PEPTIDE HYDROLASES OF THE BRUSH BORDER MEMBRANE FROM HUMAN SMALL INTESTINE

by

Erwin E Sterchi, M.I.Biol

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Biochemistry Research Laboratory
Biological Sciences Department
University of Keele

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ABBREVIATIONS

Abbreviations in this thesis comply with the policy of the Biochemical Journal.

All amino acids in free or peptide-form, are of the L-configuration.

ABSTRACT

A method has been developed for the purification of brush border membranes from human small intestine. The method did not involve the use of EDTA-buffers or disruption of brush borders with high concentrations of Tris. On average, a 24-fold increase in specific activity for α-glucosidase (brush border marker) was obtained in the final preparation which contained only traces of enzyme markers from other cellular organelles.

The homogenates of human small intestinal mucosa were shown to contain enzymes capable of hydrolysing di-, tri- and oligopeptides as well as 2-naphthylamides. Distribution studies indicated that all of the oligopeptidase activity towards peptides, four and more amino acids in length and activity towards leucine-2-naphthylamide were located exclusively in the brush border. A large proportion of activity towards α-glutamic acid-2-naphthylamide (aminopeptidase A), γ-glutamic acid-2-naphthylamide (γ-glutamyltransferase) and glycyl-proline-2-naphthylamide (dipeptidyl peptidase IV) were also recovered in the brush border membrane fraction. Depending on the substrate used, 33-87% of tripeptidase activity was located in the brush border membrane. An estimated 58-87% of dipeptidase activity, on the other hand, was recovered in the soluble fraction.

Solubilisation of brush border membrane proteins by sodium dodecyl sulphate, Triton X-100 and papain followed by polyacrylamide gel electrophoresis revealed seven different peptide hydrolases. These included the specific enzymes aminopeptidase A, dipeptidyl peptidase IV, γ-glutamyltransferase and aminopeptidase M which were clearly separable on polyacrylamide gels after solubilisation with

Triton X-100 or papain. Activity recovered in the aminopeptidase M peak in the above gel system could be resolved into two distinct peptidases in addition to aminopeptidase M, by SDS-gel electrophoresis. One of these peptidases was most active towards aliphatic tripeptides (peptidase 1) while the other appeared to be specific for dipeptides. A further peptidase (aminopeptidase M') was resolved by isoelectric focusing in polyacrylamide gels. The role of these brush border peptide hydrolases in the absorption of protein by the gut is discussed.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 The Morphology of the epithelium of the mammalian small intestine

Any substance to be absorbed from the intestinal lumen into the circulation of the body has to pass across the epithelium of the gut. Equally, substances to be transferred to the lumen of the gut have to pass through the epithelial lining or are secreted by the cells of the epithelium. The necessity to deal with absorption, secretion and excretion alike in maintaining nutrition and homeostatis of the organism requires a morphologically complex organ which contains various highly differentiated cell types.

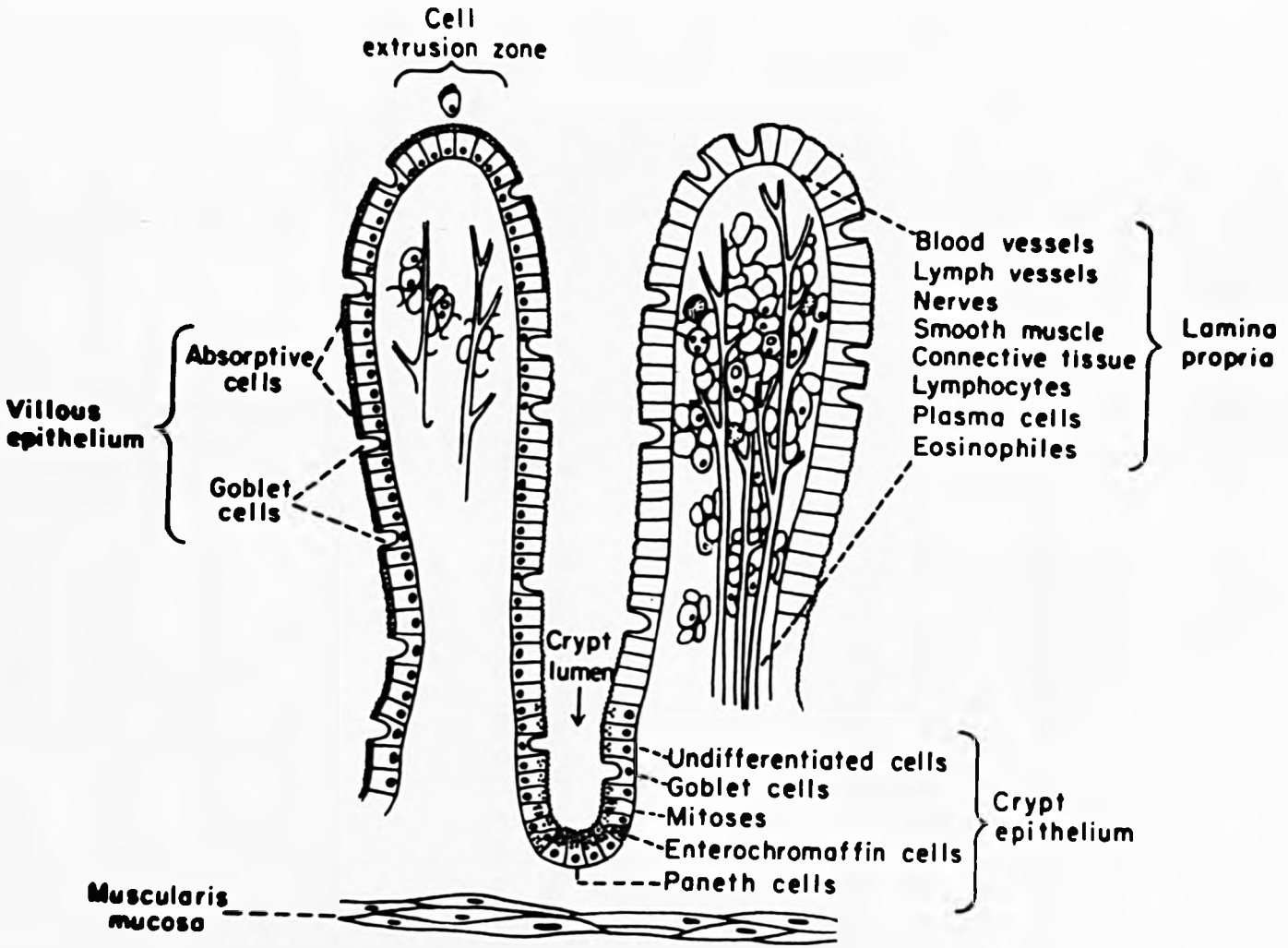
With the introduction of new techniques such as jejunal biopsy and electron microscopy, the structure of the small intestinal mucosa could be thoroughly investigated.

1.1.1 Architecture of the small intestinal mucosa

The mucosa of the small intestine may be divided into three layers (Fig 1.1). At the outermost lies the Muscularis mucosa, a thin sheet of smooth muscle 3-10 cells in thickness, which separates the mucosa from the submucosa.

The middle layer is a continuous sheet of connective tissue, the lamina propria, which together with the epithelium forms the villi and the crypts. The lamina propria contains different cell types including fibroblasts, macrophages, plasma cells, lymphocytes, eosinophils and mast cells. It also contains blood and lymph vessels, unmyelinated nerve fibres and strands of smooth muscle.

