## *Proteolysis using enzymes in vitro*

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# PROTEOLYSIS USING ENZYMES *IN VITRO*

by

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## ABSTRACT

A variety of methods were tested for their ability to completely hydrolyse proteins to free amino acids *in vitro*. The most successful method was found to be that using the enzyme mixture papain, prolidase and aminopeptidase-M, which together completely hydrolysed the test substrates ovalbumin, BSA and insulin (B chain).

A range of enzymes were coupled to insert supports, and although such immobilization simplified handling of the enzymes, the coupled enzymes proved less efficient for proteolysis than their soluble counterparts.

Amino acid analysis and SDS polyacrylamide gel electrophoresis were used to identify the end products of proteolysis. The use of a pH-stat was found unsuitable for the measurement of proteolysis.

*In vitro* proteolysis was used to study the digestibility of glycoprotein II, purified from *Phaseolus vulgaris*. Denatured glycoprotein II was fully digested by the enzymic method, although in its native form, the glycoprotein was only partially digested. The digestibility of trypsin inhibitor, purified from *Phaseolus vulgaris* was also studied. Both in its native form, and after heat treatment, the trypsin inhibitor was poorly hydrolysed. It was also found that native trypsin had some resistance to digestion conferred upon it by the presence of the inhibitor. Active trypsin inhibitor was detected in the faeces of rats whose diet included the inhibitor. The significance of these findings *in vitro* and *in vivo* were discussed.

The use of proteolysis *in vitro* to measure the availability of amino acids was found comparable with standard assays for the measurement of available lysine and methionine.

The method was also used to measure the levels of glutamine and asparagine in storage proteins extracted from peas, *Pisum sativum*, at various stages of germination. The only change detected was a decrease in the level of glutamine present in the storage protein, legumin.

# ABBREVIATIONS

The abbreviations used in this thesis are in accordance with the recommendations of the *Biochemical Journal* (1981) 193, 4-27. In addition, the following abbreviations are also used:-

| | |
|---|---|
| AH | acid hydrolysis |
| ATEE | N-acetyl-L-tyrosine ethyl ester |
| BAEE | Nα-benzoyl-L-arginine ethyl ester |
| BANA | Nα-benzoyl-DL-arginine β-napthylamide |
| bis-acrylamide | N,N'-ethylene-bis-acrylamide |
| BSA | bovine serum albumin |
| BV | biological value = $\frac{\text{retained nitrogen}}{\text{absorbed nitrogen}}$ % |
| CMC | carboxymethylcellulose |
| CMCMT | 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulphate |
| Cys A (or CysA) | cysteic acid |
| Cys/2 | half cystine |
| EDAC | 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide |
| EH | enzymic hydrolysis |
| nd | not determined |
| N-Leu | nor leucine |
| SDS | sodium dodecyl sulphate |
| T | trypsin |
| TCA | trichloracetic acid |
| TD | true digestibility = $\frac{\text{food N-(faecal N-metabolic N)}}{\text{food N}}$ % |
| Temed | N,N,N',N'-tetramethylethylenediamine |
| (Thr+Ser+Gln+Asn) | combined total of the amino acids Thr, Ser, Gln and Asn |
| TI | trypsin inhibitor |

| | |
|---|---|
| TNBS | 2,4,6-trinitrobenzene sulphonic acid |
| Tris | tris(hydroxymethyl)aminomethane |

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# 1.0 INTRODUCTION

The work reported in this thesis was prompted in response to the regular statements in the literature that legume proteins are of low digestibility (for example Marquez and Lajolo, 1981). The work concerns the development and use of a suitable method with which to study the digestibility of some proteins present in the legume *Phaseolus vulgaris*.

Study of the nutritional value of plant proteins is important now, because as world population increases, the demand for protein will no longer tolerate the inefficient conversion of plant protein to animal protein before human consumption, and plants must provide a greater proportion of the protein in human diets. Communities which already obtain sufficient protein in their diet without relying, whether from choice or necessity, on animal proteins, usually make use of legumes as a source of protein. Thus legumes comprise an important part of the diet of populations living in third world countries; the *per capita* consumption of bean protein is highest in South America, where it provides up to 30% of the total protein intake.

Of the available legumes, *Phaseolus vulgaris* is the most commonly used in South American countries, and is a daily feature of the diet, particularly of low income groups (Bressani and Elias, 1977).

Tobin and Carpenter (1978) in their review of nutritional evaluation studies on *Phaseolus vulgaris* found that most values for the True Digestibility (TD) of whole, cooked beans, in rats, were within the range 70-85%. This level is low compared with the TD of protein sources such as milk and egg. In view of the present and future importance of legumes as a source of protein, the cause of this low digestibility must be determined.

In attempting to assess the digestibility of legume proteins, there are many other factors such as protease inhibitors, haemagglutinins and polyphenolics, which are present in whole beans and may complicate their digestion. Therefore, to test whether the storage proteins themselves have an inherent resistance to hydrolysis, it is necessary for them to be purified and studied in isolation from the rest of the bean.

Some studies have been made into the digestibility of purified legume proteins *in vivo*, measured in the rat. Ideally however, the digestibility of proteins intended for human consumption should be determined in humans, but the nitrogen balance experiments required are not conducted routinely because of their inherent cost, and instead, the rat is most commonly used for determining the nutritional value of proteins intended for human consumption. Yet the results obtained from rat assays are not necessarily applicable to humans (a problem to be described more fully in the discussion section).

An alternative to these assays is to use an enzyme system *in vitro* to study the digestibility of proteins. The advantages of such a system are its relative simplicity and the possibility of isolation and characterisation of the products of hydrolysis. However, the digestibility of a protein *in vitro* can only be relevant to its digestibility *in vivo*, if the enzymic system used is of similar capability to that *in vivo*.

Proteolysis using enzymes *in vitro* has already been used to study the digestibility of glycoprotein II, the major storage protein of *Phaseolus vulgaris*. From their experiments, Romero and Ryan (1978), Vaintraub *et al* (1979) and Liener and Thompson (1980), have all claimed that native glycoprotein II is resistant to digestion. These findings have been based upon measurements of the extent of hydrolysis of

glycoprotein II when incubated with a limited number of specific enzymes, which in any case could not be expected to hydrolyse all the peptide bonds present in the glycoprotein. A more fair test of the digestibility of glycoprotein II would be to measure the extent of its hydrolysis by an enzymic system known to be capable of fully hydrolysing other proteins *in vitro*.

This was the first object of the present work: to find an enzymic method, capable of achieving the complete hydrolysis of proteins *in vitro*.

The method was first used to study the digestibility of glycoprotein II, purified from *Phaseolus vulgaris*.

As well as their low digestibility, a second limiting factor with legumes is that they are of low Biological Value (BV). Feeding trials on rats fed with cooked beans have reported BV values ranging from 44% to 68% (Tobin and Carpenter, 1978). The BV of a legume depends upon its level of sulphur amino acids, since in rats at least, these are the first limiting amino acids. Cystine is not an essential amino acid for mammals, since it can be produced by them from methionine. Therefore, the total sulphur amino acid requirement of the rat could be satisfied by methionine alone, or by a combination of methionine and cystine. As cystine is added to the diet, so the requirement for methionine can be reduced, and the extent to which cystine can "spare" methionine in this way is approximately (for rats) at a level of 60-70% of the total sulphur amino acid requirement (Sowers *et al*, 1972). For this reason, supplementation with cystine in legume based diets for rats has been found to be less effective in improving nutritional quality than supplementation with methionine (James and Hove, 1980).

It has been shown in rat feeding tests using a variety of heat-treated legumes containing similar amounts of methionine and cystine, that there can be large differences in the availability of the sulphur amino acids (Evans and Bandemer, 1967), and Sawar *et al* (1977) have shown that the TD of the sulphur amino acids in legumes is less than that of the protein as a whole. These experiments suggest that there may be some problem with the availability of the small amount of sulphur amino acids present in legumes.

Between 30% and 40% of the total cystine of a legume seed may be present in the trypsin inhibitors (Kakade *et al*, 1969) although these only comprise about 25% of the total protein of the seed. The disulphide bonding which would be produced by this high level of cystine could result in a very stable molecule, perhaps resistant to proteolytic attack. It was for this reason that a trypsin inhibitor, purified from *Phaseolus vulgaris* was tested for its possible resistance to hydrolysis by enzymes *in vitro*.

The susceptibility to hydrolysis of trypsin inhibitor in the presence of trypsin was also tested, because *in vivo*, active trypsin inhibitor may be present in the gut in complex with such a protease. Also, in an attempt to test whether trypsin inhibitors are digested *in vivo*, samples of faecal extracts of rats whose diet included the inhibitor were assayed for trypsin inhibitory activity.

As well as providing a guide to the extent to which whole proteins may be digested *in vivo*, enzymic hydrolysis *in vitro* may also be useful for the determination of individual amino acids. Using acidic or alkaline hydrolysis to determine the amino acid profile of a protein, the harsh reaction conditions may release amino acids which could not be released by enzymes. If an amino acid is present in a

protein, but involved in some bonding making it resistant to enzymic cleavage, the amino acid is rendered non-available. Also, if acidic or alkaline hydrolysis are used to determine the amino acid content of a foodstuff, these conditions may encourage interaction of amino acids with other components of the food, in particular with the carbohydrates. This problem does not occur when pure proteins are analysed, yet the amino acid content as present in the entire food should be known in order to estimate a nutritional value.

The chemical natures of most of the enzyme-resistant bonds formed in foods are obscure, except for the Maillard reactions which have been well studied and in foods, usually involve the reaction of sugars such as glucose and fructose with amino acids. Lysine is the amino acid most frequently modified by such reactions, although arginine, tyrosine and methionine may also be involved to a lesser extent (Feeney *et al*, 1975). In the initial stage of the Maillard reaction, the free amino group of, for example, lysine, reacts with the aldehyde group of, for example, glucose, to form, after several intermediates and isomerisation, fructoselysine. The lysine part of this compound would be released by acidic hydrolysis, but not by enzymic hydrolysis, and so by using acidic hydrolysis to analyse the protein, a falsely high level for nutritionally available lysine would be produced.

Since lysine is the amino acid most adversely affected by the heat processing of foods (Evans and Butts, 1949), measurement of available lysine has been shown to be a useful guide to the quality of a protein after heat processing (Hackler, 1976). Measurement of lysine is also important because it is the first-limiting amino acid in many foods. For example the prolamins, which together with the glutelins constitute the bulk of the proteins of most cereals, contain very low amounts of lysine.

In many countries, dried milk provides a useful protein source, high in lysine, to supplement diets which, if poorly balanced and based on cereals, may be deficient in lysine. However, the presence of reducing sugars in milk has meant that in the processing required for the production of skim-milk and milk powders, the lysine is made unavailable by the Maillard reaction. There are already efficient methods for the measurement of available lysine and methionine, but in this work, the total hydrolysis method using enzymes was tested for its efficiency as a simple assay, able to measure available lysine and methionine by one procedure.

The selective ability of enzymes to specifically hydrolyse peptide bonds while leaving other bonds intact allows the individual determination of asparagine and glutamine, which under the conditions used for acidic or alkaline hydrolysis of proteins, are always deamidated to aspartic acid and glutamic acid. For this reason, the amide contents of proteins are rarely measured and it is customary to report only the combined values of (aspartic acid + asparagine) and (glutamic acid + glutamine) measured as aspartic and glutamic acids after acid hydrolysis.

Enzymic hydrolysis may therefore be useful for the individual measurement of these four amino acids in proteins in which they are of particular interest. For example, it is thought that during the germination of legume seeds the developing seedling is supplied with nitrogen provided by the removal of amino groups from the storage proteins of the cotyledon (Bewley and Black, 1978). In this work, the enzymic hydrolysis method was used to measure the individual levels of asparagine and glutamine in samples of the major storage proteins extracted from pea, *Pisum sativum*, at various stages during germination.

The first task of the present work was to find a suitable method for the enzymic hydrolysis of a protein *in vitro*. There already exist many methods designed to assess nutritional value by enzymic hydrolysis *in vitro*, but none of these achieve complete hydrolysis of the protein.

One of the earliest developed of these enzymic assays for the nutritional evaluation of food proteins is the Pepsin Digest Residue (PDR) index of Sheffner (1956). Because of the limited digestion achieved by pepsin, the hydrolytic products needed to be measured by microbial assay. The PDR index was first modified by Akeson and Stahmann (1964) to include a digestion with pancreatin as well as pepsin, giving the Pepsin Pancreatic Digest (PPD) index, and further modified by Mauron (1970) to include a dialysis of the digestion mixture, giving the Pepsin Pancreatin Digest Dialysate (PPDD) index.

According to these three methods (and others developed along similar lines), when proteins are digested *in vitro* with one or more enzymes, differences in the essential amino acid patterns of the proteins appear in the hydrolysates, which are not evident from their total essential amino acid content. In each method, a reference protein (usually egg) must be simultaneously digested by the same procedure as the test protein. Index values are then calculated which relate the pattern of amino acids released enzymatically and the pattern of amino acids in the remainder of the protein, with the corresponding values for the reference protein.

These indices have been used in a few studies to give considerable amounts of data (Sheffner, 1967; Mauron, 1973), and have been shown to correlate with the biological values of proteins determined *in vivo*.

However, these methods have not found wide acceptance as alternatives to rat bioassay (Marshall *et al*, 1979) and seem to offer little advantage in estimating nutritional value over indices computed simply from amino acid composition (Marable and Sanzone, 1980).

More recently other methods for nutritional evaluation by enzymic hydrolysis *in vitro* have been devised. These involve the measurement of the initial rate of hydrolysis of proteins by enzymes. The first of these methods was introduced by Hsu *et al* (1977) with a multienzyme technique for measuring protein digestibility. It was shown that the fall in pH of a protein suspension after 10min of digestion with trypsin, chymotrypsin and a peptidase mixture, was highly correlated with measurements of digestibility in rats. The method was further developed by Hsu *et al* (1978) into the C-PER (Computed Protein Efficiency Ratio) assay which combined the essential amino acid profile with *in vitro* protein digestibility data to predict protein quality. For 23 foods tested, the correlation between *in vitro* protein digestibility and apparent digestibility measured in rats was 0.9. However the method was incapable of accurately predicting the degree of *in vivo* protein digestibility of foods such as whole egg or beef which although fully digested *in vivo*, were said to be resistant to attack by these proteolytic enzymes *in vitro*. Wolzak *et al* (1981) have tested the efficiency of the C-PER method and found that it agreed well with PER and NPR values determined in rats for unprocessed cereal grains and oilseeds, but the values given by the C-PER method for leguminous seeds and animal proteins did not show any significant correlation with the rat values.

Bodwell *et al* (1980) tested the original method of Hsu, and a modification by Satterlee *et al* (1979) to include pronase in the digestion mixture, for their ability to predict the digestibility of a variety of plant and animal proteins. For both types of protein,

high correlations were found between the *in vitro* estimates and true or apparent human and rat digestibilities, but these were only achieved when different equations were used for predicting the digestibilities of plant and animal proteins. It was found that for food sources containing both plant and animal proteins, the *in vitro* enzymic procedures could not predict the *in vivo* digestibility for humans. In similar experiments, Pedersen and Eggum (1981) found that these *in vitro* procedures could not predict the digestibilities of animal proteins. Marshall *et al* (1979) measured protein digestibility *in vitro* by a method similar to that of Hsu *et al* (1977) but using human, rat or pig pancreatin as the enzyme source. In a test of the method, Rich *et al* (1980) found that it varied in its ability to predict protein digestibility.

From these many recent experiments to find a rapid *in vitro* assay, although individual assays have correlated well with some *in vivo* measurements of digestibility, it appears that no single assay is yet suitable for predicting the digestibility of all types of proteins and foods.

Furuya *et al* (1979) have developed a method for the estimation of digestibility using intestinal fluid freshly extracted from a pig fitted with a cannula in the upper jejunum; the correlations achieved between values determined by this method and digestibility values determined *in vivo* (Sakamoto *et al*, 1980) are no greater than those of the methods described above using purified enzymes, and do not justify the procedure involved. A further disadvantage of the method is that the enzymes present in the intestinal lumen of an animal will vary greatly according to the protein content of the diet of the animal (Corring and Saucier, 1972).

Although all the above assays have been shown to give results which correlate to some extent with levels of digestibility determined *in vivo*, all these assays can only give values which are relative to some reference protein, which is digested at the same time as the test protein. This reference protein is necessary because none of these methods achieve complete enzymic hydrolysis. Because digestion does not continue to completion, these methods cannot be used to determine the total numbers of individual amine acids within the protein which are available to enzymic release. One of the advantages of a procedure for complete proteolysis is therefore to give direct values of the number of available amino acids.

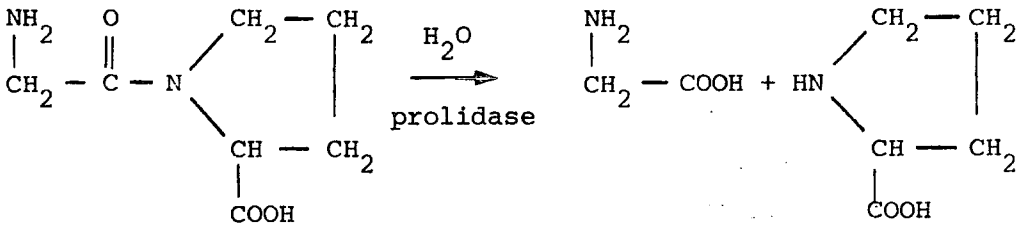
Although there are many proteolytic enzymes which show broad substrate specificities, none is known which can hydrolyse, at a reasonable rate, all the types of peptide bonds found in proteins. Therefore for the total enzymic hydrolysis of proteins it is necessary to use a mixture of enzymes of different specificities.

One of the first attempts to achieve total enzymic hydrolysis was by Frankel (1916) who used impure mixtures of pancreatic, gastric and intestinal enzymes for the digestion of several proteins. Similar methods were subsequently used for the isolation of acid or alkali labile constituents of proteins, for example, the polysaccharide moiety from ovalbumin (Neuberger, 1938) and haem containing peptides from cytochrome c (Tuppy, 1958) were isolated from enzymic digests.

The first method for the total enzymic hydrolysis of proteins by the use of purified enzymes was introduced by Hill and Schmidt in 1962. The first part of the digestion method involved the incubation of the protein with an endopeptidase to break the protein into small peptides: Hill and Schmidt used papain which was the purest and most active endopeptidase then available. After inactivation of the papain,

hydrolysis was completed by the addition of leucine aminopeptidase and prolidase, both of which were freshly prepared from pig kidney.

Leucine aminopeptidase is capable of hydrolysing peptide bonds formed by L-amino acids adjacent to a terminal amino group, although the rates of hydrolysis vary over a range of several thousandfold (Hofmann *et al*, 1962), with the poorest rate shown towards peptide bonds involving the imino group of proline, for example as contained in glycylproline:-




glycylproline glycine proline

Prolidase is specific for this type of peptide bond and was therefore included in the digestion mixture with leucine aminopeptidase.

This method was used by Hill and Schmidt for the hydrolysis of corticotrophin, papain, carboxypeptidase and enolase. The amino acid compositions of the enzymic hydrolysates agreed well with the compositions determined by acidic hydrolysates, but in some cases certain amino acids were not detected in theoretical amounts, perhaps because of incomplete hydrolysis or degradation of amino acids, and some yields were greater than expected, perhaps because of enzyme autolysis.

The method of Hill and Schmidt is the only procedure for total proteolysis to have been used by other authors for the hydrolysis of some other proteins: horse heart cytochrome c (Margoliash *et al*, 1962), human myoglobin (Perkoff *et al*, 1962), BSA (Dvorak, 1968) and horse muscle acyl phosphatase (Ramponi *et al*, 1971).

One problem with the Hill and Schmidt method is the necessity for purification of the leucine aminopeptidase and prolidase from pig kidney. Commercially prepared leucine aminopeptidase has since become available, together with purified aminopeptidase M, which is also present in the microsomal fraction prepared from pig kidney. Aminopeptidase M is said to show greater rates of hydrolysis over a wider range of dipeptides than does leucine aminopeptidase (Light, 1967). Purified prolidase is also now available commercially, but the relatively impure prolidase preparation used by Hill and Schmidt was known to contain prolinase and aminopeptidase activities which may be necessary for the complete hydrolysis of dipeptides.

In this work, the Hill and Schmidt method was tested with aminopeptidase M and with both freshly prepared and commercially prepared prolidase.

A few other methods for the total enzymic hydrolysis of proteins have been published, and most follow a pattern similar to the method of Hill and Schmidt, but with the replacement or addition of one or more enzymes. Pronase, a mixture of enzymes isolated from *Streptomyces griseus*, has a very wide range of specificities and is commonly used in enzymic digestion mixtures; Sletten (1968) used pronase in combination with leucine aminopeptidase; Holt *et al* (1971) used pronase, prolidase and leucine aminopeptidase for the hydrolysis of wool proteins, and Garner *et al* (1974) used pronase, carboxypeptidase C and leucine aminopeptidase for the hydrolysis of sperm whale myoglobin. Rayner and Fox (1976) claimed successful hydrolysis of rapeseed meals using only pronase, and Winkler and Schön (1979) described total enzymic hydrolysis of seed proteins achieved with pronase in combination with proteinase-K.

In this work, the efficiency of pronase, alone and in concert with proteinase-K and prolidase, was tested as a method of achieving complete hydrolysis.

According to Royer *et al* (1973) and Chin and Wold (1974), it is possible to achieve the complete hydrolysis of some proteins by using several different proteases covalently attached to insoluble supports.

To test this, in this study a variety of enzymes were coupled to supports, and their subsequent stability and efficiency at hydrolysis of large and small proteins were determined.

As well as finding a suitable method with which to achieve enzymic hydrolysis, it was also necessary to establish useful means to assay the extent of digestion. The efficiency of the pH-stat to follow digestion was examined, and useful methods for the identification of the end products of digestion were also determined.

# 2.0 MATERIALS

## 2.1 ENZYMES

The enzymes used, with their Enzyme Commission numbers (or in the case of crude preparations, descriptions of the types of enzymic activities which they contain), the source of each enzyme, the form in which it was supplied, and the reference number of the enzyme preparation. Unless stated otherwise, enzymes were supplied by the Sigma Chemical Company, Fancy Road, Poole, Dorset, U.K. BH17 7NH.

Acylase (N-Acylamino acid amidohydrolase), E.C. 3.5.1.14, from porcine kidney, lyophilized, type A3010

Aminopeptidase-M, E.C. 3.4.11.2, from porcine kidney microsomes, in suspension in $(NH_4)_2SO_4$ (3.5M) and $MgCl_2$ (10mM), type L5006

Carboxypeptidase A, E.C. 3.4.12.1, from bovine pancreas, crystalline, type C6510

Carboxypeptidase B, E.C. 3.4.12.3, from porcine pancreas, crystalline, type C7261

α-Chymotrypsin, E.C. 3.4.21.1, from bovine pancreas, crystalline, type C4129

Elastase (Pancreopeptidase), E.C. 3.4.21.11, an aqueous suspension prepared from porcine pancreas, type E1250

Intestinal peptidase mixture. A mixture of enzymes containing general proteolytic activity and aminopeptidase activity, an aqueous suspension prepared from porcine intestinal mucosa, type P7500

Leucine aminopeptidase, E.C. 3.4.11.1, from porcine kidney, in suspension in 2.9M $(NH_4)_2SO_4$ 0.1M Tris - 5mM $MgCl_2$ solution, pH 8.0, type L9876

Pancreatin. A dried extract of porcine pancreas which contains many types of enzymes, including amylase, trypsin and protease activities, type P1750

Papain, E.C. 3.4.22.2, from papaya latex, supplied in 0.05M sodium acetate, pH 4.5, type P3125

Pepsin, E.C. 3.4.23.1, from porcine stomach mucosa, crystalline, type P7012

Prolase. A crude extract from papaya which contains protease activity. A lyophilized powder, type P4880

Prolidase (Aminoacyl-L-proline hydrolase, proline dipeptidase, imidodipeptidase), E.C. 3.4.13.9, from porcine kidney, in suspension in 70% $(NH_4)_2SO_4$, Batch 23A. Supplied by Miles Laboratories Ltd., P.O. Box 37, Stoke Poges, Slough, U.K. SL2 4LY

Pronase. A protease mixture extracted from *Streptomyces griseus*. Narahashi and Yoda (1979) state that this consists of six different enzymes:-

Neutral proteinase, stable at pH 5.0 - 9.0, in the presence of $Ca^{++}$

Alkaline proteinase B, stable at pH 5.5

Alkaline proteinases A and C, stable at pH 4.0 - 6.5

Aminopeptidase and Carboxypeptidase, both stable at pH 5.0 - 8.0, in the presence of $Ca^{++}$, and inactivated by EDTA.

Supplied as a lyophilized powder, type P5130

Proteinase-K, A protease extracted from *Tritirachium album*, crystalline, type Protease XI. A protease of strong proteolytic activity towards denatured and native proteins (Ebeling *et al*, 1974)

Trypsin, E.C. 3.4.21.4, from bovine pancreas, crystalline, type T8253

## 2.2 OTHER BIOLOGICAL MATERIALS

Seeds of *Phaseolus vulgaris* (L.) c.v. Dwarf White and *Vigna unguiculata* c.v. Prima, were obtained from the Tyneside Seed Co. Ltd., Sunderland.

Trypsin inhibitor purified from *Phaseolus vulgaris* and samples of the faeces of rats, some of whose diets incorporated the inhibitor, were a gift from Dr D. Phillips, University of Durham. Protein extracts and purified proteins from *Pisum sativum* (L.) c.v. Feltham First, were a gift from Dr M. Tyler, University of Durham.

Fish meals of known available lysine and methionine contents were a gift from Dr J.E. Ford, National Institute for Research in Dairying, University of Reading.

Skim-milk powder was that sold under the brand name "Marvel", and manufactured by Cadbury Typhoo, Bournville, Birmingham, U.K., B30 2NA.

Oxidised bovine insulin (B chain) was obtained from The Boehringer Corporation (London) Ltd., Bell Lane, Lewes, East Sussex, U.K., BN7 1LG.

Hen egg white ovalbumin (crystallized and lyophilized, grade VI).

Bovine serum albumin (crystallized and lyophilized)

Lyophilized porcine kidney acetone extract

Lima bean trypsin inhibitor

Soybean trypsin inhibitor

were obtained from the Sigma Chemical Company, Fancy Road, Poole, Dorset, U.K., BH17 7NH

## 2.3 CHEMICALS AND REAGENTS

With the exceptions listed below, all chemicals and reagents were obtained from British Drug Houses (B.D.H.) Ltd., Poole, Dorset, U.K., BH12 4NN, and were of analytical grade or the best available.

From the Sigma Chemical Company, Fancy Road, Poole, Dorset, U.K., BH17 7NH, were obtained:

Acrylamide

Amino acids (purified samples of each amino acid and a standard mixture of amino acids)

ATEE

BAEE

BANA

Bis-acrylamide

CMCMT

Cyanogen bromide

EDAC

EDTA

Glutathione

Gly-L-Pro

L-leucinamide

Leucine-p-nitroanilide

Picryl sulphonic acid (TNBS)

Pro-L-Ala

Pro-L-Gly

Pro-L-Val

SDS

Temed

Trizma base (Tris)

Val-L-Pro

## 2.4 OTHER MATERIALS

Activated CH-Sepharose, Sepharose-4B, Sepharose-6B and Sephadex G-100 were obtained from Pharmacia (G.B.) Ltd., Prince Regent Road, Hounslow, Middlesex, U.K., TW3 1NE

Carboxymethylcellulose was obtained from Whatman Lab Sales, Springfield Mill, Maidstone, Kent, U.K., ME14 2LE

Controlled pore glass was obtained from Pierce and Warriner (U.K.) Ltd., 44 Upper Northgate Street, Chester, U.K., CH1 4EF

Visking dialysis tubing was obtained from Medicell International Ltd., 239 Liverpool Road, London N1, U.K.

# 3.0 METHODS

## 3.1 THE PURIFICATION OF PROLIDASE

Prolidase was purified from pig kidney acetone powder, as recommended by Dvorak (1968), according to the method of Davis and Smith (1957).

Pig kidney acetone powder was extracted with 0.02M $MnCl_2$, pH 6.2 (25ml $MnCl_2$/g powder) for 1h at 40°C. The suspension was cooled and centrifuged for 30min at 4°C and 10000g (MSE 18, MSE Ltd., Crawley, Sussex). After centrifugation the sediment was discarded and solid ammonium sulphate added to the supernatant to 50% saturation (312g $(NH_4)_2SO_4$ per litre at 4°C). When all the ammonium sulphate had dissolved, the solution was left to stand for 1h at 4°C. The precipitate which formed was separated by centrifugation (15min, 10000g, 4°C), dissolved in distilled water, and dialysed against distilled water (4°C) to remove the sulphate. Solid $MnCl_2$ was added to the dialysed solution to give a concentration of 0.02M, and bring the solution to pH 5.3. The solution was incubated at 40°C for 20min, after which an inactive precipitate was removed by centrifugation (15min, 10000g). The solution was cooled to 0°C and the temperature gradually lowered to -10°C as acetone, pre-cooled to -20°C, was added (43ml acetone/100ml supernatant). After 1h, the suspension was centrifuged (20min, 10000g, 4°C), the precipitate collected, suspended in a small amount of water, and dialysed against 100 vol of 0.01M $MnCl_2$, pH 8.0, for 24h at 4°C.

Prolidase of this purity was not lyophilized since this caused loss of activity (Hill and Schmidt, 1962). The enzyme is most stable if stored at pH 6-8 in a 0.01M $MnCl_2$, frozen at -10°C.

## 3.2 TECHNIQUES FOR THE IMMOBILIZATION OF ENZYMES

### 3.2.1 The covalent coupling of enzymes to agarose

This method, using cyanogen bromide for the activation of agarose, was described by March *et al* (1974). The agarose used was Sepharose 4B or Sepharose 6B (Pharmacia).

### 3.2.2 The covalent coupling of pronase to porous glass

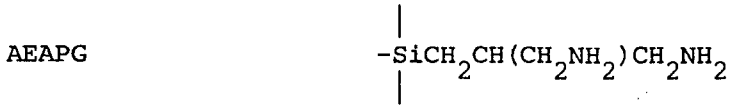
Pronase was coupled to porous glass by Schiff base coupling. The method used was a modification of that described by Mason and Weetall (1972). It depended upon the reaction between the aldehyde group of a support material with a primary amino group of an enzyme, to form an imine coupling:-

$$\text{(support)-CHO} + H_2N\text{-(enzyme)} \rightleftharpoons \text{(support)-CH=N-(enzyme)} + H_2O$$

The two types of support used for coupling pronase were aminopropyl glass (APG) and aminoethylaminopropyl glass (AEAPG). The formulae of their amino containing side groups are:-

APG

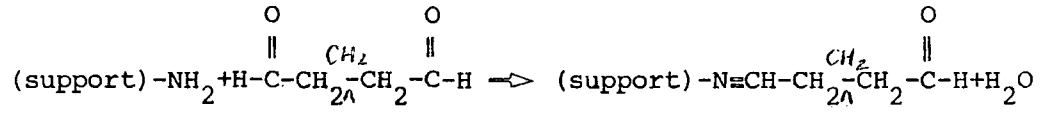
AEAPG



Coupling of the enzyme to the glass involved the side groups on the glass being formed into reactive aldehyde intermediates, which were then capable of Schiff base coupling with amino groups of the enzyme.

a. Formation of the reactive aldehyde intermediate

This was formed by reacting glutaraldehyde with the amino support:-



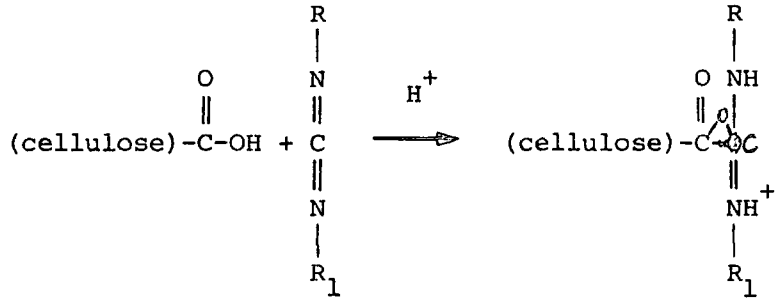
The reaction was carried out at room temperature. 10ml of a 2.5% (v/v) glutaraldehyde solution in water were added to 1g of alkyl amino glass, and the slurry was degassed. The reaction was continued for 1h, after which the glutaraldehyde was decanted, and the glass washed five times with distilled water.

b. Schiff base coupling of the pronase to the reactive aldehyde intermediate

100mg of pronase were dissolved in 40ml 0.1M sodium phosphate buffer, pH 7.0, and the glass added to form a slurry. The slurry was degassed and coupling continued for 1h at $4^{o}C$. The glass was then washed well with distilled water and stored at $4^{o}C$ as a wet cake. Determination of the absorbance at 280nm of the water used for washing allowed estimation of the amount of pronase which had been coupled.

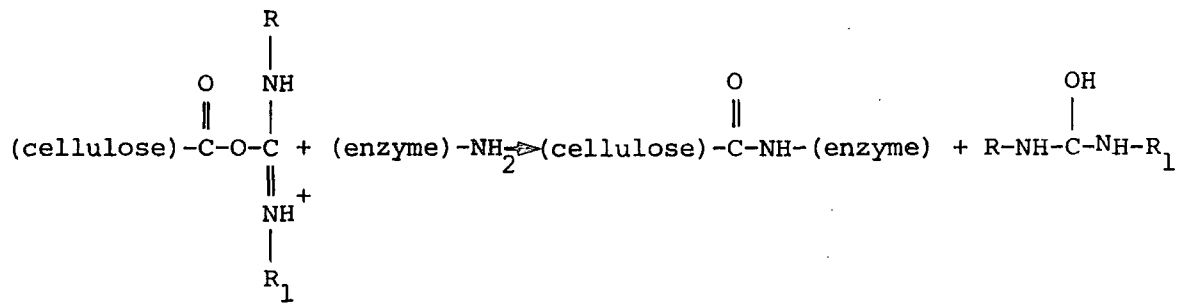
3.2.3 The covalent coupling of pronase to carboxymethylcellulose (Weetall, 1975)

The carboxymethylcellulose was first reacted with carbodiimide to form a pseudourea, the reactive intermediate required for coupling:-



pseudourea

The excess carbodiimide was removed from the reaction and a solution of the enzyme added, to complete the coupling:-



a. Formation of pseudourea

1g of swollen, washed carboxymethylcellulose was added to 50mg of ethylenediamine carbodiimide (EDAC) dissolved in 10ml of water. The reaction mixture was adjusted to pH 4.5 with 6M HCl (approximately 0.5ml) and rotated for 3h at room temperature. The support was then washed five times with ice-cold water, and resuspended in 10ml of water. In some preparations, the carbodiimide was supplied by cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulphate, (CMCMT), rather than by EDAC.

b. Coupling of the enzyme

50mg of pronase were dissolved in 2ml water and added to the support. The mixture was adjusted to pH 4.5, or the chosen value of pH, and left rotating overnight at 4°C. After this time the reaction solution

was decanted and the product was washed with water. Coupled pronase-carboxymethylcellulose was stored at 4°C in 0.05M Tris-HCl buffer, 0.002% sodium azide.

3.2.4 The covalent coupling of enzymes to activated CH-Sepharose 4B

Activated CH-Sepharose 4B contains a 6-carbon spacer group and an active ester group (N-OH succinimide) to allow spontaneous coupling to the amino groups of proteins. A stable amide linkage is formed between the spacer group and the protein.

