some fixed distance in pectins of different degrees of esterification and randomly distributed carboxyl groups. The lines were constructed as described in the text.
1. n = 1; 2. n = 2; 3. n = 3.

2; equal to the slope of the experimental lines in Fig. 35. It may be concluded that for both Pectinesterase I and II, 2 carboxyl groups at a certain distance in the pectin chain are required for an optimal enzyme-substrate complex formation. At what distance is unknown. Because (crude) orange pectinesterase is only inhibited by galacturonic acid oligomers with a degree of polymerization of 8 or more (Termote et al., 1977), 6 monomers in between 2 monomers with free carboxyl groups seems likely. However, then it is difficult to understand how a pectinesterase can proceed linearly along the pectin chain producing blocks of free carboxyl groups as indicated by the results of several workers (Schultz et al., 1945; Hills et al., 1949; Heri et al., 1961; Kohn et al., 1968). For such a mechanism the attack of 2 carboxyl groups next to each other seems more likely.

Solms & Deuel (1955) found an opposite relationship between the K_m and the degree of esterification of the substrate as presented in Table 20. They also reported an increasing V_{max} with decreasing degree of esterification of the substrate. Probably their results were affected strongly by suboptimal and variable concentrations of cations in the reaction mixtures (0.05-0.1 mol/l).

The reduced affinity at low pH values (Table 21) may partly be explained by the decreased dissociation of the free carboxyl groups of the pectin. The pK value for pectin is about 4.0 (Speiser et al., 1945). But the pH will also have its effect by protonation of acidic groups on the pectinesterases as may be concluded from the change in V_{max} . At lower pH, the pectate inhibition also increases. This increase cannot be understood if the relationship $(1/K_m)$ = a (% free carboxyl groups)^2 holds for all degrees of esterification. For Pectinesterase I it can be deduced from Table 20 that the $(1/K_m)$ does not increase below 50% esterification of the substrate. Thus the affinity for polygalacturonic acid at pH 4 (where about 50% of the free carboxyl groups are dissociated) may be the same as at pH 7.0. The affinity for the substrate is greatly reduced at pH 4.0 so that the apparent K_i value is lower and the polygalacturonic acid

is more effective as inhibitor.

The broadening of the pH-activity profile at the acid side by a substrate with a lower degree of esterification may also be ascribed to the decrease of affinity at low pH (Figs 26-29). At equal substrate concentrations, the enzyme remains more saturated with the substrate for which it has the highest affinity.

Rexová-Benková et al. (1976) suggested that the best method is to express a K_m of pectinesterase as a concentration of methyl-D-galactopyranosyluronate residues. My opinion is that this is not true for citrus pectinesterase and presumably also not for other plant pectinesterases. Expression of the K_m on basis of the pectin concentration together with a good description of the pectin (which includes anhydrogalacturonic acid content, degree of esterification, distribution of carboxyl groups and degree of polymerization) is more meaningful.

The velocity of Pectinesterase I on highly esterified substrates increases after some substrate was saponified because of increase in affinity for the changed substrate (Fig. 36). Apparently new points of attack are created by and for the pectinesterase. The increase in affinity cannot be explained by a trace of free carboxyl groups which may be formed by spontaneous de-esterification at pH 7.0. Perhaps the enzyme starts at a point of attack and moves in one direction. One or more of the carboxyl groups produced in the beginning can be a part of the point of attack in the opposite direction. This would imply to some extent a multichain mode of attack mechanism. Otherwise only increasing product inhibition would have been observed.

The pectinesterases are unable to de-esterify pectin completely (Figs 37 and 38). This was also observed by Solms & Deuel (1955). Also the alkali pre de-esterified pectin with the lowest degree of esterification could not be de-esterified further than a degree of esterification of 11%. Possibly these methoxyl groups are located close to side chains or other irregularities in the pectin chain. Another possibility is that besides free carboxyl groups also a methoxyl group is required for the enzyme-substrate complex, a group outside the active centre which is not de-esterified.

The highly esterified pectin (degree of esterification of 95.6%) could only be de-esterified to a degree of esterification of about 50%. Separation of reaction products of pectinesterases with this substrate de-esterified to a degree of esterification of 60%, revealed that about 30% of the substrate was unaffected by the pectinesterase (Fig. 39), presumably because these substrate polymers contained more side chains as was revealed by neutral sugar analysis. But also the distribution of free carboxyl groups over the polymer will have played a role.

Although 2 carboxyl groups at a certain fixed distance are required for optimal enzyme-substrate complex formation, another enzyme-substrate complex must be possible. Highly esterified pectin with a degree of esterification of 95% contains 0.25% units of 2 carboxyl groups at some fixed distance. Pectin with a degree of polymerization of 80 contains less than 20% pectin chains suitable for optimal enzyme-substrate complex formation. The other 80% of substrate chains could not be attacked if 2 carboxyl groups as described above would be required. However, it can be seen in Figs 37, 38 and 39B that more than 20% of the pectin chains are attacked. Thus another type of enzyme-substrate complex should be possible.

Upon doubling the amount of enzyme when the reaction rate had decreased to nearly zero, the reaction rate was more than doubled (Figs 37, 38). This result could be caused by inhibitory sites produced in the pectin chain for which the pectinesterase has a much higher affinity than for other sites still present and not yet used for an attack. Whitaker (1972) mentioned adsorption of the enzyme to the surface of the reaction container or the relative shortage of an essential cofactor as the cause of such a phenomenon.

The stimulation by endo-pectate lyase and endo-polygalacturonase (both splitting between free carboxyl groups) can presumably be ascribed to the removal of the reaction product: blocks of free carboxyl groups acting as competitive inhibitor. Pectinesterase I is also stimulated by the other pectic enzymes (Fig. 40), whereas this enzyme is hardly inhibited by polygalacturonic acid (Table 19). It must be kept in mind that the inhibition by a competitive inhibitor increases when the affinity for the substrate decreases. The enzyme has a low affinity for the highly esterified substrate. Moreover, the results in Table 19 were produced at pH 7.0, whereas the experiments in combination with the other pectic enzymes were done at pH 6.0. Pectinesterase II, which is more efficiently inhibited by polygalacturonic acid, is stimulated more by the other pectic enzymes.

Calcium ions also stimulate pectinesterases (Fig. 40). Presumably the blocks of free carboxyl groups bind calcium (Kohn, 1975) in a way that makes them less accessible to the pectinesterases. Thus calcium ions also reduce the competitive inhibition of the reaction product.

8 Stability of pectinesterases

Stability studies of pectinesterase under technologically relevant conditions, reported in the literature, were always done with crude pectinesterase preparations, presumably composed of several forms of pectinesterase. Manabe (1973a) tested the heat stability of a purified citrus pectinesterase at nearly optimal conditions for activity and stability, which are different from the conditions prevailing in citrus juice. Factors known to affect pectinesterase stability in citrus juices are the pH (Rouse & Atkins, 1953a) and the pulp content (Rouse & Atkins, 1952). As mentioned in Section 6.1.1, discrepancies between heat stability of purified pectinesterases and the original crude preparation made me aware of the technological importance of the high molecular weight pectinesterase.

8.1 EXPERIMENTS AND RESULTS

8.1.1 Stability during storage, assay and orange cloud stability tests

Stability during storage The loss of activity of purified Pectinesterase I and II in solutions of 0.005 mol/l sodium phosphate, pH 7.5, with 0.1 mol/l sodium chloride was less than 15% after storage for 2 years at 4 °C.

Stability during activity measurements at pH 4.0 and 7.0 Pectinesterases were incubated in pectin solutions of pH 4.0 and 7.0, under conditions similar to the conditions during the kinetic experiments described in Chapter 7. At time intervals samples were taken and the residual activity was measured by recorded titration at standard assay conditions. Fig. 42 shows the residual activities upon incubation of Pectinesterase I and II in media of both pH values. At pH 7.0 both enzymes were stable for at least 24 h. Pectinesterase I was also stable at pH 4.0 but Pectinesterase II lost all activity within 6 h. For this reason Pectinesterase II was not used for some of the experiments at low pH values described in Section 7.1. The curves for the crude enzyme (not shown) ran in between the curves of Pectinesterase I and II, for example at pH 4.0 the residual activity dropped to about 70% within 5 h and remained constant for the next 12 h.