Apart from serving a structural role, there is evidence that the lamina propria may help the body combat harmful, foreign



Schematic diagram of 2 villi and a crypt to illustrate the histological organization of the mucosa of the small intestine.

Figure 1.1

substances that have penetrated the epithelial barrier. Phagocytic cells are certainly present in the lamina propria and plasma and lymphoid cells present contain significant amounts of immunoglobulins (Rubin et al 1965).

The third layer of the intestinal mucosa is the epithelium, a continuous sheet of a single layer of cells covering both villi and crypts. The surface of the villi is covered by absorptive, goblet and a few enterochromaffin cells. The villi of the duodenum and jejunum of man have a height of 0.5-1mm, thus greatly increasing the surface area of the absorptive epithelium. Villi may show a normal range of variation, depending on individual and race, from finger shape to elongated leaf (or tongue) forms, to convolutions and finally, in some disease states, to a totally flat mucosa. These variations are now mostly recognised when assessing villous patterns for diagnostic purposes in diseases of the small intestine.

1.1.2 Dynamics of the epithelium

The epithelium of the intestinal mucosa is a dynamic, actively proliferating tissue which renews itself rapidly and regularly. The crypts have been identified as the site for cell proliferation. Pulse-labelling studies with ^{3}H-thymidine have shown that DNA synthesis only takes place within the cells of the crypts (Leblond et al 1958) where differentiated cells are produced from precursor cells. The mitotic cycle has been timed and estimated to take about 24 hours in man. The DNA synthesis phase is thought to last for 11-14 hours while actual mitosis lasts only one hour (Shorter et al 1964).

After proliferation, the differentiated cells migrate up the

villi and are finally lost from extrusion zones at the tip of the villi (Creamer 1967). The time required to reach the top, ie to replace the whole epithelium, is known as the turnover rate. This is very constant under normal conditions and is three to five days in man. The rate of formation and extrusion of villous epithelial cells may be altered together or separately, resulting in an alteration of general architecture as well as the morphology of the individual epithelial cells. Nutritional states will affect the balance of renewal and loss of cells, resulting in either a lengthening or a shrinking of the villi. Radiation or antimitotic drugs stop cell production in the crypts while cell loss continues, resulting in a progressive shrinkage of the villi (Sherman and Quastler 1960, Trier and Browning 1966, Trier 1962). Abnormal cell proliferation may well play an important part in coeliac disease where villi are markedly reduced in height, or absent altogether, while the depth of the crypts is greatly increased. It has been established that the number of mitotic cells are greatly increased in the crypts of coeliac patients and it has been suggested that this might reflect a compensatory increase in cell proliferation to counteract increased cell loss (Yardley et al 1962, Hendrix and Yardley 1964).

1.1.3 Fine structure of the absorptive cells of the epithelium

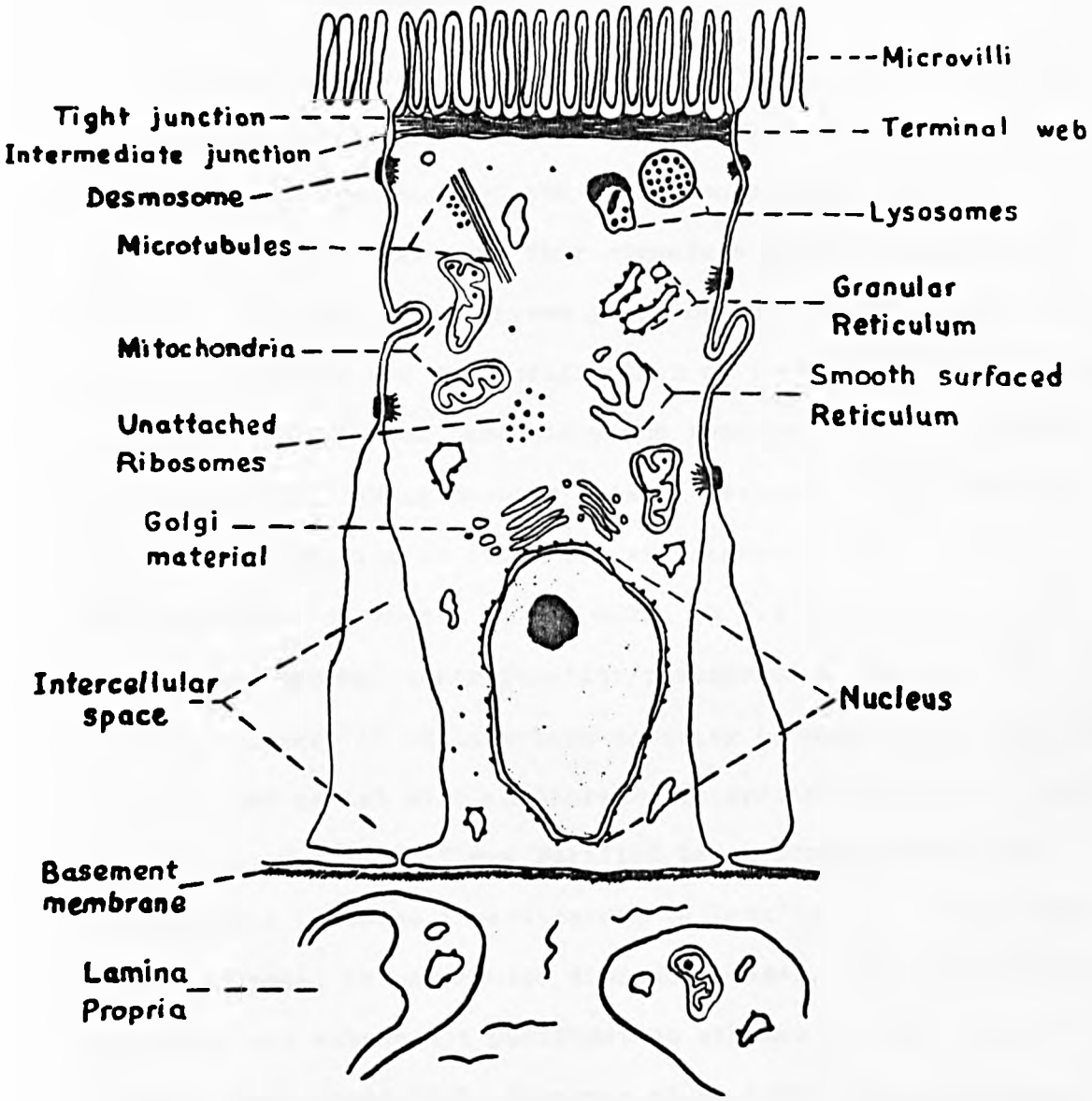
The absorptive cells or enterocytes cover the villi with a continuous sheet which is only broken at the extrusion zones of cells on the villous tip. Mammalian enterocytes are tall, columnar cells with basally located nuclei, while the Golgi apparatus, the endoplasmic reticulum, mitochondria and lysosomes are located in the

apical part of the cell. The cell base rests on the basement membrane, a fibrillary structure of glycoprotein. The apical plasma membrane shows the brush border arrangement typical of many epithelial cells. The microvilli of the enterocyte brush border average about 1μm in length and 0.1 to 0.2μm in width and are strengthened by a bundle of fibrils which are rooted in the terminal web of the cell. The outer layer of the brush border membrane is covered with the "fuzzy" coat or glycocalyx which appears to be firmly attached to the membrane. The appearance of the glycocalyx has been described as anything from fibrillar (Ito 1965) to amorphous, to containing knobs (Johnson 1969), and probably depends on the technique used for fixing. Evidence suggests that the glycocalyx consists of glycoprotein synthesised in the Golgi and that it is continuously renewed (Forstner 1969). It has been suggested that the glycocalyx may serve as a relatively impenetrable barrier against substances in the gut lumen such as bacteria and other foreign materials (Ito 1965), that it plays an important role in selective binding of substances prior to their absorption into the cell (Fawcett 1964), or that it might even be an important site for the actual digestion of nutrients prior to their absorption (Fawcett 1964, Dobbins 1969).