Coupling of the enzyme

1g of dried N-OH succinimide CH-Sepharose 4B was swollen in 1mM HCl and washed well. 500mg of enzyme were dissolved in 10ml $NaHCO_3$,0.1M, pH 8.0, 0.5M in NaCl. This suspension was rotated for 4h at 4°C. Excess ligand was then removed by washing firstly in 200ml of the coupling buffer, and then with three cycles of 200ml each of 0.1M sodium acetate, 1M NaCl, pH 4.0 and 0.1M Tris buffer, 1M NaCl, pH 8.0. After washing, the coupled enzyme was stored at 4°C in the Tris buffer.

3.3 ENZYME ASSAY PROCEDURES

Spectrophotometric assays

Spectrophotometric assays of enzyme preparations were carried out in cuvettes of 1cm light path, incubated at a constant temperature of 37°C in a Pye Unicam SP8-150 double beam spectrophotometer. Sufficient buffer was added to maintain a constant total volume in each cuvette, depending upon the total volume of substrate and enzyme used. Reference cuvettes contained buffer in place of enzyme.

### 3.3.1 Assay of prolidase activity

This assay depends upon the decrease in absorbance at 242nm as the peptide bond of glycyl-L-proline is hydrolysed. For maximum activity, prolidase requires the presence of $Mn^{++}$ and glutathione. Activation of prolidase requires some time to reach maximum activity (Davis and Smith, 1957), and therefore enzyme preparations were made up in activation medium and incubated for 1h at 40°C prior to assay.

Table 1 Contents of the activation medium for prolidase activity

| Contents | Vol. (ml) | Final concentration (M) |
|---|---|---|
| 0.20M $MnCl_2$ | 0.4 | 0.032 |
| 0.03M glutathione | 0.1 | 0.001 |
| 0.05M Tris-HCl buffer, pH 8.0 | 2.4 | 0.048 |
| Enzyme | 0.1 | - |

Table 2 Contents of the assay system for prolidase assay

| Contents | Vol. (ml) | Final concentration (M) |
|---|---|---|
| 0.025M Gly-L-Pro in buffer | 2.7 | 0.023 |
| 0.200M $MnCl_2$ | 0.2 | 0.013 |
| Enzyme | 0.1 | - |

The reaction was initiated by addition of the enzyme to the test cuvette, after which the rate of decrease in $A_{242}$ was recorded over 10min.

Unit Definition: One Enzyme Unit is that amount of activity which catalyzes the hydrolysis of 1μmol of Gly-L-Pro per minute at 37°C. The molar absorption coefficient of the peptide bond of Gly-L-Pro is $0.0254 \times 10^3$.

Calculation of the proteolytic coefficient, $C_1$

To allow comparison with prolidase preparations used in enzymic digestion mixtures by other authors, the $C_1$ values of the freshly prepared and commercially available enzymes were determined. The hydrolysis of Gly-L-Pro by prolidase is a first order reaction, and the enzymic activity of a preparation may be expressed in terms of the first order proteolytic coefficient, $C_1$. This was calculated from the first order rate constant, k, and the enzyme concentration, E (in mg of protein N per ml of test solution), according to the equation $C_1 = \frac{k}{E}$.

3.3.2 Assay of aminopeptidase activity

a. Using L-leucinamide as substrate

This assay depends upon the decrease in $A_{238}$ as L-leucinamide is hydrolysed:

$$\text{L-leucinamide} + H_2O \longrightarrow \text{L-leucine} + NH_3$$

Table 3 Aminopeptidase assay system using L-leucinamide

| Contents | Vol (ml) | Final concentration (M) |
|---|---|---|
| 0.06M L-leucinamide, in buffer | 2.5 | 0.049 |
| 0.50M Tris-HCl buffer, pH 8.5 | 0.4 | 0.066 |
| Enzyme | 0.1 | - |

After addition of the enzyme to the test cuvette, the decrease in absorbance at 238 nm was measured over 10min.

Unit Definition: One Enzyme Unit is that amount of activity which catalyzes the hydrolysis of 1µmol of L-leucinamide per minute at 37°C. The molar absorption coefficient of the amide bond of L-leucinamide is $0.011 \times 10^3$.

b. Using leucine-p-nitroanilide as substrate

Hydrolysis of leucine-p-nitroanilide leads to the production of p-nitroaniline which absorbs at 405 nm (Tuppy *et al*, 1962).

$$R\text{-}CH(NH_2)\text{-}C\text{-}ONH(C_6H_4\text{-}NO_2) + H_2O \rightleftharpoons R\text{-}CH(NH_2)\text{-}COOH + H_2N\text{-}C_6H_4\text{-}NO_2$$

leucine-p-nitroanilide leucine p-nitroaniline

Leucine-p-nitroanilide required about 1h and gentle heating to dissolve in the buffer used.

Table 4 Aminopeptidase assay system using leucine-p-nitroanilide

| Contents | Vol (ml) | Final concentration (M) |
|---|---|---|
| 0.06M phosphate buffer, pH 7.0 | 0.7 | 0.0420 |
| 0.002M leucine-p-nitroanilide, in buffer | 0.2 | 0.0004 |
| Enzyme | 0.1 | - |

After addition of enzyme to the test cuvette, the increase in absorbance at 405 nm was measured over 15min.

Unit Definition: One Enzyme Unit is the amount of enzyme which produces 1µmol of p-nitroaniline per min at 37°C. The molar absorption coefficient of p-nitroaniline is $9.62 \times 10^3$.

3.3.3 Assay of soluble and coupled pronase

For the assay of coupled pronase, the activity of 1mg of coupled pronase was expressed as a percentage of the activity of 1mg of the enzyme in free solution.

1. Assay of pronase coupled to agarose and cellulose supports

a. pH-stat assay of proteolytic activity

The pH-stat was used as described in Section 3.4.1.

Table 5 The conditions for assay of pronase using the pH-stat

| Contents of vessel | Concentration (M) | Vol (ml) | Final concentration after addition of BAEE (M) |
|---|---|---|---|
| KCl | 0.5 | 1.0 | 0.125 |
| enzyme | - | 0 - 2.0 to a final volume of 4.0 | - |
| water | - | | |
| BAEE | 0.1 | 1.0 | 0.125 |

pH-stat conditions: 37°C, 5mM NaOH as titrant, $N_2$ bubbled over the surface of the reaction mixture, end point set at pH 7.0.

The reaction was initiated by the addition of BAEE, and the rate of addition of alkali was measured over the following 7min.

To use ovalbumin as substrate, the BAEE was replaced by 1ml of a $1mgml^{-1}$ aqueous solution of ovalbumin.

b. Spectrophotometric assay of aminopeptidase activity

CH-Sepharose-pronase was assayed for aminopeptidase activity as described in Section 3.3.2.b, using volumes of coupled enzyme of up to 25µl.

2. Assay of pronase coupled to porous glass supports

The glass-coupled pronase was assayed by a discontinuous method, by taking samples at intervals from a digestion mixture consisting of the enzyme and ovalbumin as substrate, and using TNBS to assay these samples. The digestion mixtures consisted of 1ml of coupled enzyme, or enzyme in free solution, 1ml of ovalbumin ($2.5 mg ml^{-1}$ in water) and 0.1M $KH_2PO_4$, pH 8.0, to a final volume of 4ml. These mixtures were incubated, together with relevant controls, for 3h, 37°C, in rotated glass vials to ensure that the entire solution came into contact with the glass.

Assay method: 0.1ml samples were taken, at 30min intervals, from the surface of the digestion mixture (the glass beads quickly settle to the bottom of the vial making centrifugation unnecessary), and added to 0.9ml 0.1M $Na_2B_4O_7$, and 0.1ml 0.03M TNBS. These were incubated for 1h at 37°C, after which time the reaction was quenched by the addition of 2ml 0.1M $NaH_2PO_4$, 1.5mM $Na_2SO_3$ and absorbance measured at 420 nm.

3.3.4 Assay of soluble and coupled proteinase-K

Soluble and coupled proteinase-K were assayed by using a pH-stat to measure the decrease in the pH value of the medium as ATEE was hydrolysed.

pH-stat conditions: 37°C, 5mM NaOH as titrant, $N_2$ over the surface of the reaction mixture, end point at pH 9.0

Assay system: samples of the coupled enzyme or native enzyme were placed in the reaction vessel of the pH-stat, together with water to a total volume of 3ml. After this mixture had been brought to pH 9.0, and allowing a few minutes for temperature equilibration, the reaction

was initiated by addition of 1.0ml of ATEE, 0.01M in 50% v/v methanol. The rate of addition of hydroxide was measured over 14min, after the initial fast addition of hydroxide to neutralise the acidity of the ATEE solution. Comparison with the activity of the native enzyme allowed the activity of the coupled proteinase-K to be expressed in terms of mg of enzymic activity per ml of coupled enzyme.

3.3.5 Assay of coupled prolidase

Coupled prolidase was assayed by a discontinuous method based on that described in Section 3.3.1. Samples of the coupled prolidase or native prolidase were incubated with 1.0ml glycyl-L-proline, 0.025M, in 0.05M Tris-HCl, pH 8.0, and additional Tris buffer was added to give a total volume of 2.0ml. Appropriate controls were also incubated. The absorbances at 242nm of these solutions, after centrifugation, were measured at the start of the experiment and at 20min interals, for 3h. After measurement, the samples were resuspended with the coupled enzyme. Activity of the coupled prolidase was expressed in mg of native enzymic activity per ml of coupled enzyme.

3.3.6 Assay of soluble and coupled papain

Papain was assayed using a pH stat, as described in Section 3.4.1, to follow the hydrolysis of BAEE. EDTA is required in the assay system as an activator of papain.

Table 6 The conditions for assay of papain using the pH-stat

| Contents | Concentration (M) | Vol (ml) | Final concentration after addition of BAEE (M) |
|---|---|---|---|
| EDTA | 0.005 | 2 | 0.002 |
| KCl | 0.500 | 1 | 0.100 |
| Enzyme | - | 0 - 1 | - |
| Water | - | to a final vol. of 5 | - |
| BAEE | 0.100 | 1 | 0.020 |

pH-stat conditions: 37°C, 5mM NaOH as titrant, $N_2$ bubbled over the surface of the reaction mixture, end point set at pH 8.2.

The reaction was initiated by the addition of BAEE, and the rate of addition of alkali was measured over the following 14min.

Activity of the coupled papain was expressed in mg of native enzymic activity per ml of coupled enzyme.

### 3.3.7 Assay of coupled trypsin

Sepharose-trypsin was assayed using a pH-stat to measure the hydrolysis of BAEE as described in Section 3.3.6. Activity of the coupled enzyme was expressed in mg of native enzymic activity per ml of coupled trypsin.

## 3.4 THE USE OF THE pH-stat

### 3.4.1 Use of the pH-stat to follow enzymic reactions

The pH-stat may be used to measure the hydrolysis of peptide bonds, as the release of hydrogen ions is followed by continuous titration, adding sufficient alkali to maintain a constant pH value.

The volume of alkali added therefore serves as a measure of the extent of hydrolysis.

The reaction to be studied is carried out in a water jacketed, stirred vessel. Also in the vessel are electrodes to monitor pH, and tubes delivering alkali from the autoburette, and leading nitrogen over the surface of the reaction mixture. To maintain the contents of the vessel at a constant value of pH, the auto-titrator is set with this as end-point. A decrease in the pH-value of the contents of the vessel will therefore cause delivery of alkali from the autoburette until the chosen pH value is reached. The volume of alkali delivered by the autoburette is recorded on the chart recorder. The concentration of the alkali must be high enough to minimize dilution effects, but not so concentrated that "over-shooting" beyond the end-point becomes a problem. Also, speed of delivery from the burette must not be too slow, leading to a falsely low rate of reaction, but not so fast as to over-shoot the end-point. In practice, it is found best to select a fairly high speed at the start of a reaction, changing to a lower speed when the rate of reaction has decreased.

When the substrate solution is at a constant pH, the enzyme solution may be added from a syringe through a hole in the stopper of the vessel. Because the pH value of the enzyme solution may differ from the pH of the substrate solution, a control must be carried out in which the same volume of enzyme is added to water in the vessel. This is more satisfactory than prior adjustment of the pH of the enzyme solution to that of the reaction mixture.

### 3.4.2 Use of the pH-stat to measure the extent of hydrolysis of ovalbumin by a variety of enzymes

The enzymes were used in sufficient concentrations to ensure that reactions were substrate limited, and allowed the maximum rate of reaction to be achieved. Whether enzyme or substrate were rate limiting was determined by adding an extra aliquot of substrate solution; if enzyme was in excess then an increase in reaction rate occurred.

The extent of autolysis of the enzyme or enzymes being used was measured by their addition to the pH-stat vessel containing water in place of substrate. Any activity measured was subtracted from the level of activity found in the presence of substrate. Reactions were initiated by the addition of enzyme to substrate solution, and allowed to continue until no further alkali was being added to the vessel

pH-stat conditions: $37^{o}C$, 5mM NaOH as titrant, end point set at pH 7.0.

Substrate: Ovalbumin in aqueous solution at a concentration of $5mgml^{-1}$ and denatured by boiling for 10min at pH 10.0. 1ml used for assay.

Initial reaction volume: water was added to the pH-stat vessel such that after the addition of enzyme(s), the total volume was 5ml.

Table 7 The concentrations of enzymes used for the hydrolysis of ovalbumin

| Enzyme(s) | Solution | Concentration in reaction mixture $mg.ml^{-1}$ |
|---|---|---|
| α-Chymotrypsin | HCl,1mM : $CaCl_2$,20mM | 0.1 |
| Elastase | Water | 0.1 |
| Intestinal peptidase mixture | HCl,1mM : $CaCl_2$,20mM | 0.1 |
| Pancreatin | HCl,1mM : $CaCl_2$,20mM | 0.2 |
| Pronase | HCl,1mM : $CaCl_2$,20mM | 0.2 |
| Pepsin | Water | 0.1 |
| Papain | Water | 0.1 |
| Trypsin | HCl,1mM : $CaCl_2$,20mM | 0.1 |
| Carboxypeptidase A | Water | 0.04 |
| Carboxypeptidase B | Water | 0.04 |
| Leucine aminopeptidase | $MgCl_2$,0.05M,pH 8.0 | 0.04 |

Pepsin has an acidic optimum pH, therefore the ovalbumin was predigested for 1h at pH 3.0 with the pepsin, after which the solution was raised to pH 8.0 and pronase added.

Leucine aminopeptidase was activated by incubation in 0.05M $MgCl_2$ solution, at 40°C for 1h prior to use.

Calculation of results: the extent of enzymic hydrolysis was calculated from the number of moles of hydroxide which were needed to maintain a constant value of pH, and were therefore equivalent to the number of moles of hydrogen ions released into solution by the breakage of peptide bonds.

3.4.3 Use of the pH-stat as a recording pH meter

As well as measuring the volume of hydroxide added from the autoburette over a period of time, it was possible to use the system as a recording pH meter, and measure either the change in pH with volume of hydroxide added, or the change in pH with time. The latter method was used to follow the change in pH of a reaction when a constant pH value was not being maintained by addition of alkali from the autoburette.

3.4.4 Use of the pH-stat for the production of titration curves

The buffering capacity of a given solution was measured between two values of pH, by setting the end-point to the higher pH value and adjusting the pH of the solution to the lower value. The change in pH was then recorded as hydroxide was added at a constant rate.

3.5 THE USE OF TNBS TO MEASURE PROTEOLYSIS

3.5.1 To determine separately the levels of amino acids and proteins in a mixture (Mokrasch, 1967)

The sample (less than 0.2µmol of amino acid or less than 0.4mg of protein) was made up to 1.0ml with 0.1M $K_2B_4O_7$, and 0.1ml of 0.03M TNBS, in solution in the buffer, was added. These were mixed, and incubated at 40$^{o}$C for 10min. After this time, the reaction was quenched by the addition of 0.2ml of 6M HCl. Extraction was then carried out with 3 x 1ml portions of toluene. The absorbance of the toluene layer was measured at 340nm, to estimate amino acids. To the residual aqueous phases, 2ml of 88% (v/v) formic acid were added, and the absorbances were read at 340nm, to estimate protein content.

3.5.2 The use of TNBS for routine assay (Romero and Ryan, 1978)

The sample (containing up to 40µg of amino acid) was added to 0.1M $Na_2B_4O_7$, pH 9.0, to give a final volume of 1.0ml. 100µl of TNBS (0.03M in 0.1M $Na_2B_4O_7$) were added, mixed well, and incubated at 37°C. After 1h the reaction was quenched by the addition of 2.0ml of 0.1M $NaH_2PO_4$ containing 1.5mM $Na_2SO_3$. Absorbance at 420nm required to be read within the next two hours. A blank was also prepared, containing buffer in place of sample, and was assayed with TNBS following the same procedure.

Assay of protein digestion mixtures

Absorbance values of no-enzyme controls and enzyme-only controls were subtracted from the absorbance values of the appropriate samples.

A mixture of amino acids corresponding to the published amino acid composition of ovalbumin (McReynolds *et al*, 1978) was made up, for use as a calibration curve for these assays. Samples of this mixture had been freeze-dried so that a constant reference was used in all experiments. This reference mixture was compared with an acid hydrolysis of ovalbumin. Both amino acid mixtures gave the same values when assayed with TNBS.

3.6 METHODS FOR THE IDENTIFICATION OF THE PRODUCTS OF PROTEOLYSIS

3.6.1 Gel filtration chromatography

Protein digests were fractionated on a column of Sephadex G-100 (58 x 3)cm, using 0.1M sodium phosphate buffer, pH 7.0, 0.1M NaCl, as eluant. A constant flow rate (81.4ml.$h^{-1}$) was maintained by use of a peristaltic pump. Absorbance of the effluent was monitored continuously at 280nm (Uvicord II, LKB Ltd).

1.0ml samples containing 20mg of the protein or protein digests were loaded on to the column, eluted, and 7.0ml fractions collected. Samples of these fractions were assayed with TNBS to measure free amino groups.

Column calibration

A solution containing Blue Dextran and NaBr (0.5ml, each at 1.0mg.ml$^{-1}$) was used; elution of the dextran giving the void volume ($V_o$), and NaBr giving the total elution volume. For molecular weight calibration, 1.0ml of a solution containing 5mg of each of BSA, ovalbumin, cytochrome C and myoglobin was applied to the column, and their elution volumes ($V_e$) noted. The calibration curve is shown in Fig. 1.

3.6.2 The use of gel filtration to follow the degradation of glycoprotein II by enzymic hydrolysis

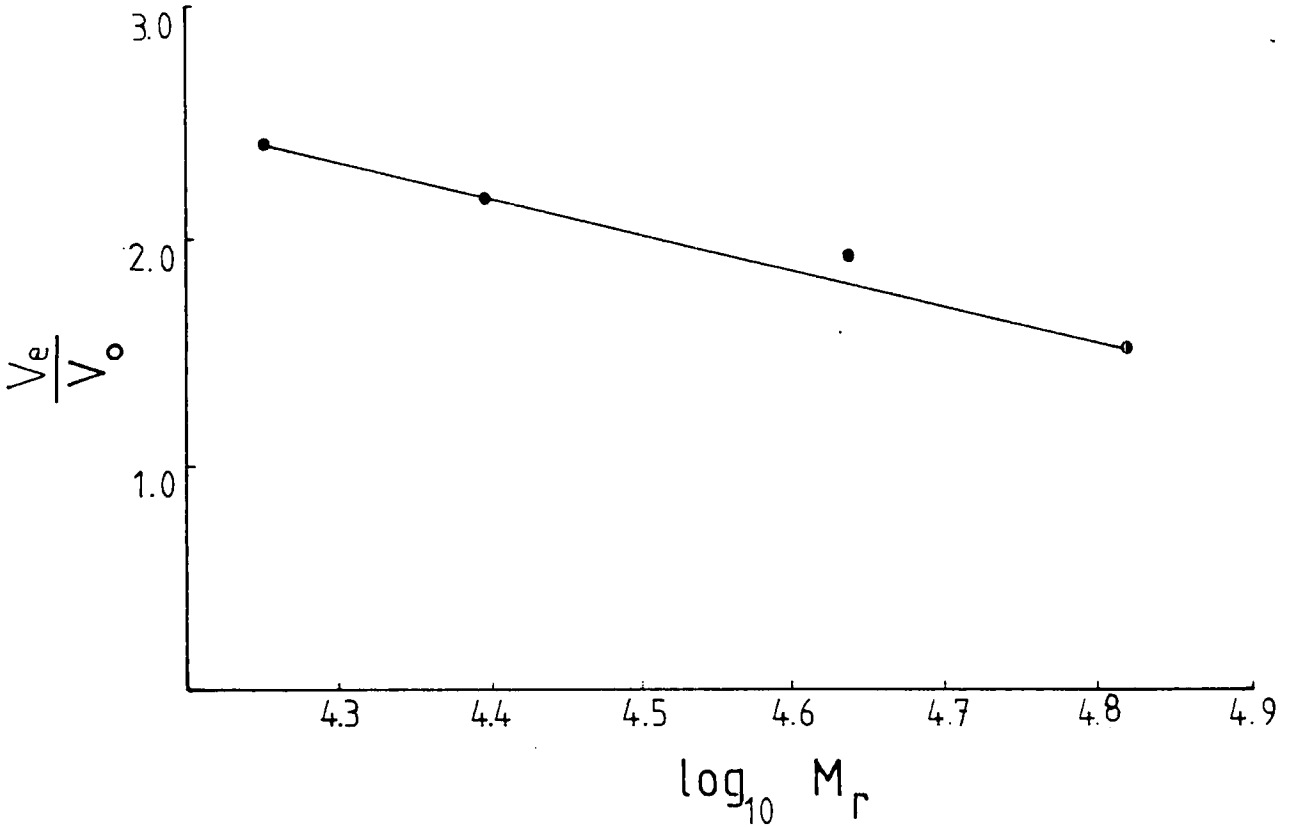
Glycoprotein II was digested with trypsin at a substrate to enzyme ratio of 10:1, in 0.1M sodium phosphate buffer, pH 7.5, 50mM $CaCl_2$, at a final glycoprotein II concentration of 20mg.ml$^{-1}$. Identical preparations were incubated at 37$^{o}$C for the chosen time intervals, and then stored at -20$^{o}$C. A portion of glycoprotein II was denatured as described in Section 3.6.4. 1ml samples of the glycoprotein II (20mg.ml$^{-1}$) or of the digests were applied to the surface of the column.

Preparation of the column, and the collection and assay of fractions are described above.

3.6.3 SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gels were developed according to the method of Lämmli (1970). Polyacrylamide gels (17% acrylamide containing 0.1% bisacrylamide) were made in 0.1% SDS, 0.375M Tris-HCl at pH 8.8. Gels

FIGURE 1 CALIBRATION CURVE FOR G-100 GEL FILTRATION COLUMN



were polymerized with 50mg ammonium persulphate and 33µl Temed per 100ml acrylamide solution. Running buffer was 0.025M Tris, 0.2M glycine and 0.1% SDS.

Storage proteins were stained in a solution of 0.025% Coomassie Brilliant Blue R-250, 7% acetic acid and 25% methanol. An identical solution without the dye was used for destaining.

Formaldehyde fixation

In order to stain proteins of high solubility, e.g. samples of low molecular weight from protein digests, or trypsin inhibitors, the proteins were covalently linked to gels by formaldehyde fixation, according to the method of Steck *et al* (1980). Proteins were then fixed and stained by immersing the gel for 1h in a solution of 180ml ethanol, 420ml distilled water, 100ml 35% formaldehyde and 0.8g Coomassie Brilliant Blue R-250. To avoid gel shrinkage, the gels were stained for a further 1h in a solution of 250ml ethanol, 750ml distilled water, 10ml 35% formaldehyde and 1.2g Coomassie Brilliant Blue R-250. The same solution without the dye was used for destaining overnight. The gels were calibrated with chymotrypsinogen ($M_r$25000), lysozyme ($M_r$14300) and thyrocalcitonin ($M_r$3300).

3.6.4 The use of SDS polyacrylamide gel electrophoresis to visualise the degradation of glycoprotein II by enzymic hydrolysis

Solutions of glycoprotein II (16mg.ml$^{-1}$ in 0.1M Tris-HCl buffer, pH 7.5) and the enzyme to be used (1.6mg.ml$^{-1}$ in 0.05M $CaCl_2$, pH 6.0) were mixed in equal volumes to give a final substrate to enzyme ratio of 10 : 1, and incubated at 37°C. Samples taken at timed intervals after the start of incubation were immediately added to SDS sample buffer and boiled for 2min, then stored at -20°C. Controls consisting only of buffer and substrate or enzyme were also prepared.

Samples containing 20µg of substrate protein were loaded on to 17% acrylamide gels, prepared and run as described in Section 3.6.3.

A sample of denatured glycoprotein II was prepared by dissolving 20mg of glycoprotein II in 10ml of water. The solution was adjusted to pH 7.5, and incubated in a boiling water bath for 1h.

## 3.7 AMINO ACID ANALYSIS

Amino acids were determined on a Locarte, single column, automatic loading amino acid analyser.

### 3.7.1 The preparation of acid hydrolysates for amino acid analysis

Samples containing between 0.5 - 2.5mg protein were hydrolysed in 1.5ml 6M HCl, at 105°C, for 22h, *in vacuo*, according to the method of Evans and Boulter (1974). For accurate determination of the acid labile amino acids (threonine, tyrosine, serine), hydrolysis was carried out on identical samples for 22h, 50h and 90h, and the following equation used to calculate the amounts present at $t_o$:

$$\log A_o = \left[\frac{t_2}{t_2-t_1}\right] \log A_1 - \left[\frac{t_1}{t_2-t_1}\right] \log A_2$$ (Moore and Stein, 1954)

where $A_o$ represents the amount of a given amino acid at time $t_o$ and $A_1$ and $A_2$ represent the amounts at times $t_1$ (22h), and $t_2$ (50h). Isoleucine is only slowly released during hydrolysis, and the value used was that measured after 90h of hydrolysis.

### 3.7.2 The preparation of enzymic hydrolysates for amino acid analysis

The lyophilized product from an enzymic hydrolysis was dissolved in water such that a 0.5ml aliquot of the solution would contain 0.5 - 2.5mg

of protein. One aliquot was made up in sample buffer (0.1M sodium citrate buffer, pH 2.2), with a final nor-leucine concentration of $0.05\mu mol.ml^{-1}$. This sample was applied directly to the amino acid analyser. A second sample was taken for subsequent acid hydrolysis.

### 3.7.3 Determination of cystine

Cystine was determined by the method of Moore (1963), by oxidation to cysteic acid with performic acid prior to acid hydrolysis.

### 3.7.4 Determination of asparagine and glutamine

To allow the separate elution of these amino acids from threonine and serine, the sodium citrate buffers normally used for amino acid analysis were replaced by lithium citrate buffers, as described by Glazer *et al* (1975).

### 3.7.5 Calculation of results from amino acid analysis

Unless otherwise stated, all hydrolyses were carried out in duplicate, and the values given in the Results section are the mean values from each pair after amino acid analysis. Where possible, results were expressed in the form which allowed comparison with other known data. The most simple form in which to express results was as μmoles of amino acids per mg of sample, allowing a correction for the moisture content of the sample. For pure proteins whose expected amino acid composition was known, the results from amino acid analysis were calculated to give the number of amino acids per molecule of the protein, using the molecular weight of the protein and correcting the weight of the sample used for moisture, and (if relevant) carbohydrate content. For some other samples, the expected amino acid composition was known in terms of grammes of amino acids per 16 grammes of N. Therefore to express the results from this work in the same form, it was necessary to determine the N and moisture content of these samples.

## 3.8 OTHER ANALYTICAL METHODS

### 3.8.1 Determination of moisture content and dry weight

Weighed triplicate samples were dried at 105°C until they reached constant weight. When the samples had cooled over silica gel, they were reweighed to determine their water content.

### 3.8.2 Determination of total nitrogen content

The N contents of samples were determined according to the micro-Kjeldahl method described by Evans and Boulter (1974).

## 3.9 THE PURIFICATION OF GLYCOPROTEIN II

Dwarf white beans, *Phaseolus vulgaris*, were dried and milled to pass through a 335µm mesh sieve, and then defatted with ice-cold n-hexane. The powder was air dried and extracted for 2h at 4°C with 50mM sodium borate buffer, pH 8.0, with a meal to buffer ratio of 1 : 20, w/v. The mixture was centrifuged at 20000g, and 4°C, for 30min. The supernatant was retained and ammonium sulphate crystals were slowly added to the stirred solution to achieve a final saturation of 90% at 0°C. After stirring for a further hour, the contents were centrifuged at 20000g, and 4°C for 30min. The supernatant was retained and dialysed against 10 vol of 50mM sodium borate buffer, pH 8.0, at 4°C for 60h, with three changes of buffer, after which time the purified globulins were lyophilized. Purity of the sample was checked by SDS polyacrylamide gel electrophoresis.

## 3.10 PROTEOLYSIS *IN VITRO* ACCORDING TO THE METHOD OF HILL AND SCHMIDT (1962)

### 3.10.1 The modified Hill and Schmidt procedure

The sample (1 - 10mg protein) was dissolved in 3.0ml of water adjusted to pH 7.0; if the sample was to be denatured before hydrolysis, unless stated otherwise, it was incubated at 100°C for 10min. The solution was cooled and the following added:- 1.5ml of 0.2M sodium acetate buffer, pH 5.2; 0.3ml of 0.1M sodium cyanide, pH 5.2; 0.03ml of papain (686.0 Enzyme Units/ml) in 0.2M sodium acetate buffer, pH 5.2; and 0.015ml of toluene. Nor-leucine in solution in 0.1M sodium citrate buffer, pH 2.2, was used as an internal standard such that aliquots taken for amino acid analysis at the end of the digestion procedure would each contain 0.25µmol of nor-leucine.

The digestion mixture was incubated, rotating, at 37°C for 24h, after which time the pH was lowered to 2.0, to inactivate the papain. After filtration, the sample was lyophilized.

The lyophilized digest was dissolved in 0.5ml of water and adjusted to pH 8.0 with 1M NaOH. To this were added the following:- 0.3ml of 0.02M Tris-HCl buffer, pH 8.0; 0.3ml of 0.02M $MnCl_2$, pH 8.0; 0.01ml of prolidase (314.9 Enzyme Units/ml) in suspension in 70% saturated ammonium sulphate; 0.01ml of aminopeptidase M (4.51 Enzyme Units/ml) in 3.5M ammonium sulphate solution, pH 7.7, containing 10mM $MgCl_2$; and 0.015ml toluene. The digestion mixture was incubated for a second time, with rotating at 37°C for 24h. After this time the sample was transferred to a centrifuge tube, the pH was lowered to 2.0, and it was incubated in a boiling water bath for 10min. After cooling, the tube was spun at 10000g for 10min, and the supernatant removed and lyophilized.

Control experiments were also carried out, in which enzymic digestion was performed in the absence of any substrate.

3.10.2 Estimation of the loss of amino acids during the enzymic hydrolysis procedure

The error introduced during the enzymic digestion procedure was measured by subjecting a mixture of amino acids of known concentration to the identical procedure used for the hydrolysis of proteins. Half of the samples were also acid hydrolysed after the enzymic hydrolysis.

The amino acid mixture consisted of Cys A, Ala, Arg, Asp, Glu, Gly, His, Ile, Leu, N-Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val, all at a concentration of 2.5mM, and Cys at 1.25mM, in citrate buffer, pH 2.2. Ten 0.2ml samples of this solution were treated according to the modified Hill and Schmidt enzymic hydrolysis method. Five of the final solutions were further acid hydrolysed under the same conditions used for the hydrolysis of proteins (Section 3.7.1). The final levels of amino acids were measured by amino acid analysis. Control solutions containing only N-leu were treated in the same way.

3.10.3 The enzymic hydrolysis of BSA

BSA was hydrolysed by enzymes *in vitro* by two slightly different methods. In the first method, BSA was hydrolysed by the Hill and Schmidt procedure using standard (commercial) preparations of each enzyme. In the second method, BSA was hydrolysed by the same procedure, except that the standard prolidase was replaced by a fresh preparation from pig kidney acetone extract. Otherwise, the method described in Section 3.10.1 was followed, using 10mg samples of BSA, except that nor-leucine was not added until the lyophilized digest products were prepared for amino acid analysis.

For use in the second enzymic hydrolysis method, the final precipitate from the prolidase preparation (Section 3.1) was dialysed overnight against distilled water containing 0.01M $Mn^{++}$. The inactive precipitate which formed was removed by centrifugation, and the activity of the supernatant assayed. A volume of supernatant (0.75ml) with activity equal to that of the commercial prolidase was used in the enzymic digestion mixtures.

Duplicate hydrolysates were prepared, and half of each of the final digest products (5mg) was acid hydrolysed according to the method in Section 3.7.1. Duplicate enzyme controls containing no added substrate were treated similarly.

3.10.4 The enzymic hydrolysis of glycoprotein II

Enzymic hydrolysis by the modified Hill and Schmidt procedure (Section 3.10.1) was carried out separately on 15mg portions of the glycoprotein II preparation (Section 3.9). Half of the samples were heat denatured prior to hydrolysis, by adjusting 10% aqueous solutions to pH 7.5 and incubating in a boiling water bath for 10min. Each digestion was carried out in triplicate, together with control digests consisting only of the enzymes, incubated in the absence of any added substrate.

3.10.5 The enzymic hydrolysis of trypsin inhibitor and trypsin

Samples of trypsin inhibitor and trypsin were hydrolysed by the method described in Section 3.10.1. Samples of trypsin inhibitor were hydrolysed in both the native form and after heat treatment, which involved the boiling of an aqueous solution of the inhibitor for 30min.

When trypsin inhibitor and trypsin were to be hydrolysed together, equal weights (1mg) of each were preincubated together at 37°C,

for 15min. After this time, they were treated according to the normal procedure for denaturation and hydrolysis. A similar experiment was also carried out, but with the trypsin and trypsin inhibitor both undergoing the denaturation procedure before their preincubation together.

Before the final step of the enzymic hydrolysis procedure, *i.e.* the precipitation of the enzymes, samples were taken for SDS polyacrylamide gel electrophoresis. Aliquots of the final digest products were used for amino acid analysis to measure the amino acids released by enzymic hydrolysis. Control incubations containing enzymes in the absence of any added substrates were also analysed.

### 3.10.6 The enzymic hydrolysis of skim-milk powder

Samples of skim-milk powder (50mg) were adjusted to a moisture content of 10%, and incubated in sealed containers at 37°C for 50 days. At intervals, samples were removed and stored at -20°C. At the end of the incubation period, weighed portions were digested by the modified Hill and Schmidt procedure (Section 3.10.1), but with the omission of the denaturation step. The levels of free amino acids in the enzymic digests were determined.

### 3.10.7 The enzymic hydrolysis of proteins purified from cotyledons of germinating pea, *Pisum sativum*

Samples of the proteins were hydrolysed by the modified Hill and Schmidt procedure. The levels of Thr, Ser, Asp, Asn, Glu and Gln present in the enzymic digest were determined by amino acid analysis using a $Li^+$ buffer system (Section 3.7.4). The moisture and N contents of the protein preparations were determined.

### 3.10.8 The enzymic hydrolysis of fish meals

Samples of the fish meals (4mg) were hydrolysed by the modified Hill and Schmidt procedure, but with the omission of the denaturation step at the start of the procedure. The levels of Met and Lys were determined by amino acid analysis under normal conditions. The moisture contents of the fish meals were determined.