Effect of orange juice constituents on pectinesterase stability The concentration of some orange juice constituents was varied to test its effect on pectinesterase stability. The pectinesterases (initially about 40 units/ml) were incubated in the media listed in Table 24 in tubes sealed with Parafilm for 2 weeks both at 30 °C and 6 °C. At time intervals, the tubes were stirred and samples were taken to measure the residual activity under standard assay conditions. The log (% residual activity) was plotted against incu-

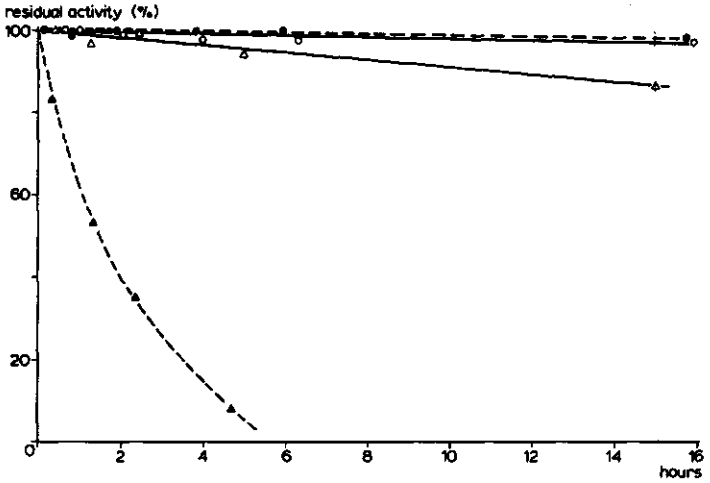


Fig. 42. Stability of purified Pectinesterase I and II under the reaction conditions as used in the kinetic experiments. Pectinesterases (about 40 units/ml) were incubated at 30 °C with green ribbon pectin (1 mg/ml), 0.1 g/l sodium azide, 0.03 mol/l potassium phosphate pH 7.0 or 0.03 mol/l potassium citrate pH 4.0, potassium chloride was added to give an ionic strength of 0.1. At time intervals samples were taken to measure activity under standard assay conditions. Pectinesterase I at pH 7.0 (o———o); Pectinesterase I at pH 4.0 (●- - - -●); Pectinesterase II at pH 7.0 (Δ———Δ); Pectinesterase II at pH 4.0 (▲- - - -▲).

Table 24. Incubation media[1] for pectinesterase stability tests.

No.	Orcat[2]	Sugars[3]	Pectin[4]
1	1 ×	20 g/l each	1 g/l
2	3 ×	20 g/l each	1 g/l
3	1 ×	60 g/l each	1 g/l
4	3 ×	60 g/l each	1 g/l
5	1 ×	20 g/l each + 40 g/l sucrose	1 g/l
6	single strength reconstituted Valencia orange juice concentrate		
7	as medium No. 6, 3 × concentrated.		

1. Sodium azide 0.1 g/l and adjusted to pH 4.0 with potassium hydroxide.
2. Orange cations according to Krop (1974): 0.03 mol/l potassium citrate, 0.08 mol/l potassium chloride, 0.43 mmol/l sodium chloride, 2.4 mmol/l calcium chloride and 5.2 mmol/l magnesium chloride.
3. Sucrose, glucose and fructose.
4. Green ribbon pectin.

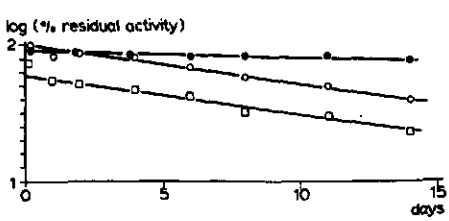


Fig. 43. Stability of pectinesterases in an orange juice-like environment. Pectinesterases (about 40 units/ml) were incubated at 30 °C in medium No. 5 from Table 21. At time intervals samples were taken to measure the residual activity under standard assay conditions. Pectinesterase I (o); crude Navel orange pectinesterase (□); high molecular weight pectinesterase (●).

bation time as demonstrated in Fig. 43 for 3 pectinesterases in one medium at 30 °C. From these destruction curves the decimal reduction values (*D* values) were calculated as is common practice in thermobacteriology (Leniger & Beverloo, 1975). The *D* values for all experiments are listed in Table 25. As can be seen in Fig. 43, the lines of the crude pectinesterase and the high molecular weight pectinesterase do not run through the point $t = 0$, log (% residual activity) = 2, because these preparations are composed of more than a single form of pectinesterase, with different stabilities. The amount of relatively stable pectinesterase in these preparations was calculated from the intercept with the vertical axis and is listed between brackets in Table 25. In the crude preparation the labile fraction will mainly be Pectinesterase II. In the high molecular weight pectinesterase there are 2 small heat labile fractions (see Section 8.1.2) and presumably the initial drop in activity can be ascribed to both of them. The *D* values over about 50 days are inaccurate because of the relatively short incubation time of 14 days.

It can be seen from Table 25 that Pectinesterase II is very labile, especially at 30 °C. Pectinesterase I is 100-200 × more stable at most incubations conditions, but the high molecular weight pectinesterase is even more stable. Generally, the *D* values of the crude enzyme resemble the *D* values of Pectinesterase I, because after the loss of Pectinesterase II, about 90% of the residual activity is Pectinesterase I. The orange ions in 3-fold concentration reduced the stability, whereas the sugars increased the stability of the pectinesterases. A 3-fold ion concentration combined with a 3-fold sugar concentration reduced the stability of the pure enzymes considerably. The same applies to a 3-fold concentrated juice. Remarkably, the crude enzyme followed these trends only partially.

In preliminary experiments it appeared that raising the pectin concentration in medium No. 2 (Table 24) from 1 to 3 g/l did not affect pectinesterase stability (Höcker, 1978).

8.1.2 Heat stability

Residual activities after 5 min at different temperatures Pectinesterases (0.1 ml, 5 units) were added to 5-ml test tubes with 1 ml of a medium (1.1 × final strength), cooled in ice. For these experiments and also for the experiments described hereafter, the tubes were selected to be equal in length, diameter and weight. After mixing the contents, the tubes were incubated for 5 min in a waterbath at various temperatures and cooled in water with melting ice. Immediately, the residual activity was measured under standard assay conditions. Fig. 44 shows the stabilities in 0.01 mol/l potassium phosphate buffer pH 7.5, Fig. 45 in orange cation solution, pH 4.0 (medium as in Footnote 2 in Table 24, this is without sugars, pectin and sodium azide) and Fig. 46 shows the stabilities in single strength reconstituted Valencia orange juice concentrate, pH 4.0. In addition, Fig. 46 shows the stability of the pectinesterase present in freshly pressed Valencia orange juice. For this purpose prechilled Valencia oranges were halved, pressed on a rotary kitchen citrus press and filtered through a 1 mm sieve. All handlings were done at 4 °C. The juice (pH 3.85) was adjusted with 1 mol/l potassium hy-

Table 25. Stability of pectinesterases incubated in media of pH 4.0 at 30 °C and 6 °C.

Pectinesterase	Decimal reduction values (days) in various media[1]													
	1		2		3		4		5		6		7	
30 °C														
I	36		2.1		121[3]		5.8		33		21		8.2	
II	0.16		0.01		0.42		0.02		0.18		0.22		0.02	
high molecular weight	185	(91%)[2]	173	(87%)	171	(85%)	125	(91%)	208	(91%)	166	(87%)	147	(85%)
crude	35	(55%)	7.9	(56%)	120	(55%)	30	(46%)	35	(60%)	45	(56%)	43	(58%)
6 °C														
I	150		150		210		73		133		400		400	
II	4.9		0.28		10		0.66		5.8		4.5		2.9	
high molecular weight	> 400	(92%)	> 400	(91%)	> 400	(92%)	> 400	(91%)	> 400	(91%)	> 400	(91%)	> 400	(91%)
crude	210	(63%)	> 400	(63%)	210	(71%)	58	(63%)	210	(66%)	> 400	(63%)	> 400	(63%)

1. The media are listed in Table 24.
2. Approximate part in the preparation having this decimal reduction time.
3. Values over 50 days are approximate values.

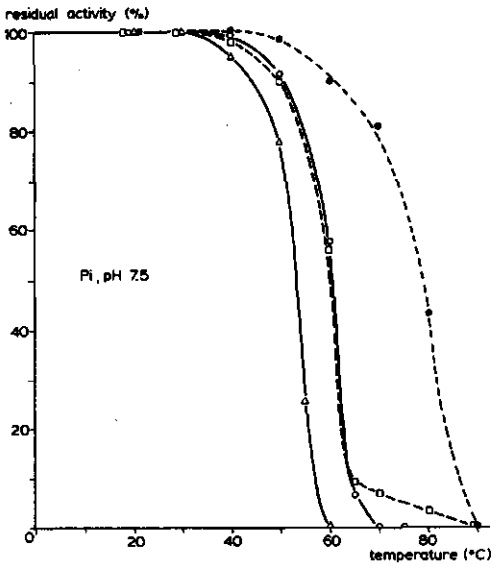


Fig. 44. Heat stability of pectinesterases in phosphate buffer of pH 7.5. Pectinesterases (about 5 units/ml) were incubated in 0.01 mol/l potassium phosphate buffer (pH 7.5) for 5 min at various temperatures and the residual activity was determined under standard assay conditions. Pectinesterase I (o——o); Pectinesterase II (Δ——Δ); crude Navel orange pectinesterase (□——□); high molecular weight pectinesterase (●----●).