Adjacent enterocytes are closely apposed and locked together. The so-called junctional complex consists of a tight junction where the membranes actually appear to fuse, an intermediate zone and a number of desmosomes, thickened areas beneath the cell membrane. The cells show frequent infoldings in adjacent lateral plasma membranes which might allow for expansion of individual cells as the need arises. Whether or not tight junctions can open and what can pass through them is still under some dispute; it seems likely though, that at

least water and sodium may pass through (Machen et al 1972, Schultz and Frizzell 1972).

The lateral cell membranes may be seen adjacent or widely separated. There is evidence that this space opens up during absorption to expose the lateral cell membrane. Figure 1.2 is a schematic diagram of an intestinal enterocyte.



Schematic diagram of a villous absorptive cell.

Figure 1.2

1.2 Purification of brush border membranes from epithelial cells of the small intestine

As investigators became increasingly aware of the importance of the brush border membrane in the terminal digestion of nutrients and subsequent transport of the digestion products into the enterocytes, it became clear that this structure had to be studied more closely. Over the last fifteen years or so, several groups have published methods for the purification of intestinal microvillous membranes, mainly from laboratory and domestic animals. Miller and Crane (1961) using hamster tissue, devised a simple method for the purification of brush border fragments based on the homogenisation of mucosa in 5mM EDTA, pH 7.4 in a Waring blender followed by several centrifugation/resuspension cycles. They were able to recover 75% of invertase activity (a marker for brush border) in the final pellet with an increase in specific activity over the homogenate of 10-15. These purified brush borders were used successfully by these investigators to localise and study brush border enzymes, in particular disaccharidases, and the method became the basis for subsequent purification schemes by other workers (Eichholz and Crane 1965, Forstner et al 1968). Hübscher et al (1965) purified brush borders from guinea pig and rabbit intestinal mucosa. They homogenised the tissue in a Teflon homogeniser and the medium (0.3M sucrose, pH 7.4) did not contain EDTA. The centrifugation/resuspension cycles following homogenisation were very similar to those used by Miller and Crane (1961). They obtained a brush border/nuclei fraction which contained large amounts of alkaline phosphatase and invertase activities but was contaminated (in addition to nuclear material) by lysosomal hydrolases. Porteous

and Clark (1963) also produced a brush border/nuclear fraction from rabbit small intestine, containing most of the alkaline phosphatase activity. Boyd et al (1968), homogenising hamster, rat, chicken and pigeon tissue in 0.3M sucrose - 5mM EDTA followed by centrifugation and resuspension cycles in solutions of decreasing molarity, claimed to obtain brush borders free from contamination but no data of enzyme assays were included in the paper. All these techniques yielded partly purified brush borders which were contaminated by other cellular components. To improve the purification, Forstner et al (1968) further fractionated their "purified" brush borders by a complex method of adding water, leaving the mixture for an exact time, then adding EDTA to a final concentration of 4.5mM. After high speed centrifugation steps, membrane fractions designated I and II and a fibrillar fraction were obtained. 70% of invertase activity was recovered in the two membrane fractions with an increase in specific activity of 29 over the original homogenate. Eichholz and Crane (1965) used high concentrations of Tris to disrupt brush borders prepared essentially by the method of Miller and Crane (1961) and fractionated the disrupted brush border fragments by glycerol gradient centrifugation. They obtained a fraction of microvillous membranes quite similar to that obtained by Forstner et al (1968).

Brush border membranes from human small intestine were prepared by Welsh et al (1972) using basically the same method as Eichholz and Crane (1965). In two fractions from glycerol gradient they found a large proportion of disaccharidase activity with increases in specific activity of 12-16 times that of the homogenate. Alkaline phosphatase activity was also found to a large proportion in those fractions. Human brush borders seem more fragile than those of rat,

hamster or guinea pig and intact brush borders are rather difficult to prepare. Additionally, fresh human tissue is more difficult to obtain so that frozen tissue has to be used. The process of freezing usually on dry-ice, has been reported to decrease the yield of brush borders in a preparation (Welsh et al 1972) but despite this disadvantage, these workers concentrated on the direct preparation of microvillous membranes from frozen tissue. Their purified brush border membranes were contaminated substantially with microsomal material which is very difficult to separate from human brush border fragments. A different method was subsequently published by Schmitz et al (1973) from the same laboratory but again using frozen tissue. This method was based on the aggregation of microsomal vesicles by calcium followed by a low speed centrifugation step to separate these aggregated vesicles from the unaffected brush border fragments. The brush border fragments were then spun down at a higher speed and further separated by glycerol gradient centrifugation following Tris disruption to yield a pure fraction of microvillous membrane free from major contamination by other subcellular components. This method was the basis for the purification scheme used in this study and is described in more detail in Chapter 3.2.

Table 1.1 Summary of methods used for the purification of brush border membranes from intestine

Investigator	Animal	Medium used	Purification factor disaccharidase	Contamination
Miller & Crane (1961)	Hamster	5mM EDTA pH 7.4	10-15	Cytosol Microsomes
Porteous & Clark (1963)	Rabbits	150mM NaCl-5mM EDTA pH 7.4		Nuclei
Hübscher et al (1965)	Guinea pigs Rabbits	300mM Sucrose pH 7.4	4	Nuclei
Eichholz & Crane (1965)	Hamster	5mM EDTA pH 7.4		
Forstner et al (1968)	Rats	5mM EDTA pH 7.4	17.5(purified brush borders) 29 (Membrane fraction)	Microsomes?
Boyd et al (1968)	Hamster Rat Chicken Pigeon	300mM Sucrose 5mM EDTA pH 7.4.		None
Welsh et al (1972)	Human	5mM EDTA 5mM Tris pH 7.3	12-16	Microsomes
Schmitz et al (1973)	Human	50mM Mannitol 2mM Tris pH 7.1	26	Traces

1.3 Absorption of peptides from mammalian intestine

The digestion and absorption of protein has been under serious investigation since the second half of the last century, when it was generally believed that proteins were absorbed in the form of "peptones" (oligopeptides) (Kühne 1867). These oligopeptides could not, however, be demonstrated in the blood by investigators at that time. At the beginning of this century it was shown that "erepsin" in the intestinal wall and the intraluminal fluid could hydrolyse proteins to amino acids (Cohnheim 1901). Van Slyke and Meyer (1912, 1913-14), by the relatively specific nitrous acid method for α-amino nitrogen, observed a large increase in amino nitrogen in the plasma during protein and amino acid absorption. Later, Dent and Schilling (1949), by using paper chromatography demonstrated an increase of free amino acids in portal plasma during absorption of protein but were unable to detect any peptides. Such evidence led to the assumption that protein was hydrolysed completely to its constituent amino acids in the intestinal lumen followed by the uptake into the blood of these free amino acids. This classical hypothesis of protein absorption was supported by the view held in the first half of this century that amino acids were absorbed by simple diffusion which would be unfavourable for the absorption of larger molecules, and later by the discovery of specific transport mechanisms for amino acids in intestinal mucosa (Gibson and Wiseman 1951). The findings by Agar *et al* (1953) that dipeptides could not pass across the wall of inverted sacs of small intestine *in vitro* except in traces, brought additional support for the hypothesis.