## 3.11 PROTEOLYSIS *IN VITRO* USING COUPLED ENZYMES

### 3.11.1 Hydrolysis of ovalbumin and insulin by coupled pronase, prolidase and proteinase-K

Each preparation of coupled enzyme was washed well in buffer, and assayed before use to ensure consistent activity. For hydrolysis of 25mg of substrate, coupled pronase of activity equivalent to 1mg of native pronase was used. Enzyme and substrate were rotated for 72h at 37°C in 5ml of buffer, 0.1M sodium borate, pH 8.0, 0.05% Azide. At the end of the digestion period, the mixture was filtered through sintered glass, and the Sepharose-pronase washed with 0.1M $NH_4HCO_3$. Filtrate and washings were then combined and freeze dried. Samples of the freeze dried product were further incubated using the same conditions with Sepharose-proteinase-K and/or Sepharose-prolidase, using coupled enzyme equivalent to 0.05mg of native enzyme per 1mg of substrate digest. After filtration and washing of the Sepharose-enzyme, samples were freeze dried and then redissolved in citrate buffer, pH 2.2, at a concentration suitable for amino acid analysis.

Controls consisting of enzymes with no substrate were treated by the same procedure, and the levels of amino acids present in these controls were subtracted from the levels of amino acids released from enzyme-substrate mixtures.

3.11.2 The incorporation of coupled papain into the Hill and Schmidt procedure, used for the hydrolysis of ovalbumin and insulin

This enzyme hydrolysis method was similar to the modified Hill and Schmidt procedure (Section 3.10.1) except that the soluble papain used in the first part of the procedure was replaced with a volume of Sepharose-papain (prepared as described in Section 3.2.1) containing activity equivalent to that of the soluble papain (20 Enzyme Units). Before use, the coupled papain was washed with water to remove the buffer in which it had been stored.

The Hill and Schmidt procedures using soluble and coupled papain were both used to hydrolyse ovalbumin and insulin (oxidized, B chain). At the end of the 24h digestion period, the solutions of digests containing native papain were lowered to pH 2.0 and lyophilized. Digest mixtures containing coupled papain were filtered through sintered glass to remove the coupled enzyme, and the filtrate was lyophilized. The subsequent digestion procedure was identical for all samples.

Control hydrolysates consisting of enzymes incubated in the absence of any added substrates were also prepared. The levels of amino acids present in these controls were subtracted from the levels of amino acids released from the enzyme-substrate mixtures.

Samples of the final digest products were acid hydrolysed according to the method described in Section 3.7.1.

3.12 PROTEOLYSIS *IN VITRO* ACCORDING TO THE METHOD OF WINKLER AND SCHÖN (1979)

This method depended upon a single incubation of an SDS extract of a seed meal with the enzymes proteinase-K and pronase.

Preparation of the substrate

Cowpeas (*Vigna unguiculata)* were dried, and after removal of their testas, were ground to a fine meal by use of a ball mill. 10mg samples were mixed with 0.8ml 0.1M NaOH and 0.2ml 15% (w/v) SDS, and incubated for 2h at 37°C. After this time, the samples were centrifuged (10000g, 5min), and the supernatants retained for hydrolysis.

Preparation of the enzymes

Proteinase-K coupled to Sepharose-4B (Section 3.2.1) was used. The preparation was assayed before use, and a volume of coupled enzyme with activity equivalent to 1mg of native proteinase-K was used for the hydrolysis of 20mg of meal. The coupled enzyme was washed free of its storage buffer and made up in an alkaline medium of 1.2% (w/v) SDS and 10% (v/v) ethanol.

The pronase used for hydrolysis had previously been dialysed against 0.1M sodium borate buffer, pH 8.3, 0.001M $CaCl_2$, for 40h at 37°C. The pronase was assayed, and a volume containing activity equivalent to 1mg of enzyme was used.

Conditions for hydrolysis

The substrate extract and the enzymes were made to a total volume of 5ml, by the addition of ethanol to a final concentration of 10% (v/v), and 0.1M sodium borate buffer, pH 8.3, 0.001M $CaCl_2$. These were incubated for 50h at 37°C, after which time the samples were centrifuged at 10000g for 15min and the supernatants retained.

Amino acid analyses

The final enzymic hydrolysate solutions were lowered to pH 1.8 and analysed using both the $Na^+$ and $Li^+$ buffer systems (Section 3.7).

Samples of the original meal (2.5mg) were also acid hydrolysed (Section 3.7.1) and analysed using the $Na^+$ buffer system.

## 3.13 THE DETECTION OF TRYPSIN INHIBITORS IN FAECAL EXTRACTS FROM RATS

### 3.13.1 The preparation of faecal extracts for trypsin inhibitor assay

0.5g of dried faeces from trypsin inhibitor and control fed rats were extracted for 48h at $4^{o}C$, in 10ml of 0.05M Tris-HCl, 0.02M $CaCl_2$, pH 8.2. After this time the extracts were centrifuged at 15000g for 10min. The supernatants were lyophilized and made up in 0.25ml of the Tris buffer. 10µl samples of these preparations were loaded on to the gel for assay of trypsin inhibitory activity (see below).

### 3.13.2 The use of immobilised trypsin for the assay of trypsin inhibitory activity (Gatehouse and Gatehouse, 1979)

Sepharose coupled trypsin was incorporated into agarose gel slabs and samples to be assayed were placed in wells cut in the slabs. During electrophoresis, trypsin inhibitors moved through the gel towards the anode until they combined with trypsin bound Sepharose. Therefore, when the gel was later incubated with a histochemical substrate for trypsin, stain was produced throughout the gel except where trypsin had been inactivated by combination with trypsin inhibitor. Clear "rockets" were therefore visible arising from those wells which contained trypsin inhibitor, with the height of the rocket proportional to the amount of inhibitor present.

Method

Assay gels were prepared by dissolving agarose in boiling water and adding buffer to give a final concentration of 1% agarose in 0.05M Tris-HCl, 0.02M $CaCl_2$, pH 8.2. Trypsin coupled Sepharose (method for preparation in Section 3.2.1) was diluted with distilled water to form a slurry, and mixed with the agarose solution at a concentration of 2.5ml slurry per 100ml of 1% agarose. Slab gels (volume 30ml) were cast on oven dried, glass plates (110 x 205mm), precoated with 0.5% agarose in 50% aqueous methanol. Electrophoresis was carried out on flatbed apparatus using barbitone-sodium buffer, pH 8.6. Gels were run overnight at 5V per cm, after which they were rinsed in distilled water and air dried. They were then incubated in the following: 30mg fast blue B salt dissolved in 30ml sodium acetate, 0.1M, pH 6.5; 24ml sodium chloride, 0.15M, and 3ml potassium cyanide, 0.02M, containing 24mg BABA dissolved in 3ml of methanol. After staining for 30min, the gels were finally rinsed and air dried.

Preparation of samples for electrophoresis

Samples of trypsin inhibitors were made up in 0.05M Tris-HCl, 0.02M $CaCl_2$, such that a loading of 10µl would give the required concentration of inhibitor.

3.13 Affinity chromatographic purification of trypsin inhibitor

A column (15 x 2cm) of Sepharose-4B to which trypsin had been coupled by cyanogen bromide activation (Section 3.2.1) was used. This was equilibrated with 0.1M sodium acetate, pH 4.0 with a flow rate of 60ml/h. The sample (1.0ml) was applied to the surface of the column,

and the absorbance of the effluent monitored continuously at 280nm (Uvicord II, LKB Ltd). After elution of the first protein peak, the column was washed first with 0.3M NaCl, 0.01M $CaCl_2$, and then with 0.01M HCl, 0.3M NaCl, 0.01M $CaCl_2$, pH 2.1. The peak produced was collected and lyophilized.

3.13.4 Preparation of faecal extracts for affinity chromatography

0.5g of dried faeces collected from trypsin inhibitor fed rats was extracted for 48h at 4°C, in 10ml of 0.05M tris-HCl, 0.02M $CaCl_2$, pH 8.2. The mixture was then centrifuged at 15000g for 10min. The supernatant was lyophilized and made up in 1.0ml pH 4.0 buffer for application to the column.

3.13.5 Preparation of faecal extracts for protein analysis by SDS polyacrylamide gel electrophoresis

100mg of dried faeces from either trypsin inhibitor fed, or control fed rats, were extracted for 48h at 4°C, in 1.0ml of 0.05M Tris-HCl, 0.02M $CaCl_2$, pH 8.2. After extraction the samples were centrifuged and the supernatants lyophilized. Samples were redissolved in 0.1ml SDS sample buffer, of which 0.025ml was loaded on to gels.

# 4.0 RESULTS

## 4.1 THE CHOICE AND PREPARATION OF ENZYMES FOR PROTEOLYSIS *IN VITRO*

### 4.1.1 The purification and assay of prolidase

The purification of prolidase was continued to the end of "step 2" of the method of Davis and Smith (1957), as recommended by Dvorak (1968). By this stage a 9-fold increase in the specific activity of the preparation had been achieved (Table 8).

To ascertain whether the freshly prepared enzyme contained any enzymic activity of a specificity not present in the commercially available preparation, both preparations were assayed using a range of substrates (Table 9). Both preparations contained prolidase activity as shown by their hydrolysis of peptides of the type X-Pro (Gly-L-Pro and Val-L-Pro). Both preparations also hydrolysed Pro-L-Gly, indicating the presence of some prolinase (iminodipeptidase) type activity. Prolinase was present in the commercial preparation at a level of 0.38% of the prolidase activity, and in the freshly made preparation, prolinase activity amounted to 0.92% of the prolidase activity. No hydrolysis of Pro-L-Ala or Pro-L-Val was evident after 30min of assay. Both preparations caused a low level of hydrolysis of leucine-p-nitroanilide, and must therefore contain some amino-peptidase activity.

According to their calculated $C_1$ values (Fig. 2 and Table 10), the commercial prolidase had an activity 4.0 times that of the freshly purified preparation.

Table 8 The purification of prolidase from pig kidney acetone extract

| Fraction | Total activity $\Delta A_{242}$ $min^{-1}$ | Activity recovered % | Specific activity $\Delta A_{242} min^{-1} mg^{-1}$ protein |
|---|---|---|---|
| First supernatant | 15.00 | 100.0 | 0.018 |
| Precipitate after ammonium sulphate | 8.19 | 54.6 | 0.039 |
| Supernatant after magnesium chloride | 6.84 | 45.6 | 0.069 |
| Final preparation | 3.28 | 21.8 | 0.168 |

Samples were taken throughout the purification of prolidase and assayed for protein content and enzyme activity. Prolidase activity is expressed as the decrease in absorbance at 242nm due to the hydrolysis of Gly-L-Pro, measured over 7min.

Table 9 Enzymic activities present in commercial and freshly prepared prolidase preparations

| Substrate | Prolidase: Commercial preparation Enzyme Units per mg protein | Prolidase: Fresh preparation Enzyme units per mg protein |
|---|---|---|
| Gly-L-Pro | 31.49 | 6.52 |
| Val-L-Pro | 27.84 | 5.84 |
| Pro-L-Gly | 0.12 | 0.06 |
| Pro-L-Val | 0 | 0 |
| Pro-L-Ala | 0 | 0 |
| Leu-p-nitroanilide | 0.02 | 0.01 |

Assays were carried out as described for prolidase assay, substituting the appropriate substrates in place of Gly-L-Pro

FIGURE 2 DETERMINATION OF THE FIRST ORDER RATE CONSTANT, k, FOR FRESHLY PREPARED AND COMMERCIAL PREPARATIONS OF PROLIDASE

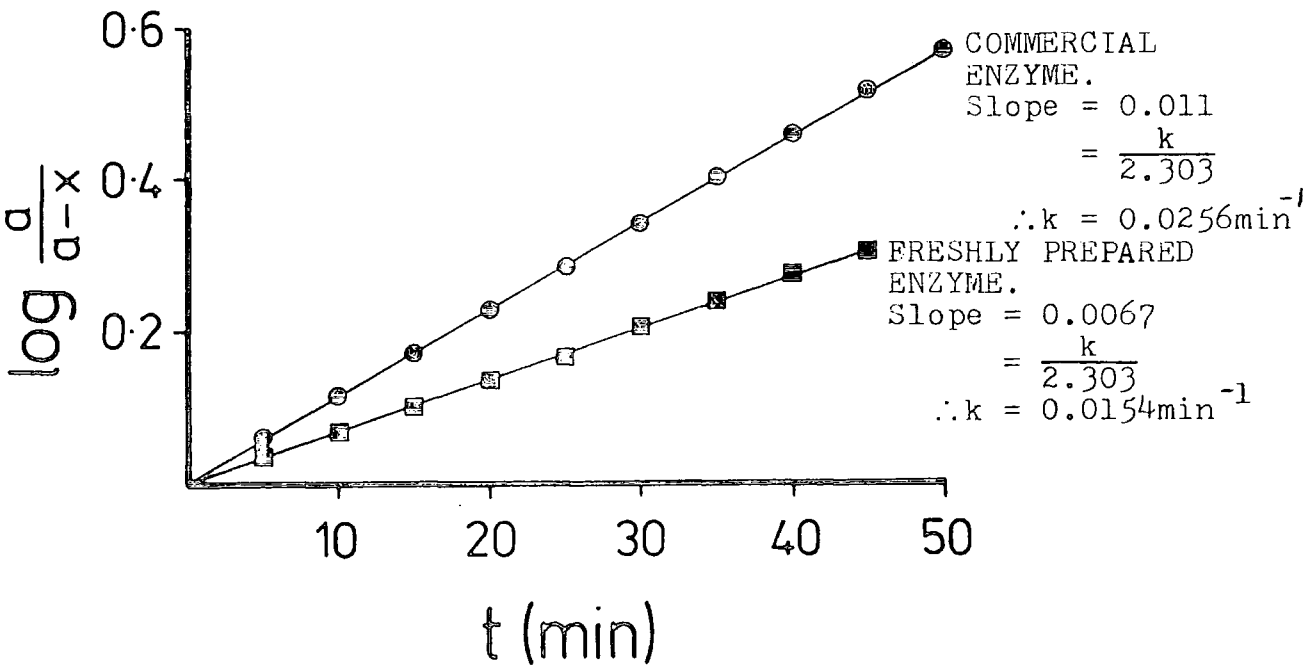



TABLE 10 DETERMINATION OF THE FIRST ORDER PROTEOLYTIC COEFFICIENT, $C_1$ , FOR FRESHLY PREPARED AND COMMERCIAL PREPARATIONS OF PROLIDASE

| Preparation | Volume used (ml) | Protein content (mg) | Nitrogen content (mg) | k ($min^{-1}$) | $C_1$ |
|---|---|---|---|---|---|
| Commercial | 0.001 | 0.0100 | 0.0016 | 0.0256 | 16.0 |
| Fresh | 0.005 | 0.0242 | 0.0039 | 0.0154 | 3.95 |

### 4.1.2 Assay of a range of enzymes for prolidase, prolinase and aminopeptidase-type activities

The enzymes assayed and their levels of activity are shown in Table 11. Apart from prolidase, acylase was the only enzyme found to contain prolidase-type activity; acylase, like prolidase, is extracted from pig kidney microsomes. No enzyme preparation other than prolidase showed any prolinase-type activity. Pronase showed a high level of aminopeptidase activity, four times that of the aminopeptidase-M preparation.

Table 11 Assay of a range of enzymes for prolidase, prolinase and aminopeptidase-type activities

| Type of activity: | Prolidase | Prolinase | Aminopeptidase |
|---|---|---|---|
| Substrate: | Gly-L-Pro | Pro-L-Gly | Leucine-p-nitroanilide |
| Enzyme | Activity (Enzyme units $mg^{-1}$ protein) | Activity (Enzyme units $mg^{-1}$ protein) | Activity (Enzyme units $mg^{-1}$ protein) |
| Acylase | 1.80 | 0 | nd |
| Aminopeptidase-M | 0 | 0 | 4.20 |
| Elastase | 0 | 0 | nd |
| Intestinal peptidases | 0 | 0 | nd |
| Pancreatin | 0 | 0 | nd |
| Papain | 0 | 0 | nd |
| Prolase | 0 | 0 | nd |
| Prolidase (commercial preparation) | 31.49 | 0.12 | 0.02 |
| Pronase | 0 | 0 | 17.00 |
| Proteinase-K | 0 | 0 | nd |

Assays for prolidase, prolinase and aminopeptidase activity were carried out as described in the Methods section.

Systematic names and sources of the enzymes are given in the Materials section.

nd = not determined.

4.1.3 The use of the Hill and Schmidt method for the enzymic hydrolysis of BSA

Shown in Table 12 are the recoveries of amino acids after enzymic hydrolysis of BSA by the modified Hill and Schmidt procedure ("Enzymic hydrolysis method 1"), together with the recoveries of amino acids after replacement of the commercial prolidase preparation used in the modified procedure, by a freshly purified sample of prolidase ("Enzymic hydrolysis method 2"). Acidic hydrolysis of the enzymic hydrolysates revealed little or no increase in the levels of amino acids for either of the enzymic methods, indicating that complete enzymic hydrolysis of the BSA had been achieved in both cases.

However, both enzymic methods gave slightly different results for the levels of amino acids present in the BSA, and acidic hydrolysis of a sample of the original BSA gave higher yields for all amino acids. There has therefore been a loss of amino acids during the enzymic process. The enzymic procedure used in this experiment differed from that subsequently used, since in this experiment an internal standard (nor-leucine) was only added at the end of the enzymic hydrolysis, and could therefore not reflect losses which occurred during the precipitation and filtration steps of the procedure.

Each hydrolysis was carried out in duplicate and for each pair of results, the coefficient of variation was calculated by expression of the standard deviation as a percentage of the mean. For the acid hydrolysates, the average coefficient of variation was 2.1%. For enzymic hydrolysis using Method 1, the average coefficient of variation was 4.4%, and for Method 2, the average coefficient of variation was 4.6%.

It can be seen from Table 12 that enzymic hydrolysis allowed the determination of tryptophan, which was completely destroyed during acid hydrolysis. Because the $Na^+$ buffers used during the amino acid analysis in this experiment do not allow the complete separation of Thr, Ser, Gln and Asn which are present in enzymic hydrolysates, values are given for the total recoveries of these four amino acids. This problem is to be discussed more fully below.

Table 12 The acidic and enzymic hydrolysis of bovine serum albumin (BSA)

| Amino acid | Acid hydrolysis | Enzymic hydrolysis : method 1 | | | Enzymic hydrolysis : method 2 | | |
|---|---|---|---|---|---|---|---|
| | | EH | EH + AH | $\frac{(EH)}{(EH + AH)}$ % | EH | EH + AH | $\frac{(EH)}{(EH + AH)}$ % |
| Cys A | 0 | 0.25 | 1.41 | - | 6.09 | 9.22 | - |
| Asp | 42.71 | 18.59 | 20.83 | - | 19.71 | 17.41 | - |
| (Thr+Ser+Gln+Asn) | - | 29.23 | - | - | 31.83 | - | - |
| Thr | 23.14 | - | 14.42 | - | - | 19.19 | - |
| Ser | 20.80 | - | 14.20 | - | - | 13.38 | - |
| Gln | - | - | - | - | - | - | - |
| Asn | - | - | - | - | - | - | - |
| ~~Gln~~Glu | 55.55 | 24.16 | 26.34 | - | 24.54 | 23.34 | - |
| Pro | 32.95 | 21.34 | 22.04 | 96.8 | 17.92 | 18.37 | 97.6 |
| Gly | 13.16 | 6.55 | 6.17 | 106.2 | 5.21 | 4.91 | 106.1 |
| Ala | 35.62 | 20.08 | 20.01 | 100.3 | 21.12 | 22.30 | 94.7 |
| Cys/2 | 22.36 | 13.24 | 17.00 | - | 15.32 | 15.91 | - |
| Val | 24.23 | 11.16 | 10.12 | 110.2 | 12.72 | 9.52 | 106.3 |
| Met | 3.58 | 2.89 | 0.74 | - | 2.90 | 0.82 | - |
| Ile | 11.59 | 7.97 | 8.49 | 93.9 | 6.99 | 6.77 | 103.2 |
| Leu | 45.45 | 22.53 | 23.72 | 95.0 | 23.34 | 21.17 | 112.0 |
| Tyr | 17.70 | 11.30 | 12.49 | 90.5 | 10.26 | 10.62 | 96.6 |
| Phe | 31.02 | 25.35 | 24.46 | 103.6 | 23.93 | 24.91 | 96.1 |
| His | 15.16 | 11.08 | 12.05 | 89.4 | 11.60 | 12.50 | 92.8 |
| Lys | 33.56 | 19.93 | 17.25 | 115.5 | 13.13 | 16.51 | 79.5 |
| Trp | 0 | 2.38 | 0 | - | 3.70 | 0 | - |
| Arg | 15.25 | 10.75 | 11.40 | 94.3 | 7.44 | 8.25 | 90.2 |

EH = enzymic hydrolysis, EH + AH = enzymic hydrolysis followed by acidic hydrolysis.
Samples of the BSA, and of the enzymic hydrolysates, were acid hydrolysed, *in vacuo* for 22h at 105°C.
In method 1, enzymic hydrolysis was carried out by the modified Hill and Schmidt procedure using standard preparations of all enzymes. In method 2, the standard prolidase was replaced by a fresh preparation from pig kidney acetone extract.
Results are expressed as numbers of amino acids per BSA molecule, and are corrected for a moisture content of 9.74%.

4.1.4 The use of the modified Hill and Schmidt method for the enzymic hydrolysis of insulin (B chain) and ovalbumin

Insulin (oxidized, B chain) and ovalbumin were used to test the efficiency of the modified Hill and Schmidt procedure, and the results are shown in Tables 13 and 14, together with the published compositions of these proteins.

For the insulin, enzymic hydrolysis gave the correct composition (when rounded to the nearest whole numbers) for the molecule, except for the levels of Thr, Ser, Gln and Asn, which were not separated when the amino acid analyser was used with buffers in the $Na^+$-form. However, the enzymic hydrolysates did give the correct yields of Asp and Glu. The deamidation of Asn and Gln during acid hydrolysis meant that these amino acids appeared only as part of the total Asp and Glu levels in acid hydrolysates. Subtraction of the Asp and Glu values found after enzymic hydrolysis from those found after acid hydrolysis therefore gave individual values for Asp, Asn, Glu and Gln. The combination of information from both enzymic and acidic hydrolysates thus gave values of 0.73 and 1.17 for Asn and Gln, which corresponded with the correct composition of the insulin molecule.

The levels of amino acids released after enzymic hydrolysis have been expressed as percentages of the levels of amino acids found after acid hydrolysis of the enzymic digests. If the enzymic digestion has incompletely hydrolysed the protein, an increase in amino acids may be expected after a subsequent acid hydrolysis. However, Asn, Cys, Gln, Ser, Thr, Trp and Tyr, may not be compared by this method since they are acid labile.

For most of the amino acids of insulin, the levels after enzymic hydrolysis were within ± 10% of those found after acid hydrolysis.

The exceptions were Leu and His, where the levels found in the acid hydrolysate were higher than found in the composition of the molecule, and Arg, where a very low level was recovered in the acid hydrolysate.

The enzymic hydrolysis of ovalbumin (Table 14) achieved a similarly high yield of amino acids, with the levels of most amino acids after enzymic hydrolysis within ± 10% of those found after acid hydrolysis, except for Ala (88.53%) and Arg (110.34%). The values of Asp and Glu found after enzymic hydrolysis were only 74% and 77% of those expected. However, after subsequent acid hydrolysis of the enzyme hydrolysate, the total (Asp + Asn) and (Glu + Gln) levels were 97.5% and 93% of those expected. These results indicate that compared with the known composition of ovalbumin presented, this sample of ovalbumin contained greater proportions of aspartate and glutamate in the amide forms.

Table 13 Amino acid composition of oxidized insulin (B chain), and the yields of amino acids after enzymic hydrolysis using the modified Hill and Schmidt procedure

| Amino acid | Composition of insulin (oxidized B chain) | Enzymic hydrolysis (EH) | Acid hydrolysis of enzymic hydrolysate (EH + AH) | $\frac{(EH)}{(EH + AH)}$ % |
|---|---|---|---|---|
| CysA | 2 | 2.00 | 2.03 | 98.52 |
| Asp | 0 | 0.16 | 1.33 | - |
| (Thr,Ser,Gln,Asn) | - | 2.73 | - | - |
| Thr | 1 | - | 0.99 | - |
| Ser | 1 | - | 1.10 | - |
| Gln | 1 | - | 0 | - |
| Asn | 1 | - | 0 | - |
| Glu | 2 | 2.01 | 2.73 | - |
| Pro | 1 | 1.22 | 1.31 | 92.97 |
| Gly | 3 | 3.16 | 3.20 | 98.49 |
| Ala | 2 | 2.35 | 2.28 | 103.06 |
| Cys/2 | 0 | 0 | 0 | - |
| Val | 3 | 2.44 | 2.43 | 100.44 |
| Met | 0 | 0 | 0 | - |
| Ile | 0 | 0 | 0.04 | - |
| Leu | 4 | 4.03 | 4.30 | 93.72 |
| Tyr | 2 | 1.63 | 1.65 | 98.49 |
| Phe | 3 | 3.14 | 3.41 | 92.16 |
| His | 2 | 1.66 | 2.23 | 74.74 |
| Lys | 1 | 1.05 | 1.14 | 92.31 |
| Trp | 2 | 1.56 | 0 | - |
| Arg | 1 | 0.50 | 0.31 | 155.14 |

EH Enzymic hydrolysis

AH Acidic hydrolysis

Results are expressed as the number of amino acids per insulin molecule.
Samples of the enzymic digests were further acid hydrolysed *in vacuo*, for 22h at 105°C.
The composition of insulin is according to Ryle *et al* (1955).

Table 14 Amino acid composition of ovalbumin and the yields of amino acids after enzymic hydrolysis using the modified Hill and Schmidt procedure

| Amino acid | Composition of ovalbumin | Enzymic hydrolysis (EH) | Acid hydrolysis of enzymic hydrolysate (EH + AH) | $\frac{(EH)}{(EH + AH)}$ % |
|---|---|---|---|---|
| CysA | - | 1.67 | 0.20 | - |
| Asp | 14 | 10.44 | 29.93 | - |
| (Thr,Ser,Gln,Asn) | - | 46.99 | - | - |
| Thr | 15 | - | 13.69 | - |
| Ser | 38 | - | 28.52 | - |
| Gln | 15 | - | - | - |
| Asn | 17 | - | - | - |
| Glu | 33 | 25.48 | 44.48 | - |
| Pro | 14 | 12.73 | 12.91 | 98.60 |
| Gly | 19 | 17.57 | 18.94 | 92.76 |
| Ala | 35 | 27.24 | 30.77 | 88.53 |
| Cys/2 | 6 | 2.44 | 0 | - |
| Val | 31 | 33.48 | 31.10 | 107.65 |
| Met | 16 | 13.65 | 3.85 | - |
| Ile | 25 | 20.71 | 22.71 | 91.19 |
| Leu | 32 | 27.87 | 30.79 | 90.51 |
| Tyr | 10 | 7.53 | 8.31 | 90.61 |
| Phe | 20 | 17.00 | 16.68 | 101.92 |
| His | 7 | 6.75 | 7.43 | 90.85 |
| Lys | 20 | 17.32 | 17.98 | 96.32 |
| Trp | 3 | 2.38 | 0 | - |
| Arg | 15 | 14.40 | 13.05 | 110.34 |

Results are expressed as the number of amino acids per ovalbumin molecule. Samples of the enzymic hydrolysates were further acid hydrolysed *in vacuo*, for 22h at 105°C.
The composition of ovalbumin is according to L. McReynolds *et al*, 1978.

4.1.5 The recovery of amino acids from a standard mixture after enzymic hydrolysis by the Hill and Schmidt method, and acid hydrolysis

The recoveries of the amino acids after the enzymic hydrolysis, and subsequent acid hydrolysis procedures are shown in Table 15. After enzymic hydrolysis, the recovery of all amino acids was within ± 10% of the original value, except for CysA, Arg and Pro. After enzymic and acid hydrolysis of the samples, there were also reduced recoveries of the acid labile amino acids, Ser, Cys, Met and Trp.

When the recoveries after enzymic hydrolysis were expressed as percentages of the recoveries after both enzymic and acidic hydrolysis, the mean value (excluding acid-labile amino acids and proline) was 102.6%, with the largest error (among the non acid-labile amino acids) shown in the recovery of alanine; 113.4%.

Table 15 Recovery of amino acids from a standard mixture after enzymic and acidic hydrolysis

| Amino acid | ENZYMIC HYDROLYSIS | | | ENZYMIC AND ACIDIC HYDROLYSIS | | | |
|---|---|---|---|---|---|---|---|
| | Amino acids recovered (µmoles) mean | S.D. | % error of mean | Amino acids recovered (µmoles) mean | S.D. | % error of mean | $\frac{(EH)}{(EH + AH)}$ % |
| CysA | 0.0218 | 0.0014 | 12.78 | 0.0200 | 0.0010 | 19.88 | 108.9 |
| Asp | 0.0258 | 0.0021 | 3.24 | 0.0246 | 0.0023 | 1.67 | 105.0 |
| Thr | 0.0268 | 0.0019 | 7.04 | 0.0270 | 0.0009 | 8.14 | 99.0 |
| Ser | 0.0243 | 0.0034 | 2.96 | 0.0182 | 0.0012 | 27.37 | 133.6 |
| Glu | 0.0230 | 0.0028 | 8.16 | 0.0258 | 0.0028 | 1.55 | 89.1 |
| Pro | 0.0218 | 0.0005 | 12.76 | 0.0186 | 0.0084 | 25.56 | 117.2 |
| Gly | 0.0269 | 0.0016 | 7.40 | 0.0238 | 0.0026 | 4.96 | 113.0 |
| Ala | 0.0263 | 0.0026 | 5.24 | 0.0232 | 0.0002 | 9.28 | 113.4 |
| Cys/2 | 0.0232 | 0.0024 | 7.05 | 0.0110 | 0.0006 | 55.93 | 210.5 |
| Val | 0.0274 | 0.0015 | 9.56 | 0.0274 | 0.0017 | 9.47 | 100.1 |
| Met | 0.0249 | 0.0020 | 0.56 | 0.0110 | 0.0020 | 55.84 | 225.1 |
| Ile | 0.0273 | 0.0009 | 9.28 | 0.0271 | 0.0014 | 8.22 | 100.9 |
| Leu | 0.0274 | 0.0032 | 9.77 | 0.0283 | 0.0012 | 13.28 | 96.9 |
| Tyr | 0.0227 | 0.0033 | 9.24 | 0.0216 | 0.0034 | 13.56 | 105.0 |
| Phe | 0.0262 | 0.0010 | 4.80 | 0.0260 | 0.0010 | 4.04 | 100.7 |
| His | 0.0245 | 0.0020 | 2.08 | 0.0240 | 0.0018 | 3.83 | 102.0 |
| Lys | 0.0258 | 0.0056 | 3.24 | 0.0271 | 0.0016 | 8.39 | 95.3 |
| Trp | 0.0234 | 0.0113 | 6.32 | 0 | - | - | - |
| Arg | 0.0208 | 0.0031 | 16.68 | 0.0224 | 0.0016 | 10.62 | 93.2 |

0.025µmoles of each amino acid were present at the start of the experiment.
Samples were put through the modified Hill and Schmidt enzymic digestion procedure, followed by acid hydrolysis *in vacuo*, for 22h at 105°C.
The mean values represent the averages of five samples.
The Standard Deviation (S.D.) is calculated using N - 1 weighting.
The "% error of the mean" is the difference of the mean from the ideal recovery, 0.025µmoles, expressed as a percentage.

$\frac{(EH)}{(EH + AH)}$ %. is the recovery of amino acids after the enzymic hydrolysis procedure, expressed as a percentage of the recovery of amino acids after both enzymic and acidic hydrolysis.

4.1.6 The preparation of immobilized enzymes

1. Assay of immobilized enzymes

Spectrophotometric assay of immobilized enzymes was often made difficult due to light scattering caused by the support. This was overcome in some cases by discontinuous measurement; removing aliquots from a digestion mixture at intervals, and assaying after filtration. pH-stat methods provided a convenient means for continuous assay of immobilized enzymes. Use of the pH-stat required a substrate which would cause a change in pH value of the mixture as the reaction proceeded, and reproducible results were produced as long as the immobilized enzyme was kept well suspended by stirring. This method proved unsuitable for enzymes coupled to porous glass because of the friability of the glass beads.

2. The immobilization of papain, prolidase, proteinase-K and trypsin

Papain, proteinase-K and trypsin were all bound to Sepharose by means of cyanogen bromide activation (Table 16). Prolidase was only successfully coupled to CH-Sepharose if previous dialyzed against a manganese solution: $Mn^{++}$ ions are necessary for activation of the enzyme.

The activities of these enzymes were proportional to the amount of enzyme bound, by contrast with the coupling of pronase (see below), where preparations with a relatively good binding of enzyme showed only a low retention of activity.

Table 16 The immobilization of papain, prolidase, proteinase-K and trypsin

| Enzyme | Support | Reagent used for activation of the support | Modification to standard conditions | Activity $mg.ml^{-1}$ |
|---|---|---|---|---|
| Papain | Sepharose-4B | CNBr | - | 1.69 |
| Prolidase | Sepharose-6B | CNBr | - | 0 |
| Prolidase | Activated CH-Sepharose | - | Prolidase dialysed against 0.01M $Mn^{++}$ for 24h before immobilization | 0.75 |
| Proteinase-K | Sepharose-6B | CNBr | - | 0.73 |
| Trypsin | Sepharose-4B | CNBr | - | 1.22 |

The activity of the coupled enzyme is expressed in terms of mg of active enzyme per ml of swollen gel.

3. The immobilization of pronase

Pronase was coupled to a variety of supports, but in each case the coupled enzyme was of low activity (Table 17), even though binding of the enzyme to the support may have been relatively high. Pronase coupled to Sepharose by the commonly used cyanogen bromide method was found to have little activity. Pronase coupled to porous glass was difficult to assay, and quickly lost activity.

Preparations of pronase bound to carboxymethylcellulose showed relatively high enzymic activities. Variation of the pH values of the coupling media (Fig. 3) was found to affect the extent of binding and the activity of the coupled enzyme: a coupling medium of pH 6-7 was found to give the optimum binding and enzyme activity. The greatest amount of binding and activity was achieved by use of E.D.A.C. to activate carboxymethylcellulose for coupling to pronase, however, Fig. 4 shows how the preparation lost over half its activity within the first week of storage. Pronase coupled to activated CH-Sepharose was the only preparation which retained the original level of activity throughout storage, and although the extent of coupling was low, that which was bound was of relatively high activity. This coupled pronase still retained its ability to hydrolyse large substrates (ovalbumin) and small substrates (BAEE), and the aminopeptidase activity was still present (Table 18).