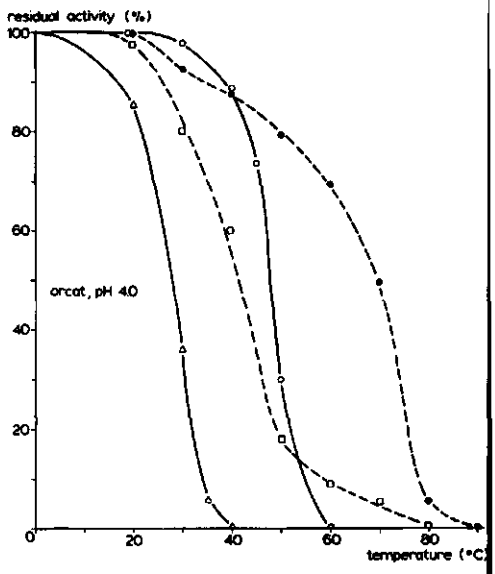


Fig. 45. Heat stability of pectinesterases in orcat solution pH 4.0 Same conditions and procedures as in Fig. 44, except that the pectinesterases were incubated in orcat solution (orange cations) as described in the text. Pectinesterase I (o——o); Pectinesterase II (Δ——Δ); crude Navel orange pectinesterase (□----□); high molecular weight pectinesterase (●----●).

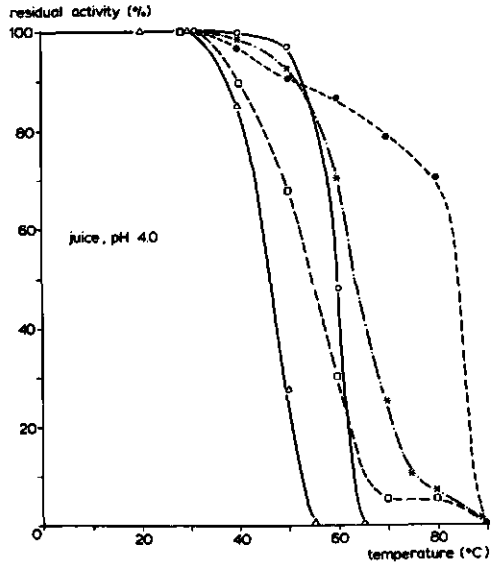


Fig. 46. Heat stability of pectinesterases in orange juice of pH 4.0. Same conditions and procedures as in Fig. 44, except that the pectinesterases were incubated in single strength reconstituted Valencia orange juice concentrate. In addition also fresh Valencia orange juice (adjusted to pH 4.0) was used with its natural occurring pectinesterase. Pectinesterase I (o——o); Pectinesterase II (Δ——Δ); crude Navel orange pectinesterase (□----□); high molecular weight pectinesterase (●----●); fresh Valencia orange juice with its pectinesterase (*—·—·—·—*).

droxide to pH 4.0. The juice was 13.3 °Brix and contained 1.23 units pectinesterase per ml. The juice was held at 0 °C and used within a few hours for heat inactivation studies. Because of the low initial activity, 10 ml portions were incubated and assayed.

Figs 44 - 46 show that for inactivation of Pectinesterase I a 10 - 20 °C higher temperature is required than for Pectinesterase II. The crude pectinesterase contains a fraction, only about 5% of the total activity, which requires a 20 - 30 °C higher temperature for inactivation than both Pectinesterase I and II. This heat stable fraction is the high molecular weight pectinesterase. The figures clearly show the relatively great heat resistance of the high molecular weight pectinesterase. This pectinesterase showed 2 small peaks (HM I and HM III) and a major peak (HM II) upon chromatography on cross-linked pectate (Chapter 6, Fig. 22). Incubation of these 3 peaks separately in juice as in Fig. 46 for 5 min at 80 °C resulted in complete loss of the activity of Pectinesterases HM I and HM III. The major form, Pectinesterase HM II, retained 73% of its activity and no differences were observed between different fractions of this broad peak. The initial small loss in activity at rather low temperatures of the high molecular weight pectinesterase in Figs 45 and 46 can be ascribed to the pectinesterases in peaks HM I and HM III.

It can be seen in Fig. 46 that the native pectinesterase in Valencia orange juice is more heat stable that the crude Navel orange pectinesterase but considerably less heat stable than the high molecular weight pectinesterase. The warming up time for the 10 ml Valencia juice portions is longer than the warming up time in the 1.1 ml portions with the other pectinesterases. Presumably under identical heating conditions the curves for crude pectinesterase and Valencia juice would be somewhat closer to each other. Moreover, the Valencia orange juice appeared to contain a pectinesterase fraction with a heat stability less than that of the high molecular weight pectinesterase but higher than that of Pectinesterase I, as found in the experiments hereafter. It is estimated from Fig. 46 that the pectinesterase in the fresh Valencia juice consists of about 10% of the high molecular weight pectinesterase or an enzyme with a similar heat stability.

The pectinesterases are more stable in the reconstituted Valencia juice (Fig. 46) than in an orcat (orange cation) solution (Fig. 45), probably because of the natural sugar content of the orange juice.

D and Z values Pectinesterases (0.1 ml, 10 units) were added to test tubes with 1 ml juice reconstituted from Valencia concentrate to 1.1 times single strength and treated as in the heat inactivation experiments described above, but incubation times at the different temperatures were varied. The log (% residual activity) was calculated and plotted against incubation time in destruction curves as shown for the high molecular weight pectinesterase in Fig. 47 and for crude Navel orange pectinesterase in Fig. 48. For the experiments with a single form of pectinesterase the decimal reduction values (*D* values) at the different temperatures were read from the figures (not shown for Pectinesterase I and II) and used for thermal destruction curves (Fig. 49). The *Z* values (the raise in temperature (°C) necessary to observe a ten times faster heat inactivation) were taken from and are indicated in this figure. The warming up time was eliminated from the *D* values by using only the straight part of the lines in

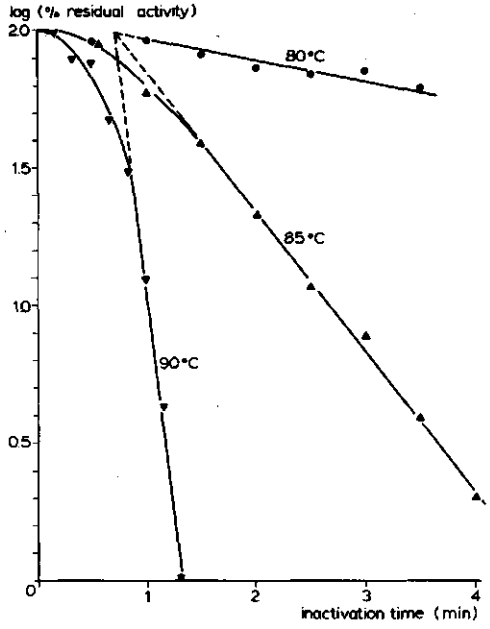


Fig. 47. Heat stability of high molecular weight pectinesterase in orange juice of pH 4.0. High molecular weight pectinesterase (10 units/ml) was incubated in single strength reconstituted Valencia orange juice concentrate, adjusted to pH 4.0, for several time intervals at several temperatures as indicated in the figure. Residual activities were measured at pH 7.0 under standard assay conditions.

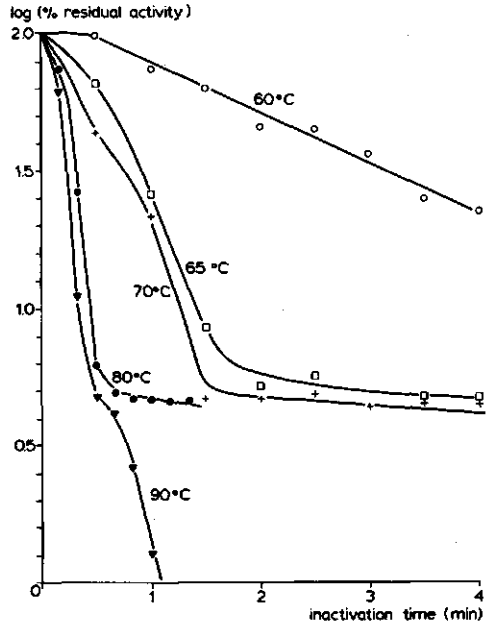


Fig. 48. Heat stability of crude Navel orange pectinesterase in orange juice of pH 4.0. Crude Navel orange pectinesterase (10 units/ml) was incubated and assayed as in Fig. 47.

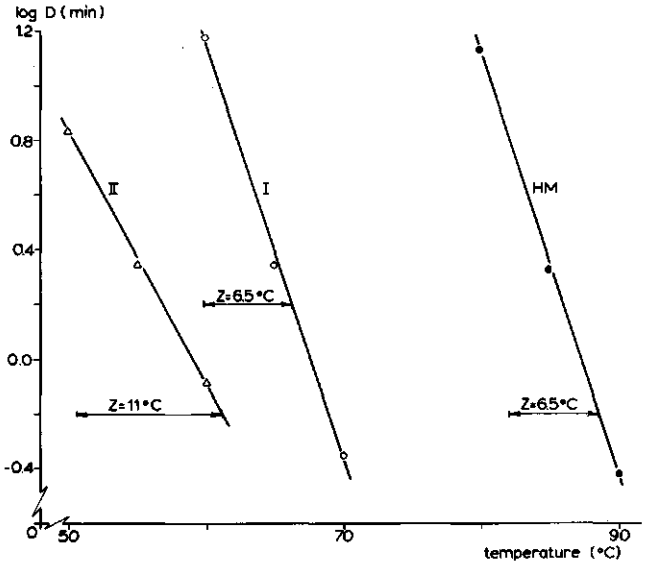


Fig. 49. Thermal destruction curves for pectinesterases in orange juice of pH 4.0. Methods and conditions of the determinations as in Fig. 47. Pectinesterase I, II and the high molecular weight pectinesterase (HM) are indicated in the figure.

the destruction curves as shown in Fig. 47.