Experimental results which did not fit into the concept of the classical hypothesis of protein absorption were published very early

on but such was the overwhelming support for the hypothesis, their importance was not recognised until much later. In 1922 Folin and Berglund found a very high level of nitrogen in plasma during protein absorption which could not be attributed to amino acids, proteins or urea and they suggested that peptides may be present in the portal blood. Gupta et al (1958) showed that peptides could be absorbed as fast as amino acids in the rat, and Goldberg and Guggenheim (1962) reported that during the absorption of protein in the rat, lysine concentrations reached a maximum in portal blood before the maximum in the intestinal lumen was obtained. There was confirmation of early reports (Cohnheim 1901) that "erepsin" activity was mainly associated with the mucosa itself (Wright et al 1940, Florey et al 1941) and not with the intraluminal fluid, the peptidase activity of which was too low to account for the amount of peptides taken up from the luminal side. Fisher (1954) strongly criticised the classical hypothesis of protein absorption pointing out that protein digestion to free amino acids by known digestive proteases in vitro was too slow to account for absorption in this way.

1.3.1 Uptake of peptides into the mucosal cells

The classical hypothesis of protein absorption finally collapsed when in vitro experiments showed that small quantities of glycyl-glycine and glycyl-leucine crossed the intestinal wall intact (Agar 1954). There followed the work of Newey and Smyth (1959) who found that some peptides were taken up intact by the intestinal mucosa. These workers reported that peptidase activity in the intestinal lumen was insufficient to account for the amount of peptides disappearing

(1960) and concluded (1962) that products of protein digestion could be transported into the mucosal cells in the form of peptides followed by intracellular hydrolysis. At that time, the concept of peptide absorption was strongly influenced by the discovery of the mechanisms by which disaccharides are taken up into the cells. It was believed that absorption of dipeptides took place by a mechanism analogous to that for disaccharides ie. surface hydrolysis followed by uptake into mucosal cells of free amino acids by specific transport mechanisms, and that the concept of peptide uptake as a further mechanism for protein absorption was probably insignificant (Crane 1968). This view had to change in the face of further evidence which established that amino acids were absorbed faster from peptides than from equivalent mixtures of free amino acids. Craft et al (1968) found that glycine was absorbed faster when administered orally as the di- or tripeptide than in the free form. They concluded that peptides must have been taken up intact by the intestinal mucosa because if surface hydrolysis of peptides preceded transport, the amino acids could not have been absorbed more rapidly. This phenomenum has now been observed in man (Craft et al 1968, Adibi and Phillips 1968, Hellier et al (a) and (b) 1972, Silk et al 1973), rat (Matthews et al 1968 (a) and (b), Cheng et al 1971, Burston et al 1972) and hamster (Matthews et al 1974) using a variety of techniques in vitro and in vivo, including tolerance tests, intestinal perfusion, disappearance from tied loops of intestine and transport by everted sacs and rings of small intestine.

When amino acids which compete for transport into the mucosal cells are present as a peptide, competition is partly or completely avoided. This was shown studying the absorption of mixed peptides

of methionine and glycine and equivalent mixtures of methionine and glycine. Glycine was absorbed at a lower rate when methionine was present but this competition between the two amino acids was abolished when glycyl-methionine or methionyl-glycine were presented. In addition, both amino acids were taken up faster from the peptides than from the equivalent amino acid mixtures (Cheng et al 1971). If surface hydrolysis preceded amino acid transport, competition between the two amino acids could not be avoided and therefore, these findings were taken as additional evidence in favour of the existence of a specific peptide transport system.

A survey recently conducted of the absorption of 22 dipeptides containing basic, acidic and neutral amino acids by everted rings of rat ileum, showed that in 18 of the tested dipeptides, one or both amino acids were taken up faster than from equivalent free amino acid mixtures (Burston et al 1972). More information was gained from an investigation of the uptake of methionine from methionyl-methionine by rings of everted rat ileum in vitro, about the mechanisms of uptake of amino acids from peptides (Cheng et al 1971). The authors found that at low concentrations, uptake of methionine from equivalent solutions of methionine and methionyl-methionine was about equal, while at higher concentrations, uptake was greater from methionyl-methionine. Also, uptake of methionine from methionyl-methionine continued to increase when the uptake mechanism for free methionine was saturated suggesting that uptake of methionine and methionyl-methionine involves independent mechanisms. Indeed, more recent findings have shown that mucosal uptake of dipeptides is substantially independent of mucosal uptake of free amino acids. This independence of dipeptide and amino acid

uptake was clearly shown in patients with Hartnup disease, a congenital defect in intestinal amino acid transport in which many amino acids are very poorly absorbed owing to a defective carrier. Affected amino acids are taken up freely by the mucosa of Hartnup patients when presented as dipeptides (Asatoor et al 1970). In cystinuria the loss of active transport or arginine is also adequately compensated by peptide transport systems (Asatoor et al 1972).

Investigations using carnosine (β-alanyl-histidine) established that amino acids or amino acid mixtures did not affect uptake of this peptide significantly. It was, however, inhibited by glycyl-glycine, glycyl-sarcosine, glycyl-proline, methionyl-methionine, prolyl-hydroxyproline. The most powerful inhibitor of uptake was glycyl-proline and the kinetics of inhibition were shown to be competitive; carnosine uptake was not affected by lysyl-lysine and α-glutamyl-glutamic acid (Addison et al 1974). It was therefore suggested that there may be several transport systems for dipeptides. More concrete evidence came from earlier experiments with glycyl-sarcosine, a peptide readily taken up by intestinal mucosa but poorly hydrolysed by its peptidases. After a 20 minute incubation of jejunal rings with glycyl-sarcosine an intracellular fluid:medium concentration gradient of over 3:1 was observed. Accumulation of glycyl-sarcosine in the tissue was abolished by anoxia, cyanide and DNP and was also inhibited by replacement of medium Na^+, by K^+, or Li^+ (Addison et al 1972). These findings endorsed Newey and Smyth's suspicion (1962) that glycyl-glycine might enter mucosal cells by an active system.

More recently, similar results were obtained with carnosine using the same technique (Addison et al 1973, Matthews et al 1974)

and like glycyl-sarcosine, carnosine appeared in the serosal fluid of everted sacs of intestine. It appears then, that both glycyl-sarcosine and carnosine are actively taken up into the mucosal cell and it is probable that many dipeptides enter the mucosal cell by an active transport mechanism.

1.3.2 Maximum size of peptide taken up by the intestinal mucosa

Early investigations with peptides of glycine showed that glycyl-glycyl-glycine was absorbed more rapidly than glycyl-glycine and very much more rapidly than the equivalent glycine (Craft et al 1968). These findings were interpreted as indicating entry of tripeptides as well as dipeptides into mucosal cells by an active transport system. Another possibility is that tripeptides are split into dipeptides and amino acids at membrane level, followed by uptake into the cells in these forms. There are some reports of glycyl-sarcosyl-sarcosine and β-alanyl-glycyl-glycine being taken up actively (Addison et al 1974) and Evered and Wass (1970) found that glutathione (γ-Glu-Cys-Gly) was transferred to the serosal side of everted sacs of rat small intestine in vitro.