Table 17 Retention of enzymic activity on coupling pronase to a variety of supports

| Support | Reagent used for activation of support | Modification to standard conditions | Extent of coupling, % | Activity of 1mg coupled enzyme, % |
|---|---|---|---|---|
| Sepharose-4B | CNBr | - | 46.0 | 2.6 |
| Sepharose-4B | CNBr | Urea replaced by 0.1M sodium acetate | 25.6 | 5.1 |
| aminopropyl glass | glutaraldehyde | - | 30.0 | 8.1 |
| aminoethyl-aminopropyl glass | glutaraldehyde | - | 36.0 | 12.5 |
| carboxylmethyl cellulose | E.D.A.C. | - | 56.3 | 18.8 |
| carboxymethyl cellulose | C.M.C.M.T. | coupling buffers of varied pH (see Fig. 3) | 18.4-35.5 | 0-10.77 |
| Activated CH-Sepharose-4B | - | - | 20.0 | 15.4 |

Extent of coupling is expressed as the percentage of the total protein used which has been bound to the support.
Activity of 1mg of the coupled enzyme is expressed as a percentage of the activity given by 1mg of native enzyme.
The activities of glass coupled enzymes are measured by TNBS assay, using ovalbumin as substrate.
The activities of Sepharose and cellulose coupled enzymes are measured by pH-stat assay, using BAEE as substrate.

FIGURE 3 AMOUNT OF PRONASE COUPLED, AND ACTIVITY RELATIVE TO NATIVE PRONASE, OF CM-CELLULOSE-PRONASE PREPARATIONS, MADE OVER A RANGE OF pH VALUES BETWEEN pH 3 AND pH 9

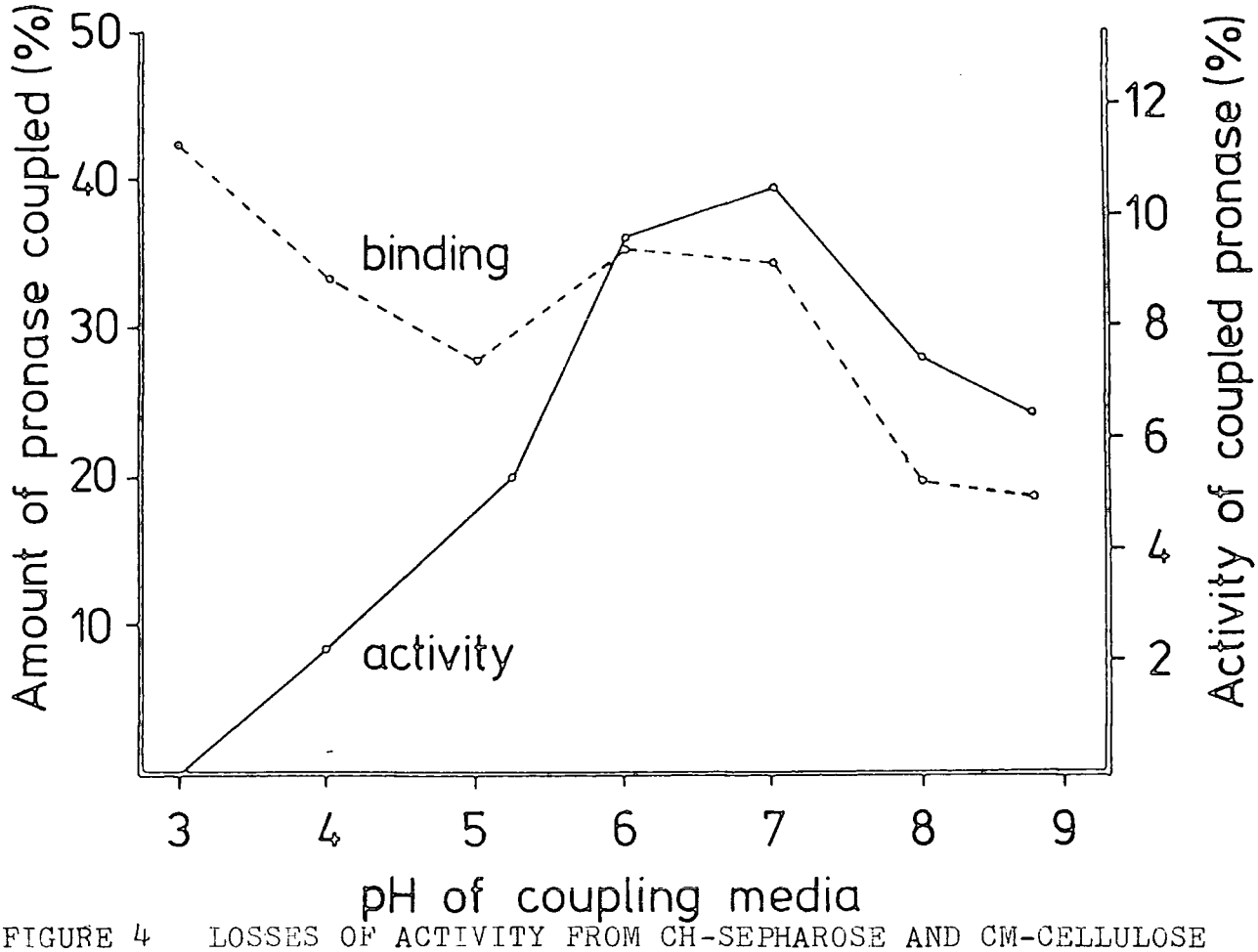
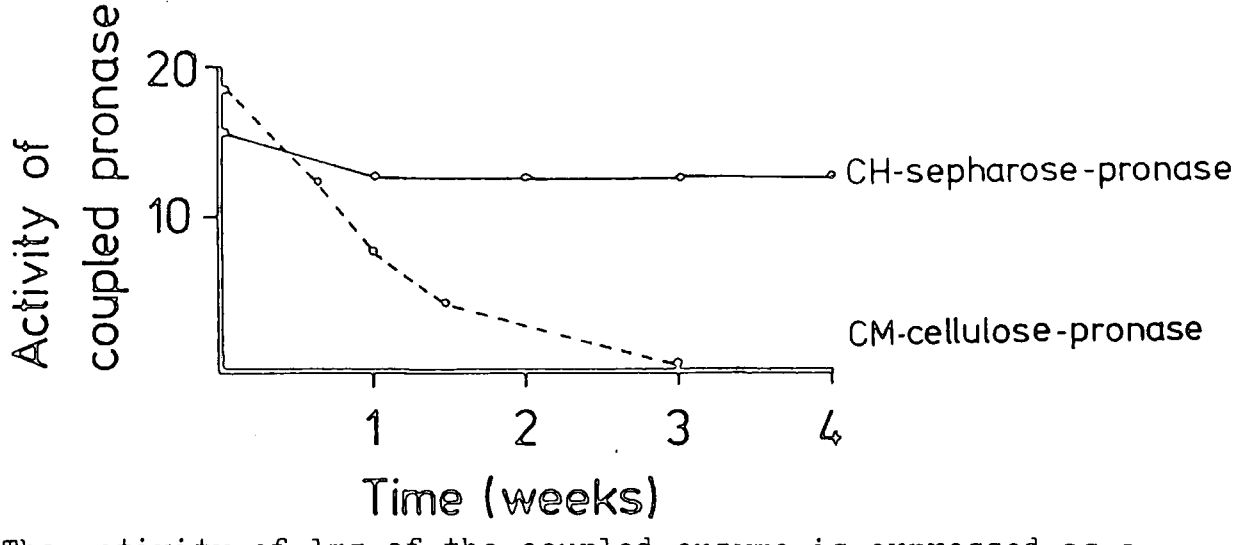



FIGURE 4 LOSSES OF ACTIVITY FROM CH-SEPHAROSE AND CM-CELLULOSE COUPLED PRONASE PREPARATIONS




The activity of 1mg of the coupled enzyme is expressed as a percentage of the activity given by 1mg of the native enzyme.

Table 18 Specificity of immobilized pronase towards different types of substrate

| Substrate | Type of enzymic activity present | Activity of soluble pronase (%) | Activity of CH-Sepharose-pronase (%) |
|---|---|---|---|
| L-leucine-p-nitroanilide | amino peptidase | 100 | 16.0 |
| B.A.E.E. | peptidase | 100 | 15.4 |
| Ovalbumin | protease | 100 | 14.2 |

The enzymic activity shown by 1mg of immobilised pronase towards a given substrate is expressed as a percentage of the activity of 1mg of soluble pronase towards the same substrate.

4.1.7 The use of a mixture of immobilized enzymes for the hydrolysis of ovalbumin and insulin (B chain)

The hydrolysis of ovalbumin by coupled enzyme mixtures is shown in Table 19. Hydrolysis with Sepharose-pronase resulted in the release of approximately 66% of the amino acids of ovalbumin, and this percentage of amino acids in the free form increased as coupled protinease K or prolidase were included in the digestion procedure, to a maximum recovery of 85% using all three coupled enzymes. Pro was only released in the presence of prolidase. The yields of Ala, Val, Tyr and Lys were greater than expected from the published ovalbumin composition. However, acid hydrolysis of the same preparation of ovalbumin (Table 19b) also showed an increased level of these amino acids. (The published ovalbumin composition was used in Table 19 for comparison with the enzymic hydrolysate, because separate values for Asp, Asn, Glu and Gln were required.) While enzymic hydrolysis using all three coupled enzymes gave a good yield of most amino acids, there was a relatively poor recovery of the acid and hydroxyl amino acids.

The recovery of amino acids using the same coupled enzymes to hydrolyse the B chain of insulin (Table 20) was also good for most amino acids, but, as with ovalbumin, the poorest yield was of the acidic amino acids (see Table 21), with, for example, Glu having a recovery of 15%. In case this effect was due to these amino acids binding to the Sepharose supports, a variety of buffers of varying salt concentration and pH value were used to wash the Sepharose after the digestion procedure, but none of these was effective in increasing the yield of acidic amino acids.

Table 19 The yield of amino acids from ovalbumin after hydrolysis by coupled enzymes

| Amino acid | Ovalbumin composition | Enzymes used | | | |
|---|---|---|---|---|---|
| | | Sepharose-pronase | Sepharose-pronase + Sepharose-proteinase-K | Sepharose-pronase + Sepharose-prolidase | Sepharose-pronase + Sepharose-proteinase-K + Sepharose-prolidase |
| CysA | - | 1.1 | 0.7 | 0.6 | 0.7 |
| Asp | 14 | 5.1 | 4.1 | 5.0 | 5.0 |
| (Thr,Ser, Gln,Asn) | 85 | 48.9 | 41.9 | 54.0 | 58.5 |
| Glu | 33 | 5.3 | 6.3 | 13.0 | 13.8 |
| Pro | 14 | 0 | 0 | 6.7 | 10.4 |
| Gly | 19 | 4.7 | 6.5 | 10.0 | 12.0 |
| Ala | 35 | 31.6 | 31.5 | 37.6 | 41.7 |
| Cys/2 | 6 | 4.9 | 4.2 | 1.7 | 2.6 |
| Val | 31 | 32.8 | 34.4 | 33.6 | 31.6 |
| Met | 16 | 14.6 | 16.5 | 16.2 | 16.0 |
| Ile | 25 | 18.8 | 21.5 | 21.9 | 22.0 |
| Leu | 32 | 23.9 | 26.6 | 29.0 | 28.8 |
| Tyr | 10 | 10.9 | 12.3 | 11.7 | 11.9 |
| Phe | 20 | 16.2 | 17.7 | 18.5 | 18.4 |
| His | 7 | 6.5 | 7.2 | 8.4 | 7.4 |
| Lys | 20 | 15.2 | 22.5 | 23.2 | 25.1 |
| Arg | 15 | 10.8 | 13.7 | 12.9 | 14.6 |
| Total number of residues | 382 | 251.3 | 267.6 | 304.0 | 320.5 |
| Expressed as % of total composition | 100 | 65.8 | 70.1 | 79.6 | 83.9 |

Ovalbumin composition according to McReynolds *et al* (1978)
Results are given as the number of amino acids per ovalbumin molecule.

Table 19b Amino acid composition of hen egg white ovalbumin after acid hydrolysis

| Amino acid | 22h | | 50h | | 90h | | final value | expected composition |
|---|---|---|---|---|---|---|---|---|
| | mean | coefficient of variation, % | mean | coefficient of variation, % | mean | coefficient of variation, % | | |
| Asp | 37.8 | 6.1 | 37.0 | 2.9 | 36.8 | 0.2 | 37 | 31 |
| Thr | 14.8 | 4.9 | 14.1 | 1.1 | 11.7 | 1.4 | 15 | 15 |
| Ser | 35.3 | 4.1 | 31.1 | 10.7 | 15.7 | 1.3 | 39 | 38 |
| Glu | 58.8 | 4.2 | 54.5 | 2.6 | 56.7 | 2.6 | 57 | 48 |
| Gly | 19.4 | 6.1 | 18.8 | 2.0 | 20.1 | 1.2 | 19 | 19 |
| Ala | 40.1 | 5.9 | 47.6 | 5.1 | 40.8 | 2.6 | 43 | 35 |
| Val | 31.8 | 3.5 | 31.0 | 4.1 | 32.9 | 0.1 | 32 | 31 |
| Met | 15.7 | 3.9 | 14.9 | 2.3 | 16.1 | 7.2 | 16 | 16 |
| Ile | 24.3 | 0.3 | 23.7 | 1.9 | 25.4 | 4.1 | 25 | 25 |
| Leu | 35.0 | 2.3 | 34.1 | 1.1 | 34.2 | 2.1 | 34 | 32 |
| Tyr | 11.0 | 1.3 | 9.9 | 9.5 | 5.7 | 4.4 | 12 | 10 |
| Phe | 20.3 | 3.8 | 19.5 | 9.5 | 21.5 | 4.5 | 20 | 20 |
| His | 6.8 | 7.6 | 6.2 | 1.5 | 6.6 | 0.3 | 7 | 7 |
| Lys | 25.6 | 5.7 | 22.3 | 1.5 | 18.5 | 0.2 | 22 | 20 |
| Arg | 15.0 | 4.3 | 15.1 | 0.3 | 12.8 | 6.1 | 14 | 15 |

Mean values are the averages of two hydrolysates, hydrolysed and analysed under identical conditions.
Coefficient of variation is the standard deviation expressed as a percentage of the mean.
For Thr, Ser and Tyr, the final value was determined after correction for destruction during hydrolysis, using the equation of Moore and Stein (see Methods). For Ile, the value after 90h hydrolysis was taken as the final value.
For all other amino acids, the final value was taken as the mean of the values found at 20, 50 and 90h.
Final values are rounded to the nearest whole number.
Expected composition from McReynolds *et al* (1978).

Table 20 Amino acid composition of oxidised insulin (B chain), and yields of amino acids after acid hydrolysis and hydrolysis using coupled enzymes

| Amino acid | Insulin composition (oxidised B chain) | Composition expected after acid hydrolysis | Composition found after acid hydrolysis | Composition found after enzymic hydrolysis |
|---|---|---|---|---|
| CysA | 2 | 2 | 2.1 | 0.6 |
| Asp | 0 | 1 | 0.8 | 0.1 |
| (Thr,Ser,Gln,Asn) | - | - | - | 1.5 |
| Thr | 1 | 1 | 0.7 | - |
| Ser | 1 | 1 | 0.6 | - |
| Gln | 1 | 0 | - | - |
| Asn | 1 | 0 | - | - |
| Glu | 2 | 3 | 2.7 | 0.3 |
| Pro | 1 | 1 | 0.7 | 0.4 |
| Gly | 3 | 3 | 3.1 | 0.7 |
| Ala | 2 | 2 | 1.7 | 1.2 |
| Cys/2 | 0 | 0 | 0 | 0 |
| Val | 3 | 3 | 2.6 | 2.2 |
| Met | 0 | 0 | 0 | 0 |
| Ile | 0 | 0 | 0 | 0 |
| Leu | 4 | 4 | 4.4 | 3.6 |
| Tyr | 2 | 2 | 1.7 | 1.6 |
| Phe | 3 | 3 | 2.5 | 2.3 |
| His | 2 | 2 | 2.0 | 1.5 |
| Lys | 1 | 1 | 1.1 | 0.8 |
| Trp | 2 | 0 | 0 | - |
| Arg | 1 | 1 | 0.9 | 0.8 |

Insulin composition according to Ryle *et al* (1955).

Enzymic hydrolysis achieved using Sepharose-pronase, Sepharose-proteinase-K and Sepharose-prolidase.

Table 21 Recovery of amino acids after enzymic hydrolysis of ovalbumin and insulin (B chain)

| Amino acid | Amino acids released after enzymic hydrolysis / Numbers of amino acids in protein % | |
|---|---|---|
| | Ovalbumin | Insulin (B chain) |
| CysA | - | 30.0 |
| Asp | 35.7 | - |
| (Thr,Ser,Gln,Asn) | 68.8 | 37.5 |
| Glu | 41.9 | 15.0 |
| Pro | 71.4 | 40.0 |
| Gly | 63.1 | 23.3 |
| Ala | 119.2 | 60.0 |
| Cys/2 | 42.7 | - |
| Val | 102.0 | 73.3 |
| Met | 106.2 | - |
| Ile | 88.2 | - |
| Leu | 89.9 | 90.0 |
| Tyr | 118.6 | 80.0 |
| Phe | 92.1 | 76.7 |
| His | 105.6 | 75.0 |
| Lys | 125.7 | 80.0 |
| Arg | 97.5 | 80.0 |

The presence of Gln and Asn in enzymic hydrolysates prevents the resolution of Thr and Ser, and an average value based on the total of these four amino acids is given.

Asp, Cys, Met and Ile are not present in the B chain of bovine insulin.

### 4.1.8 The incorporation of immobilized papain with the Hill and Schmidt enzymic hydrolysis procedure

According to the modified Hill and Schmidt hydrolysis method, the substrate must first be incubated with papain, which is then precipitated by acid before the addition of prolidase and aminopeptidase M. The use of immobilized papain simplified this procedure since the Sepharose-papain could be completely removed from the mixture by filtration, requiring no changes in the pH value of the solution. The levels of amino acids released from control mixtures containing the added substrates (Table 24) were also slightly lower for the mixture containing coupled enzyme.

Results for the enzymic hydrolysis of insulin B chain and ovalbumin, using immobilised papain, are shown in Tables 22 and 23, together with the recoveries of amino acids after acid hydrolysis of the enzymic hydrolysates.

After incorporation of immobilised papain into the Hill and Schmidt procedure, the yields of amino acids after enzymic hydrolysis of the insulin B chain were similar to those produced using the procedure with soluble papain (Table 13). Again, the percentage recoveries of Leu and His were low, due to the levels of these amino acids in the acid hydrolysates being higher than found in the composition of the molecule. The levels of amino acids found by this enzymic hydrolysis method agree with the known composition of the insulin.

Although the enzymic hydrolysis of ovalbumin by the Hill and Schmidt procedure using soluble papain (Table 14) achieved a high yield of amino acids, this was much reduced when immobilized papain replaced the soluble form (Table 23). In this case there were large increases in the levels of amino acids after acid hydrolysis, indicating that complete hydrolysis was not achieved by this enzymic method.

Table 22 The amino acids released from insulin (oxidized, B chain), using immobilised papain in the enzymic hydrolysis procedure

| Amino acid | Enzymic hydrolysis using immobilized papain (EH) | Acid hydrolysis of enzymic hydrolysate (EH + AH) | $\frac{(EH)}{(EH + AH)}$ % |
|---|---|---|---|
| CysA | 1.21 | 1.97 | 61.46 |
| Asp | 0.09 | 1.20 | - |
| (Thr,Ser,Gln,Asn) | 3.20 | - | - |
| Thr | - | 1.02 | - |
| Ser | - | 1.02 | - |
| Gln | - | - | - |
| Asn | - | - | - |
| Glu | 2.47 | 3.42 | - |
| Pro | 1.14 | 1.20 | 94.77 |
| Gly | 3.57 | 3.97 | 90.20 |
| Ala | 2.43 | 2.41 | 100.80 |
| Cys/2 | 0 | 0 | - |
| Val | 2.87 | 3.15 | 90.80 |
| Met | 0 | 0 | - |
| Ile | 0 | 0 | - |
| Leu | 4.48 | 5.11 | 87.61 |
| Tyr | 1.62 | 1.51 | 107.34 |
| Phe | 3.47 | 3.60 | 96.65 |
| His | 1.62 | 2.35 | 68.66 |
| Lys | 1.06 | 1.14 | 92.83 |
| Arg | 0.42 | nd | - |

Results are expressed as the number of amino acids per insulin molecule.
Enzymic hydrolysis was by the modified Hill and Schmidt procedure.
Samples of the enzymic digests were further acid hydrolysed, *in vacuo*, for 22h at 105°C.
nd = not determined.

Table 23 The amino acids released from ovalbumin using immobilized papain in the enzymic hydrolysis procedure

| Amino acid | Enzymic hydrolysis using immobilized papain (EH) | Acid hydrolysis of enzymic hydrolysate (EH + AH) | $\frac{(EH)}{(EH + AH)}$ % |
|---|---|---|---|
| CysA | 0.67 | 0 | - |
| Asp | 4.57 | 30.41 | - |
| (Thr,Ser,Gln,Asn) | 16.57 | - | - |
| Thr | - | 4.35 | - |
| Ser | - | 26.32 | - |
| Gln | - | - | - |
| Asn | - | - | - |
| Glu | 9.12 | 35.19 | - |
| Pro | 1.20 | 10.46 | 11.48 |
| Gly | 5.81 | 15.41 | 37.70 |
| Ala | 11.32 | 30.52 | 37.10 |
| Cys/2 | 0 | 0 | - |
| Val | 9.88 | 18.71 | 52.82 |
| Met | 3.10 | 3.74 | - |
| Ile | 1.38 | 18.64 | 7.41 |
| Leu | 5.59 | 22.33 | 25.01 |
| Tyr | 1.25 | 7.09 | 17.61 |
| Phe | 3.87 | 13.62 | 28.42 |
| His | 1.95 | 5.03 | 38.74 |
| Lys | 4.16 | 17.69 | 23.52 |
| Arg | 1.58 | nd | - |

Results are expressed as the number of amino acids per ovalbumin molecule.
Enzymic hydrolysis was by the modified Hill and Schmidt procedure.
Samples of the enzymic digests were further acid hydrolysed, *in vacuo*, for 22h at 105°C.
nd = not determined.

Table 24 Background levels of amino acids in enzymic hydrolysis mixtures

| Amino acid | Enzyme Systems | |
|---|---|---|
| | native papain + native prolidase + native aminopeptidase µmoles | Immobilized papain + native prolidase + native aminopeptidase µmoles |
| Cys A | 0 | 0 |
| Asp | 0.033 | 0.032 |
| Thr | 0.057 | 0.044 |
| Ser | 0.010 | 0 |
| Glu | 0.014 | 0.009 |
| Pro | 0 | 0 |
| Gly | 0.049 | 0.041 |
| Ala | 0.026 | 0.019 |
| Cys/2 | 0 | 0 |
| Val | 0.037 | 0.030 |
| Met | 0 | 0 |
| Ile | 0.014 | 0 |
| Leu | 0.006 | 0 |
| Tyr | 0.013 | 0 |
| Phe | 0 | 0 |
| His | 0 | 0 |
| Lys | 0.013 | 0.018 |
| Trp | 0 | 0 |
| Arg | 0 | 0 |

Results are expressed as the total number of µmoles of amino acids released in the absence of any added substrate.

### 4.1.9 The enzymic hydrolysis of legume protein according to the method of Winkler and Schön (1979)

The recoveries of amino acids after the hydrolysis of cowpea (*Vigna unguiculata*) meal are shown in Table 25. In the enzymic hydrolysate, no Cys or Pro were detected, although both of these amino acids were present in the acidic hydrolysate. The recoveries of some non acid-labile amino acids were greater in the enzymic hydrolysate than in the acidic hydrolysate; these "extra" amino acids could have arisen from the enzymes used for hydrolysis since, according to the method, no control was used to measure the amino acids arising as a result of enzymic autolysis.

Table 25 The enzymic hydrolysis of legume protein, according to the method of Winkler and Schön (1979)

| Amino acid | Yields of amino acids after enzymic hydrolysis µmoles | Yields of amino acids after acidic hydrolysis µmoles | $\frac{\text{Enzymic hydrolysis}}{\text{Acidic hydrolysis}}$ % |
|---|---|---|---|
| CysA | 0 | 0 | - |
| Asp | 0.017 | 0.222 | - |
| Thr | 0.026 | 0.122 | - |
| Ser | 0.050 | 0.104 | - |
| Gln | 0.026 | - | - |
| Asn | 0.087 | - | - |
| Glu | 0.034 | 0.154 | - |
| Pro | 0 | 0.036 | 0 |
| Gly | 0.066 | 0.076 | 86.2 |
| Ala | 0.062 | 0.071 | 87.3 |
| Cys/2 | 0 | 0.008 | 0 |
| Val | 0.063 | 0.056 | 112.5 |
| Met | 0.021 | 0.013 | 0 |
| Ile | 0.046 | 0.047 | 97.9 |
| Leu | 0.087 | 0.077 | 112.9 |
| Tyr | 0.024 | 0.022 | 109.2 |
| Phe | 0.055 | 0.045 | 122.2 |
| His | 0.034 | 0.023 | 147.8 |
| Lys | 0 | 0 | 0 |
| Trp | 0 | 0 | 0 |
| Arg | 0.042 | 0.052 | 80.7 |

Results are expressed as the number of µmoles of amino acids released per mg of cowpea, *Vigna unguiculata*, and are the mean levels of duplicate hydrolysates.
The levels of Thr, Ser, Gln and Asn in the enzymic hydrolysates were determined by rerunning the samples on the amino acid analyser using the $Li^+$-buffer system (see Methods section).

## 4.2 THE MEASUREMENT OF PROTEOLYSIS

### 4.2.1 A test of the efficiency of the pH-stat: results and discussion

Table 26 shows the apparently low levels, as measured by the pH-stat, of hydrolysis of ovalbumin by enzymes with a wide range of specificities. The use of alternative forms of assay has shown that these results are low. For example, the extent of hydrolysis of ovalbumin by pronase, measured over one hour, is shown in Fig. 5. While the number of free amino groups measured by TNBS assay has continued to increase throughout the incubation, the reaction, according to the pH-stat, is apparently complete after 15min with no more alkali being required. This anomaly may be explained by consideration of the changing buffering capacity of the contents throughout digestion.

The hydrolysis of the peptide bond may be represented by the equation:-

$$R_1 - NH - CO - R_2 + H_2O \longrightarrow \begin{matrix} R_1 - NH_2 \\ \downarrow\uparrow \\ R_1 - NH_3^+ \end{matrix} + H^+ + \begin{matrix} R_2 - COO^- \\ \downarrow\uparrow \rightarrow H^+ \\ R_2 - COOH \end{matrix}$$

Following hydrolysis, the extent of ionization of the carboxyl and amino groups will depend upon the pH of the medium, and the dissociation constants of the groups involved.

$$\begin{matrix} R \\ | \\ CHNH_3^+ \\ | \\ COOH \end{matrix} \underset{Ka_1}{\rightleftharpoons} \begin{matrix} R \\ | \\ CHNH_3^+ \\ | \\ COO^- \end{matrix} \underset{Ka_2}{\rightleftharpoons} \begin{matrix} R \\ | \\ CHNH_2 \\ | \\ COO^- \end{matrix}$$

FIGURE 5 HYDROLYSIS OF OVALBUMIN BY PRONASE

Hydrolysis was measured over 1h, by means of pH-stat assay and TNBS assay

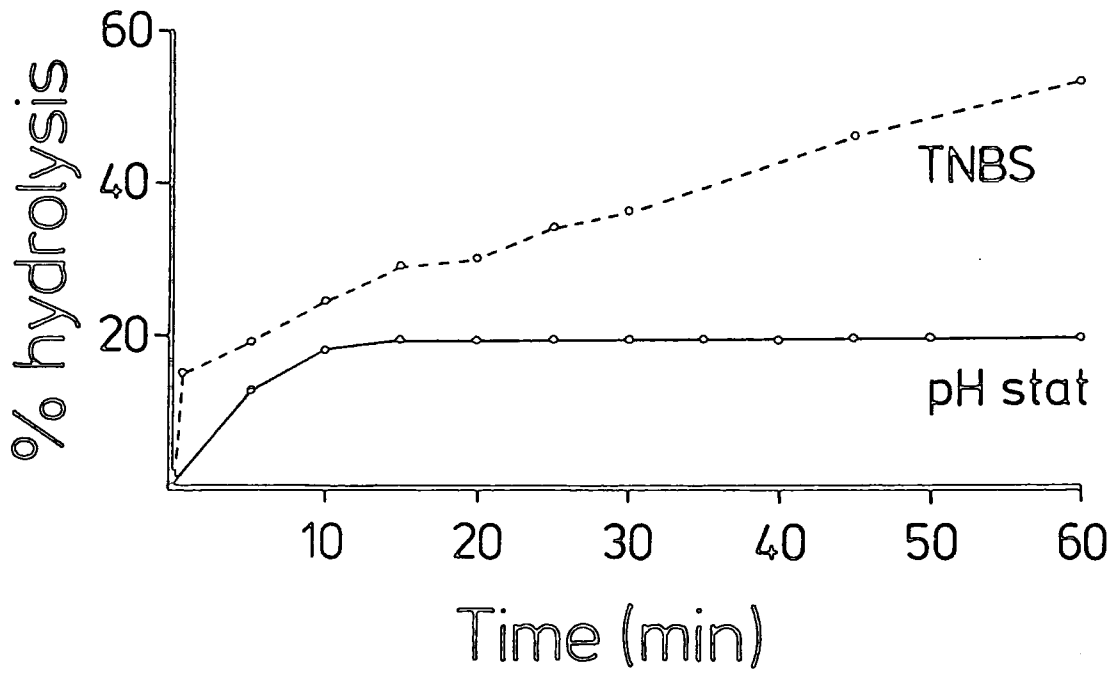



FIGURE 6 CHANGE IN pH OF THE UNBUFFERED REACTION MIXTURE AS OVALBUMIN IS HYDROLYSED BY PRONASE

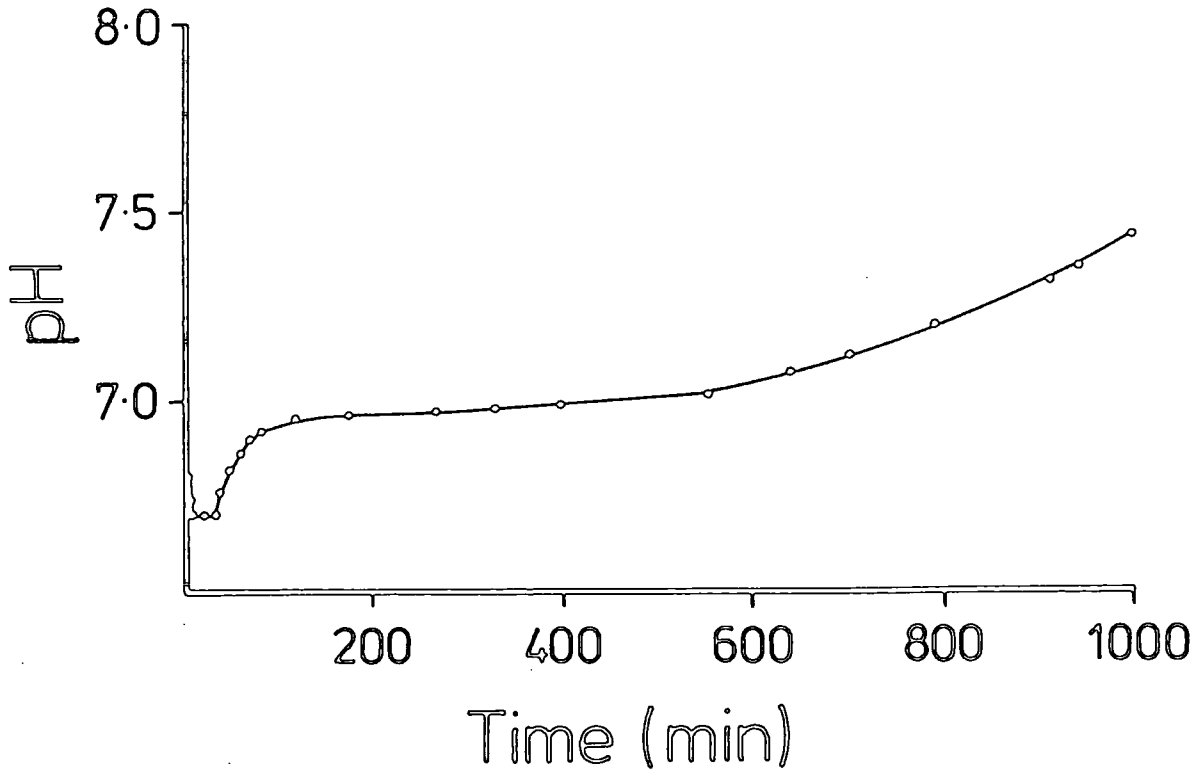


Table 26 Apparent extent of hydrolysis of ovalbumin achieved by a variety of enzymes, as measured using the pH-stat

| Enzyme(s) | Maximum hydrolysis (expressed as % of total peptide bonds cleaved) |
|---|---|
| α-Chymotrypsin | 8.0 |
| Elastase | 2.0 |
| Intestinal peptidase mixture | 8.0 |
| Pancreatin | 17.0 |
| Papain and pronase | 19.8 |
| Pepsin predigestion, followed by pronase | 20.2 |
| Pronase | 19.9 |
| Trypsin | 3.7 |
| Trypsin, α-chymotrypsin and intestinal peptidase mixture | 7.6 |
| Trypsin, α-chymotrypsin, intestinal peptidase mixture and elastase | 11.1 |
| Trypsin, α-chymotrypsin, carboxypeptidases A and B, and leucine aminopeptidase | 10.3 |

Percentages of total peptide bonds cleaved were calculated from the volumes of alkali required to maintain a constant pH value. Reactions were allowed to continue until apparently complete, i.e. no further alkali was added.

The dissociation constants $K_{a_1}$ and $K_{a_2}$ are measures of the tendencies of the carboxyl and amino groups to dissociate, for example:-

$$K_{a_2} = \frac{[NH_2][H^+]}{[NH_3^+]}$$

$pK_a$ values are the logarithmic transformations of $K_a$ values:

$$pK_{a_2} = \log \frac{1}{K_{a_2}} = -\log K_{a_2}$$

pH and $pK_a$ values are related by the Henderson-Hasselbalch equation:

$$pH = pK_{a_2} + \log \frac{[NH_2]}{[NH_3^+]}$$

Therefore, the $pK_{a_2}$ value is numerically the same as the pH at which the concentration of $NH_2$ and $NH_3^+$ are equal.

If an enzymic reaction is being carried out at pH 8.0, then at this pH all the carboxyl groups will be fully ionized, but because this pH is near the $pK_a$ values of the amino groups, these groups will be present in both protonated and unprotonated forms.

Only when the amino group is present in the unprotonated form, is a proton released into solution. Therefore, as the polypeptide chain of a protein is cleaved, the number of free amino groups is increased, and as peptides become shorter, their $pK_{a_2}$ values increase (see Table 27), and protons are taken up from solution to form $NH_3^+$ groups. This explains the change in pH shown during the digestion of ovalbumin by pronase, as shown in Fig. 6, where the initial, large drop in pH levels off and is followed by an increase in pH as protons are taken up from solution. In this way, the protein, as it is cleaved and more amino

Table 27 $pK_a^1$ values for alanine and di, tri and tetra-alanine, and the concentration of $NH_3^+$ and $NH_2$ groups which will be present at pH 8.0, calculated using the Henderson-Hasselbalch equation

| | $pK_{a_1}^1$ | $pK_{a_2}^1$ | $[NH_3^+]$ % | $[NH_2]$ % |
|---|---|---|---|---|
| alanine | 2.34 | 9.69 | 98.00 | 2.00 |
| dialanine | 3.30 | 8.14 | 58.00 | 42.00 |
| trialanine | 3.39 | 8.03 | 51.73 | 48.27 |
| tetraalanine | 3.42 | 7.94 | 46.55 | 53.44 |

$pK_a^1$ values refer to the negative logarithm, to the base 10, of an equilibrium constant based on molar concentrations.