The inactivation of the crude pectinesterase follows a complicated pattern (Fig. 48) and no real *D* values can be obtained. At 60 °C mainly Pectinesterase II but also a considerable part of Pectinesterase I will be rapidly inactivated. At 70 °C, after the inactivation of Pectinesterase I and II, only the high molecular weight pectinesterase remains after heating for 1½ min. This pectinesterase is very slowly inactivated (from this figure estimated to be about 5% of the total activity).

The heat inactivation of the pectinesterases in fresh Valencia orange juice also followed a complicated pattern (results not shown). It appeared to contain about 10% of the high molecular weight pectinesterase (or a pectinesterase with a similar heat stability) and 20% of a pectinesterase with a stability less than the high molecular weight pectinesterase but higher than Pectinesterase I.

Fig. 49 gives a quantitative illustration of the differences in heat stability between different forms of pectinesterase.

8.2 DISCUSSION

The heat stabilities of the pectinesterases in potassium phosphate buffer (pH 7.5) may be compared with the results of Manabe (1973a) for purified and crude *Citrus natsudaidai* pectinesterase. Manabe determined the heat stabilities under identical conditions. Both the purified and the crude Natsudaidai pectinesterase were completely inactivated after 5 min at 80 °C. Apparently neither the crude nor the purified enzyme contained a

detectable quantity of high molecular weight pectinesterase. The crude Natsudaidai pectinesterase seemed to contain about 15% of (a) pectinesterase(s) with a stability between Pectinesterase I and the high molecular weight pectinesterase, whereas in the purified Natsudaidai pectinesterase this fraction was reduced to about 7% of the total activity.

In older literature irregular heat inactivation patterns are also shown (Bissett et al., 1953; Rouse & Atkins, 1952; Rouse & Atkins, 1953). From the third publication it can be estimated that about 7% high molecular weight pectinesterase (or a pectinesterase with a similar heat stability) was present in both the Hamlin and Pineapple orange pectinesterase and about 20% in the Marsh Seedless grapefruit pectinesterase. The relatively high amount of high molecular weight pectinesterase in grapefruit was also found in Section 6.1.2, Table 18. Remarkably, in some recent work no irregularities in heat stability of pectinesterases of citrus juice are reported or shown (Eagerman & Rouse, 1976; Nath & Ranganna, 1977a). From the results presented it can be deduced that all pectinesterase was rather stable. The values obtained with citrus juice by these authors and with purified pectinesterase preparations in this work were recalculated with the following formula to facilitate comparison:

$$\log (D/D \text{ ref.}) = (T \text{ ref.} - T)/Z \quad \text{(Leniger \& Beverloo, 1975)}$$

The recalculated values together with the incubation environments are listed in Table 26. It can be seen in Table 26 that both authors found rather high heat stabilities, more or less the stability of the high molecular weight pectinesterase. The pectinesterase in the mandarin juice had also remarkably high Z values. It may be possible that the mandarins contain considerable amounts of a pectinesterase not encountered in my work. In this work some pectinesterase forms were found in mandarins which were not evident in

Table 26. Comparison of the heat stability of pectinesterases.

Source of enzyme	Juice for incubation				Stability	
	origin	pH	pulp content (ml/100 ml)	°Brix	D_{90} °C (min)	Z (°C)
Pectinesterase II Navel	Valencia	4.0		12.6	0.0015	11.0
Pectinesterase I Navel	Valencia	4.0		12.6	0.00037	6.5
High molecular weight pectinesterase Navel	Valencia	4.0		12.6	0.375	6.5
Hamlin (orange)[1]	Hamlin	4.1	12	12.8	0.151	4.8
Pineapple (orange)[1]	Pineapple	4.1	12	12.8	0.177	5.1
Valencia (orange)[1]	Valencia	4.1	12	12.8	0.500	6.8
Duncan (grapefruit)[1]	Duncan	3.6	10	10.5	0.069	5.2
Mandarin[2]	Mandarin	3.6	16	12.0	0.793	11.4
Mandarin[2]	Mandarin	4.0	16	12.0	1.11	10.1

1. Calculated from Eagerman & Rouse (1976).
2. Calculated from Nath & Ranganna (1977a).

grapefruit and orange (Section 6.1.2, Fig. 24). Further a relatively high amount of pectinesterase was found which migrated slowly (R_f < 0.55) in pH gradient electrophoresis (Section 6.1.2, Table 18). Nothing is known as yet about these forms of pectinesterase.

Pectinesterase extracted from the whole orange may be very different from the pectinesterase which can be found in the juice. But the experiments with the freshly prepared Valencia juice revealed that no more than 10% of the pectinesterase had a heat stability comparable with the high molecular weight pectinesterase. It is difficult to explain why the authors cited in Table 26 did not observe irregularities in the heat stability determinations. But it seems likely that processes based on the D and z values recommended by these authors and the total pectinesterase activity present in the juice as a base, result in overpasteurized juice. The problem of heat inactivation of pectinesterase in most citrus juices may be reduced to inactivation of the high molecular weight pectinesterase, which represents only a part of the total activity. The other pectinesterases are easily inactivated below 80 °C.

At increased sugar concentrations the stability of purified Pectinesterase I and II at 30 °C is increased and at increased ionic strength, the stability is decreased (Table 25). The destabilization by the ionic strength was stronger than the stabilization by the sugars. This effect could also be observed in 3-fold concentrated orange juice. The stability of the high molecular weight pectinesterase at 30 °C was too high to observe such an effect if at all present (Table 25). The positive effect of sugars on the heat stability may be presumed by comparing Figs 45 and 46. It may be much easier to inactivate the high molecular weight pectinesterase in concentrated juice than in single strength juice. Bissett et al. (1953) indeed observed differences in heat stability of pectinesterases and cloud stability of Valencia juice concentrates depending on the concentration factor of the juice. The relations were complicated. The pectinesterase was less heat stable in 2-4 fold concentrated juice than in single strength juice, but more heat stable in 6-fold concentrated juice. Nevertheless cloud stability was most easily obtained in 6-fold concentrated juice (heat treatment less than 1 min at 71 °C). Inhibition of the pectinesterase by the increased sugar concentration (see also Section 2.2.5) will also help to increase cloud stability.

9 Cloud loss of orange juice

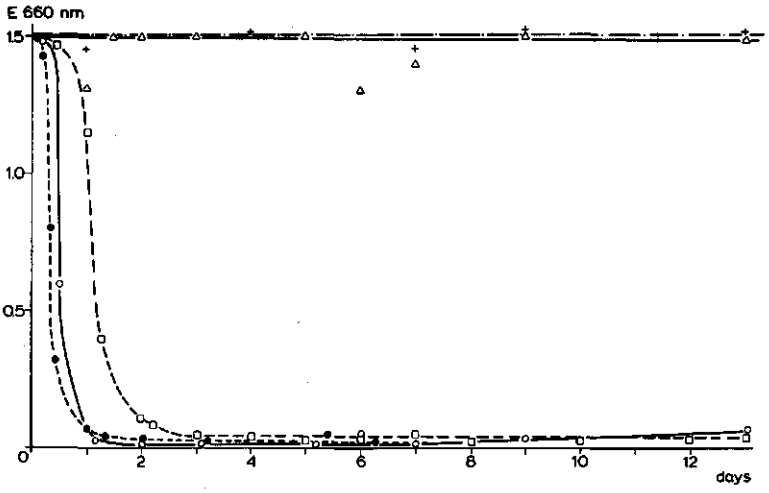
Destabilization of the cloud of citrus juice has been known for a long time as an effect of pectinesterase activity. Krop (1974) reviewed the literature on this subject and studied the mechanism of cloud loss phenomena in orange juice. Up till now unpasteurized juices or pasteurized juices with added crude pectinesterase preparations were used in pectinesterase - cloud loss studies. In addition to pectinesterase, some other enzymes may play a role, for example by making the substrate more accessible for pectinesterase. Therefore in the experiments described below, purified Pectinesterase I and II were tested, but also the crude Navel orange pectinesterase and the (impure) high molecular weight pectinesterase.