There are some biologically active peptides which are absorbed from the intestine into the body including some peptide antibiotics, toxins and hypothalamic hormone regulating factors. Little is known about the mechanisms by which these peptides are absorbed from the gut. A peptide which is resistant to gastric acid and to gastric and intestinal proteases might be absorbed from the small intestine by several different ways:

(1) By mechanisms responsible for absorption of macromolecules and intact proteins ie. pinocytosis. Although the ability of the

intestine to absorb whole proteins on a large scale by pinocytosis decreases after the first few weeks of life, there is evidence that it may persist into adult life (Morris 1968, Walker et al 1972). Immunological evidence certainly suggests that many proteins may be taken up whole in small quantities.

(2) If lipid-soluble, by diffusion through the plasma membrane of the mucosal cells. It has been suggested that many lipid-soluble drugs are absorbed in this way.

(3) If water-soluble, by diffusion through aqueous pores in the plasma membrane of mucosal cells. The plasma membrane of the absorptive cells appears to have regions specially permeable to water and the possibility of the absorption of small water-soluble peptides through these regions of the membrane can not be excluded.

(4) By a specific carrier mechanism. There is little evidence that large peptides and proteins are taken up by an active transport mechanism but the possibility may exist.

It is unlikely that these possible uptake mechanisms play a significant part in nutrition.

1.3.3 Possible mechanisms involved in the mucosal uptake of peptides

As a rule peptides, with possibly a few exceptions, do not pass right through to the portal blood. The precise site of hydrolysis of peptides within the intestinal mucosal cell has yet to be defined. There are two possible mechanisms by which peptide material might be taken up into the epithelial cells:

(1) Superficial hydrolysis on the luminal side followed by uptake of amino acids by specific transport carriers. This view is held by, among others, Ugolev and his colleagues (1965, 1972) and is supported

by the detection of free amino acids in the intestinal lumen during dipeptide absorption in animals (Matthews et al 1968, 1969) and man (Adibi 1971, Silk et al 1973, 1973, 1974). The avoidance of competition between amino acids when peptides are presented and the finding of intact peptides in the intestinal mucosa in vitro (Addison et al 1972, 1973) can not be explained by such a scheme. (2) Uptake of intact peptide into the mucosal cell followed by intracellular hydrolysis. The findings that several peptides appear intact in the mucosal cells and that poorly hydrolysed peptides may be concentrated in the tissue by an active mechanism lend strong support to this concept. Further evidence in favour of the second concept comes from the Hartnup and cystinuria investigations (Asatoor et al 1970, 1972) and from subcellular fractionation studies which suggest that the bulk of dipeptide hydrolases in the intestinal mucosa is located in the cytosol (Robinson 1963, Josefsson and Sjöström 1966, Peters 1970).

It seems most probable that peptides are handled by a combination of both systems or that some peptides are handled mainly by one and some peptides mainly by the other of the two mechanisms. Much more work remains to be done to establish the transport mechanisms of peptides in the small intestine. Most work has been on the uptake of dipeptides and relatively little is known about the transport (and/or hydrolysis) of tri- and oligopeptides.

1.4 Peptidases of the mammalian small intestine

According to Bergman (1942) all enzymes that split peptide bonds may be classed as either endopeptidases or exopeptidases.

Endopeptidases are defined as enzymes capable of hydrolysing centrally located peptide bonds in a protein as well as terminal bonds. On the basis of their active centres they may be divided into three distinctive groups:

a) Serine endopeptidases eg. chymotrypsin, trypsin, elastase and thrombin. All these enzymes have a serine residue at their active centre.

b) Thiol endopeptidases eg. papain, ficin and some cathepsins. Enzymes of this group appear to require the presence of free thiol groups for their activity.

c) Acid endopeptidases eg. pepsin, rennin and some cathepsins. The distinguishing feature of the enzymes in this group is their low pH optima (pH 2 to 4).

Exopeptidases hydrolyse peptide bonds adjacent to N-terminal and C-terminal amino acids and are thus designated amino peptidases and carboxypeptidases respectively. The enzymes in this group include di- and oligopeptidases and the carboxypeptidases of the pancreatic juice.

The ability of the intestinal mucosa to hydrolyse peptides has been recognised since the beginning of this century (Cohnheim 1901). With the availability over the past fifteen years or so of a large number of peptide and synthetic substrates, a great deal of work has been carried out on the hydrolysis of peptides by mucosal extracts from the intestine. Tables 1.2 and 1.3 list di-, tri- and oligopeptides used in such investigations.

Table 1.2 Substrates used in studies of dipeptide hydrolysis

Substrate	Reference
Ala-Glu	Peters 1970; Dolly *et al* 1971.
Ala-Gly	Rhodes *et al* 1967; Heizer and Laster 1969; Kim *et al* 1974.
Ala-Phe	Heizer and Laster 1969.
Gln-Pro	Rubino *et al* 1969.
Glu-Ala	Dolly *et al* 1971.
Glu-Glu	Donlon and Fottrell 1972.
Glu-Gly	Donlon and Fottrell 1972.
Glu-Pro	Dolly *et al* 1971.
Glu-Trp	Dolly *et al* 1971; Donlon and Fottrell 1972.
Glu-Tyr	Dolly and Fottrell 1969; Dolly *et al* 1971; Donlon and Fottrell 1972.
Glu-Val	Dolly and Fottrell 1969; Dolly *et al* 1971; Dolly *et al* 1971.
Gly-Ala	Heizer and Laster 1969.
Gly-Glu	Dolly and Fottrell 1969; Peters 1970; Dolly *et al* 1971; Donlon and Fottrell 1972.
Gly-Gly	Rhodes *et al* 1967; Heizer and Laster 1969; Peters 1970; Donlon and Fottrell 1972.
Gly-His	Donlon and Fottrell 1972; Das and Radhakrishnan 1973.
Gly-Pro	Heizer and Laster 1969; Rubino 1969; Donlon and Fottrell 1972.

contd.

Table 1.2 (continued)

Substrate	Reference
Gly-Phe	Heizer and Laster 1969; Heizer *et al* 1972; Dolly *et al* 1971; Kim *et al* 1972; Das and Radhakrishnan 1973; Donlon and Fottrell 1972.
Gly-Met	Peters 1970.
Gly-Leu	Heizer and Laster 1969; Peters 1970; Dolly *et al* 1971; Das and Radhakrishnan 1973; Donlon and Fottrell 1972; Kim *et al* 1972.
Gly-Trp	Dolly *et al* 1971; Heizer and Laster 1969; Donlon and Fottrell 1972; Das and Radhakrishnan 1973; Fujita *et al* 1972.
Gly-Val	Dolly *et al* 1971; Das and Radhakrishnan 1973.
Leu-Ala	Kim *et al* 1972; Heizer and Laster 1969; Dolly *et al* 1971; Donlon and Fottrell 1972.
Leu-Gly	Rhodes *et al* 1967; Robinson 1963; Peters 1970; Fujita *et al* 1972; Maroux *et al* 1973; Heizer and Laster 1969; Dolly *et al* 1971; Das and Radhakrishnan 1973; Kim *et al* 1972; Donlon and Fottrell 1972.
Leu-Leu	Rhodes *et al* 1967; Peters 1970; Dolly *et al* 1971; Kim *et al* 1972; Donlon and Fottrell 1972; Das and Radhakrishnan 1973.
Leu-Phe	Heizer and Laster 1969.
Leu-Tyr	Heizer and Laster 1969; Dolly *et al* 1971; Donlon and Fottrell 1972.

contd.