$pK_a^1$ values from Dawson *et al* (1959).

groups become free to buffer, increases its buffering capacity as shown in Fig. 7. By contrast, Fig. 8 shows the change in pH as BAEE (an analogue dipeptide) is hydrolysed by pronase. In this case there is the expected drop in pH due to proton release during hydrolysis, but this is not followed by a rise in pH, because of the simplicity of the products, involving no complex buffering effects.

Since the pH-stat follows the extent of hydrolysis according to the amount of alkali required to maintain a constant value of pH, if the buffering activity of a protein being digested is causing the pH of the reaction mixture to be held constant or increase, no further alkali will be added, and according to this measure, the reaction must have finished. Also, at any time during hydrolysis, the degree of ionization of the range of components present cannot be known, and therefore the extent of hydrolysis cannot be calculated. Some authors (Samuelsson, 1964, Ronca *et al*, 1975) have introduced correction factors to cope with this problem. The assumption that at pH 8.0 amino acids will be half ionized, so that equivalents of alkali added represent half the true extent of hydrolysis, depends upon the protein having been completely hydrolysed to free amino acids and, as described above, the pH-stat fails to provide a reliable measure of when the reaction is actually complete.

In a search for some reagent which might react with free amino groups and cause their full ionization, it seemed that formaldehyde may fulfil this role. According to Levy and Li (1955) an uncharged amino group may combine with either one or two molecules of formaldehyde, but a charged amino group does not combine.

FIGURE 7 THE BUFFERING CAPACITY OF DIGESTED OVALBUMIN

A. The total buffering capacity of separate solutions of ovalbumin and pronase.

B. The buffering capacity of the ovalbumin after incubation with the pronase for 1h.

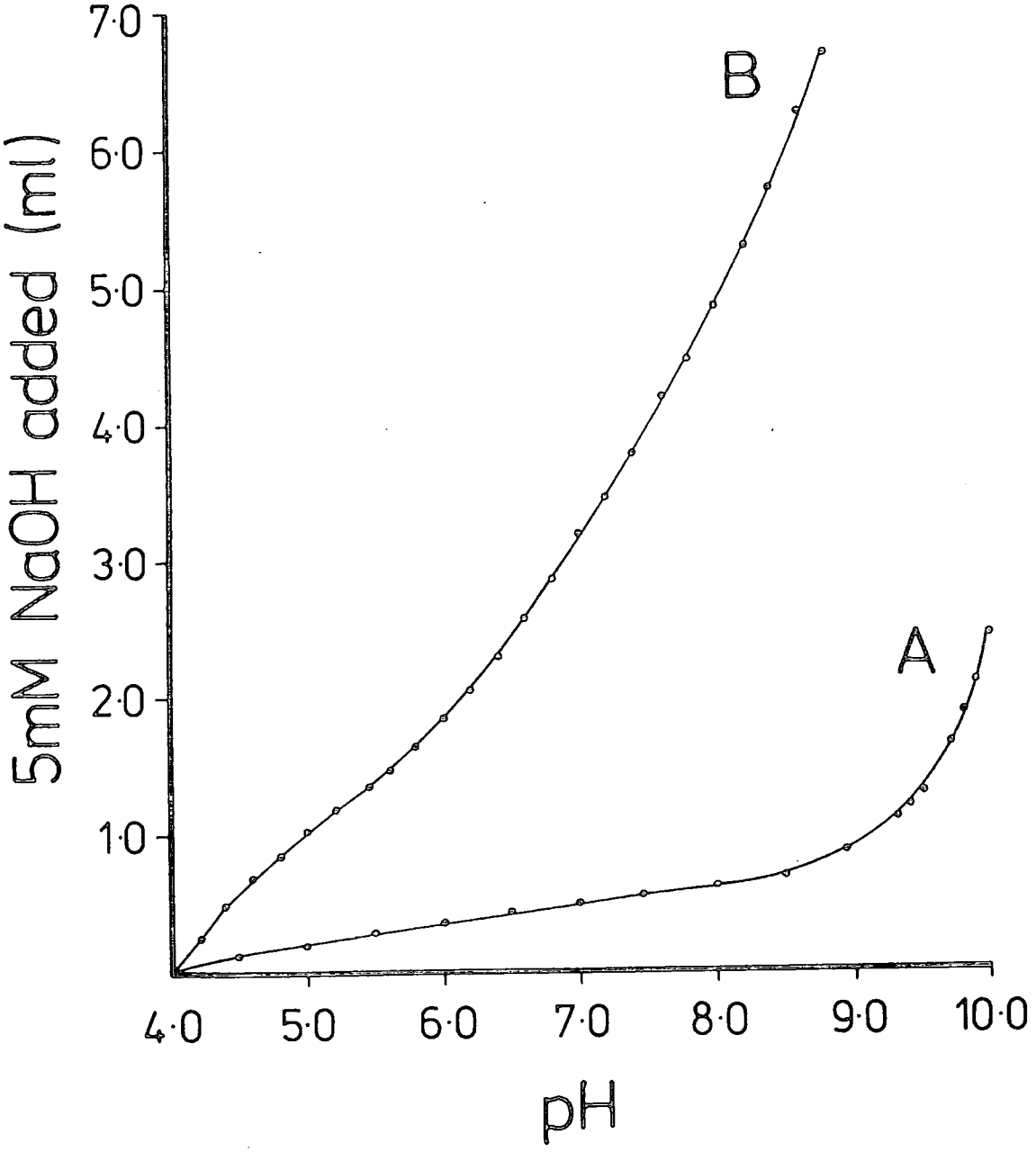


FIGURE 8 THE CHANGE IN pH OF THE REACTION MIXTURE AS BAEE IS HYDROLYSED BY PRONASE

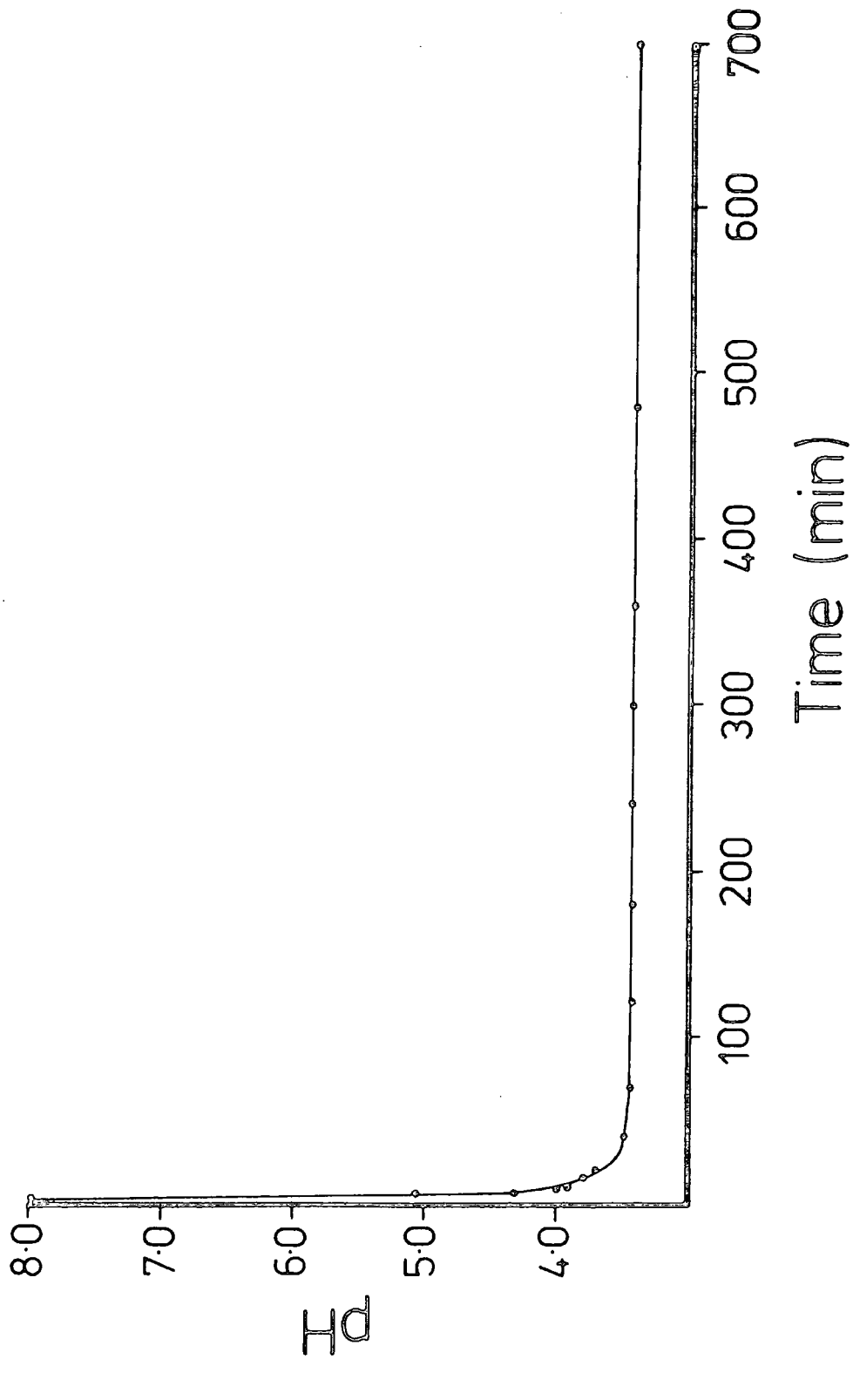
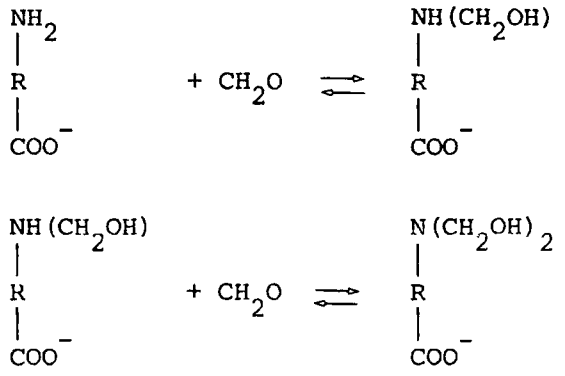


Fig. 9 Reaction of uncharged amino groups with formaldehyde



Reaction of $-NH_2$ groups with formaldehyde and their effective removal from solution causes further dissociation of $-NH_3^+$ groups, with the release of protons. Formaldehyde therefore serves to lower the $pK^1_{a_2}$ value of an amino group of an amino acid.

Initially the effect of formaldehyde on the titration of glycine was investigated. Glycine has a $pK^1_{a_2}$ value of 9.6, therefore at pH 9.6, half of the amino groups must be in the charged form, $-NH_3^+$, with protons available for release. However, at pH 7.6, two pH units below $pK^1_{a_2}$, 98% of amino groups are still in the charged form. From the results (Table 28), it can be seen that after the addition of formaldehyde to a solution of glycine at pH 7.6, the release of protons (as measured by the volume of hydroxide required to maintain constant pH) was more than double the release of protons measured when the experiment was repeated at pH 9.6. This is explained by the greater proportion of amino groups present in the form $NH_3^+$ at pH 7.6, and therefore the greater number of protons to be released by them as they ionized to maintain equilibrium as $NH_2$ groups were taken up from free solution by formaldehyde.

It was hoped that by addition of formaldehyde, full proton release could be measured during the hydrolysis of proteins. However, it was found that in the presence of formaldehyde, enzymic activities

were lowered (Table 29). The only way in which formaldehyde could be incorporated was by its addition at the end of each assay, but then the original advantage of the pH-stat, to follow the course of a reaction, was lost. The pH-stat was therefore found inadequate for measuring the extent of proteolytic reactions.

Table 28 The effect of the presence of formaldehyde upon the titration of glycine

| pH | Water (ml) | Glycine (ml) | Formaldehyde (ml) | $OH^-$ added (ml) | ∴ volume of $OH^-$ required for the titration of glycine (ml) |
|---|---|---|---|---|---|
| 7.6 | 4.0 | - | 0.5 | 1.5 | - |
| 7.6 | 3.0 | 1.0 | 0.5 | 9.8 | 8.3 |
| 9.6 | 4.0 | - | 0.5 | 6.9 | - |
| 9.6 | 4.0 | 1.0 | 0.5 | 10.3 | 3.4 |

Titrations were carried out in the vessel of the pH-stat, under nitrogen, and with sodium hydroxide (5mM) added by the autoburette to maintain a constant pH value.

The water, or water and glycine ($3.753 mg ml^{-1}$; $50 \mu mol.ml^{-1}$ in water), were inserted first, and raised to pH 7.6 or 9.6. Formaldehyde solution was then added, and the volume of hydroxide required to maintain the pH was recorded.

Table 29 The use of formaldehyde in the measurement of the release of protons during hydrolysis

| Experiment | Water (ml) | Ovalbumin $5mgml^{-1}$ in water (ml) | Pronase $1mgml^{-1}$ in water (ml) | Formaldehyde (ml) | Total $OH^-$ added (ml) |
|---|---|---|---|---|---|
| Control | 4.0 | - | - | 1.0 | 0.02 |
| Control | 3.0 | 1.0 | - | 1.0 | 0.04 |
| Control | 3.0 | - | 1.0 | 1.0 | 0.03 |
| Formaldehyde added at the end of the reaction | 3.0 | 1.0 | 1.0 | 1.0 | 2.14 |
| Formaldehyde present during the reaction | 2.0 | 1.0 | 1.0 | 1.0 | 1.40 |

The ovalbumin was incubated with pronase in the vessel of the pH stat at pH 8.0, 37°C, and under nitrogen. NaOH (5mM) was added by the autoburette. A formaldehyde solution which had already been adjusted to pH 8.0 was used. In the first digestion experiment, the reaction was continued until no further alkali was being added, after which the formaldehyde was added, and further alkali uptake was recorded. In the second experiment, formaldehyde was present in the reaction mixture before addition of the enzyme.

4.2.2 The measurement of proteolysis by the use of TNBS

For assays concerned with measurement of the hydrolysis of proteins, a procedure in which TNBS could be used to assay for free amino acids and proteins separately seemed appropriate.

Such a procedure has been described by Mokrasch (1967) and Table 30 shows that it could be used to measure individually free amino acids and protein from a mixture of glycine and ovalbumin. The method was also used to assay samples taken over one hour, from a digestion mixture of ovalbumin and pronase (Fig. 10). After assay, the organic layer, containing amino acids, shows an increase in amino acid content of 3½ fold, while the protein layer also shows a very slight increase in content, presumably due to the presence of an increased number of polypeptides in this fraction.

However, for use as a routine assay, this method is unsatisfactory; proteins tended to form emulsions with the toluene, and a centrifugation step had to be introduced.

It was therefore decided to use TNBS simply to measure the increase in free amino groups during the course of protein hydrolysis. For routine assay, a modification of the method described by Romero and Ryan (1978) was found most suitable.

FIGURE 10 THE HYDROLYSIS OF OVALBUMIN BY PRONASE
Samples were taken at intervals from a digestion mixture consisting of 5mg ovalbumin and 1mg pronase, in a total volume of 5ml 0.1M sodium borate buffer, pH 8.0, and assayed according to the method of Mokrasch (1967), as described in the methods section.

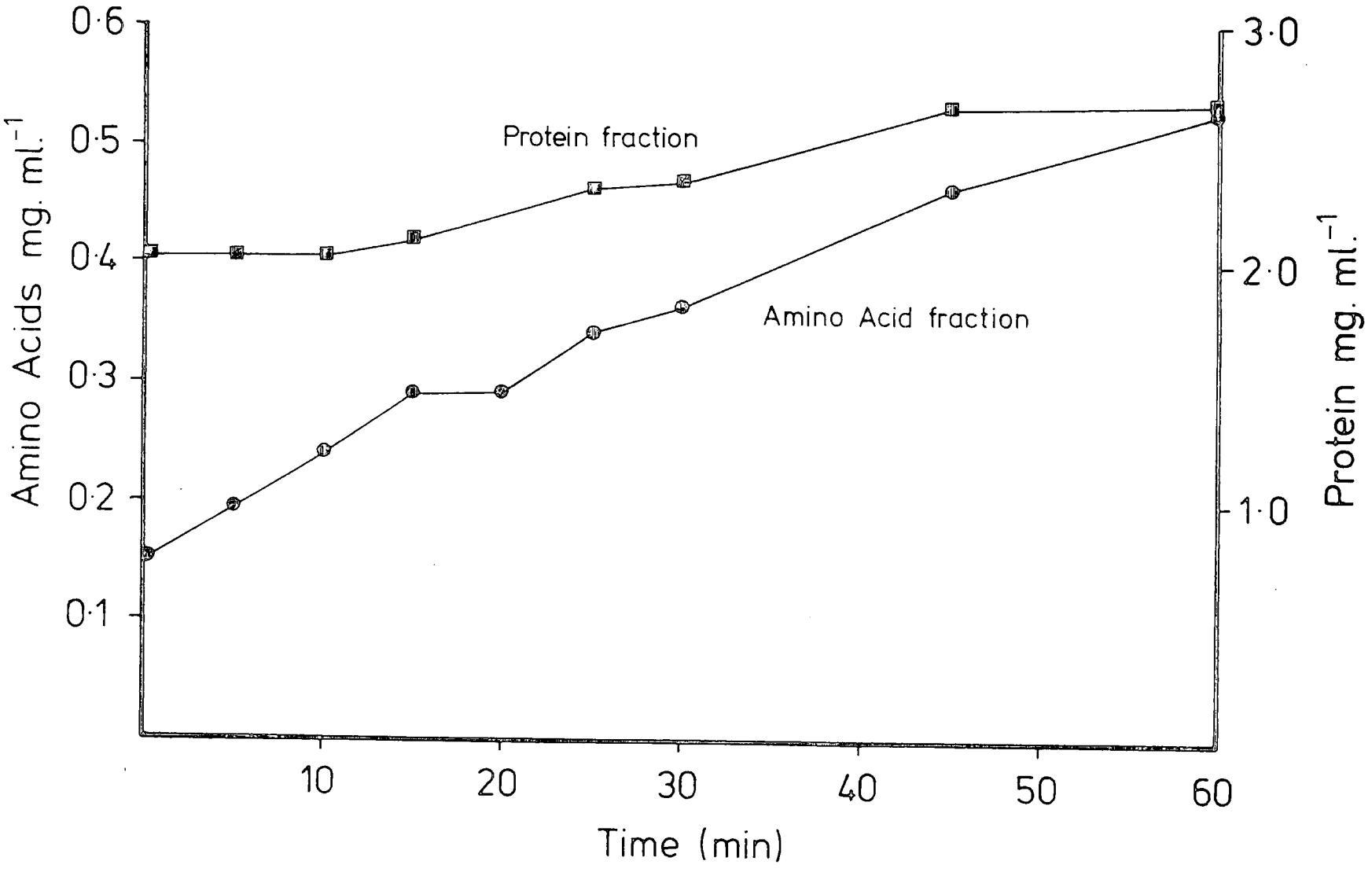


Table 30 The use of TNBS and a partitioning procedure to measure separately the protein and amino acid contents of a mixture

| Buffer<br>ml | Ovalbumin ($2.5 mg ml^{-1}$, in buffer)<br>ml | Glycine ($0.2 \mu mol.ml^{-1}$ in buffer)<br>ml | $A_{340}$ of protein (aqueous) layer | $A_{340}$ of amino acid (toluene) layer |
|---|---|---|---|---|
| 1.0 | - | - | 0 | 0 |
| 0.8 | 0.2 | - | 0.09 | 0 |
| 0.5 | - | 0.5 | 0 | 0.56 |
| 0.3 | 0.2 | 0.5 | 0.16 | 0.49 |

Samples of the mixtures were reacted with TNBS, after which the reaction was quenched, and extraction was carried out using toluene. Details in Methods section.

## 4.3 THE DIGESTIBILITY OF GLYCOPROTEIN II PURIFIED FROM *PHASEOLUS VULGARIS*

### 4.3.1 The purification of glycoprotein II

Glycoprotein II was purified from *Phaseolus vulgaris* as described in the Methods section. Polyacrylamide gel electrophoresis of a sample of this preparation showed the characteristic three banded pattern of glycoprotein II (Fig. 11, track 3), with major bands at molecular weights 53000 and 47000, and a minor band at 44000. Amino acid analysis after acidic hydrolysis revealed an amino acid composition corresponding with the known composition of glycoprotein II (Table 31).

### 4.3.2 The use of SDS polyacrylamide gel electrophoresis to visualise the degradation of glycoprotein II by enzymic hydrolysis

Native glycoprotein II was incubated with trypsin, and samples taken throughout the incubation were run on SDS polyacrylamide gels (Fig. 11). In the first sample taken 10s after the addition of trypsin, diffuse bands are visible, centred at molecular weights 26800 and 22500. These newly generated bands were the result of proteolytic cleavage of glycoprotein II since they did not appear in controls containing only enzyme or substrate. After 5min, the original bands of high molecular weight protein are no longer visible, while the bands of lower molecular weight ~~protein~~ peptides remain. After 30min, these low molecular weight proteins are visible as three distinct bands; one feint, representing proteins of molecular weight 26000, and two major bands at $M_r$ values of 24000 and 22500. This pattern remains the same for all subsequent samples taken over 24h.

Incubation of the native glycoprotein II with pronase resulted in a different pattern of degradation (Fig. 13). As with

Figure 11

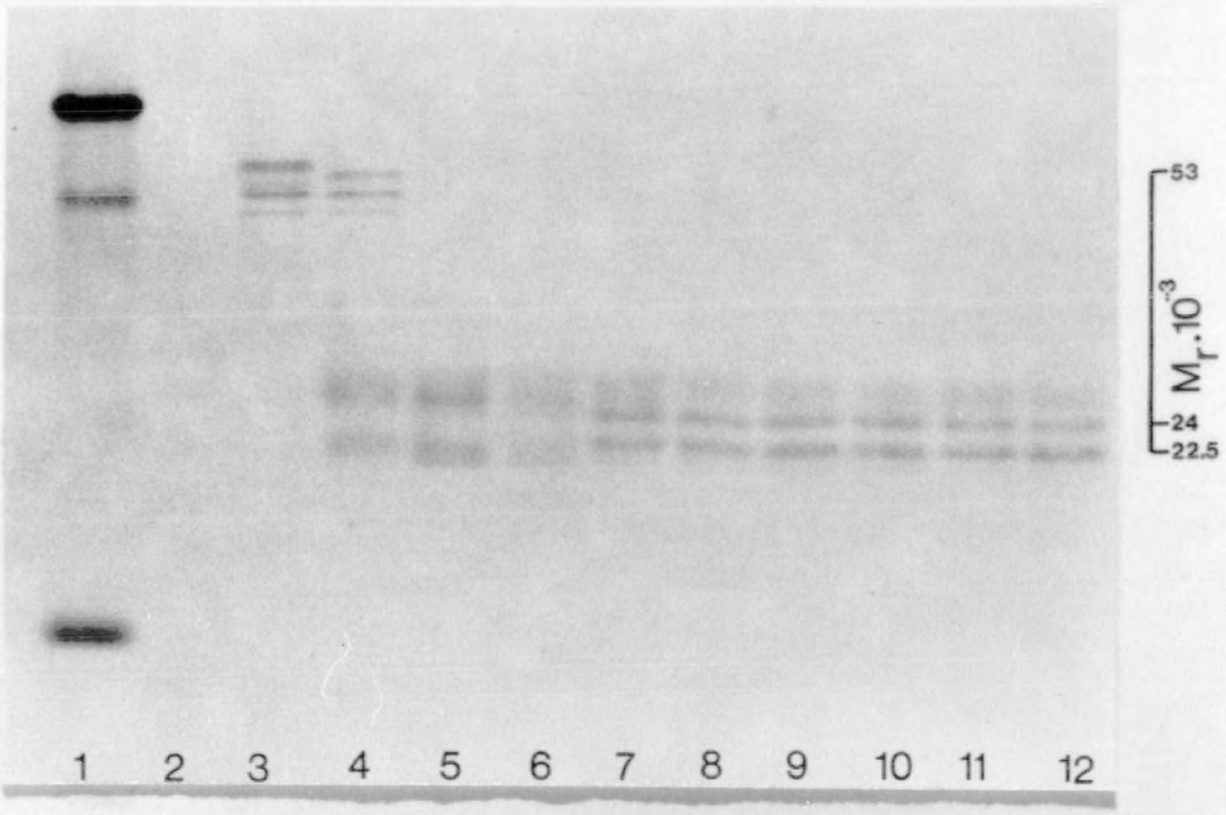
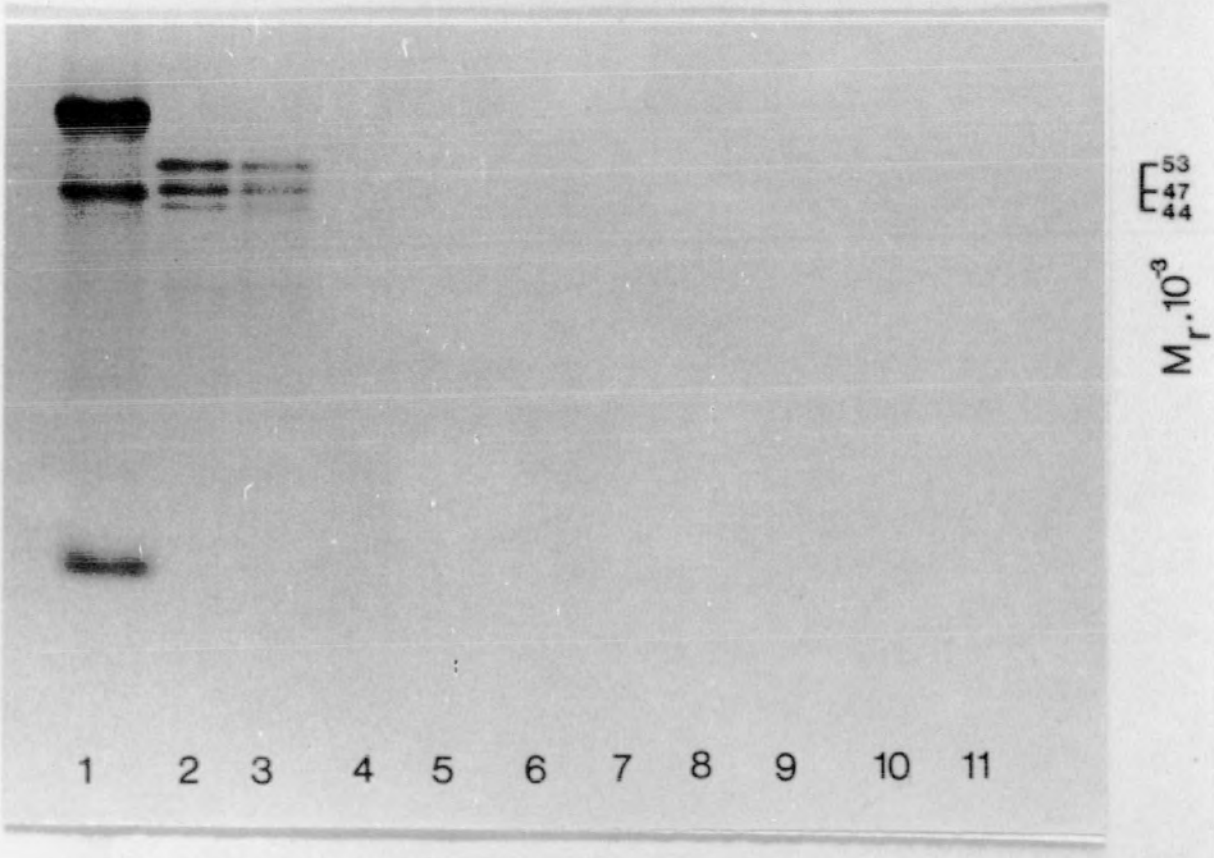



Figure 12



trypsin, protein fragments remained even after 20h of incubation, but these were smaller after hydrolysis with pronase, with the most prominent bands at molecular weights of 25000, 19500 and 16000.

Native glycoprotein II was more completely degraded in the presence of proteinase-K (Fig. 14). While the tracks containing samples of glycoprotein II incubated just with trypsin (tracks ~~6,7,8~~ 7,8,9) show the expected three banded pattern, no distinct bands are visible in the tracks ~~1~~ 2 - ~~5~~ 6 containing glycoprotein II incubated with either proteinase-K or proteinase-K plus trypsin. Hydrolysis of the glycoprotein with proteinase-K therefore resulted in degradation to some products of molecular weight less than 12000, since proteins smaller than this diffuse readily from the gels and therefore cannot be stained.

4.3.3 The use of gel filtration to follow the degradation of glycoprotein II by enzymic hydrolysis

The Sephadex G100 used for the gel filtration column has a fractionation range for proteins of $M_r$ 4000 - 150000. Glycoprotein II of $M_r$ 140000 was eluted just after the void volume of the column (Fig. 15a). A sample of native glycoprotein II which had been digested by trypsin for 30min showed a similar elution profile (Fig. 15b), but with some small peaks after the initial main peak, which was slightly reduced in area. In the elution profile of the sample of glycoprotein II which had been digested with trypsin for 24h (Fig. 15c), the main peak was still present, but TNBS assay of free amino groups revealed an increase in the number of smaller proteins. These proteins came off the column at elution volumes between 260 and 380ml, and from the calibration curve (Fig. 1), these proteins must have molecular weights within the range 4000 - 25100.

Figure 13

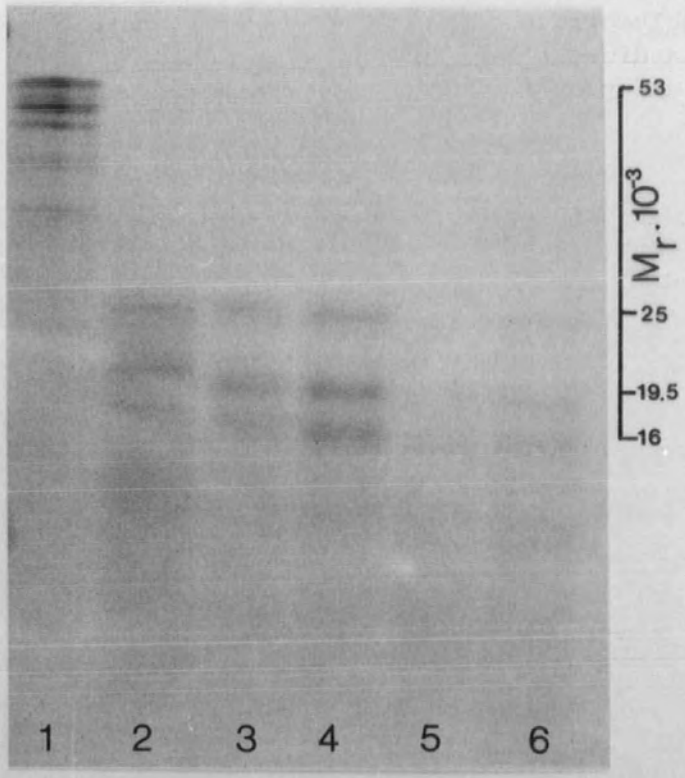
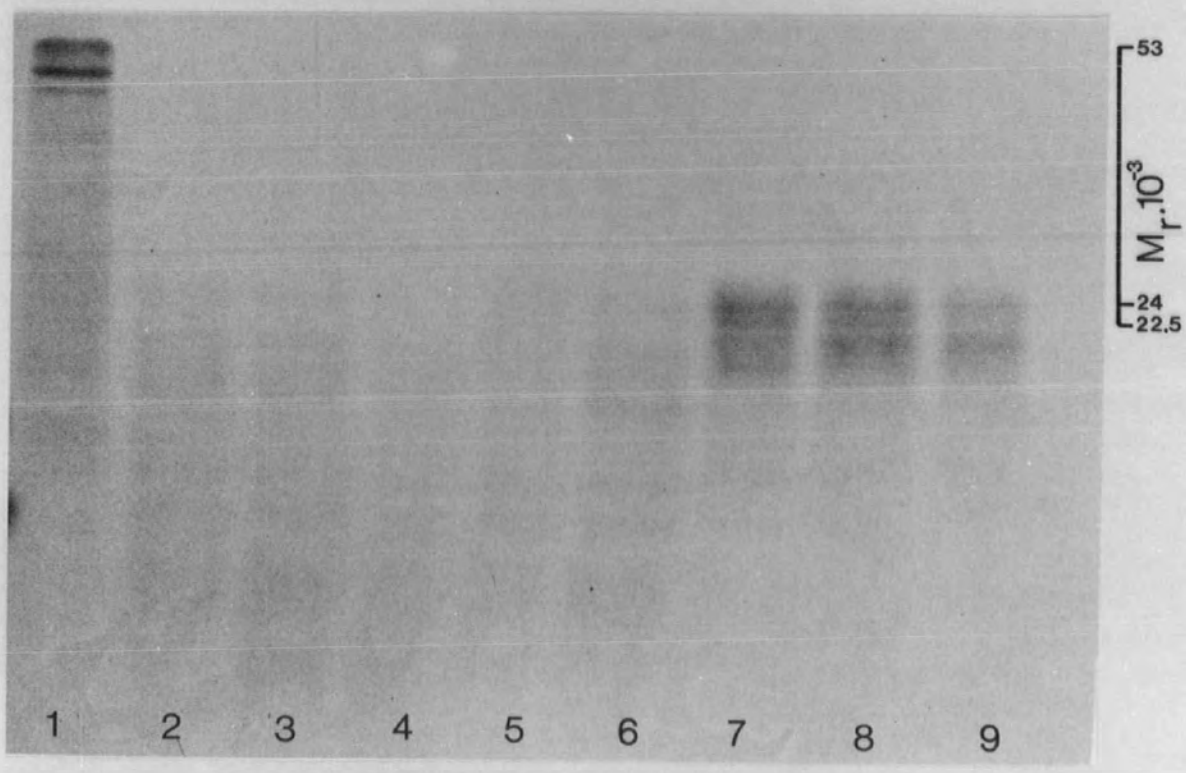
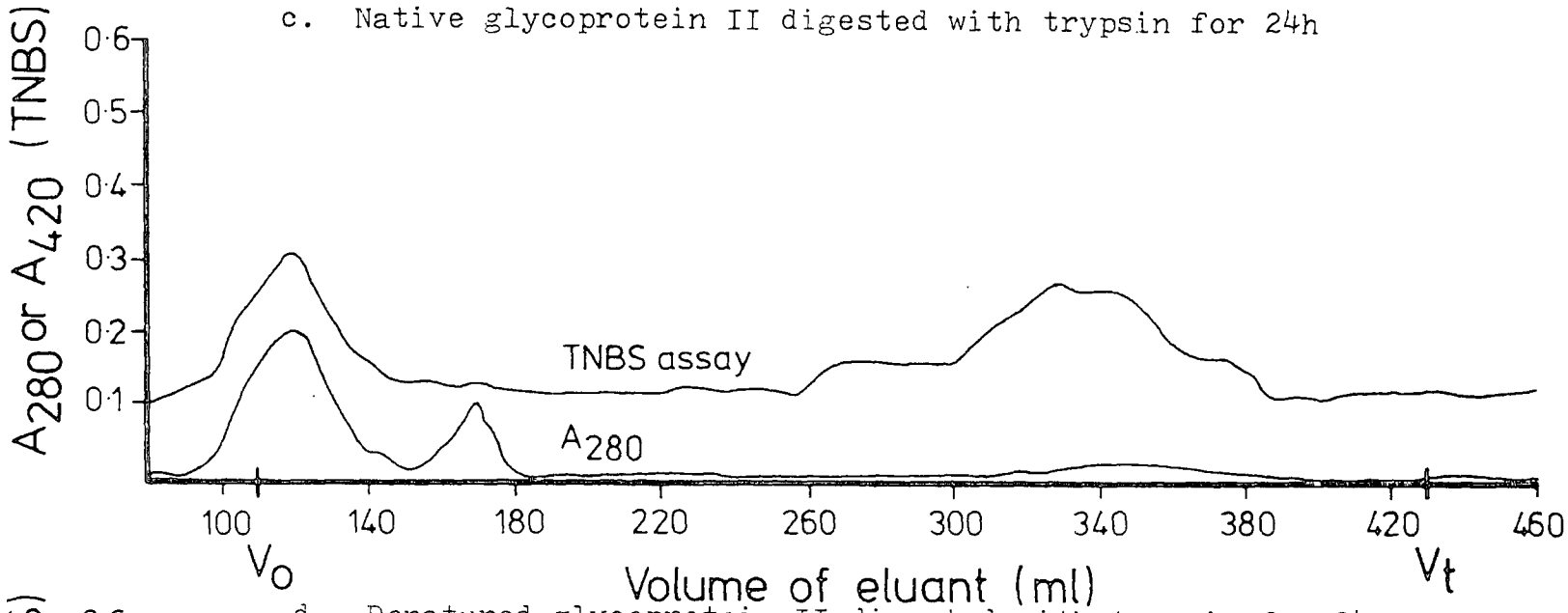


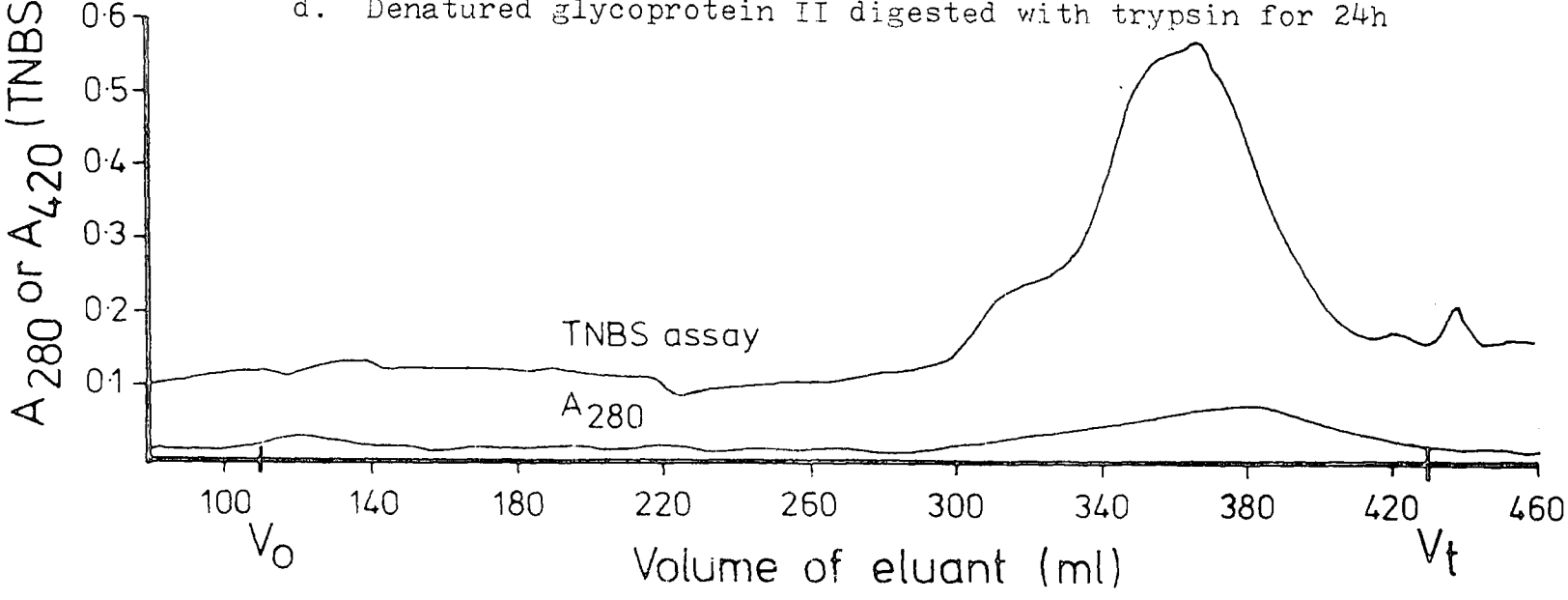

Figure 14



c. Native glycoprotein II digested with trypsin for 24h




d. Denatured glycoprotein II digested with trypsin for 24h




$V_o$ = void volume $V_t$ = total volume

Fractions were monitored at 280nm as they were eluted from the column, and later assayed with TNBS to measure the level of free amino groups.