9.1 EXPERIMENTS AND RESULTS

9.1.1 Pectinesterases and the cloud loss at 30 °C and 5 °C

Pectinesterase inactive Valencia orange juice concentrate was diluted with 2 volumes of distilled water, potassium metabisulphite was added to give a final concentration of 1000 mg/l sulphur dioxide and the pH was adjusted to 4.0 with 1 mol/l potassium hydroxide under vigorous stirring. The volume was adjusted to give 5/4 x single strength. To 40 ml of this solution, propanol-2 was added to give a final strength of 25 mg/l, 50 units of pectinesterase were added and the volume was adjusted to 50 ml. The juices were incubated in glass stoppered bottles at 30 °C or 5 °C. The juices for the 5 °C incubation were prepared at 0 °C. In these experiments preservation with sulphur dioxide was preferred over sodium azide. Browning of the juice upon incubation with the latter preservative was not prevented. In preliminary experiments with crude pectinesterase, it was established that 25 mg/l propanol-2 affected neither cloud stability nor pectinesterase activity. Also there was no difference between the 2 preservatives in this respect. At time intervals, 3 ml samples were removed after inverting the bottles 10 times. Immediately the turbidity was measured as describes in Chapter 3. The sediment and the supernatant of the sample were remixed and kept in ice till used for gas chromatographic methanol assay (see Chapter 4), which was done within 2 h after removal of the sample from the incubation bottle. Fig. 50 A shows the cloud stability of orange juice upon incubation with different pectinesterases at 30 °C and Fig. 50 B shows the methanol development in the same samples. Figs 51 A and 51 B show the same for samples incubated at 5 °C.

All pectinesterase preparations, except the purified Pectinesterase II, destabilized the cloud of orange juice. At 30 °C Pectinesterase II stopped before a concentration of 20 mg/l methanol was reached. At 5 °C a concentration of 35 mg/l methanol was reached,



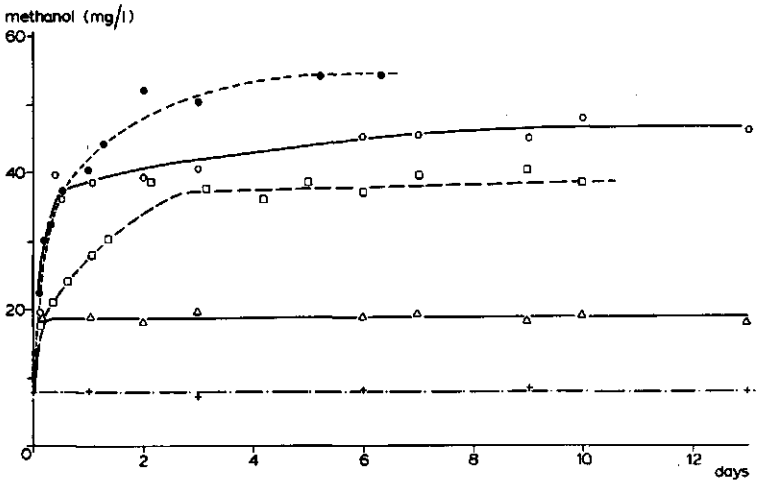
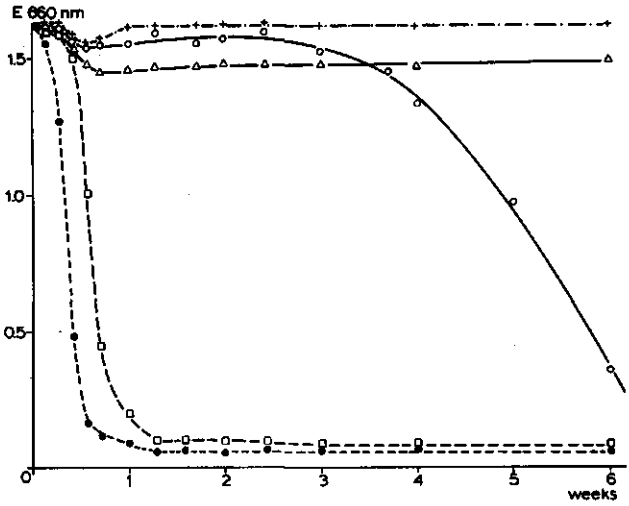


Fig. 50. Cloud stability of (A) and methanol formation in (B) orange juice incubated with pectinesterases at 30 °C. Single strength reconstituted Valencia orange juice concentrate, pH 4.0 with 1000 mg/l sulphur dioxide was incubated with several forms of pectinesterase (1 unit per ml). Pectinesterases: crude Navel orange pectinesterase (□– – –□); purified Pectinesterase I (o——o); purified Pectinesterase II (Δ——Δ); high molecular weight pectinesterase (•– – –•); no pectinesterase (+— · — · — · — · —+).

but the cloud was not destabilized. With the other pectinesterases, cloud destabilization became apparent when about 30 mg/l methanol was attained. At 5 °C, the initial activity of all pectinesterase preparations, except that of Pectinesterase I, was still rather high. The activity of Pectinesterase I at 5 °C was about one hundredth of that at 30 °C. At 30 °C a concentration of 30 mg/l methanol was reached within 6 h, whereas at 5 °C it lasted about 25 days before this concentration was built up. Consequently it lasted



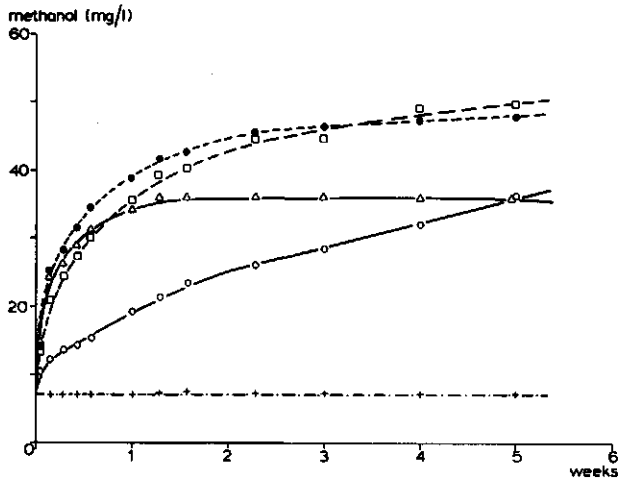


Fig. 51. Cloud stability of (A) and methanol formation in (B) orange juice incubated with pectinesterases at 5 °C. Conditions (except temperature) as in Fig. 50. Pectinesterases: crude Navel orange pectinesterase (□----□); purified Pectinesterase I (o———o); purified Pectinesterase II (Δ———Δ); high molecular weight pectinesterase (●----●); no pectinesterase (+ —·—·—·—·— +).

nearly 4 weeks before cloud destabilization became apparent with Pectinesterase I. If after 3 days incubation at 5 °C a bottle was further incubated at 30 °C, the cloud was completely lost within 24 h (result not shown).

9.1.2 Cloud stabilization by polygalacturonase

Crude Navel orange pectinesterase and purified Pectinesterase I were combined with partially purified yeast endo-polygalacturonase in the cloud stability tests. Several concentrations of polygalacturonase were tested. The samples were incubated only at 30 °C. The conditions and analyses were the same as described in Section 9.1.1. Fig. 52 shows the results for the crude pectinesterase and Fig. 53 shows the results for Pectinesterase I.

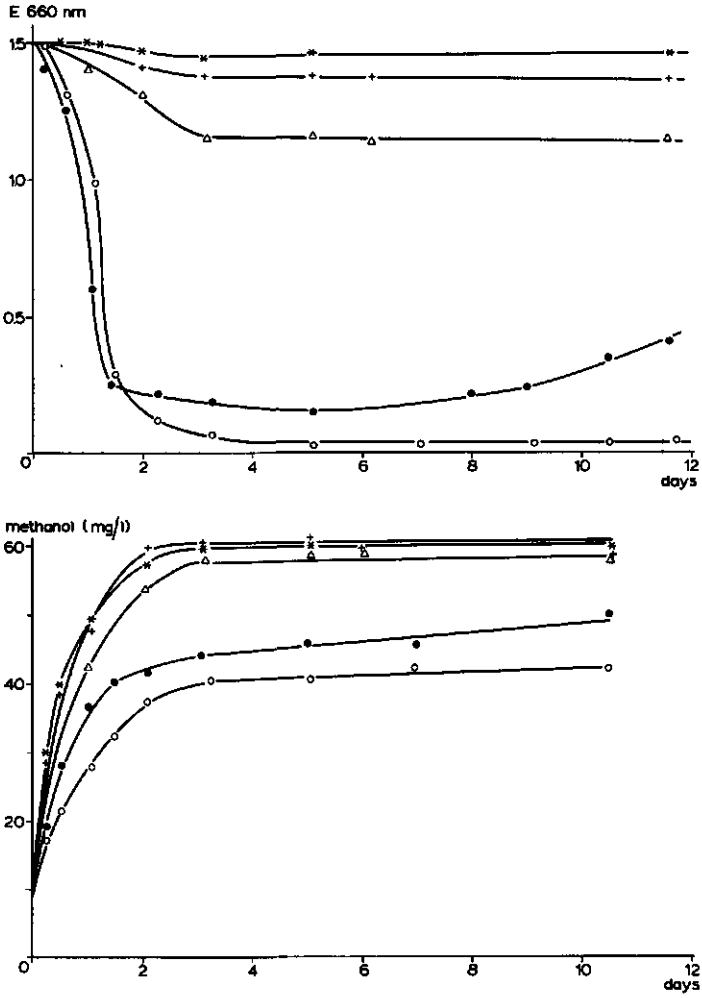


Fig. 52. Cloud stability of (A) and methanol formation in (B) orange juice incubated with crude pectinesterase and different concentrations of polygalacturonase. Single strength reconstituted Valencia orange juice concentrate, pH 4.0 with 1000 mg/l sulphur dioxide was incubated with crude Navel orange pectinesterase (1 unit per ml) and several concentrations of polygalacturonase at 30 °C. Polygalacturonase concentrations: no polygalacturonase (o); 10^{-4} unit per ml (•); 5×10^{-4} unit per ml (Δ); 10^{-3} unit per ml (+); 10^{-2} unit per ml (*).