Table 1.2 (continued)

Substrate	Reference
Leu-Pro	Dolly *et al* 1971; Donlon and Fottrell 1972; Kim *et al* 1972.
Met-Leu	Kim *et al* 1972.
Met-Phe	Heizer and Laster 1969.
Phe-Gly	Heizer and Laster 1969; Donlon and Fottrell 1972; Fujita *et al* 1972; Kim *et al* 1972.
Phe-Leu	Fujita *et al* 1972; Kim *et al* 1972.
Phe-Met	Heizer and Laster 1969.
Phe-Phe	Heizer and Laster 1969.
Phe-Pro	Heizer and Laster 1969; Donlon and Fottrell 1972.
Pro-Gly	Heizer and Laster 1969; Peters 1970; Das and Radhakrishnan 1973.
Pro-Leu	Dolly and Fottrell 1969; Donlon and Fottrell 1972; Kim *et al* 1972.
Pro-Phe	Kim *et al* 1972.
Trp-Leu	Das and Radhakrishnan 1973.
Val-Leu	Donlon and Fottrell 1972; Fujita *et al* 1972; Kim *et al* 1972.

Table 1.3 Substrates used in studies of oligopeptide hydrolysis

Substrate	Reference
Ala-Ala-Ala	Auricchio *et al* 1971.
Ala-Gly-Gly	Peters 1970; Maroux *et al* 1973; Kim *et al* 1974.
Ala-Leu-Gly	Fujita *et al* 1972.
Gly-Gly-Gly	Peters 1970; Maroux *et al* 1973; Kim *et al* 1974.
Gly-Gly-Leu	Fujita *et al* 1972.
Gly-Leu-Phe	Fujita *et al* 1972.
Gly-Leu-Tyr	Rhodes *et al* 1967.
Gly-Ala-Phe	Heizer and Laster 1969.
Leu-Leu-Leu	Rhodes *et al* 1967; Kim *et al* 1972; Donlon and Fottrell 1972.
Leu-Gly-Gly	Rhodes *et al* 1967; Peters 1970; Auricchio *et al* 1971; Donlon and Fottrell 1972; Fujita *et al* 1972.
Phe-Gly-Gly	Fujita *et al* 1972.
Trp-Gly-Gly	Peters 1970; Donlon and Fottrell 1972.
Tyr-Gly-Gly	Peters 1970; Donlon and Fottrell 1972.
Tyr-Tyr-Tyr	Dolly *et al* 1971; Donlon and Fottrell 1972.
Ala-Ala-Ala-Ala	Auricchio *et al* 1971.
Ala-Gly-Gly-Gly	Kim *et al* 1974.
Gly-Gly-Gly-Gly	Peters 1973; Kim *et al* 1974.
Leu-Trp-Met-Arg-Phe-Ala	Kim *et al* 1974.

contd.

Table 1.3 (continued)

Substrate	Reference
Gly-Gly-Gly-Gly-Gly-Gly	Peters 1973.
Val-Ala-Ala-Lys-Ile-Val-Gly	Maroux et al 1973.

1.4.1 Methods for measuring peptide hydrolase activity

Several methods are available for the determination of peptide hydrolase activity, a selection of which are discussed in this section.

a) Measurement of peptide bond absorbance:

The peptide bond has an absorbance at wavelengths (λ 220nm) in the low ultra-violet range and is therefore suitable for spectrophotometric studies. When peptide bonds are hydrolysed, a decrease in absorbance proportional to the number of bonds broken is observed. The presence of other UV-absorbing substances such as protein in most assay systems, necessitates the inclusion of a precipitation step (Noren et al 1973). The method is applicable to di- and oligopeptide hydrolysis. Provided absorption from other components in the assay system is controlled, this method is very specific and has been used in purification studies of dipeptide hydrolases from the intestine (Das and Radhakrishnan 1972, Noren et al 1973) and in clinical studies on biopsy material (Heizer and Laster 1969, Dahlqvist

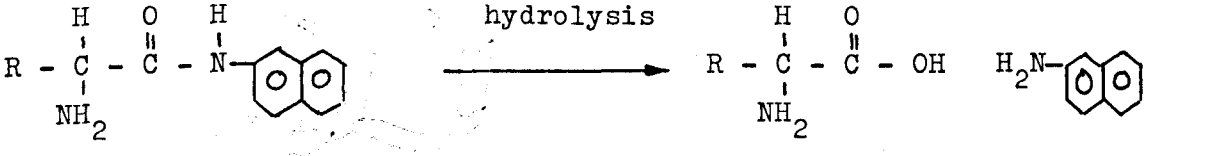
et al (1970). The method is unsuitable for the study of hydrolysis of peptides containing aromatic amino acids as these have a strong absorbance in the UV range.

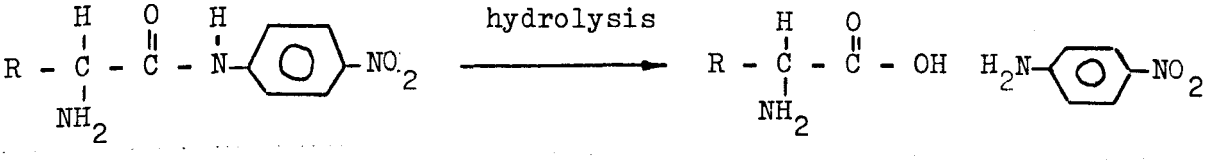
b) Measurement of released amino groups:

The increase in N-terminal amino groups after peptide hydrolysis may be determined by specific reagents such as ninhydrin. Ninhydrin is particularly useful when used as a spray after paper electrophoresis or thin layer chromatography to identify degradation products. One of the limitations of the method is the interference in the reaction of NH_3 and Tris. The method is, however, fairly sensitive and thus allows the measurement of small amounts of peptidase activity. It was used successfully in the work described in this thesis as a follow-up to the assaying of gel slices after polyacrylamide gel electrophoresis.

c) The use of chromogenic substrates:

An amino acid or peptide may be coupled to a chromogen which may be measured spectrometrically after its release by hydrolysis. Two of the most widely used chromogens are 2-naphthylamide and p-nitroaniline.





2-naphthylamide may be measured directly by fluorimetry or may be converted to an azo dye which can be measured by spectrometry in the visible range. p-nitroaniline is a yellow compound and may be directly measured spectrophotometrically. Artificial chromogenic substrates have been used by many investigators (Peters et al 1972, Auricchio et al 1972) and the enzymes capable of hydrolysing them have been designated naphthylamidases, arylaminopeptidases or arylamidases (the name preferred by many).

d) The use of amino acid oxidase to determine liberated amino acids:

L-amino acid oxidase from snake venom can oxidise many L-amino acids but has no effect on di- or oligopeptides. Amino acids liberated by hydrolysis of peptides, undergo oxidative deamination yielding hydrogen peroxide which in turn oxidises a further compound, such as o-dianisidine, to a coloured form. The method was extensively used in the work discussed in this thesis and is dealt with in more detail in Chapter 4.2. The method has been applied to zymogram studies (Dolly and Fottrell 1969) and to the quantitative determination of peptidases (Heizer and Laster 1969, Auricchio et al 1971, Fujita et al 1972).