Fig. 15d shows the effect of denaturation of the glycoprotein II before incubation with trypsin for 24h. After this digestion, no high molecular weight protein remained, but TNBS assay revealed high levels of free amino groups in fractions eluted late from the column. The peak occurred at an elution volume of 370ml: from the calibration curve this corresponds with proteins of $M_r$ less than 18000.

4.3.4 The use of amino acid analysis to follow the degradation of glycoprotein II by enzyme hydrolysis

The recoveries of amino acids after enzymic hydrolysis of native and heat denatured glycoprotein II are given in Table 31. Enzymic hydrolysis of denatured glycoprotein II gave a composition which, for the non acid-labile amino acids, agreed well with the known composition of the glycoprotein, while enzymic hydrolysis of the glycoprotein in its native form resulted in very low yields of free amino acids. From the totals of those amino acids which may be compared between hydrolysates, the mean recovery of amino acids after enzymic hydrolysis of the denatured protein was 91.4%, and of the native protein 12.7%, of the yield of amino acids after acid hydrolysis of the same preparation of glycoprotein II. The levels of Thr, Ser, Asn and Gln were individually determined in the enzymic hydrolysate of the denatured glycoprotein II, by use of the $Li^+$ buffer system for amino acid analysis. In the enzymic hydrolysate, the levels of (Asn + Asp) and (Gln + Glu) were 76% and 54% of the levels of Asp and Glu, respectively, in the acidic hydrolysate.

Table 31 The amino acid composition (g $16g^{-1}N$) of glycoprotein II as given after acidic hydrolysis, and after enzymic hydrolysis of the native and heat-denatured forms of the glycoprotein

| Amino acid | Composition published (of an acidic hydrolysate) | Acidic hydrolysis | Enzymic hydrolysis of heat-denatured glycoprotein II | | Enzymic hydrolysis of native glycoprotein II | |
|---|---|---|---|---|---|---|
| | | | | $EH/_{AH}$ % | | $EH/_{AH}$ % |
| Asp | 13.6 | 13.8 | 3.6 | - | 0 | - |
| (Thr,Ser,Gln,Asn) | - | - | - | - | 1.5 | - |
| Thr | 3.6 | 2.7 | 2.5* | - | - | - |
| Ser | 6.6 | 4.6 | 4.3* | - | - | - |
| Gln | - | - | 4.3* | - | - | - |
| Asn | - | - | 6.9* | - | - | - |
| Glu | 20.5 | 20.4 | 6.9 | - | 1.3 | - |
| Pro | 2.8 | 3.1 | 3.2 | 103.2 | 0.8 | 25.8 |
| Gly | 3.0 | 2.9 | 2.7 | 93.1 | 0.6 | 20.7 |
| Ala | 3.3 | 3.3 | 3.0 | 90.9 | 0.5 | 15.2 |
| Cys/2 | 0.4 | 0 | 0.5 | - | 0 | - |
| Val | 4.9 | 5.6 | 4.8 | 85.7 | 0.2 | 3.6 |
| Met | 0.9 | 1.1 | 0.9 | 81.8 | 0 | 0 |
| Ile | 4.5 | 5.4 | 4.4 | 81.5 | 0.1 | 1.8 |
| Leu | 9.9 | 9.0 | 8.4 | 93.3 | 1.0 | 11.1 |
| Tyr | 3.9 | 3.2 | 2.8 | 87.5 | 0.6 | 18.8 |
| Phe | 6.0 | 6.7 | 6.3 | 94.0 | 1.2 | 17.9 |
| His | 2.7 | 2.7 | 2.6 | 96.3 | 0.3 | 11.1 |
| Lys | 7.4 | 6.4 | 6.3 | 98.4 | 0.9 | 14.1 |
| Arg | 5.6 | 8.6 | 5.8 | - | 0 | - |

EH = enzymic hydrolysis, AH = acidic hydrolysis.
The published composition of glycoprotein II is from Phillips *et al* (1981).
The acid hydrolysis of glycoprotein II used in this experiment took place *in vacuo*, for 22h at 105°C.
The enzymic hydrolysis was by the modified Hill and Schmidt procedure.
Each result represents the mean value of three identical hydrolysates.
* Determined by the use of $Li^+$-buffers during amino acid analysis.

## 4.4 THE DIGESTIBILITY OF TRYPSIN INHIBITOR PURIFIED FROM *PHASEOLUS VULGARIS*

### 4.4.1 *In vitro* enzymic hydrolysis of trypsin inhibitor from *Phaseolus vulgaris*

The levels of amino acids released from the *Phaseolus vulgaris* trypsin inhibitor after hydrolysis by the modified Hill and Schmidt enzymic procedure, and the total amino acid content of the trypsin inhibitor determined by acid hydrolysis, are shown in Table 32.

Comparison with the total yields of amino acids after acidic hydrolysis shows that enzymic hydrolysis released only 34.34%, and after further denaturation of the trypsin inhibitor, 38.42% of the total amino acids. Neither cystine nor cysteic acid were released by enzymic hydrolysis.

Table 32 *In vitro* enzymic hydrolysis of trypsin inhibitor from *Phaseolus vulgaris*

Comparison of the yields of amino acids after enzymic hydrolysis of the trypsin inhibitor in its active form and after further denaturation, with the yields after acid hydrolysis of the trypsin inhibitor.

| Amino acid | 22h acid hydrolysis of 1mg of trypsin inhibitor µmoles | Enzymic hydrolysis of 1mg of trypsin inhibitor µmoles | Recovery of non acid-labile amino acids $\frac{EH}{AH}$ % | Enzymic hydrolysis of 1mg of trypsin inhibitor, further denatured before digestion µmoles | Recovery of non acid-labile amino acids $\frac{EH}{AH}$ % |
|---|---|---|---|---|---|
| Asp | 0.9273 | 0 | | 0 | |
| (Thr,Ser,Gln,Asn) | - | 0.2495 | | 0.3299 | |
| Thr | 0.5663 | - | | - | |
| Ser | 0.5202 | - | | - | |
| Glu | 0.7799 | 0.2025 | | 0.1419 | |
| Pro | 0.2618 | 0.0275 | | 0.0455 | |
| Gly | 0.5212 | 0.3575 | | 0.3607 | |
| Ala | 0.5193 | 0.3081 | | 0.3036 | |
| Cys/2 | 0.6086* | 0 | | 0 | |
| Val | 0.2937 | 0.2172 | 73.95 | 0.2181 | 74.26 |
| Met | 0.0560 | 0.0628 | | 0.0292 | |
| Ile | 0.2297 | 0.0530 | 23.07 | 0.0516 | 22.46 |
| Leu | 0.3072 | 0.1105 | 35.97 | 0.1245 | 40.53 |
| Tyr | 0.1271 | 0.0593 | 46.66 | 0.0885 | 69.63 |
| Phe | 0.1787 | 0.0902 | 50.48 | 0.1732 | 96.92 |
| His | 0.1864 | 0.1268 | 68.03 | 0.1393 | 74.73 |
| Lys | 0.5108 | 0.3924 | | 0.4358 | |
| Trp | 0 | 0.1152 | | 0.0934 | |
| Arg | 0.3307 | 0.1205 | | 0.1020 | |
| Total recovery of amino acids (excluding Trp) | 6.9249 | 2.3778 | | 2.5438 | |
| Recovery of amino acids $\frac{EH}{AH}$ % | | 34.34 | | 38.42 | |

EH = enzymic hydrolysis AH = acidic hydrolysis

* Determined as cysteic acid by the method of Moore (1963).

4.4.2 Analysis of faeces of rats whose diets incorporated the trypsin inhibitor

1. Analysis of faecal extract for protein

Track 1 of the SDS polyacrylamide gel in Fig. 16 shows a sample of the *Phaseolus vulgaris* trypsin inhibitor. A sample of Tris-HCl extract prepared from the faeces of rats whose diet incorporated the inhibitor (track 2) shows stained proteins at positions corresponding to $M_r$ 22600, 20400 and 26000, with feint bands at 6000 and 4200. An identically prepared faecal extract from control fed rats (track 3) shows no stained protein.

2. Assay of faecal extract for trypsin inhibitory activity

Samples of the extract were assayed for inhibitor by the method of Gatehouse and Gatehouse (1979), in which inhibitory activity is determined using immobilized trypsin in 1% agarose gel. The gel is shown in Fig. 17.

Soybean trypsin inhibitor, run at each end of the gel in wells 1 - 5 and ~~18~~ 20 - ~~20~~ 22, was used as a control. Wells 6 and 7 show the activity of a pure sample of *Phaseolus vulgaris* trypsin inhibitor, and wells 8 and 9 contained samples of the preparation used in rat feeding trials and all the above experiments. From the height of the rockets produced by these samples, it can be seen that both preparations are of equal activity. Pure trypsin inhibitor from Lima beans *(Phaseolus lunatus)* was run in wells ~~16~~ 18 and 19 and ~~17~~.

From the rockets produced from wells 10 and 11, which contain faecal extract of inhibitor fed rats, the extract does contain trypsin inhibitory activity. Calculated from the height of the rocket and

Figure 16

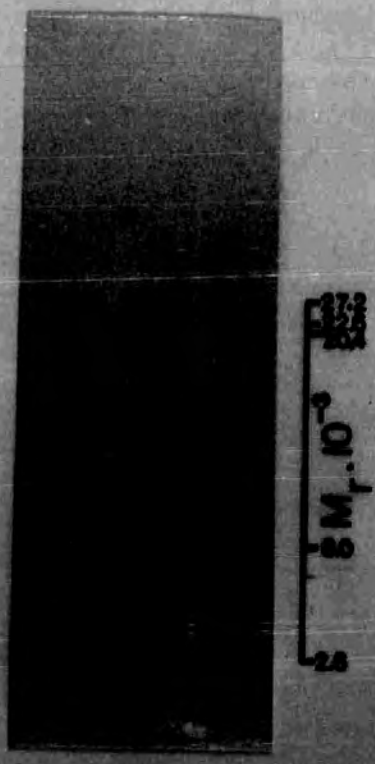



Figure 19

Figure 17

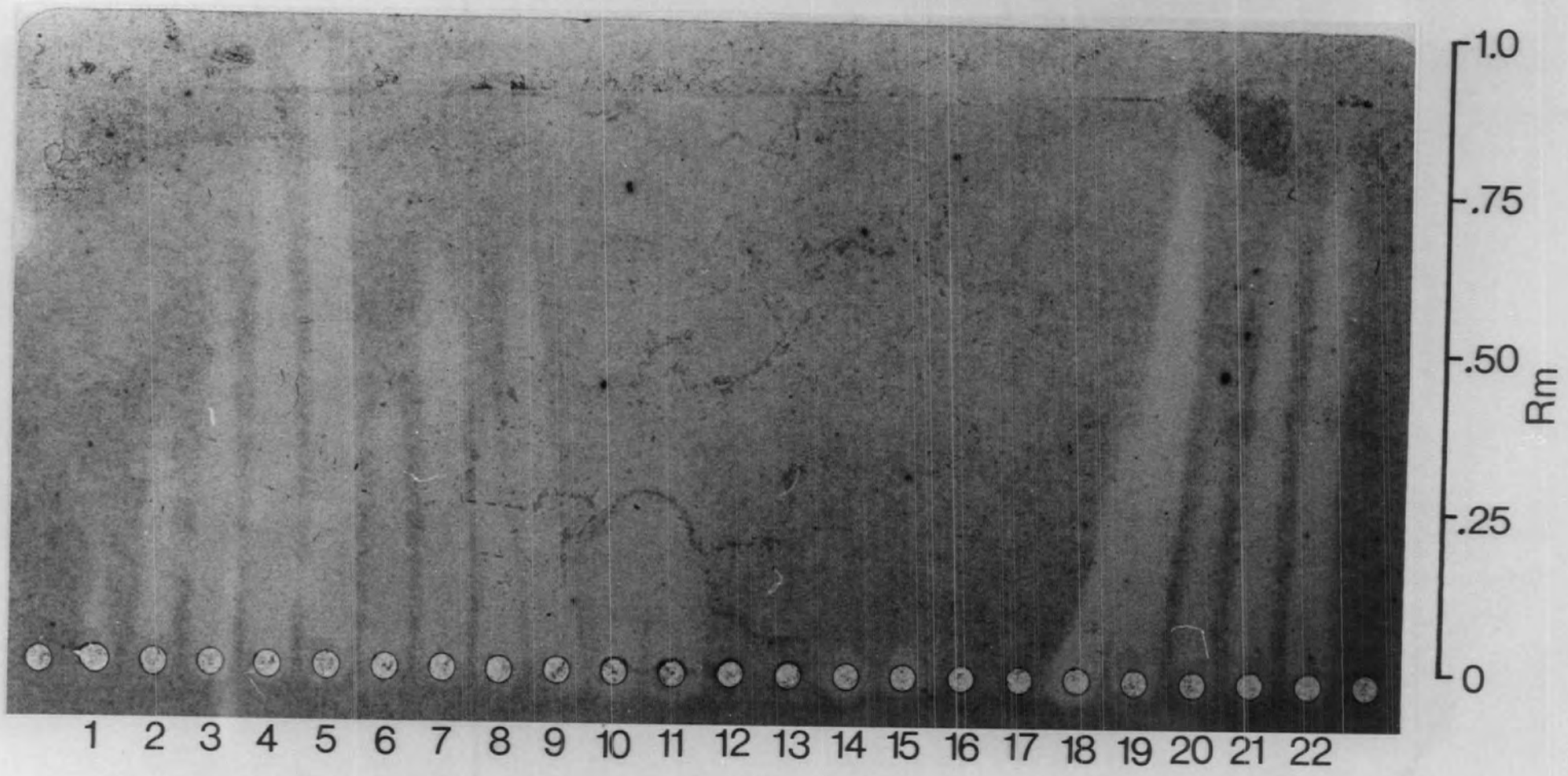
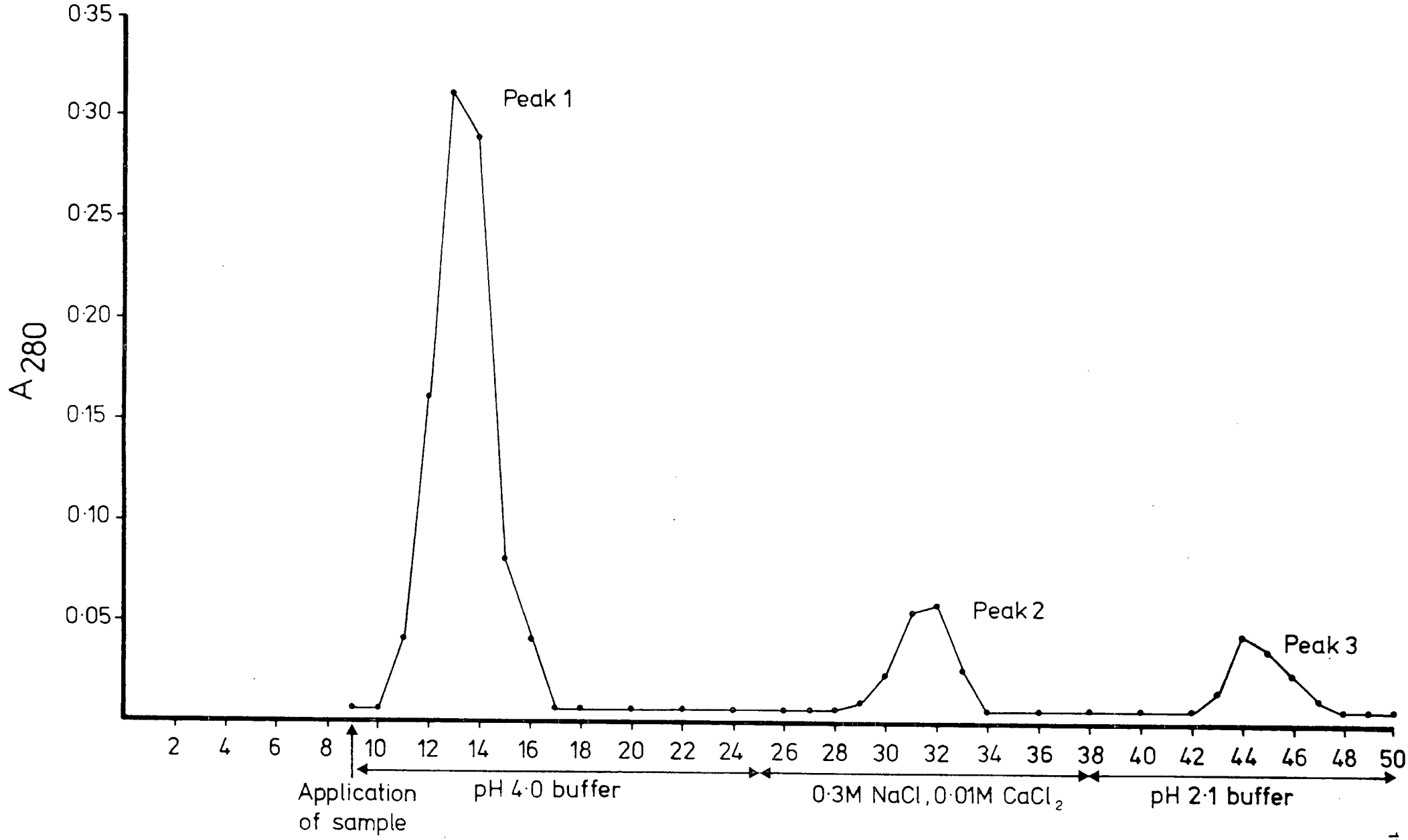


FIGURE 18 ELUTION PROFILE OF TRYPSIN AFFINITY COLUMN



the volume of extract used, trypsin inhibitory activity is present at a concentration of 0.254mg per 1g of dried rat faeces, The faecal extract of control fed rats (wells 12 and 13) shows no trypsin inhibitory activity.

Affinity chromatography was carried out on a sample of the faecal extract of inhibitor fed rats, using a column containing Sepharose bound trypsin. The elution profile from this column is shown in Fig. 18. After elution of unbound protein from the column (peak 1), the column was washed with unbuffered medium of low pH and a small peak emerged (peak 2). A sample of this peak was inserted in wells 16 and 17 of the Sepharose gel (Fig. 17): no trypsin inhibitory activity was present. The column was finally eluted with buffer at pH 2.1 and a further small peak (peak 3) appeared. (Any trypsin inhibitor present would be expected to be eluted by this low value of pH.) Samples of peak 3 were inserted into wells 14 and 15 of the Sepharose gel (Fig. 17), where a low level of trypsin inhibitory activity can be seen.

From the amount of inhibitory activity present, and the total volume of the redissolved peak (a relatively large volume was required to redissolve the high salt concentration present), trypsin inhibitor capable of ~~building~~ binding to the trypsin in this affinity column is present at a concentration of 0.1mg per 1g of dried faeces.

### 4.4.3 The use of amino acid analysis to measure the extent of the *in vitro* enzymic hydrolysis of bovine trypsin

Table 33 shows the results of 22h acid hydrolysis of the trypsin, and the yield after use of the modified Hill and Schmidt procedure for enzyme hydrolysis of denatured trypsin. The recovery of amino acids after enzymic hydrolysis gave a yield of 100.64% of the recovery after acidic hydrolysis.

Table 33 *In vitro* enzymic hydrolysis of bovine trypsin

Comparison of the yields of amino acids after enzymic hydrolysis of trypsin with the yields of amino acids after acidic hydrolysis of trypsin

| Amino acid | 22h acidic hydrolysis of 1mg trypsin<br>µmoles | Enzymic hydrolysis of 1mg of denatured trypsin<br>µmoles | Recovery of non acid-labile amino acids<br>$\frac{EH}{AH}$ % |
|---|---|---|---|
| Asp | 0.9128 | 0.3958 | |
| (Thr,Ser,Gln,Asn) | - | 2.4476 | |
| Thr | 0.3679 | - | |
| Ser | 1.0411 | - | |
| Glu | 0.7098 | 0.1585 | |
| Pro | 0.4565 | 0.4246 | |
| Gly | 1.1574 | 1.2143 | |
| Ala | 0.5913 | 0.6457 | |
| Cys/2 | 0.3810* | 0.4182 | |
| Val | 0.7524 | 0.7352 | 97.71 |
| Met | 0.0740 | 0.0589 | |
| Ile | 0.6078 | 0.7012 | 115.37 |
| Leu | 0.6614 | 0.7052 | 106.62 |
| Tyr | 0.3994 | 0.4145 | 103.78 |
| Phe | 0.1488 | 0.1278 | 85.89 |
| His | 0.1062 | 0.1067 | 100.47 |
| Lys | 0.5445 | 0.5746 | |
| Trp | 0 | 0.2038 | |
| Arg | 0.2403 | 0.0361 | |
| Total recovery of amino acids, excluding Trp | 9.1526 | 9.1676 | |
| Recovery of amino acids $\frac{EH}{AH}$ % | | 100.16 | |

* Determined as cysteic acid by the method of Moore (1963)

4.4.4 The use of amino acid analysis to measure the extent of the *in vitro* enzymic hydrolysis of trypsin in the presence of trypsin inhibitor

Table 34 shows the yield of amino acids measured after the enzymic hydrolysis of trypsin in the presence of trypsin inhibitor. Active trypsin inhibitor and native trypsin had been preincubated together at 37°C, before heating at 100°C. for 10min and hydrolysis by the modified Hill and Schmidt enzymic method, as described in the Methods section.

The recovery of amino acids was only 41.77% of that expected, and the recovery of amino acids from trypsin and trypsin inhibitor together (4.9μmoles) was less than the yield from hydrolysis of trypsin alone (9.4μmoles).

In Table 35 are shown the yields of amino acids after enzymic hydrolysis of trypsin together with trypsin inhibitor, following a preincubation period before which both components had separately been heated to 100°C for 10min. The yields of amino acids are shown together with the yields which could be expected from the sum of individual hydrolysates of denatured trypsin and trypsin inhibitor. It can be seen that the values found agreed with those "expected", with an actual yield 101.63% of that expected.

Therefore, as shown in Table 36, when trypsin and trypsin inhibitor are preincubated in their native states, the yield of amino acids after subsequent enzymic hydrolysis is 40.60% of that achieved when the two components are separately denatured before their incubation together.

Table 34 *In vitro* enzymic hydrolysis of bovine trypsin in the presence of trypsin inhibitor from *Phaseolus vulgaris*, following preincubation together in their active forms

| Amino acid | Enzymic hydrolysis of 1mg T (Table 33) µmoles | Enzymic hydrolysis of 1mg TI (Table 32) µmoles | Sum of EH of T + EH of TI "expected" µmoles | Enzymic hydrolysis of 1mg T + 1mg TI "found" µmoles | $\frac{EH(T+TI)}{EH(T)+EH(TI)}$ * % |
|---|---|---|---|---|---|
| Asp | 0.3985 | 0 | 0.3985 | 0.1233 | 30.94 |
| (Thr,Ser,Gln,Asn) | 2.4476 | 0.2495 | 2.6971 | 0.7959 | 29.51 |
| Glu | 0.1585 | 0.2025 | 0.3610 | 0.0841 | 23.30 |
| Pro | 0.4246 | 0.0275 | 0.4521 | 0.0862 | 19.07 |
| Gly | 1.2143 | 0.3575 | 1.5718 | 0.7688 | 48.91 |
| Ala | 0.6457 | 0.3081 | 0.9538 | 0.3968 | 41.55 |
| Cys/2 | 0.4182 | 0 | 0.4182 | 0.1638 | 39.17 |
| Val | 0.7352 | 0.2172 | 0.9524 | 0.3198 | 33.58 |
| Met | 0.0589 | 0.0628 | 0.1217 | 0.0552 | 45.36 |
| Ile | 0.7012 | 0.0530 | 0.7542 | 0.3162 | 41.93 |
| Leu | 0.7052 | 0.1105 | 0.8152 | 0.4311 | 52.85 |
| Tyr | 0.4145 | 0.0593 | 0.4738 | 0.2681 | 56.59 |
| Phe | 0.1278 | 0.0902 | 0.2180 | 0.1277 | 58.58 |
| His | 0.1067 | 0.1268 | 0.2335 | 0.1353 | 57.94 |
| Lys | 0.5746 | 0.3924 | 0.9670 | 0.5701 | 58.95 |
| Trp | 0.2038 | 0.1152 | 0.3190 | 0.1828 | 57.30 |
| Arg | 0.0361 | 0.1205 | 0.1566 | 0.1306 | 83.40 |
| Total recovery of amino acids | 9.3714 | 2.4930 | 11.8644 | 4.9553 | 41.77 |

EH = Enzymic hydrolysis
T = Trypsin
TI = Trypsin inhibitor
* If, when trypsin and trypsin inhibitor are incubated together, each retains its original susceptibility to enzymic hydrolysis, then after enzymic hydrolysis of the mixture, the total yield of amino acids may be expected to equal the sum of the yields from individual enzymic hydrolysis of trypsin and trypsin inhibitor; the "expected" yield.

Table 35 *In vitro* enzymic hydrolysis of bovine trypsin in the presence of trypsin inhibitor from *Phaseolus vulgaris*, both denatured before incubation together

| Amino acid | Enzymic hydrolysis of 1mg denatured T (Table 33) | Enzymic hydrolysis of 1mg denatured TI (Table 32) | Sum of (EH denatured T + EH denatured TI) "expected" | Enzymic hydrolysis of 1mg denatured T + 1mg denatured TI "found" | $\frac{EH(T+TI)}{EH(T)+EH(TI)}$ |
|---|---|---|---|---|---|
| | µmoles | µmoles | µmoles | µmoles | % |
| Asp | 0.3958 | 0 | 0.3958 | 0.4558 | 115.16 |
| (Thr,Ser,Gln,Asn) | 2.4476 | 0.3299 | 2.7775 | 2.9062 | 104.53 |
| Gln | 0.1585 | 0.1419 | 0.3004 | 0.4453 | 148.24 |
| Pro | 0.4246 | 0.0455 | 0.4701 | 0.4278 | 91.00 |
| Gly | 1.2143 | 0.3607 | 1.5750 | 1.6551 | 105.09 |
| Ala | 0.6457 | 0.3036 | 0.9493 | 1.0793 | 113.69 |
| Cys/2 | 0.4182 | 0 | 0.4182 | 0.3474 | 83.07 |
| Val | 0.7352 | 0.2181 | 0.9533 | 0.9224 | 96.76 |
| Met | 0.0589 | 0.0292 | 0.0881 | 0.0891 | 101.14 |
| Ile | 0.7012 | 0.0516 | 0.7528 | 0.7105 | 94.38 |
| Leu | 0.7052 | 0.1245 | 0.8297 | 0.8494 | 102.37 |
| Tyr | 0.4145 | 0.0885 | 0.5030 | 0.4477 | 89.01 |
| Phe | 0.1278 | 0.1732 | 0.3010 | 0.2509 | 83.36 |
| His | 0.1067 | 0.1393 | 0.2460 | 0.2172 | 88.29 |
| Lys | 0.5746 | 0.4358 | 1.0104 | 0.8708 | 86.18 |
| Trp | 0.2038 | 0.0934 | 0.2972 | 0.4399 | 148.01 |
| Arg | 0.0361 | 0.1020 | 0.1381 | 0.0892 | 64.59 |
| Total recovery of amino acids | 9.3714 | 2.6372 | 12.0086 | 12.2040 | 101.63 |

T = Trypsin
TI = Trypsin Inhibitor
EH = Enzymic hydrolysis

Table 36 Effect of denaturation prior to preincubation, upon the *in vitro* enzymic hydrolysis of bovine trypsin in the presence of trypsin inhibitor from *Phaseolus vulgaris*

| Amino acid | Enzymic hydrolysis of 1mg trypsin in the presence of 1mg trypsin inhibitor. Both heated at 100°C for 10min before preincubation together (Table 35) µmoles | Enzymic hydrolysis of 1mg trypsin in the presence of 1mg trypsin inhibitor. Both in active forms when incubated together (Table 34) µmoles | EH native (T+TI) / EH denatured (T+TI) % |
|---|---|---|---|
| Asp | 0.4558 | 0.1233 | 27.05 |
| Thr,Ser + Amides | 2.9062 | 0.7959 | 27.39 |
| Glu | 0.4453 | 0.0841 | 18.89 |
| Pro | 0.4278 | 0.0862 | 20.15 |
| Gly | 1.6551 | 0.7688 | 46.45 |
| Ala | 1.0793 | 0.3963 | 36.72 |
| Cys/2 | 0.3474 | 0.1638 | 47.15 |
| Val | 0.9224 | 0.3198 | 34.67 |
| Met | 0.0891 | 0.0552 | 61.95 |
| Ile | 0.7105 | 0.3162 | 44.50 |
| Leu | 0.8494 | 0.4311 | 50.75 |
| Tyr | 0.4474 | 0.2681 | 59.92 |
| Phe | 0.2509 | 0.1277 | 50.90 |
| His | 0.2172 | 0.1353 | 62.29 |
| Lys | 0.8708 | 0.5701 | 65.47 |
| Trp | 0.4399 | 0.1828 | 41.55 |
| Arg | 0.0892 | 0.1306 | 146.51 |
| Total recovery of amino acids | 12.2040 | 4.9553 | 40.60 |

4.4.5 The use of SDS polyacrylamide gels to follow the *in vitro* enzymic hydrolysis of *Phaseolus vulgaris* trypsin inhibitor

Comparison of the gel pattern of the original trypsin inhibitor with that after the enzymic hydrolysis procedure (tracks 2, 4 and 5, Fig. 19) reveals that there was a change in the molecular weight of the component bands after enzymic hydrolysis, with the disappearance of the 27200 band of the original trypsin inhibitor, and the appearance of bands at 23500, 19200 and 17000. Also these bands corresponding with $M_r$ 19200 and 17000 are more prominent in the sample which was denatured before enzymic hydrolysis, than in the sample in which the native form was hydrolysed, in which the band at $M_r$ 23500 is predominant.

While amino acid analysis has shown that there is only a relatively low release of amino acids after enzymic hydrolysis of the trypsin inhibitor, the gel reveals that large ~~protein~~ peptide components do remain, although of lower molecular weight than those in the original trypsin inhibitor.

4.4.6 The use of SDS polyacrylamide gels to follow the *in vitro* enzymic hydrolysis of bovine trypsin

Pure bovine trypsin is shown in track 3 of Fig. 19, which reveals a large number of bands. Denatured trypsin ~~inhibitor~~ was subjected to the enzymic digestion procedure, and a sample of the resulting hydrolysate is shown in track 8. No bands of stained protein are present, agreeing with the results from amino acid analysis that trypsin is not resistant to enzymic hydrolysis.