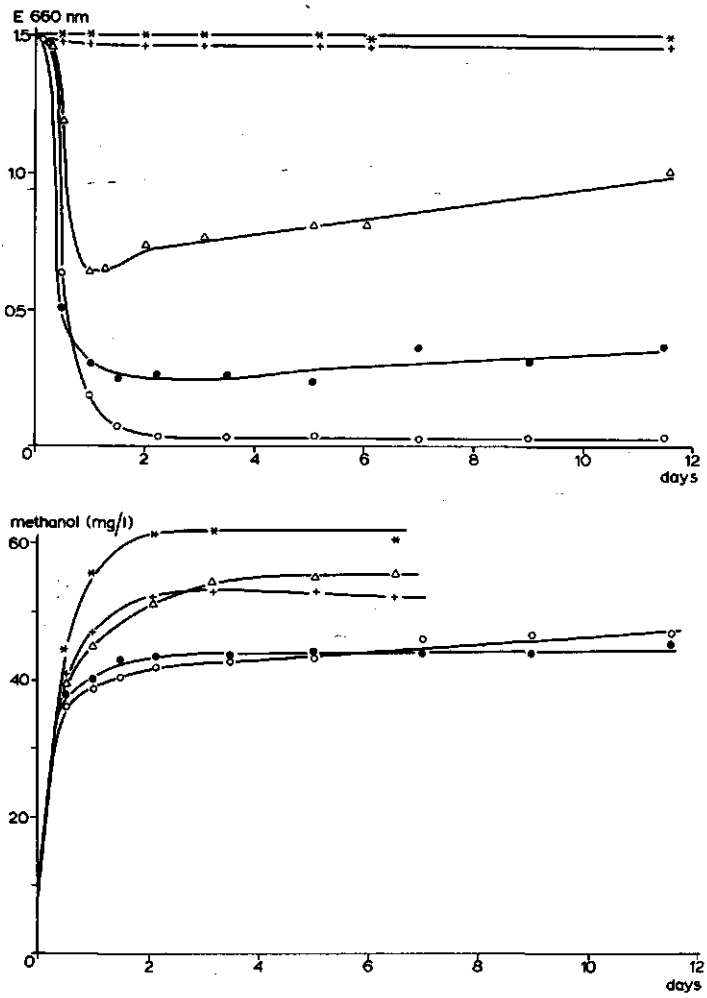


Fig. 53. Cloud stability of (A) and methanol formation in (B) orange juice incubated with purified Pectinesterase I and different concentrations of polygalacturonase. Conditions as in Fig. 52, except that Pectinesterase I (1 unit per ml) was used. Polygalacturonase concentrations: no polygalacturonase (o); 10^{-4} unit per ml (●); $5x10^{-4}$ unit per ml (Δ); 10^{-3} unit per ml (+); 10^{-2} unit per ml (*).

It can be seen in the figures that the polygalacturonase stimulated the methanol production for both pectinesterases. In spite of this increased pectinesterase activity, the orange juice cloud stability was increased. At the two highest levels of polygalacturonase activity, the cloud was not destabilized at all.

To determine the total free and bound methanol in the juice, 30 ml reconstituted juice (5/3 x single strength) was adjusted to pH 11.0 with 2 mol/l sodium hydroxide and incubated for 1 h in a glass stoppered flask at room temperature. The solution was neutralized with 2 mol/l hydrochloric acid, 5 ml of a solution of 250 mg/l propanol-2 was added and the volume was adjusted to 50 ml. The methanol concentration was determined

gas chromatographically (see Chapter 4) and was 62 mg/l. The free methanol concentration was 9 mg/l and 53 mg/l methanol could be released by the alkaline de-esterification. With an anhydrogalacturonic acid content of 574 mg/l (Krop, 1974), the average degree of esterification of the pectin in the juice is calculated to be 51%.

9.2 DISCUSSION

It was not possible to clarify the orange juice with Pectinesterase II at 30 °C. At 30 °C the enzyme was inactivated (see Table 25) before it could de-esterify the pectin to an extent that clarification occurred. It could not raise the free methanol concentration to over 20 mg/ml, whereas with the other enzymes clarification started when a concentration of about 30 mg/l methanol was reached. The *D* value for Pectinesterase II inactivation at 30 °C in juice is about 5 h (see Section 8.1.1, Table 25). However, at 5 °C, the enzyme is sufficiently stable (*D* value for inactivation about 4½ days) to build up a concentration of 35 mg/l methanol. In spite of this, the juice was not clarified. This may indicate a mode of action on the substrate in the juice which is different of that of Pectinesterase I and that of the high molecular weight pectinesterase. Possibly shorter blocks of free carboxyl groups are formed than with the other pectinesterases. For juice clarification the pectate chains must have blocks of 16 or more monomers with free carboxyl groups (Termote et al., 1977). This does not necessarily mean that Pectinesterase II removes methoxyl groups at random from pectin as suggested for the pectinesterase from *Aspergillus japonicus* (Ishii et al., 1979). Krop (1974) observed differences in the clarification of fresh juices from different orange varieties, which could not be related to overall pectinesterase activity. Krop (1974) suggested differences in the mode of action of the pectinesterases as one of the possible explanations.

Krop (1974) observed only a slight increase in the initial pectinesterase activity when it was incubated in the juice together with polygalacturonase. It now appears that both the initial activity of the pectinesterases and the ultimate methanol concentration are increased with increasing polygalacturonase concentration (Figs 52 B and 53 B). The stimulation of pectinesterase activity must be ascribed to hydrolytic splitting of longer sequences of galacturonic acid monomers (removal of end product inhibition). At the same time clarification of the juice is prevented by this degradation of the longer sequences of galacturonic acid monomers. The remaining galacturonic acid oligomers do not precipitate with calcium ions.

The crude pectinesterase used in this study appeared to be slightly more effective in orange juice cloud destabilization than the pectinesterase preparation used by Krop (1974). Also in this work more polygalacturonase was needed to stabilize the cloud than in the experiments of Krop (1974) (compare Fig. 52 A from this work with Fig. 39 B from Krop). Because the same juice and polygalacturonase were used in both experiments, the differences must be ascribed to the pectinesterase used. Krop (1974) used another orange cultivar for the pectinesterase preparation and also the extraction procedure was somewhat different (Krop used less salt and did not reextract the press cake).

Both at 30 °C and at 5 °C, the high molecular weight pectinesterase was the most

effective cloud destabilizer. At 30 °C the difference with the purified Pectinesterase I was very small. The fact that the purified Pectinesterase I could clarify orange juice, proves that no other enzymes are needed in the process of clarification. It is possible that in the clarification with the high molecular weight pectinesterase one or more other enzymes were involved, as this enzyme was not pure at all. In Chapter 5, Table 16 several enzyme activities are listed which were found to be present in the crude enzyme preparation.

By alkali de-esterification of the juice, a methanol concentration of 62 mg/l was measured. When combined with polygalacturonase, pectinesterase could nearly reach this same methanol concentration (Figs 52 B and 53 B). Apparently all methyl ester groups were eventually de-esterified by the pectinesterases. This was not expected because it was impossible to de-esterify the last 10% of the methoxyl groups, as described in Section 7.1.2. But in those experiments no polygalacturonase had been used.

10 General discussion and conclusions

The gas chromatographic methanol assay proved its usefulness in many experiments with pectinesterases at low pH values.

The presence of several (12) molecular forms of pectinesterase in citrus was established. The different forms can be distinguished by their mobility in pH gradient electrophoresis. Only three forms, representing 85-95% of the total activity in all citrus fruits, were further investigated. These forms differ in one or more of the following properties: isoelectric point, amino acid composition, molecular weight, pH-activity profile, K_m for pectin, K_i for polygalacturonic acid, stability in orange juice, heat stability, orange juice clarification properties and temperature-activity relationship.

Two forms, Pectinesterase I and II can be called isoenzymes. These two forms represented 60% or more of the total activity in various citrus fruits. The high molecular weight pectinesterase was also detected in all citrus fruits examined. Not all 12 molecular forms were detected in all of the citrus fruits, but up to 10 forms were found in a single fruit: the grapefruit. The relative amounts of the forms of pectinesterase varied with orange cultivar and citrus species. It is possible that all forms of pectinesterase were present in all citrus fruits examined, but that sometimes their quantitiy was below the detection limit of the technique used. Forms representing less than 2% of the total activity could usually not be detected.