1.4.2 Characterisation and specificity of peptide hydrolases in mammalian intestine

The data published by the various investigators in this field are very confusing for a number of reasons. Firstly, large numbers of different sustrates, chosen randomly in many cases, have been used. Secondly, various different fractions from intestinal mucosa have been employed for the determination of enzyme activity. Thirdly, activities have been expressed in many different ways. These factors

make comparison of results obtained by different workers very difficult, if not impossible. From evidence obtained over the past decade however, some conclusions may be drawn about peptide hydrolysis in the intestinal mucosa. The following enzyme or enzyme groups have been demonstrated in the intestinal mucosa:

1) dipeptidases, 2) tripeptidases, 3) oligopeptidases, 4) arylamidases and 5) endopeptidase(s).

a) Dipeptidases:

By far the most work has been carried out using dipeptide substrates. Hydrolase activities against a great many dipeptides have been found in the intestine. Most workers used a soluble fraction as the source for the enzymes. Dipeptidases seem to have a number of properties in common, such as their inhibition by chelating agents (eg. EDTA) and activation by divalent metal ions (Heizer and Laster 1969, Peters 1970, Fottrell et al 1972, Noren 1974). They are inhibited by sulphydryl reagents, eg. p-chloromercuribenzoate (PCMB) but are not affected by diisopropylfluorophosphate (DFP) (Noren 1974). Several investigators have isolated and purified enzymes capable of hydrolysing dipeptides. A leucine-aminopeptidase (substrate:leucinamide) has been purified from swine kidney (Spackman et al 1955, Himmelhoch and Peterson 1968) with a molecular weight of around 300,000. It was metal dependent and had a broad specificity towards many dipeptides. Activity towards leucinamide has since been reported in the intestinal mucosa by several groups (Rhodes et al 1967, Heizer and Laster 1969, Kim et al 1972, Dolly et al 1971). Maroux et al (1973) purified an amino-peptidase from hog intestinal brush borders (substrate:aminoacyl-2-naphthylamide) which had very similar properties to the kidney enzyme.

The use of two different substrates, leucinamide and leucine-2-naphthylamide, led to considerable confusion which was resolved by the demonstration by Paterson et al (1963) that activity against 2-naphthylamides was distinct and could be separated from leucine-aminopeptidase in extracts of ascites cells. Activities against 2-naphthylamides have thus been grouped together and designated aryl-amidases. Recently, two dipeptide hydrolases against glycyl-leucine have been purified from monkey (Das and Radhakrishnan 1973) and pig small intestine (Noren 1971). The molecular weights were reported to be 107,000 and 104,000 respectively and both enzymes dissociated into two equal subunits of 54,000 and 52,000 molecular weight respectively. Both enzymes were inhibited by EDTA and PCMB and Noren's enzyme was reported to be unaffected by DFP. Quite a large number of dipeptides were hydrolysed by the enzyme and Das and Radhakrishnan (1973) referred to it as a versatile "master" dipeptidase. Both groups reported low or no activity towards dipeptides containing proline and towards glycyl-glycine. Sjöström (1971, 1973) purified an aminoacyl-proline hydrolase from pig intestinal mucosa which is distinct from the glycyl-leucine hydrolase. Several other workers using the zymogram technique found that aminoacyl-proline substrates were hydrolysed by a special enzyme (Dolly et al 1970, Kim et al 1972, Donlon and Fottrell 1972). The enzyme purified by Sjöström, also referred to as prolidase, showed a narrow substrate specificity (Sjöström et al 1973). Over the years there have been reports of other distinct enzyme activities towards dipeptide substrates but more work on purified enzymes is necessary to provide more insight into this possibility. Zymogram studies of soluble peptidases of rat and guinea pig intestinal mucosa showed

multiple forms with broad substrate specificity (Kim et al 1972, Donlon and Fottrell 1972) but conclusions with regard to number and specificity are difficult to make from such studies. It is now clear, that the intestinal mucosa can hydrolyse almost every dipeptide tested and specificity studies indicate that there must be more enzymes than those already characterised (Noren 1973).

b) Aminotripeptidase:

An aminopeptidase specific towards tripeptides has been purified from calf thymus (Fruton et al 1948). Smith and Bergman (1944) studied a similar enzyme from pig intestinal mucosa. Activity was restricted to tripeptides showing no activity against di- and tetrapeptides. Several workers have found tripeptidases in the intestinal mucosa but invariably they hydrolysed dipeptides also. Kim et al (1972) distinguished between soluble and brush border bound tripeptidase activity (substrate:leu-leu-leu) using a zymogram technique. They found the brush border enzyme pattern coincided with arylamidase activity (substrate:leu-2-nap).

c) Oligopeptidase:

Relatively little is known about the oligopeptidase activity in the intestinal mucosa. Several groups have reported hydrolysis by mucosa of tetra-, penta- and hexapeptides (Auricchio et al 1971, Peters 1973, Kim et al 1974) and one group found activity against a heteroheptapeptide (Maroux et al 1973). Amino acids are split off sequentially from the N-terminal end as demonstrated by Kim et al (1974) using a heterohexapeptide (leu-trp-met-arg-phe-ala). Oligopeptidase activity seems confined to a particulate fraction of the mucosa, probably the brush border membrane.

d) Arylamidases:

Arylamidases are a group of enzymes defined by the use of chromogenic substrates. They are found in most organs but little is known about their physiological substrates. Several arylamidases have been isolated and purified. For example, aminopeptidase A (Glenner et al 1962) and aminopeptidase M (Wachsmuth et al 1966) were purified using chromogenic substrates. In addition to these artificial substrates, the enzymes also hydrolysed appropriate peptides ie. aminopeptidase A hydrolysed dipeptides with an N-terminal α-glutamic or aspartic acid residue and aminopeptidase M also hydrolysed di- and oligopeptides. Arylamidases therefore showed essentially an aminopeptidase character.

Aminopeptidase M from pig intestine and kidney has been studied extensively. Its reported molecular weight is 280,000 and each molecule contains 2 atoms of zinc (Wacker et al 1971, Wachsmuth et al 1966, Maroux et al 1973). The enzyme has been shown to have a broad specificity, with the alanine derivative being the most rapidly hydrolysed. Peptide substrates have been shown to be hydrolysed by this enzyme but the rates were lower than those for the corresponding chromogenic substrates. α-glutamic acid and α-aspartic acid derivatives are hydrolysed only very slowly and the γ-glutamic acid derivative not at all. Auricchio et al (1972) separated γ-glutamic acid-2-naphthylamide hydrolase activity from aminopeptidase M by column chromatography on Sephadex G200 after papain solubilisation.

γ-glutamyltranspeptidase (substrate : γ-glutamic acid-2-naphthylamide) has been shown by other workers to be a distinct enzyme of the intestinal mucosa (Donlon and Fottrell 1972) and it has been suggested that this enzyme might be involved in amino acid

transport at membrane level. A γ-glutamyl cycle has been postulated for the accomplishment of such a transport function (Meister 1973). Aminoacyl-2-naphthylamides have been used in zymogram studies confirming the difference between leucine-aminopeptidase and leucyl-arylamidase (aminopeptidase M) (Dolly et al 1971, Donlon and Fottrell 1972, Kim et al 1972).

e) Enterokinase:

Enterokinase (EC 3.4.21.9) converts trypsinogen into trypsin which then activates the other pancreatic zymogens chymotrypsinogen, procarboxypeptidase and proelastase. It is therefore a key enzyme in the intestinal tract initiating the whole process leading to protein digestion. Holmes and Lobley (1970) reported a marked rise in specific activity of enterokinase in purified brush border membranes over the mucosal homogenate which was similar to that of sucrase and the authors concluded that enterokinase was a brush border enzyme. Louvard et al (1973) found comparable enrichment of enterokinase with alkaline phosphatase and aminopeptidase in pig duodenal vesicles and Schmitz et al (1974) also reported a brush border localisation of enterokinase using human tissue. Localisation of the enzyme appears to be limited to the duodenum although some activity in a free form is found in the distal part of the small intestine.

f) Other endopeptidases

In addition to enterokinase the presence in the intestinal mucosa of an endopeptidase capable of hydrolysing the B-chain of insulin has been reported. Woodley (1969) demonstrated such an enzyme in rat brush border membranes. The endopeptidase was distinct from brush border arylamidse (substrate:leucine-2-naphthyl-

amide) as determined by inhibition studies. Fujita et al (1972) have shown that rat brush border membranes could hydrolyse the β-chain of insulin. Hydrolysis was incomplete and not sequencial, yielding a mixture of peptides and amino acids. A peptidase which hydrolyses the B-chain of insulin has also been demonstrated in human brush border membrane preparations (Welsh et al 1972).