4.4.7 The use of SDS polyacrylamide gels to follow the *in vitro* enzymic hydrolysis of trypsin inhibitor in the presence of trypsin

In track 6 of Fig. 19, the products of enzymic hydrolysis of denatured trypsin inhibitor and trypsin show bands corresponding in their prominence with those in track 4, the products of enzymic hydrolysis of denatured trypsin inhibitor. In track 6, the protein band at $M_r$ 23500 is very feint in comparison with that at 17000.

In track 7, the products of enzymic hydrolysis of native trypsin inhibitor and trypsin show a similar pattern to that in track 5, except for a very broad and prominent band centred at $M_r$ 17000. This band may have been produced by a combination of protein at $M_r$ 17000 from trypsin inhibitor, seen in tracks 4, 5 and 6, together with protein from trypsin at $M_r$ values 18000 and 17000. Track 7 also shows feint bands at positions of approximate $M_r$ 8000, 6000, 5000 and 3500. These extra bands are not visible in track 6. Also in track 7, the band corresponding with protein at $M_r$ 23500 is more prominent than in track 6.

## 4.5 THE USE OF ENZYMIC HYDROLYSIS *IN VITRO* TO MEASURE THE AVAILABILITY OF AMINO ACIDS

### 4.5.1 The use of enzymic hydrolysis *in vitro* to measure available lysine in fish meals

The levels of lysine measured by amino acid analysis following enzymic hydrolysis of the fish meals, and after acid hydrolysis of the meals, are shown in Table 37. Also shown are the results of assays of the same samples by three other methods: chick growth, microbiological assay using *Tetrahymena pyriformis*, and a dye-binding method.

The results from enzymic hydrolysis correlated most closely ($r = 0.978$) with those from the chick assay, although good correlation ($r > 0.95$) was also shown towards the dye-binding assay. Assay using *Tetrahymena* showed the lowest correlation of the three assays with the enzymic hydrolysis method.

Acid hydrolysis resulted in the highest yields of lysine from all samples except for sample E, for which *Tetrahymena* gave a higher value. Relatively low correlations ($< 0.82$) were shown between the yields of lysine after acidic hydrolysis and the yields after the four other assay methods.

### 4.5.2 The use of enzyme hydrolysis *in vitro* to measure available methionine in fish meals

Methionine was measured after enzymic hydrolysis and acidic hydrolysis of fish meals, and these results are listed in Table 38, together with the results from chick growth and *Streptococcus zymogenes* assays. The results from enzymic hydrolysis correlated most nearly

with those from chick growth assay. The levels remaining after acidic hydrolysis showed very poor correlation with the results from enzymic hydrolysis and the biological assays. Methionine sulphoxide was found to be present in the enzymic hydrolysates, and the total levels of methionine plus methionine sulphoxide are also shown in Table 38. The levels of methionine given by enzymic hydrolysis were in all cases lower than those given by the two bioassay methods, but this difference was reduced when the total methionine plus sulphoxide values of the enzymic hydrolysates were compared. However, use of the total methionine plus methionine sulphoxide values from the enzymic hydrolysate did not improve the correlation between these results and those from the other assay methods. Error in measurement of the methionine sulphoxide may have occurred because of its elution position immediately preceding aspartate; since the fish meal samples contained high levels of aspartate, the measurement of the very small methionine sulphoxide peak was made difficult.

Table 37 Use of enzymic hydrolysis to measure available lysine in fish meals: comparison with data obtained by other assay methods

| Sample | Acidic hydrolysis | Enzymic hydrolysis | Chick* | *Tetrahymena pyriformis** | Dye-binding* |
|---|---|---|---|---|---|
| A | 9.18 | 8.22 | 7.7 | 7.8 | 7.4 |
| B | 7.49 | 6.77 | 7.2 | 6.5 | 6.3 |
| C | 5.98 | 1.47 | 3.0 | 4.8 | 3.8 |
| D | 9.21 | 8.39 | 7.6 | 6.2 | 6.5 |
| E | 7.78 | 7.70 | 7.8 | 7.9 | 6.8 |
| F | 9.40 | 8.94 | 8.8 | 7.6 | 6.8 |
| G | 6.14 | 5.32 | 5.1 | 6.1 | 5.2 |
| H | 9.28 | 6.46 | 6.4 | 5.8 | 5.9 |
| Correlation with values for: | | | | | |
| Enzymic hydrolysis | 0.813 | - | 0.978 | 0.810 | 0.956 |
| Acidic hydrolysis | - | - | 0.798 | 0.514 | 0.777 |

Levels of lysine are expressed in g16g$^{-1}$N
Mean coefficient of variation of acidic hydrolysates = 9.19%
Mean coefficient of variation of enzymic hydrolysates = 9.51%

* Data supplied by Dr J.E. Ford.

Table 38 Use of enzymic hydrolysis to measure available methionine in fish meals: comparison with data obtained by other assay methods

| Sample | Acidic hydrolysis | Enzymic hydrolysis | | Chick* | *Streptococcus zymogenes** |
|---|---|---|---|---|---|
| | | methionine | methionine + methionine sulphoxide | | |
| A | 3.46 | 2.40 | 3.41 | 3.24 | 3.07 |
| B | 2.99 | 1.37 | 2.36 | 2.53 | 2.81 |
| C | 2.70 | 0.63 | 0.87 | 1.10 | 1.54 |
| D | 3.44 | 2.37 | 3.06 | 3.00 | 2.80 |
| E | 2.28 | 2.30 | 2.30 | 2.65 | 2.58 |
| F | 2.75 | 2.59 | 3.39 | 2.93 | 2.76 |
| G | 2.20 | 1.68 | 2.83 | 2.01 | 2.12 |
| H | 3.32 | 1.39 | 1.39 | 2.20 | 2.26 |
| Correlation with methionine value from: | | | | | |
| enzymic hydrolysis | 0.036 | - | | 0.902 | 0.800 |
| acidic hydrolysis | - | - | | 0.116 | 0.122 |
| Correlation with methionine sulphoxide value from: | | | | | |
| enzymic hydrolysis | 0.035 | - | | 0.845 | 0.803 |

Levels of methionine and methionine sulphoxide are expressed in $g16g^{-1}N$
Mean coefficient of variation of acidic hydrolysis = 8.99%
Mean coefficient of variation of enzymic hydrolysis = 7.61%

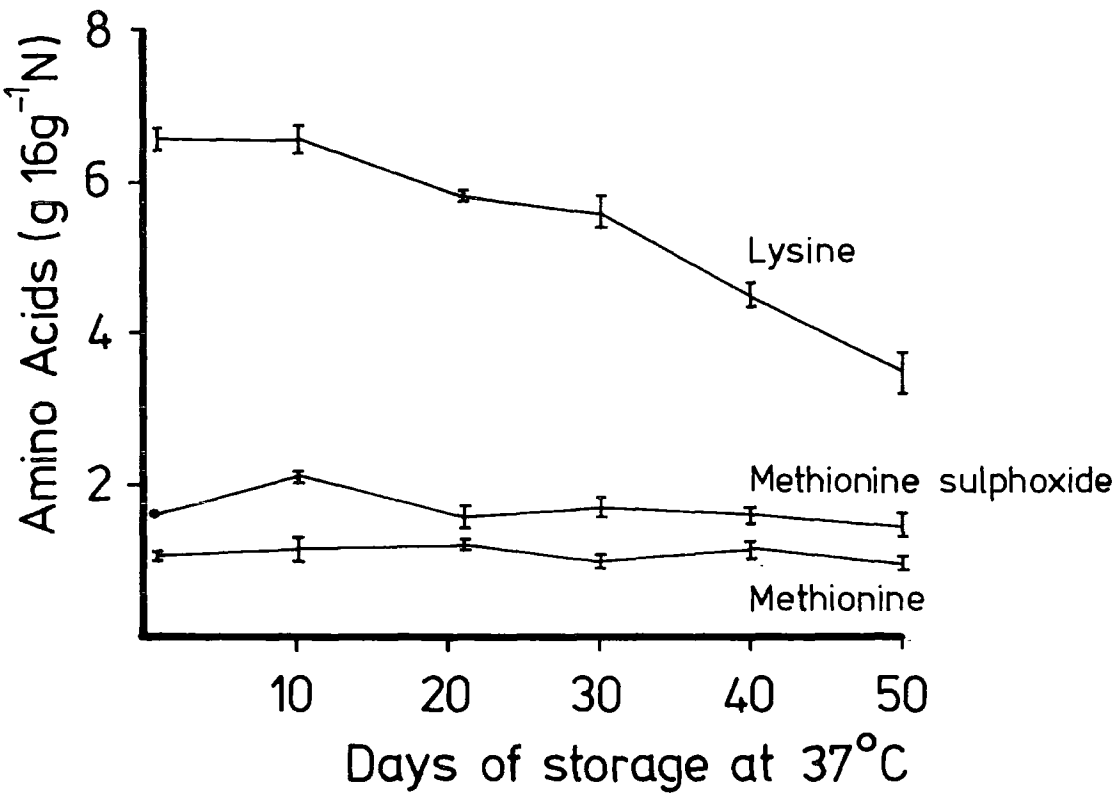
* Data supplied by Dr J.E. Ford.

4.5.3 The measurement of available lysine and methionine in skim-milk powder

The lysine levels measured in enzymic hydrolysates of skim-milk powder, which had previously been incubated at 37°C for up to 50 days, are shown in Fig. 20. The levels of lysine released by enzymic hydrolysis decreased as the period of heat treatment lengthened. The mean level of lysine in the samples taken after 50 days at 37°C was 54.3% of the original mean value.

Also shown in Fig. 20 are the levels of methionine found in the enzymic hydrolysates, together with the greater levels of methionine sulphoxide which were also present.

FIGURE 20 LEVELS OF LYSINE, METHIONINE, AND METHIONINE SULPHOXIDE, MEASURED AFTER ENZYMIC HYDROLYSIS OF SAMPLES OF SKIM-MILK WHICH HAD BEEN STORED AT 37°C FOR UP TO 50 DAYS



## 4.6 THE USE OF ENZYMIC HYDROLYSIS *IN VITRO* TO MEASURE THE AMIDE CONTENT OF PROTEINS

### 4.6.1 Analysis of proteins extracted from peas, *Pisum sativum*, at various stages of germination

SDS polyacrylamide gels of the proteins analysed are shown in Figs. 21 and 22. In Fig. 21, tracks 5 - 8 show samples of the total protein extracted from the pea cotyledons. At the onset of germination, subunits of convicilin ($M_r$ 71000), vicilin ($M_r$ 51000 and 33000) and legumin ($M_r$ 40000) were present. Samples taken at 3,5,7 and 10 days of germination showed progressively smaller amounts of these high molecular weight polypeptide bands, with increasing amounts of polypeptides of lower molecular weights.

The purified samples of vicilin (the sample of vicilin prepared from cotyledons at day 0 contained some convicilin) and legumin showed that the high molecular weight bands disappeared until little remained at 7 days after germination. Convicilin showed a more rapid degradation, with no high molecular weight polypeptides to be stained in the 5 day sample.

After enzymic hydrolysis of the proteins it was found that most of the levels of Ser, Asp, Glu and Gln showed little change following germination (Tables 39 and 40), although in legumin the level of Gln had decreased to zero by day 7. The ratios of Asn/Asp reveal that in all samples Asn was more abundant than Asp, but Gln was only present in greater concentration than Glu in the sample of legumin taken at day 0 of germination, and the sample of convicilin taken at day 3.

When the values from enzymic hydrolysis of legumin were compared with acid hydrolysates of the same samples (see Table 40), it was found that the total (Asp + Asn) of the enzymic hydrolysates amounted to 94 - 103% of the Asp in the acid hydrolysates, while the enzymically released levels of (Glu and Gln) amounted to 55 - 75% of the Glu detected in the acid hydrolysates.

Figure 21

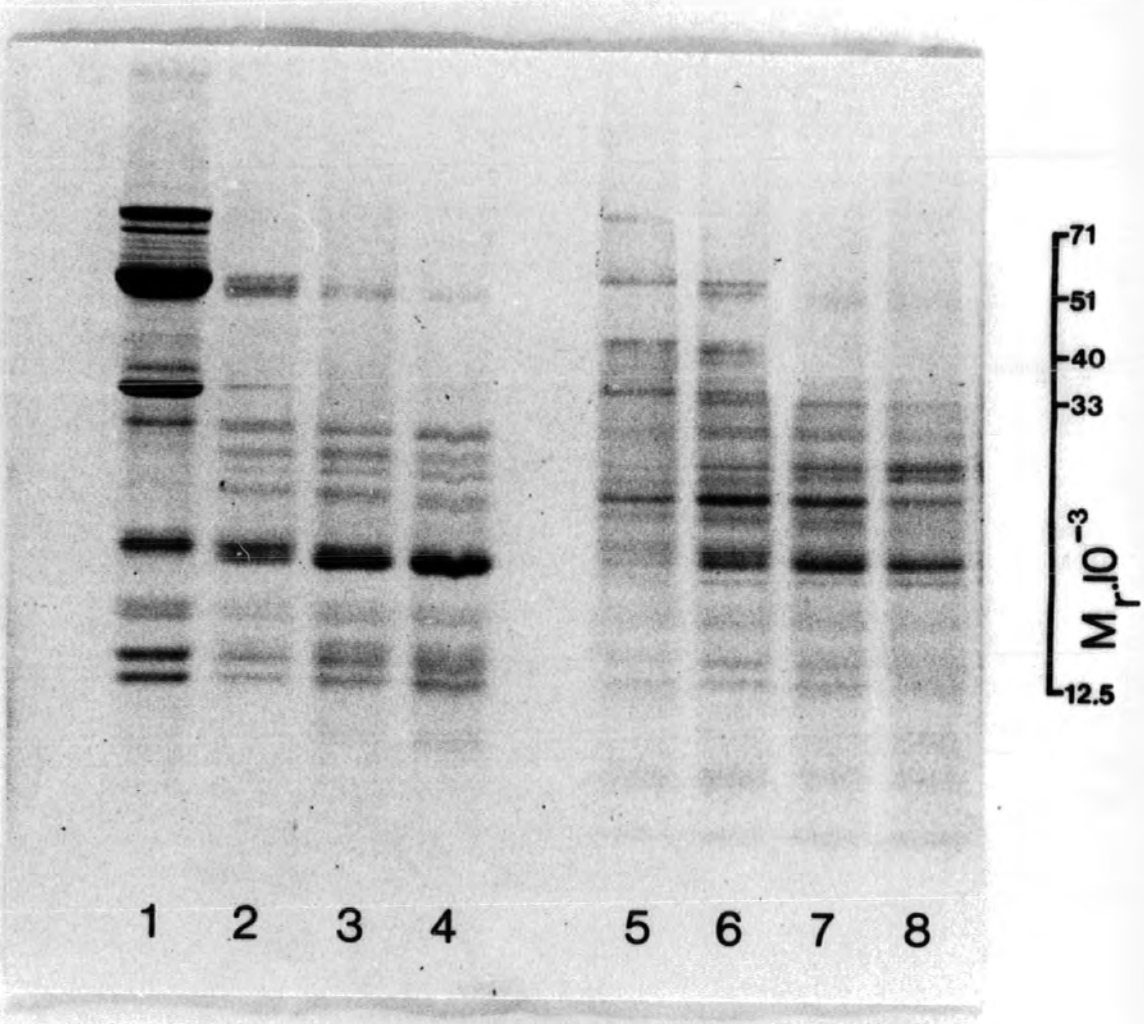



Figure 22

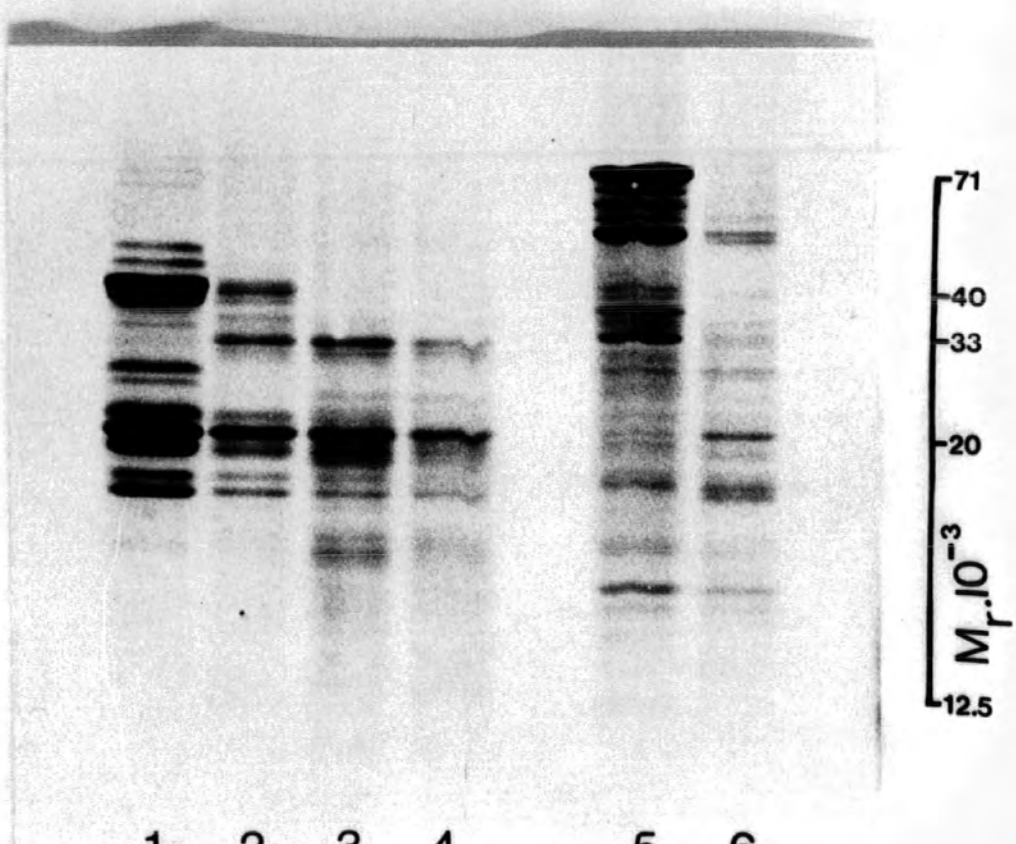


Table 39 Measurement of some amino acids after enzymic hydrolysis of convicilin and vicilin purified from peas, *Pisum sativum*, at various stages of germination

a. Vicilin

| Amino acid | Days of germination | | | |
|---|---|---|---|---|
| | 0 | 3 | 5 | 7 |
| Ser | 4.60 | 4.92 | 4.76 | 4.71 |
| Asn | 12.48 | 13.96 | 14.28 | 14.18 |
| Asp | 3.01 | 3.12 | 3.07 | 3.39 |
| Gln | 2.15 | 1.56 | 2.13 | 2.37 |
| Glu | 9.25 | 12.54 | 10.09 | 11.86 |
| Asn/Asp | 4.15 | 4.47 | 4.65 | 4.18 |
| Gln/Glu | 0.23 | 0.12 | 0.21 | 0.20 |

b. Convicilin

| Amino acid | Days of germination | |
|---|---|---|
| | 0 | 3 |
| Ser | 3.32 | 3.49 |
| Asn | 3.95 | 4.05 |
| Asp | 2.14 | 3.16 |
| Gln | 2.81 | 6.89 |
| Glu | 5.13 | 5.25 |
| Asn/Asp | 1.85 | 1.28 |
| Gln/Glu | 0.55 | 1.31 |

Levels of amino acids, measured by amino acid analysis using $Li^+$ buffers, are expressed in $g16g^{-1}N$.

Table 40 Measurement of some amino acids after enzymic hydrolysis of legumin purified from peas, *Pisum sativum*, at various stages of germination

| Amino acid | Days of germination | | | |
|---|---|---|---|---|
| | 0 | 3 | 5 | 7 |
| Ser | 3.39 | 3.85 | 3.88 | 3.88 |
| Asn | 8.54 | 8.29 | 5.30 | 8.16 |
| Asp | 3.11 | 3.30 | 3.11 | 4.81 |
| Gln | 8.38 | 5.08 | 2.22 | 0 |
| Glu | 5.76 | 5.30 | 5.45 | 4.93 |
| Asn/Asp | 2.75 | 2.51 | 1.70 | 1.70 |
| Gln/Glu | 1.46 | 0.96 | 0.41 | 0 |
| (Asn + Asp) expressed as a % of Asp recovered after acid hydrolysis | 103.3 | 102.1 | 98.8 | 94.4 |
| (Glu + Gln) expressed as a % of Glu recovered after acid hydrolysis | 75.2 | 68.0 | 55.1 | 62.2 |

Levels of amino acids, measured by amino acid analysis using $Li^+$ buffer, are expressed in $gl6g^{-1}N$.

# 5.0 DISCUSSION

## Choice of a method for proteolysis using enzymes *in vitro*

The first aim of this study was to find an efficient method for the total enzymic hydrolysis of proteins *in vitro*. Because the method described by Hill and Schmidt (1962) has provided a pattern for all subsequent methods, it was decided initially to test this method. Since the introduction of the method, commercial preparations of the enzymes used by Hill and Schmidt have become available, and were used in this study. In addition, aminopeptidase M was used in place of the leucine aminopeptidase described by Hill and Schmidt. This enzyme, although of similar activity, has the advantage that it has no cation requirement, whereas leucine aminopeptidase requires $Mn^{++}$ which tends to precipitate out of solution during incubation and assay of the enzyme.

The prolidase used by Hill and Schmidt had been freshly prepared from pig kidney and was known to contain prolidase (the ability to hydrolyse bonds of the type X-Pro), prolinase (the ability to hydrolyse bonds of the type Pro-X), and aminopeptidase activities. Therefore in this work it was necessary first to establish whether prolidase, freshly prepared from pig kidney, was necessary for complete proteolysis, or whether a commercial preparation of prolidase would suffice. Other authors following the Hill and Schmidt method have all used freshly prepared prolidase (Dvorak, 1968; Ramponi *et al*, 1971), and the need for its purification has been the reason for its omission from some enzymic digests (Rayner and Fox, 1976).

In this work, prolidase was prepared from pig kidney acetone extract according to the simplified method of Dvorak (1968), and prolidase and prolinase activities were found in both this freshly prepared

preparation and a commercial preparation of prolidase (Table 9). The commercial preparation contained greater prolidase and prolinase activity than the fresh preparation, although the prolinase was present in lower proportion. The proteolytic coefficient, $C_1$, was determined for each preparation (Table 10), and only the commercial preparation showed a value of $C_1$ within the range of $C_1$ = 10 - 20 recommended by Hill and Schmidt (1962).

For the complete hydrolysis of proteins which contain proline, both prolidase and prolinase type activities must be present to cleave all peptide bonds formed by proline. From this work, it appears that the crude preparations of "prolidase" used in enzymic digest methods are likely to have also contain prolinase activity. Only Bensusan *et al* (1971) have specified the use of separately purified prolinase and prolidase as part of the digestion mixtures which they designed for the complete hydrolysis of collagen.

Because some enzymic methods have been claimed to give the complete hydrolysis of proteins yet contain no prolidase enzyme (*eg* the methods of Sletten, 1968; Winkler and Schön, 1979), a variety of the commonly used enzymes were assayed for the presence of prolidase-type activity (Table 11). These assays showed that apart from the prolidase preparation, only one other enzyme preparation, acylase (also extracted from pig kidney), contained prolidase-type activity. Neither prolidase nor prolinase-type activities were present in any of the other enzyme preparations which are commonly used in enzymic digestion mixtures. Therefore, without the presence of prolidase in an enzymic digestion mixture, complete hydrolysis of proteins containing proline cannot be expected.

The modified Hill and Schmidt procedure was first tested for its ability to completely hydrolyse BSA. This protein was chosen as a substrate because it has previously been successfully hydrolysed by the Hill and Schmidt method (Dvorak, 1968). The yields of amino acids after enzymic hydrolysis of BSA were less than the yields after acid hydrolysis of a sample of the original BSA (Table 12). However, subsequent acid hydrolysis of the enzymic digests resulted in little change in the levels of the (non acid-labile) amino acids. These results indicate that enzymic hydrolysis achieved complete hydrolysis of the protein, but there was apparently a net loss of amino acids during the enzymic hydrolysis due to the additional handling steps involved in the procedure. To correct for this in subsequent digestions, an internal standard of nor-leucine (see methods) was added at the start of all incubations, and was therefore able to reflect any loss of amino acids which occurred during the various filtration steps of the procedure.

When incorporated into the Hill and Schmidt method, the commercial and fresh preparations of prolidase achieved similar yields of all amino acids, and the commercial preparation was therefore used as part of the modified Hill and Schmidt procedure.

This modified method was also successful in hydrolysing both ovalbumin and the oxidized B chain of insulin (Tables 13 and 14). Ovalbumin was chosen as the test protein in this study because it is known to be fully digestible *in vivo*, and is often used as a reference protein in nutritional studies (Mauron, 1970). Ovalbumin provides a good test substrate for any enzymic procedure since it is a relatively large protein containing 385 residues, one disulphide bridge, a carbohydrate moiety, and all twenty common amino acids. Also, preparations of high purity are available. The oxidized B chain of insulin was also used as a test substrate: in contrast with ovalbumin, this small polypeptide contains only twenty amino acids, with no disulphide bonding.

After enzymic hydrolysis of these two proteins, the digests were acid hydrolysed, to test whether the enzymes had achieved complete hydrolysis (Tables 13 and 14). The majority of the (non acid-labile) amino acids showed a change in level of less than 10%. However, the pattern of amino acids found after enzymic hydrolysis never exactly corresponded with that found after acid hydrolysis. Hill and Schmidt (1962) found a similar variation in the recovery of some amino acids, a complication which arises with the use of enzymes for the hydrolysis of proteins because of the possibility of amino acids being released by self-digestion of the enzymes.

To allow for this, control digests were also prepared with each digestion experiment, in which the enzymes were incubated without any added substrate. The levels of amino acids detected in these control digests were subtracted from the levels found in the sample digests. The levels of amino acids detected in the controls were between 0-0.5µmole (Table 24), which could amount to 10% of the levels of the amino acids released from the substrate during hydrolysis. However, the extent to which autolysis occurs may be greater in the absence of any added substrate, than in a digest mixture containing the test substrate. The final levels of amino acids after enzymic hydrolysis and subtraction of the controls may therefore be slightly lower than the true values. After acidic hydrolysis of both the substrate and the control digests, the problem does not arise since in both cases the enzymes will be completely hydrolysed. For this reason, it may be expected that values after acidic hydrolysis and enzymic hydrolysis of a substrate may be slightly higher than after enzymic hydrolysis alone.

Another problem in measuring the yield of amino acids after enzymic hydrolysis was that when the amino acid analyzer was used with a $Na^+$ buffer system, the glutamine and asparagine present in the enzymic

digests coeluted with threonine and serine, and the calculation of the number of residues present in this peak could therefore only be approximate. Calculation of asparagine and glutamine by subtraction of the levels of aspartic acid and glutamic acid present in the enzymic hydrolysate, from the levels present after acidic hydrolysis, gave the theoretical values of asparagine and glutamine expected in insulin, but overestimated the levels present in ovalbumin, in each case by two residues, according to the composition given by McReynolds *et al* (1978).

Arginine values were often poor in the analyses due to the elution position of arginine at the end of each sample, where an irregular trace was sometimes produced.

It has therefore been found that for the majority of non acid-labile amino acids of ovalbumin, insulin B chain and BSA, the levels of amino acids after enzymic hydrolysis changed by up to 10% after subsequent acid hydrolysis. To determine the error which may be accepted in the recovery of amino acids, a mixture of amino acids of known concentration was treated by the full enzymic and acidic hydrolysis procedures (Table 15). After enzymic hydrolysis, the recoveries of all amino acids except for cysteic acid, proline and arginine, were detected to within 10% of their original concentrations. These values were within 14% of the levels measured after subsequent acidic hydrolysis. From this, it was concluded that any protein which showed recoveries within these levels was as fully digestible *in vitro* as the test proteins ovalbumin, insulin B chain and BSA.

In an attempt to remove the problem of background levels of amino acids caused by enzymic autolysis, the possibility of using coupled enzymes was investigated. After immobilization to insoluble supports, enzymes tend to be more stable than their soluble counterparts, because the restricted movement of the enzyme makes autolysis less likely.

Other advantages are the simple removal of the enzymes from the digest mixture and the reuse of enzyme preparations. Also, it may be possible to automate a process based on immobilized enzymes.

Immobilization of enzymes to agarose supports which have been activated by cyanogen bromide (March *et al*, 1974) is one of the simplest methods available for coupling. Whilst it worked well for proteinase-K, papain and trypsin (Table 16), the pronase preparations retained little activity, although the reasons for this are not clear. Alternative methods were therefore sought for coupling pronase, and although good levels of binding were attained between pronase and a variety of supports (Table 17), using a variety of conditions, for example Fig. 3, on none except CH-Sepharose-4B did the pronase retain its activity during storage (Fig. 4). ~~Thus~~ This Sepharose is supplied in an activated form which allows very gentle conditions to be used for the coupling of the enzyme, and it would appear that such conditions are required by pronase in order to retain its activity. Thus coupled, the pronase still showed the full complement of enzymic activities which were present in the soluble enzyme (Table 18). Methods already exist for coupling pronase to porous glass ( Detar, 1975; Ohmiya, 1978), but in my hands, pronase coupled to porous glass proved difficult to assay because of the friability of the glass, and the preparations quickly lost activity. Prolidase was also successfully coupled to CH-Sepharose 4B.

The use of the coupled preparations of pronase, prolidase and proteinase-K was tested as a method of achieving complete proteolysis, using the test substrate, ovalbumin (Table 19). Used on its own, the coupled pronase released 66% of the amino acids of ovalbumin. The yields of all amino acids increased in the additional presence of proteinase-K and, as would be expected, proline was only among the amino acids released when coupled prolidase was incorporated into the

mixture. Although the recoveries of some amino acids were comparable with those achieved by the previous hydrolysis of ovalbumin using soluble enzymes (Table 14), the recoveries of the acidic and hydroxyl amino acids and glycine were very poor. Low recoveries of these amino acids and proline were also found by Holt *et al* (1971), when using pronase with prolidase and leucine aminopeptidase. These results suggest that pronase poorly hydrolyses peptide bonds involving the acidic amino acids and this may be supported by the observation of Glazer *et al*, 1975, that when several acidic amino acids occur in a cluster in a protein chain, they can be resistant to enzymic hydrolysis.

The same enzymic mixture of all three coupled enzymes was also used for the hydrolysis of the insulin B chain, and again, while most amino acids were fully recovered, the yields of glutamic acid, proline and glycine were very low (there is no aspartic acid present in this insulin molecule). The recoveries of amino acids after hydrolysis of the ovalbumin and insulin (Table 21) indicated that this method of hydrolysis was not as efficient as the previous method using soluble papain, prolidase and aminopeptidase M.

A problem of the latter method was the necessity for the precipitation and filtration of the papain at the end of the first incubation period. It was thought that this procedure would be simplified by the use of coupled papain which could easily be filtered from the digest mixture at the end of the first incubation. Hydrolysis of the two test substrates with the incorporation of the coupled papain into the papain, prolidase and aminopeptidase-M method (Tables 22 and 23) revealed that while the insulin was still well hydrolysed using the coupled papain method, ovalbumin, a much larger molecule containing carbohydrate, was poorly digested in the presence of the coupled enzyme.

This may be due to steric effects introduced by immobilization of the enzyme, making a large protein like ovalbumin difficult to hydrolyse.

The existing methods for complete enzymic hydrolysis using coupled enzymes have all used proteins of a size comparable with the insulin used in this work; Bennet *et al* (1972) used coupled trypsin, chymotrypsin and aminopeptidase M for the hydrolysis of corticotrophin peptides and oxidized ribonuclease, Chin and Wold (1974) used a mixture of coupled proteases for the hydrolysis of ribonuclease and insulin, and Royer *et al* (1977) used a mixture of enzymes coupled to glass for the hydrolysis of a dodecapeptide. Background levels of amino acids were still present in controls for the coupled enzymes, although at lower levels than found with the soluble enzymes; examples are shown in Table 24.

The final enzymic hydrolysis method to be tested in this study was that involving pronase and immobilized proteinase-K as used by Winkler and Schön (1979) for the hydrolysis of a variety of seed proteins. The method was here tested on a sample of cowpea, *Vigna unguiculata* meal, and found to be no more efficient than the previously described methods, for the following reasons:- no proline was detected in the hydrolysate (Table 25), as would be expected by the absence of any prolidase from the enzymes used; the total levels of asparagine, glutamine, aspartic acid and glutamic acid (individually determined by means of amino acid analysis using a $Li^+$ buffer system) amounted to less than 50% of the levels of aspartic acid and glutamic acid recovered after acid hydrolysis, and the levels of glycine and alanine were also low, suggesting that as with the previous method using pronase, these amino acids are poorly released from proteins by this enzyme. In contrast, the yield of most of the other amino acids after enzymic hydrolysis exceeded that found after acidic hydrolysis.

This could be explained by enzyme autolysis since the method contains no control to allow for this, although according to Winkler and Schon, this problem should not occur because of prior dialysis of the pronase and the use of proteinase-K in an immobilized form.

All the methods so far described have used amino acid analysis to measure the extent of hydrolysis. In many cases the determination of the concentration of each amino acid is the object of the experiment, but there may be instances when a simple measurement of the total number of free amino acids would provide sufficient information, for example, when testing the proteolytic activities of different enzymes. Also, if a protein is resistant to hydrolysis, amino acid analysis gives no information on the form of the remaining undigested protein, i.e. whether it is as a number of undigested peptides or as a single core of resistant protein.

Initially the pH-stat seemed a useful method of following protein hydrolysis, providing a continuous trace of the reaction and giving reproducible results. Before the introduction of amino acid analyzers, the pH-stat was often used to measure proteolysis, for example by Hauggard (1955) and Jacobsen (1957). However, it is not possible to calculate the true extent of hydrolysis of a protein, simply from the amount of alkali required to maintain a constant pH value; this limitation of the pH-stat is explained in the Results section. The pH-stat was found useful for routine enzyme assays and particularly for the assay of immobilized enzymes, which were difficult to assay by spectrophotometric methods. As described in the Introduction, a method has been designed to assess the nutritional value of a protein by use of the pH-stat to measure the fall in pH value of a digestion mixture during the first 10min of enzymic hydrolysis (Hsu *et al*, 1977). The

disadvantages of the method are, however, that it cannot be used for proteins with a strong buffering capacity (a general limitation of the pH-stat), and also that this assay rests on the assumption that the remainder of the protein is as fully digestible as that hydrolysed during the first 10min, which may not be true if, for example, the protein has a central core which is resistant to enzymic attack.

Another method used to follow the extent of proteolysis was by chemical assay with TNBS, in which chromophores are formed between TNBS and primary amino groups. A method described by Mokrasch (1967) for the separation of a mixture into amino acid and protein fractions followed by their assay by TNBS was found to be unsuitable for separating the products of a digestion mixture (e.g. see Fig. 10). TNBS was therefore simply used to measure the increase in free amino groups in samples taken at timed intervals throughout the digestion of a protein.

The sizes of the products of proteolysis were determined by gel exclusion on a column of G-100 Sephadex, or, if samples were taken at intervals from a digestion mixture and later applied to polyacrylamide gels, it was possible to follow the progressive degradation of proteins and determine the size of any proteins remaining after hydrolysis.