Though Pectinesterase I and II together represent the major part of the activity at standard assay conditions, this is not the case at lower pH values. The activity of Pectinesterase II is rapidly reduced at lower pH values. In citrus juice at 30 ^{o}C, Pectinesterase II is not technologically important because of its low activity and poor stability. Though Pectinesterase I is more active at pH 4.0 than the high molecular weight pectinesterase, at pH 2.5 the only active pectinesterase is the high molecular weight pectinesterase. It may be presumed that the natural clarification of lemon juice (Uhlig, 1978) is due to the activity of this form of pectinesterase, which represents only a small fraction of the activity under standard assay conditions.

Among the three forms investigated, the high molecular weight pectinesterase is the only one which can play a role in cloud loss phenomena of (under) pasteurized citrus juices. Pectinesterase I and II can rapidly be inactivated at temperatures below 80 ^{o}C. About this pasteurization temperature is required to avoid microbial spoilage (Bissett et al., 1953).

The differences in heat stability of pectinesterase in citrus juices at the same pH, ^{o}Brix, ion concentrations and pulp content (Rouse & Atkins, 1952; Rouse & Atkins, 1953 a; Eagerman & Rouse, 1976) now can be ascribed to differences in the pectinesterase composition, even though the heat stability of a number of forms of pectinesterase are as

yet unknown. From the work of Evans & McHale (1978) it can be seen that the pectinesterase composition differs with the part of the fruit. Therefore a form of pectinesterase being only a small part of the total activity in the fruit may be present in a relatively large quantity in the juice. The pressing method used to produce citrus juice will not only affect the pectinesterase concentration but also its composition.

At present nothing is known about the origin of the various forms of pectinesterase. If they were to be genetically determined it would seem promising to eliminate the heat stable high molecular weight pectinesterase through breeding. If Pectinesterase I or II were to be derived from the high molecular weight pectinesterase, for instance by a process in vivo, it would be attractive to study this process and try to steer it in a technologically desirable direction.

Some light was thrown on the mode of action of the pectinesterases. The rapidly increasing affinity for substrates with a lower degree of esterification led to the theory that for the optimal enzyme-substrate complex two free dissociated carboxyl groups at a fixed distance in the substrate are required. The decreasing affinity at a lower pH is in agreement with this theory. The means by which MacMillan and coworkers (Lee & MacMillan, 1970; Miller & MacMillan, 1971) reached the conclusion that half of the pectinesterase activity was initiated at the reducing end was criticized in Section 2.2.4. An important question which remains to be answered is to what extent the various pectinesterases attack their substrate by a single chain mechanism. Part of the conclusions of Solms & Deuel (1955) were questioned in Section 7.2.

The specific activity and turnover numbers of Pectinesterase I and II were calculated with the Lowry protein determination. But if the estimated $E_{1\ cm}^{1\%}$ at 280 nm are used for the protein concentrations, both values will be approximately twice as high for both pectinesterases.

I hope that the knowledge of the existence of multiple molecular forms of pectinesterase with widely different properties in citrus fruits as presented in this work will promote research towards the origin and the function of these enzymes and will provide the fruit juice technologist with useful basic information.

Summary

The purpose of this study was to obtain a better insight in the properties of pectinesterase and in the clarification of orange juice, in which pectinesterase plays a role.

After the introduction in Chapter 1, the literature on pectinesterase is surveyed and discussed in Chapter 2, covering the subjects: occurence, role in vivo, purification and properties, specifity and mode of action, inhibition and (in)activation, importance for food technology and assay and detection methods.

Chapter 3 gives the materials used and the standard methods applied in this study.

Chapters 4 to 9 describe the experiments and results. The results are discussed in each chapter.

Detailed information about a gas chromatographic alcohol assay is given in Chapter 4. Because of the need to measure pectinesterase activity at conditions under which a titrimetric assay is difficult or impossible, a gas chromatographic methanol assay was improved. In this method, alcohols are converted to their (volatile) nitrite esters and the headspace of the solution is sampled and injected. The method is rapid (about 5 min/sample), sensitive (< 1 mg/l) and reproducible (variation coefficient about 1.5%). For several biological fluids the method is directly applicable without pretreatment of the material.

Chapter 5 describes the purification of two pectinesterase isoenzymes along with their molecular properties. The pectinesterase from Navel oranges (62 500 units per kg oranges) was extracted and purified by fractionated ammonium sulphate precipitation and by gel filtration on Bio-Gel P-100. The enzyme preparation could be separated by chromatography on cross-linked pectate into a Pectinesterase I, which did not bind to this matrix and a cross-linked pectate binding Pectinesterase II. Both enzymes were further purified by chromatography on CM Bio-Gel A. Their specific activities were 694 and 762 units per mg protein, respectively. Both isoenzymes move as single bands of molecular weight 36 200 during sodium dodecyl sulphate electrophoresis, and are also single bands with isoelectric points of 10.05 and >11.0, respectively, upon isoelectric focusing. A number of cell wall degrading enzymes which were present in the crude enzyme preparation, were absent from the purified enzyme preparations. The enzymes differ in amino acid composition and they are no glycoproteins.

Chapter 6 concerns a survey of the multiple molecular forms of pectinesterase in citrus fruits. A special thin-layer pH gradient electrophoresis method was developed with which several additional forms of pectinesterase could be detected. Several orange cultivars and citrus species were assayed for their forms of pectinesterase. A total of 12 forms of pectinesterase were found in all citrus fruits together. Pectinesterase I and II together represented 60% - 90% of the total pectinesterase activity in all citrus

fruits examined. Another pectinesterase, with a higher molecular weight, which was also present in all citrus fruits examined, was also incorporated in the further studies. This high molecular weight pectinesterase was inhomogeneous and impure.

In Chapter 7 the kinetic properties are studied. On brown ribbon pectin (degree of esterification 72%) Pectinesterase I and II have optimum pH-values of 7.6 and 8.0, respectively. On green ribbon pectin (degree of esterification 62%) the pH-activity curves are about 1 pH unit broader at the acid side of the curve. The high molecular weight pectinesterase has a minimum activity at pH 3.5 and at lower pH-values the activity increases again. At pH 7.0 Pectinesterase I has a K_m value of 0.083 mg of green ribbon pectin per ml and a K_i value for polygalacturonic acid of 0.42 mg per ml. For Pectinesterase II these values are 0.0046 and 0.0016 mg per ml, respectively. For both Pectinesterase I and II the K_m value is related to the degree of esterification of the substrate in the following way: $1/K_m = a$ (% free carboxyl groups)^2 and a is about ten times higher for Pectinesterase II than for Pectinesterase I. From this relationship it was reasoned that two free carboxyl groups at a certain fixed distance in the substrate are required for the optimal enzyme-substrate complex. The V_{max} is nearly independent of the degree of esterification of the substrate. The K_m is increased at lower pH values. The lower the initial degree of esterification of the substrate, the lower the degree of esterification which could ultimately be reached by the enzymes. Pectinesterase I was more effective in this respect than Pectinesterase II, but in no case a degree of esterification of lower than 11% could be achieved. The enzymic de-esterification of highly esterified pectin (degree of esterification 95.6%) was very incomplete. Some of the substrate molecules were not de-esterified at all and these appeared to be rich of galactose. It was therefore concluded that side chains in the pectin molecule form an obstruction for pectinesterase action. Endo-polygalacturonase and endo-pectate lyase increased the rate and limit of de-esterification presumably by alleviating end product inhibition.

In Chapter 8 the stability of the pectinesterases is examined. At 30 °C in orange juice of pH 4.0, Pectinesterase I and the high molecular weight pectinesterase are stable, but Pectinesterase II is quickly inactivated (D value about 5 h). Sugars increase and ions reduce the stability of pectinesterases. The pectin concentration has no effect on the pectinesterase stability. The enzymes differ greatly in heat stability in orange juice at pH 4.0. The D values at 90 °C and Z values are 0.00037 min and 6.5 °C for Pectinesterase I, 0.0015 min and 11 °C for Pectinesterase II and 0.375 min and 6.5 °C for the high molecular weight pectinesterase. The heat stable high molecular weight pectinesterase was only a small fraction of the crude Navel orange pectinesterase preparation (5%). The pectinesterase in freshly pressed Valencia orange juice contained about 10% of the high molecular weight pectinesterase or a pectinesterase with a similar heat stability.

Chapter 9 concerns the orange juice cloud destabilizing properties of the pectinesterase at 5 °C and 30 °C. Pectinesterase II is unable to clarify orange juice, though its stability at 5 °C allows considerable de-esterification of the juice pectin. At 30 °C Pectinesterase I and the high molecular weight pectinesterase quickly destabilize the cloud of orange juice. At 5 °C the high molecular weight pectinesterase is still

effective but the activity of Pectinesterase I is hundred times less than that at 30 °C. Endo-polygalacturonase stimulates the pectinesterase activity in orange juice. In spite of this, the orange cloud is not destabilized.

In Chapter 10 the overall results are briefly discussed and some possibilities for further research are mentioned.