## Application of the enzymic hydrolysis method

It is regularly stated in the literature, for example by Antunes and Sgarbieri (1980) and Marquez and Lajolo (1981), that legume proteins are of low digestibility *in vivo*, with an inherent resistance to digestion. One or more of the few reports which support this theory are usually cited. The original suggestion of poor digestibility of legume proteins was by Johns and Finks (1920), who found that rats fed

on a raw, crude globulin fraction from navy beans, *Phaseolus vulgaris*, failed to grow, prompting Waterman and Johns (1921) to suggest that this was due to the poor digestibility of the raw globulin measured *in vitro*. It is not clear from their work exactly how the globulin fraction was prepared, but from the reported deaths of the rats fed on the raw globulin, it appears very likely that the preparation still contained active haemagglutinins and trypsin inhibitors, whose presence and physiological significance had not yet been detected at the time of their work. Because the *in vitro* measurement of the digestibility of the raw globulin was carried out by digestion with trypsin, the presence of active trypsin inhibitors would contribute towards the relatively low digestion of the globulin which was achieved. The work claiming that legume proteins are of low digestibility which is most regularly cited is that of Seidl *et al* (1969), who isolated a globulin from black beans, *Phaseolus vulgaris*, and showed that it was resistant to hydrolysis by seven different proteinases, and also inhibited the activity of these proteinases towards their respective substrates. It has since been shown by Vaintraub and Sayanova (1977) that the globulin prepared by Seidl *et al* must have contained significant levels of a TCA soluble protease inhibitor. Following Seidl, reports on the *in vitro* digestibility of glycoprotein II have been made by Romero and Ryan (1978), Vaintraub *et al* (1979) and Liener and Thompson (1980). These authors have all shown that native glycoprotein II is resistant to trypsin, and the addition of chymotrypsin and/or pepsin to the enzyme mixture gives little improvement in the extent of digestion.

In the present work, the resistance of native glycoprotein II to tryptic digestion was shown by the fact that after 24h of incubation of the glycoprotein with the enzyme, large proteins were still visible when samples of the digest were analysed on SDS gel (Fig. 11). Liener and Thompson (1980) isolated two products of the tryptic digest and

estimated them to be of $M_r$ values 30000 and 22500. In this study a fragment of $M_r$ 22500 was also detected, but it was accompanied by two other protein fragments of $M_r$ values 26000 and 24000.

These findings *in vitro*, although of academic interest, have no bearing on the possible digestibility of the protein *in vivo*. As described in the introductory section, no one enzyme, or combination of enzymes of limited specificity, is capable of hydrolysing all the types of peptide bonds formed between different amino acids in a large protein. Thus glycoprotein II resembles ovalbumin, haemoglobin and ribonuclease, which, in their native forms, have also been shown to be resistant to tryptic hydrolysis (Mihalyi, 1978) although they are fully susceptible to hydrolysis by enzyme mixtures under the right conditions.

In the present work it was shown that other enzymes could hydrolyse the native glycoprotein to a greater extent, for example, pronase (Fig. 13) produced fragments of smaller size than did trypsin, and after incubation of glycoprotein II with trypsin and proteinase-K, no products were visible on the gel after staining (Fig. 14) and they must therefore have been small enough to have diffused from the gel ($M_r$ < 12000). However, use of the modified Hill and Schmidt procedure (which involves the use of a mixture of enzymes of wide specificity) to hydrolyse native glycoprotein II still resulted in a mean recovery of free amino acids of only 13% (Table 31). Therefore, *in vitro*, glycoprotein II is relatively resistant to an enzymic hydrolysis procedure which has been shown to be capable of completely hydrolysing other proteins.

One possibility which must be considered is that the resistance shown by glycoprotein II to tryptic hydrolysis *in vitro* is due to the presence of low levels of trypsin inhibitory activity remaining in the purified glycoprotein II. However, the presence of such an inhibitor

could not account for the low digestion of glycoprotein II achieved by other enzymes such as papain (the first enzyme of the Hill and Schmidt procedure) which is known not to be susceptible to the trypsin inhibitor present in *Phaseolus vulgaris* (Pusztai, 1968).

To measure the nutritional value of raw, purified *Phaseolus* proteins *in vivo*, Liener and Thompson (1980) fed raw glycoprotein II to rats and measured a TD of 57%. Although steps had been taken to remove trypsin inhibitory and haemagglutinating activities from their preparation of glycoprotein II, the possibility exists that the remaining low levels of these factors may have been active in the raw meal. Evidence for the presence of such factors was shown by the pancreatic enlargement observed in rats fed with the raw meal, although Liener and Thompson attribute this enlargement to the presence of undigested protein in the intestinal tract.

In any case, the study of the digestibility of glycoprotein II in its native state is not pertinent to the usual situation *in vivo* when legumes are eaten by humans after some form of cooking, which renders the beans palatable and also destroys the heat-labile anti-nutritional factors present. It is therefore more realistic to study the digestibility of glycoprotein II after it has been subjected to heat treatment.

In this study it was found that after boiling glycoprotein II for 10min its susceptibility to proteolytic attack increased. This was shown by the following results:-

After heating of the glycoprotein II, it lost its resistance to tryptic hydrolysis; Fig. 12 shows that no residual proteins were visible on a gel containing samples from an incubation of the heat treated glycoprotein with trypsin. Heating of proteins provides sufficient energy to break interchain H bonding and allows a molecule

to unfold (Kauzmann, 1959). In this case, unfolding of the glycoprotein II must have revealed lysine and arginine residues for which trypsin is specific, but which were previously unavailable, allowing the protein to be cleaved to peptides which were small enough to have diffused from the gel.

The necessity for this denaturation was also shown by analysis of tryptic digests of the native and denatured glycoprotein by gel filtration (Fig. 15), where low molecular weight proteins were only produced after digestion of the denatured glycoprotein.

Using the modified Hill and Schmidt enzymic procedure for the hydrolysis of denatured glycoprotein II, the mean recovery of amino acids was increased from the 13% found with the native glycoprotein II, to 91% (Table 31). This means that within the accuracy of the method the glycoprotein has been as fully digested *in vitro* as the test substrate, ovalbumin, and suggests that like ovalbumin, the denatured glycoprotein will be fully digested *in vivo*.

This result disagrees with those of Marquez and Lajolo (1981), who produced values of digestibility, determined *in vitro* for cooked bean globulins, of between 17% and 40%. These values were determined from the number of amino acids released from the globulins after enzymic hydrolysis by the procedure of Akeson and Stahmann (1964), a method (described in the introductory section) never designed nor claimed to give the complete hydrolysis of any protein.

The level of digestibility of the denatured glycoprotein II found in this study agrees with measurements made *in vivo*: Liener and Thompson (1980) found that the TD of heated glycoprotein II fed to rats was 92% (compared with 95% for casein), and Phillips *et al* (1981) found that a purified and heat treated globulin preparation from *Phaseolus* gave a TD in rats of 95% (compared with 98% for ovalbumin).

Although these proteins may be completely digested within the animal, the values for TD may be less than 100% because of error inherent in the measurement of TD, which depends upon the measurement of ingested and faecal N, without taking into account the source of this N; for example, if non-protein N is present as part of the total faecal N, but was not part of the total N ingested, this will give an artificially low value of the TD of the protein being studied.

In summary, this work has shown that native glycoprotein II is resistant to enzyme hydrolysis *in vitro*, although after heat treatment of the glycoprotein it can be completely hydrolysed.

It is known that *in vivo*, the heated glycoprotein is of high digestibility, but the digestibility of the raw glycoprotein is still uncertain. According to Liener and Thompson (1980), the native glycoprotein has a digestibility *in vivo* 35% lower than that found after heat treatment, although the possibility of interference by other anti-nutritional factors has not been entirely eliminated. It is difficult to understand why the glycoprotein, which can be readily denatured by heat treatment, should not also be denatured by the acid (pH 2.0) conditions present in the stomach. However, a study by Jones *et al* (1981) with legumin purified from *Vicia faba* has also shown that heat treatment of the native legumin increased digestibility (measured in rats), although by a lower margin, from 86% to 92%.

The increase in susceptibility to hydrolysis of glycoprotein II after denaturation suggests that the resistance to digestion *in vitro* is due to conformational restraints. Little is known of the internal structure of glycoprotein II, but it may resemble the 7S globulin from soybean *Glycine max*, which is a glycoprotein of approximately the same size and carbohydrate content, and which shows a similar resistance to tryptic hydrolysis in its native form. This protein has been found

to be a compact molecule containing many areas of beta-sheet conformation, and with a water impenetrable hydrophobic core (Fukushima, 1968). Bozzini and Silano (1978) have suggested that the resistance to digestion may be the result of low solubility under physiological conditions, the compact molecule allowing little hydration to occur and protecting peptidase susceptible bonds from attack. Romero and Ryan (1978) suggested that resistance to trypsin could occur if a protein has a low content of lysine and arginine. Glycoprotein II has a total (lysine + arginine) content of 8.6%; about the same level as ovalbumin (8.3%), which is also resistant to trypsin, and lower than that of BSA (14.2%), which is not resistant to trypsin. If most of the susceptible residues were buried within the molecule this would explain the large protein fragments which remained after tryptic digestion, but does not explain the apparent resistance of native glycoprotein II to enzymes of broad specificity such as papain, pronase and pepsin, *in vitro*.

The greater hydrolysis of the native glycoprotein II *in vivo* than *in vitro* must be due to the denaturing conditions present in the stomach, and the wide range of proteinases, peptidases and trans-membrane peptidases which a protein encounters in the mammalian gut (Porter and Rolls, 1971). In future work it would be of interest to establish firmly whether the native glycoprotein is fully digestible *in vivo*, by use of a glycoprotein II preparation purified until free of any of the anti-nutritional factors.

As previously mentioned, a survey of nutritional evaluation studies on *Phaseolus vulgaris* revealed that most values reported for the TD of whole, cooked beans, for rats, were within the range 70-85% (Tobin and Carpenter, 1978). These values indicate that the whole beans have a TD of up to 25% less than the purified globulins.

(Humans and rats show similar values for the TD of beans, Marshall *et al*, 1979.)

Assuming that the anti-physiological activities of factors such as trypsin inhibitors and haemagglutinins are destroyed during cooking, other factors must be responsible for the low digestibility of the whole, cooked bean.

One of these factors may be the result of interaction of the protein with polyphenols, since Elias *et al* (1979) and Phillips *et al* (1981) have shown that the polyphenols present in the testas of cooked beans can account for a reduction in TD of up to 8%. Green *et al* (1973) suggested that the presence of undigested protein in the gut may lead to an increased production of pancreatic enzymes. Since these enzymes are rich in sulphur amino acids, of which legumes are deficient, increased production of pancreatic enzymes would tend to accentuate the deficiency. A further theory, proposed by Arnal-Peyrot and Adrian (1974), is that the indigestible carbohydrates present in legumes stimulate fermentation in the lower ileum, giving rise to an increased level of bacterial protein in the faeces, and indirectly decrease the measured digestibility value of the beans. Hellendoorn (1969) suggested that the increased fermentation may lead to irritation of the gut lining, resulting in increased peristalsis and less efficient digestion. Bressani and Elias (1977) have proposed that legume proteins have a high buffering capacity, which can maintain gastric pH above 3.0 for 1h longer than milk or egg. This would delay activation of the proteolytic activities, and consequently reduce the digestion of the legume proteins.

All or some of these factors may reduce the digestibility of the whole bean to a certain extent, and it is necessary to evaluate the importance of each factor. Some of the less favourable factors

may be reduced by selective plant breeding, but it is possible to overcome some of these effects by more simple measures, for example, it has been shown that digestibility can be improved by the removal of testas with a high polyphenol content (Phillips *et al*, 1981), and soaking of the beans can remove undesirable carbohydrates (Olson *et al*, 1982).

The physiological activity of trypsin inhibitors, *i.e.* their ability to inhibit a variety of proteases, may not be a major factor contributing to the low digestibility of raw legumes. Evidence for this is provided by experiments such as those of Palmer *et al* (1973), who found that when raw kidney beans in various stages of germination were fed to rats, the nutritional value of the raw kidney beans improved substantially throughout the first eight days of germination, despite a doubling of the trypsin inhibitor content of the seed during this time. Also, Abbey *et al* (1979a) showed that the depression of growth measured after feeding raw field bean (*Vicia faba*) meal to rats could not be caused by the levels of protease inhibitor present in the meal. This was proved by the incorporation of the protease inhibitor into a synthetic diet, when it was found that an intake of five times more protease inhibitor was required to give a depression of growth similar to that observed with the field bean meal.

However, a possible deleterious effect of trypsin inhibitors may be connected with the high proportion of cystine which they contain. As already mentioned in the introductory section, a second problem with *Phaseolus* beans as a food source, in addition to their relatively low digestibility, is their low BV, which is caused by a low content of sulphur amino acids. The large proportion of cystine contained in trypsin inhibitor purified from *Phaseolus vulgaris* can be seen in the amino acid profile in Table 32, column 2, where the cysteic acid content

amounts to 9% of the total amino acids. This is in contrast with the amino acid profile of purified glycoprotein II (Table 31, Column 1) where cysteic acid accounts for only 0.4% of the total amino acids. Therefore, if the inhibitor comprises, say, 2% of the total bean protein, then its cysteic acid content amounts to approximately 0.2% of the total bean protein. Similarly, if glycoprotein II comprises 50% of the total bean protein, then its cysteic acid content also only amounts to 0.2% of the total bean protein. Thus, although the trypsin inhibitor is only a minor protein constituent of the seed, it represents an important source of cystine. However, it has been suggested that trypsin inhibitors are resistant to enzymic hydrolysis (Pusztai, 1968; Kakade, 1974), perhaps as a consequence of the disulphide bonding produced because of the high cystine content. If the cystine present in the trypsin inhibitors is unavailable *in vivo*, this will tend to accentuate the already low level of sulphur amino acids present in legumes. In the present work, the digestibility of such a trypsin inhibitor was assessed by studying its susceptibility to enzymic hydrolysis *in vitro*.

The sample of trypsin inhibitor used in this work had been denatured after its preparation by heating for 1h at 100°C, however, at the time of use it was found to contain considerable levels of trypsin inhibitory activity (Fig. 17, Wells 8 and 9), and this will be referred to as the "native" sample. It is possible that the trypsin inhibitor became reactivated during storage since such a phenomenon has been observed by Lin and Shwu (1977), who demonstrated the reactivation of a trypsin inhibitor purified from potatoes. A sample of the *Phaseolus* trypsin inhibitor was also boiled immediately prior to use, and this will be referred to as the "denatured" sample.

Although the trypsin inhibitory activity of this sample was not measured, the conditions used (boiling in aqueous solution for 30min) were considered comparable with the methods which may be used for cooking beans.

The modified Hill and Schmidt procedure proved useful for the study of the relative digestibility of the trypsin inhibitor since clearly, enzymes which are susceptible to the inhibitor cannot be used to study its digestibility. However, Pusztai (1968), in a study of the protease inhibitor of *Phaseolus vulgaris*, found that even when using a fifty-times molar excess of the inhibitor, no inhibitory action was found towards papain (the endopeptidase used in the first part of the Hill and Schmidt procedure). Because this method has, in this work, already been shown capable of completely hydrolysing other proteins, it provided a guide to the relative digestibility of the trypsin inhibitor.

The native and denatured trypsin inhibitor were poorly hydrolysed *in vitro* with yields of free amino acids of 34% and 38% respectively (Table 32). The remainder of the amino acids were still present as polypeptides of $M_r$ lower than the native trypsin inhibitor, as revealed by SDS polyacrylamide gel analysis of the enzymic digests (Fig. 19). Neither cystine nor cysteic acid were among those free amino acids present in the digest, and they must therefore constitute a high proportion of the remnant protein. This may support the theory of Liener and Kakade (1969) that the resistance of trypsin inhibitors to enzymic attack is due to the stability conferred upon them by disulphide bonding.

Having shown that the trypsin inhibitor shows some resistance to enzymic hydrolysis *in vitro*, it was of interest to test whether the inhibitor might also be resistant to hydrolysis *in vivo*.

To this end, analysis was carried out on extracts of faeces collected from rats whose diet had included the heat-denatured trypsin inhibitor.

SDS polyacrylamide gel analysis of these faecal extracts revealed a number of proteins, with two prominent bands of lower molecular weight than the original inhibitor (Fig. 16), whereas the faecal extracts of rats which were not fed the inhibitor contained no such protein. Since the diets of the two groups of rats were otherwise identical, the protein present in the faecal extract of the inhibitor fed rats may either be partially digested inhibitor, or some proteins excreted as a consequence of the presence of the inhibitor.

Assay of the faecal extracts for trypsin inhibitory activity (Fig. 17) revealed the presence of such activity, and it was found only in the extracts from those rats whose diet included the inhibitor. The proteins visible on the gel (Fig. 16) may therefore be trypsin inhibitors which had been reduced in molecular weight, but still retained inhibitory activity. Affinity chromatography was also used in an attempt to concentrate the trypsin inhibitor present in the faecal extract, and the activity of the trypsin inhibitor isolated by this method is also shown in Fig. 17. Although this further proved the presence of trypsin inhibitory activity in the faecal extract, such assays can only measure that inhibitor which has retained its ability to bind to trypsin, despite its passage through the rat gut. From the gel in Fig. 16, it can be seen that proteins of a variety of molecular weights have been excreted by the inhibitor fed rats, and these proteins may represent a loss of significant nutritional value to the rat.

This work has not proved that the trypsin inhibitor fed to the rats is the source of the trypsin inhibitor excreted by them:

immunological techniques could be used to establish whether the inhibitor excreted originated from that incorporated into the diet. At present, the possibility remains that the excreted trypsin inhibitor had been manufactured within the rats, perhaps as a result of pancreatic hypertrophy caused by the presence of raw *Phaseolus* trypsin inhibitor.

The increase in pancreatic size seen in rats fed on raw legumes was originally thought to be entirely due to the presence of trypsin inhibitors (Rackis, 1965), but it has since been shown, in *Vicia faba*, that trypsin inhibitors alone cannot be entirely responsible for the hypertrophy (Abbey *et al*, 1979b), and alternative factors such as tannins have also been found to cause pancreatic hypertrophy (Griffiths and Moseley, 1980).

The level of pancreatic hypertrophy which is due to the presence of raw trypsin inhibitor may be explained by the need for the pancreas to replace proteolytic enzymes which have been effectively removed by complexing with active trypsin inhibitors. It is of interest to know whether such trypsin-trypsin inhibitor complexes are digested and reabsorbed, or whether they are resistant to digestion and cause a loss of nutritional value to the rat.

Having already shown that the *Phaseolus vulgaris* trypsin inhibitor is relatively resistant to enzymic hydrolysis *in vitro* (and perhaps *in vivo*) it was pertinent also to study the *in vitro* digestibility of the inhibitor in the presence of trypsin.

It was first established that denatured trypsin was fully digestible *in vitro*, as judged by the complete recovery of amino acids achieved after enzymic hydrolysis (Table 33), and by the SDS gel in Fig. 19 which shows the components of native trypsin (track 3) and the absence of any protein remaining after hydrolysis (track 8).

In the first experiment with both trypsin and trypsin inhibitor, the native forms were preincubated together at 37°C, after which the solution was boiled and the normal enzymic procedure followed. Because the total recovery of free amino acids (Table 34) was less than half of the yield expected from the hydrolysis of the trypsin alone, this suggests that the trypsin has had some resistance to hydrolysis conferred upon it by the presence of the trypsin inhibitor. Further evidence for this was provided by SDS polyacrylamide gel analysis of the digest (Fig. 19, track 7) which revealed bands of protein staining at $M_r$ values corresponding with those of the trypsin inhibitor and native trypsin, indicating that these species both remained intact throughout hydrolysis. The free amino acids which were recovered from the digest may have arisen from hydrolysis of the trypsin which was present in excess of the trypsin inhibitor: from the amino acid profiles obtained by acid hydrolysis of 1mg samples of the trypsin and trypsin inhibitor (Tables 33 and 32) it is evident that starting with equal weights of the preparations of trypsin and trypsin inhibitor, the trypsin must have been present in excess of the trypsin inhibitor, due to the greater purity of the trypsin preparation (both species are of similar molecular weights).

As a control, the same procedure was repeated except that the trypsin and trypsin inhibitor were both denatured before their incubation together. In this case, the recovery of free amino acids (Table 35) corresponded with the sum of amino acids released from the independent hydrolysis of these two components, and analysis of the digest on the gel (Fig. 19, track 6) only revealed feinter bands of proteins of $M_r$ values corresponding with those found after the enzymic hydrolysis of the trypsin inhibitor. It therefore appears that in this case, the trypsin was fully susceptible to hydrolysis.

The patterns of proteins seen on the gel therefore agreed with the results from amino acid analysis: when in their native forms together, trypsin and trypsin inhibitor were less susceptible to enzymic hydrolysis *in vitro* than when in their denatured forms, when trypsin was fully hydrolysed and the trypsin inhibitor retained its original low susceptibility to hydrolysis.

If a complex formed between trypsin and trypsin inhibitor was also resistant to digestion *in vivo*, this would further exacerbate the deficiency of sulphur amino acids in legumes, since as well as the high cystine content of trypsin inhibitors, proteolytic enzymes are also relatively rich in sulphur amino acids, as illustrated by the amino acid profile of trypsin (Table 33) which shows a cysteic acid content of 4.2%.

It should be pointed out that in these experiments *in vitro*, the digestibility of trypsin inhibitor from *Phaseolus vulgaris* has been studied with bovine trypsin. This was because rat trypsin is not available commercially; it would be useful to know to what extent these two enzymes resemble one another. It is known that bovine and human trypsin differ in their susceptibility to inhibition, and this is because human trypsin exists in both cationic and anionic forms so that it has been found, for example, that only the anionic form is fully inactivated by soybean trypsin inhibitor (Figarella *et al*, 1975).

Apart from the problems of their digestibility and low BV, other features of legumes require further understanding, and simple *in vitro* methods such as the one used in this work, may be suitable for their investigation, in isolation from other complicating factors. Because sources of nutrition to provide sufficient energy as well as protein are likely to be in short supply in countries where legumes

form a major part of the diet, the digestible energy content of legumes is of importance as well as the digestible protein content. To study this problem, an *in vitro* enzymic hydrolysis procedure with pancreatic amylase has been used by Shurpalekar *et al* (1979), to show that legume carbohydrates are of lower digestibility than corn starch, a problem which is due to the presence of α galactosides.

Enzymic hydrolysis *in vitro* was not only capable of estimating the digestibility of whole proteins, but was also found useful for predicting the availability of individual amino acids. In this study it was used to determine levels of available lysine and methionine.

When the levels of lysine released by the enzymic hydrolysis *in vitro* of eight fish meals were compared with the results from three other types of assay designed to measure available lysine (Table 37), the results from enzymic hydrolysis correlated most closely with the values from chick growth bioassay. This may reflect the greater similarity between the enzymic procedure *in vitro* and the digestive system of the chick, than with the conditions used in the chemical and microbiological assays. Good correlation was also achieved between the values from enzymic hydrolysis and those predicted by ~~chemical~~ dye-binding assay. This latter assay is designed to measure the difference in dye-binding capacity of a foodstuff before and after blocking of the available lysine groups, and has been shown to give reliable predictions of biologically available lysine (Hurrel *et al*, 1979). Like other chemical assays for available lysine, it provides a fast and economical means of assay, but can only be used for the measurement of lysine. The poorest correlation occurred between the values from enzymic hydrolysis and those given by microbiological assay using *Tetrahymena*; the use of this ciliate protozoan has previously been

found to be unreliable for the measurement of available lysine by Payne *et al*,(1977), and the procedure is said to be cumbersome,with frequent problems with the growth media and bacterial contamination.

From the correlations achieved between the results of the enzymic hydrolysis method and those given by accepted assays of lysine availability, the method must also be considered a useful guide to available lysine. However, no one assay can predict absolutely the level of available lysine present in a food, because the various lysine derivatives formed as a result of Maillard reactions seem to vary in their nutritional availability to different species. The lysine is most likely still to be available when the substituted groups are relatively small and, for example, although propionyl-lysine is not utilized by the rat, it can be hydrolysed to free lysine by the chick (Varnish and Carpenter, 1970). Therefore, the use of chick growth bioassay to predict the lysine available from foods containing this type of substitution would, in this case, overestimate the levels of lysine which would be available to rats.

The *in vitro* enzymic hydrolysis method was also used to measure the decrease in available lysine present in samples of skim-milk powder, throughout their period of storage at 37$^{o}$C (Fig. 20). Because lysine is the essential amino acid which is most susceptible to damage during the heating of foods (Ford and Salter, 1966), measurement of available lysine by this method could be a useful guide to the effect of processing on the nutritional value of a food protein.

An advantage of achieving the complete enzymic hydrolysis of a protein is that, unlike the other assays described above, which are limited to the measurement of lysine, the full complement of available amino acids may be simultaneously measured by this one procedure.

The levels of methionine present in the enzymic hydrolysates of the fish meals, like the levels of lysine, also correlated more closely with the results from chick growth bioassay than with the results from microbiological assay, in this case using *Streptococcus zymogenes* (Table 38), but the yields of methionine after enzymic hydrolysis were lower than those produced either by the other assay methods, or by acid hydrolysis of the samples. This was explained by the presence of methionine sulphoxide in the enzymic hydrolysates, for when they were combined, the total levels of (methionine + methionine sulphoxide) approached the yields of methionine given by the other assay methods.

During the storage of the skim-milk powder, the level of available methionine was found to remain constant (Fig. 20). This is as would be expected because methionine is resistant to heat (Erbersdobler, 1976) and would not be damaged at 37$^{o}$C. The levels of methionine sulphoxide were found to exceed those of methionine, and this may be due to the oxidation of the methionine caused by the hydrogen peroxide used in the production of skim-milk (Fox and Fosikowski, 1967).

The ability of enzymic hydrolysis to measure methionine sulphoxides is a useful advantage over acid hydrolysis which destroys the majority of any methionine sulphoxide which may be present in a protein, and converts the rest to methionine. The usual alternative is to use performic acid to oxidize methionine and methionine sulphoxide to methionine sulphone, but this technique does not allow the individual determination of the species present, and has been found to give variable yields (Rayner and Fox, 1976).

However, it is useful to be able to determine the individual concentrations of methionine sulphoxide in food proteins, because

nutritional studies have shown that the sulphoxide (but not the sulphone) can be used by some mammals in place of methionine. Erbersdobler (1976) reported that L-methionine sulphoxide was completely available to growing rats, although Gjoen and Njaa (1977) found that it was only equivalent to methionine if there was sufficient cyst(e)ine in the diet, indicating that unlike methionine the sulphoxide cannot be used to produce cysteine. The value of methionine sulphoxide in human diets is unknown. Cuq *et al* (1973) claimed that methionine sulphoxide may not be available *in vivo*, because peptides containing methionine sulphoxide showed some resistance to enzymic hydrolysis *in vitro* by pronase, but this may be explained by the poor release of the more acidic amino acids which, as suggested earlier in this work, is a characteristic of pronase.

While it is acknowledged that useful assays already exist for the measurement of available lysine and methionine, it has been shown here that enzymic hydrolysis *in vitro* is an efficient, economic and relatively simple method which allows the simultaneous measurement of both of these essential amino acids. Enzymic methods have been tested before, but none has used a procedure capable of completely hydrolysing the protein being tested, and has therefore not given the total levels of available amino acids. The more recent methods include that of Pieniazek *et al* (1975) who used elastase in the measurement of the available methionine and cystine contents of foods; although elastase achieved only a limited digestion of the foods studied, chemical assays were used to measure the amino acids present in the peptides, and rat bioassays correlated with the results of the *in vitro* estimations. Cuq *et al* (mentioned above) and Rayner and Fox (1976) used pronase digestions for the estimation of some essential amino acids. However, as shown previously in this work, pronase

is not capable of hydrolysing all the peptide bonds found in a protein and Rayner and Fox reported that the resulting values for available lysine correlated with, but were only 50-65% of, the values determined by chemical assay for available lysine.

For the nutritional evaluation of a protein, it is necessary to know the composition and availability of its amino acids. Chemical scores and indices of essential amino acids, determined by acid hydrolysis of the protein, provide a simple guide to its nutritional value, but their flaw is that they cannot predict the availability of the amino acid *in vivo*. It has been shown in this work that enzymic hydrolysis *in vitro* can evaluate the available amino acid content of a protein. Although the *in vitro* digestion is carried out in arbitrary conditions which do not fully correspond with the situation *in vivo*, it must be considered a useful alternative to the rat assays which are traditionally used for studying the nutritional value of foods intended for humans.

Rat assays are not only time consuming and expensive, but several authors have pointed out the limitations of rats when used as models for human nutrition. Firstly, because the surface to volume ratio of rats is so much greater than our own, the relatively larger food intake required to provide sufficient energy for such small, homoiothermic animals, is more likely also to supply sufficient amino acids and minor nutrients. Secondly, the essential amino acid requirements of rats and humans do differ to some extent. For example, consider the amino acid cystine. The hairy exoskeleton of rats means that they have a high requirement for cystine and are therefore particularly sensitive to its supply. For this reason, rats have been used to assess the BV of legumes, for example, by Khan *et al* (1979), because the BV of legumes, for both humans and rats, is correlated with

the sulphur amino acid content of the legume, although the high cystine requirement of the rat may mean that the supply of cystine is more critical for rats than for humans.

Apart from rats, the other organisms which have been used for the nutritional evaluation of proteins, such as protozoa and bacteria, are even less likely to have nutritional requirements and digestive systems like our own, and in this respect seem to provide little advantage over a simple *in vitro* enzymic hydrolysis procedure.

In the final part of this study, the selectivity of enzymes to only cleave peptide bonds, leaving side groups undamaged, was applied to the measurement of glutamine and asparagine (which are deamidated by acid hydrolysis) in germinating pea, *Pisum sativum*, cotyledons.

The existence of amide groups in proteins was first proved by the isolation of asparagine and glutamine from enzymic digests of gliadin and edestin (Damodaran and Ananta-Narayanan, 1938). Subsequently Hill and Schmidt (1962), Tower *et al* (1962) and Holt *et al* (1971) have all reported on the isolation of amides from enzymic digests. However, this approach has not been used in experiments to determine the amide contents of germinating seeds, which have instead depended upon measurement by electrophoresis of the increase in negative charge of a protein as amide groups are removed and allow exposure of charged carboxylic acid groups.

For example, Righetti *et al* (1977) used isoelectric focussing to reveal a heterogeneity of charge among otherwise identical proteins purified from zein. From the known glutamic acid and aspartic acid contents found after acid hydrolysis of the proteins, and the pI values observed after isoelectric focussing, the extent of

amidation of the proteins was calculated. Catsimpoolas *et al* (1968) used disc electrophoresis to show that the 7S and 11S components of soybeans became increasingly anionic during germination, and this was attributed to progressive deamidation of asparagine and glutamine. Similarly, Daussant *et al* (1969) found that α-arachin, which is a major storage protein of peanuts, *Arachis hypogaea*, changed in its electrophoretic mobility, becoming more negatively charged during germination. That this was due to deamidation was supported by the finding that the total amide content of the extracted protein was found to decrease during germination. From these results, Daussant *et al* suggested that progressive deamidation of storage proteins is the first step of their degradation, and further degradation is continued by protease systems whose activity is regulated by pH changes within the seeds.

In the present study, the levels of asparagine and glutamine in storage proteins purified from germinating peas, *Pisum sativum*, were determined. The progressive decrease in size of the component polypeptides of legumin, convicilin and vicilin are shown in the SDS gels of Figs. 21 and 22. From the amide levels of the same proteins, measured after their complete enzymic hydrolysis *in vitro* (Tables 39 and 40), the only variation in amide levels detected was the decrease in glutamine found in legumin, with none remaining in the protein extracted at seven days of germination, and this was not accompanied by a concomitant increase in the levels of glutamic acid. Acid hydrolysis of the same samples of legumin which had been hydrolysed by the enzymic method (Table 40) revealed that glutamine must have been incompletely (55-75%) recovered in the enzymic hydrolysates. This problem, which was also encountered to the same extent by Hill and Schmidt (1962) and Holt *et al* (1971), is due to the cyclization

of glutamine to pyrollidone carboxylic acid (Melville, 1935). Pyrollidone carboxylic acid does not react with ninhydrin to give a coloured product and therefore cannot be measured by amino acid analysis, but it can be measured after acid hydrolysis when, like glutamine, it is converted to glutamic acid. However, assuming that cyclization took place to a similar extent in each of the enzymic digest solutions, which were of identical pH value and concentration, it could not account for the large decrease in glutamine present in the legumin samples taken over 7 days of germination. For vicilin and convicilin (Table 39) there was no evidence for deamidation having occurred, although convicilin showed an increase in glutamine in the sample taken at 3 days of germination. Because these results are based on measurements of the amino acids present per gramme of protein, they do not indicate whether there has been a net decrease in the levels of amino acids per cotyledon.

These results in pea do not support the theory of Daussant *et al* (1969) that deamidation has a role in the initiation of protein degradation, because clearly vicilin and convicilin were being degraded in the absence of deamidation. Changes in the amidation of legumin occurred throughout the first seven days of germination, during which time protein degradation was also taking place, but the connection between these two events is unknown.

However, these results agree well with those of Basha and Beevers (1975) who, in experiments similar to those of Daussant *et al*, measured the electrophoretic mobilities of fractions prepared from pea cotyledons at successive stages of germination. Legumin showed a change in electrophoretic mobility, becoming increasingly negatively charged during germination, but no evidence for deamidation could be detected in vicilin. As in the present work, the results did not

support the theory of Daussant because, as shown by Basha and Beevers, most of the change in the electrophoretic mobility of legumin occurred between days 4 and 8 of germination which was after the start of protein depletion.

Enzymic hydrolysis is not likely to replace acidic hydrolysis for determining the amino acid compositions of proteins, but as illustrated by the measurement of amides, it can be a useful adjunct to the routine method. As well as those acid labile amino acids already mentioned, other acid-labile groups which could be measured after enzyme hydrolysis are covalently linked prosthetic groups such as glycopeptides, sulphate ester groups and phosphorylated amino acids.

Since enzymes are specific for particular conformations of amino acids, enzymic hydrolysis can be used to test for isomerization, a feature which has been used by Milligan and Holt (1978) for the identification of some enantiomers present in wool proteins, and this ability to measure the amount of amino acids recemised to their D-forms may also serve as a guide to the nutritional quality of processed foods (Friedman *et al*, 1981).

Enzymic hydrolysis is, however, much more complicated and time consuming than acid hydrolysis, and also some of the enzymes used are expensive. The enzymes must be assayed before use and control digestions are always necessary to measure the extent of enzyme autolysis. Also, the measurement of amides requires separate amino acid analysis using (expensive) lithium buffers. Additional acid hydrolysis and amino acid analysis is often required to test whether complete enzymic hydrolysis has been achieved.

The enzymic hydrolysis method could probably be more streamlined than that used in this study, where no attempt has been made to

decrease incubation periods or alter the amounts of enzymes used; the priority was to find a method capable of completely hydrolysing the test proteins. The use of immobilized enzymes may provide the key to a simpler enzymic procedure. However, insoluble food proteins would be less available to enzymes attached to the inert supports than to soluble enzymes, and some solubilization step may need to be introduced.

In conclusion therefore, the results presented in this thesis demonstrate that proteolysis using enzymes *in vitro*, according to the method developed and used in this work, will give information on the digestibility of proteins, can be used to assay the availability of such important essential amino acids as lysine, methionine and methionine sulphoxide, and is also a useful analytical method with which to determine amino acids such as glutamine, asparagine and tryptophan, which are normally destroyed by acid hydrolysis.

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