Samenvatting

Het doel van deze studie was meer inzicht te krijgen in de eigenschappen van pectine-esterase en in de destabilisatie van troebel sinaasappelsap, waarbij pectine-esterase een rol speelt.

De literatuur over pectine-esterase is samengevat en bediscussieerd in hoofdstuk 2. Hierbij worden verschillende aspecten van pectine-esterase behandeld, te weten: voorkomen, rol in de natuur, zuivering en eigenschappen, specifiteit en werkingsmechanisme, remming en (in)activering, belang voor de levensmiddelentechnologie en analysemethoden.

In hoofdstuk 3 worden de bij het onderzoek gebruikte materialen en gevolgde methoden beschreven.

De hoofdstukken 4 tot en met 9 omvatten het experimentele gedeelte, waarbij in elk hoofdstuk de resultaten worden besproken.

In hoofdstuk 4 wordt een gaschromatografische methode voor de bepaling van alcoholen beschreven. Bij deze methode worden alcoholen omgezet tot de overeenkomstige vluchtige nitrietesters. De damp boven de vloeistof wordt bemonsterd en geïnjecteerd. De methode is geschikt voor de bepaling van de pectine-esteraseactiviteit onder condities waarbij een titratiemethode moeilijk uitvoerbaar is. De gaschromatografische methode vraagt voor een methanolanalyse ongeveer 5 minuten per monster, heeft een gevoeligheid van < 1 mg/l methanol en een reproduceerbaarheid met een variatiecoëfficient van 1,5%. De methode is geschikt voor gebruik in verschillende natuurlijke vloeistoffen zonder dat de monsters voorbehandeld behoeven te worden.

Hoofdstuk 5 beschrijft de isolatie en zuivering van twee pectine-esterase-isoenzymen uit Navelsinaasappelen alsmede enige moleculaire eigenschappen van deze enzymen. De Navel sinaasappelen bevatten ruim 60 000 eenheden (µmol/min) enzym per kg vrucht. De enzymen werden geëxtraheerd met een buffer en daarna gezuiverd door gefractioneerde ammoniumsulfaatprecipitatie en gelfiltratie op Bio-Gel P-100. Het enzympreparaat kon door affiniteitschromatografie op verknoopt pectaat gescheiden worden in twee enzymen, te weten pectine-esterase I, die niet aan deze matrix gebonden werd en pectine-esterase II, die wel gebonden werd. Beide enzymen werden verder gezuiverd over een CM Bio-Gel A ionenwisselaar. Na zuivering bedroeg de specifieke activiteit van pectine-esterase I en II respectievelijk 694 en 762 eenheden per mg eiwit. Beide enzymen werden getest m.b.v. natriumdodecylsulfaatelectroforese; hierbij gaven beide enzymen één enkele band met een molecuulgewicht van 36 200 te zien. Tevens gaven experimenten m.b.v. isoelectric focusing voor beide enzymen slechts één band te zien: De isoelectrische punten van pectine-esterase I en pectine-esterase II zijn respectievelijk 10,05 en > 11,0. Tevens bleek dat de activiteit van een aantal celwandafbrekende enzymen, welke in het ruwe enzymmengsel aangetoond kon worden afwezig was in de gezuiverde preparaten. De enzymen verschillen in aminozuursamenstelling en bevatten geen suikers.

Hoofdstuk 6 betreft een onderzoek naar de verschillende vormen van pectine-esterase in citrusvruchten. Daartoe werd een speciale electroforesemethode ontwikkeld waarbij over een dunne laag gel een pH gradiënt aangelegd werd. Met deze methode werden naast pectine-esterase I en II verschillende andere vormen van pectine-esterase ontdekt. Verscheidene citrusvruchten werden onderzocht op hun pectine-esterasesamenstelling. In alle onderzochte citrusvruchten samen werden totaal 12 vormen van pectine-esterase (inclusief pectines-esterase I en II) gevonden. Tevens bleek dat pectine-esterase I en II samen 60-90% van de pectine-esterase hoeveelheid vormen in alle (onderzochte) citrusvruchten. Een andere pectine-esterase, met een hoger molecuulgewicht dan pectine-esterase I en II, werd vanwege zijn grote hittestabiliteit betrokken in het verdere onderzoek. Deze pectine-esterase, die aanwezig was in alle onderzochte citrusvruchten, werd in een niet homogene en een onzuivere staat gebruikt.

In hoofdstuk 7 worden de kinetische eigenschappen van de pectine-esterases bestudeerd. Op bruinbandpectine met een veresteringsgraad van 72% hebben pectine-esterase I en II een pH optimum van respectievelijk pH 7,6 en 8,0. Op groenbandpectine met een veresteringsgraad van 62% zijn de pH-activiteitsprofielen met ongeveer één pH eenheid verbreed aan de zure zijde van de curve. De pectine-esterase met het hoge molecuulgewicht heeft de laagste activiteit bij pH 3,5 en bij nog lagere pH waarden neemt de activiteit weer toe. Op groenbandpectine heeft pectine-esterase I bij pH 7,0 een K_m van 0,083 mg pectine per ml en een K_i waarde van 0,42 mg polygalacturonzuur per ml. Voor pectine-esterase II bedragen deze waarden respectievelijk 0,0046 en 0,0016 mg per ml. De K_m van pectine-esterase I en II is afhankelijk van de veresteringsgraad van de pectine; de volgende afhankelijkheid werd opgesteld:
$1/K_m = a \times (\%\ \text{vrije carboxyl groepen})^2$. De constante a is voor pectine-esterase II ongeveer 10 maal groter als voor pectine-esterase I. Op grond van dit verband werd beredeneerd dat 2 vrije carboxylzuurgroepen op een bepaalde afstand van elkaar in het substraat nodig zijn voor een optimaal enzym-substraatcomplex. De V_{max} is nagenoeg onafhankelijk van de veresteringsgraad van de pectine. Tevens bleek dat de K_m hoger wordt bij lagere pH. Hoe lager de initiële veresteringsgraad van het substraat, hoe lager de veresteringsgraad die door de enzymen uiteindelijk bereikt kan worden. Pectine-esterase I bereikte uiteindelijk een lagere veresteringsgraad dan pectine-esterase II; echter een lagere veresteringsgraad dan 11% kon nooit bereikt worden. De enzymatische verzeping van hoogveresterde pectine met een veresteringsgraad van 95,6% is heel onvolledig. Een aantal van de substraatmoleculen wordt in het geheel niet verzeept. Deze moleculen bleken rijk aan galactose te zijn. Daaruit kon geconcludeerd worden dat zijketens in het pectinemolecuul een belemmering vormen voor de pectine-esteraseactiviteit. Endo-polygalacturonase en endo-pectaatlyase verhoogden de verzepingssnelheid en verzepingslimiet, waarschijnlijk door produktremming op te heffen.

In hoofdstuk 8 wordt het onderzoek naar de stabiliteit van de pectine-esterases beschreven. In sinaasappelsap van pH 4,0 en 30 °C zijn pectine-esterase I en de pectine-esterase met het hoge molecuulgewicht stabiel; pectine-esterase II wordt echter snel geïnactiveerd; hierbij is een D-waarde van ongeveer 5 uur gevonden. De stabiliteit van de pectine-esterases wordt verhoogd door een mengsel van suikers en verlaagd door een mengsel van ionen. De pectineconcentratie heeft geen invloed op de pectine-esterase

stabiliteit. De enzymen verschillen sterk in hittestabiliteit in sinaasappelsap van pH 4,0: De D_{90} °C-waarden en z-waarden zijn 0,00037 min en 6,5 °C voor pectine-esterase I, 0,0015 min en 11 °C voor pectine-esterase II en 0,375 min en 6,5 °C voor de pectine-esterase met het hoge molecuulgewicht. De pectine-esterase met het hoge molecuulgewicht vormde slechts 5% van de ruwe pectine-esterase van de Navel sinaasappel. In verse geperst Valencia sinaasappelsap had ongeveer 10% van de pectine-esterase een hittestabiliteit vergelijkbaar met de hittestabiliteit van de pectine-esterase met het hoge molecuulgewicht.

Hoofdstuk 9 handelt over de sinaasappelsap klarende eigenschappen van de pectine-esterases bij 5 °C en 30 °C. Het bleek dat pectine-esterase II niet in staat is sinaasappelsap te klaren, alhoewel de stabiliteit en de activiteit van het enzym bij 5 °C voldoende zijn om een aanzienlijk deel van de pectine in het sap te verzepen. Pectine-esterase I en de pectine-esterase met het hoge molecuulgewicht geven daarentegen een snelle klaring van sinaasappelsap bij 30 °C. Bij 5 °C is de pectine-esterase met het hoge molecuulgewicht nog goed werkzaam; pectine-esterase I werkt bij 5 °C echter honderdmaal zo langzaam als bij 30 °C. Endo-polygalacturonase stimuleert de pectine-esteraseactiviteit in sinaasappelsap. Desondanks bleek de troebelingsstabiliteit van het sap verhoogd te worden.

In hoofdstuk 10 wordt het totale resultaat kort besproken en worden enige mogelijkheden voor verder onderzoek genoemd.

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