1.5 The subcellular localisation of intestinal peptide hydrolases

Undoubtedly most of the investigations on intestinal peptidases have been carried out using soluble or non-particulate fractions of intestinal mucosa. Heizer and Laster (1969) tested the peptidase activity of a high speed supernatant of rat mucosal homogenates. They recovered at least 90% of activity against some dipeptides (alanyl-phenylalanine, glycyl-phenylalanine, leucyl-phenylalanine, methionyl-phenylalanine, phenylalanyl-phenylalanine, phenylalanyl-methionine), 43% against phenylalanyl-glycine, 35% against phenyl-alanyl-amide and only 15% against leucine-2-naphthylamide in that supernatant. Das and Radhakrishnan (1973) purified a "master" dipeptidase from a soluble extract of monkey small intestinal mucosa which had a wide specificity. Some of the dipeptides not hydrolysed were glycyl-glycine, glycyl-proline, glycyl-histidine, prolyl-glycine and some peptides containing arginine and asparagine. Supernatants of human intestinal biopsy homogenates have been used by Rubino et al (1969) and Dolly and his colleagues (1969, 1971) to study peptidases. Such studies are of limited use if the subcellular distribution of peptidases is to be examined.

In order to locate an enzyme within a cell, subcellular fractionation techniques may be employed to separate the various intracellular organelles. Fractionation techniques have been applied to the study of subcellular location of enzymes, including peptidases in the intestinal mucosa. There have been two main approaches in the fractionation of intestinal mucosa, one analytical, the other preparative. An analytical system sets out to separate the various subcellular organelles according to their size by differential

centrifugation, a scheme first put forward by De Duve et al (1962). Comparison of the distribution of an enzyme of unknown subcellular location with known markers for intracellular organelles, gives an indication of its subcellular location. If the distribution is identical to that of a known marker it may be assumed that their location is identical too. An analytical method was published by Hübscher et al (1965) which has been used by other workers to determine the subcellular location of peptidases in intestinal mucosa cells (Peters 1970, Donlon and Fottrell 1972).

A preparative approach is used if the presence of a particular enzyme in a specific subcellular fraction is to be examined. Such a technique has been used by the Crane group who isolated pure brush border membranes from intestinal mucosa (Eichholz and Crane 1965) of hamster to study enzymes associated with those membranes. In order to improve the yield of a particular subcellular component in a preparative fractionation, steps may be taken to preserve that component's integrity throughout the fractionation procedure. Crane and his colleagues thus used a medium containing EDTA (5mM) in their fractionations which, they claimed, maintained the structure of brush border membranes by inhibiting autolytic processes. EDTA is however, also a powerful inhibitor of many peptidases and subcellular localisation studies of peptidases using a technique involving EDTA, should be viewed with caution. Many workers have used the method of Eichholz and Crane (1965) for the localisation of peptidases in the intestinal mucosa cells (Rhodes et al 1967, Peters 1970, 1973, Kim et al 1972, 1974). A different preparative method for rat was published by Forstner et al (1968) but it too employed an EDTA-containing medium. This method has also been used

in the study of peptidases (Heizer and Laster 1972, Auricchio *et al* 1972).

Peters (1970) used both the Hübscher and the Eichholz and Crane methods to study the subcellular localisation of di- and tripeptide hydrolases in guinea pig small intestine. Using the Hübscher method 76 to 92% of the dipeptidase activity, depending on the substrates used, was found in the soluble fraction with 4 to 12% in the brush border membrane. Nineteen to 63% of tripeptidase activity was found in the brush border membrane with the remainder in the soluble fraction. Using the Eichholz and Crane method, 95 to 99% of dipeptidase activity was found in the soluble fraction (1 to 5% in the brush border membrane) while 10 to 40% of tripeptidase activity was found in the brush border membranes (with remainder in the soluble fraction). The activity in the Eichholz and Crane homogenate against dipeptides was generally lower due, presumably, to the presence of EDTA. Tripeptidases showed an increase in specific activity in the brush border membrane over the homogenate, indicating that these activities may be associated with that membrane. Peters (1970) concluded from these results that dipeptidases were probably located in the cytosol of the epithelial cells and that any activity recovered in the brush border membrane fraction was due to adsorption of cytosol enzymes during the fractionation procedure. His results are in agreement with those obtained with soluble extracts of intestinal mucosa. In a subsequent paper, again using guinea pig tissue, Peters (1973) reported that brush border membranes contained peptidase activity against di-, tri-, tetra-, penta- and hexapeptides and that with the exception of dipeptidase, all activities were concentrated in the brush border membrane fraction. Kim *et al*

(1974) found similar results in the rat with some activity against glycyl-glycyl-glycine and alanyl-glycyl-glycine in the soluble fraction, but no activity against larger peptides. Their brush border membrane fraction hydrolysed all peptides up to five amino acids in length plus a hetero hexapeptide. Inhibition studies indicated that in the cytosol alanyl-glycine and alanyl-glycyl-glycine were hydrolysed by separate enzymes while in the brush border hydrolysis of the two substrates was by one and the same enzyme. Different heat stabilities, metal requirements and distinct kinetic properties indicated that two different groups of enzymes are present in cytosol and brush border membranes of rat intestinal mucosa (Kim et al 1974). Donlon and Fottrell (1972) on the other hand, found no such significant differences using guinea pig intestinal mucosa.

Maroux et al (1973) purified an aminopeptidase from hog intestinal brush border and reported that the purified enzyme accounted for almost all of the peptidase activity of the purified brush border membranes. It also accounted for all of the aryl-amidase activity, all activity against a heptapeptide, about half the tripeptidase and a small but significant amount of dipeptidase activity of the whole jejunal and ileal mucosa.

Auricchio (1972) was able to separate activity against L-α-glutamyl-2-naphthylamide from activity against other naphthylamide substrates in the rat brush border membrane.

1.6 Concluding remarks

With the change in views on mammalian protein absorption in recent years, it has become accepted that small peptides - mainly dipeptides and possibly tripeptides - are taken up into the epithelial cells of the intestinal mucosa intact. As peptides do not in general, enter the portal blood intact, those not hydrolysed on the luminal side of the mucosa, must be hydrolysed intracellularly or on the surface of the enterocyte. Several apparently distinct peptidases have been demonstrated in intestinal mucosa of mammals which could perform such hydrolysis. Peptidases hydrolysing leucyl-glycine and glycyl-proline have clearly been established as separate enzymes and activity towards glycyl-glycine, leucinamide, tripeptides and α-glutamyl peptides probably represent distinct enzymes.

Multiple forms of dipeptidases have been reported in rat, guinea pig and human mucosa (Dolly et al 1971, Fottrell et al 1972) while no heterogeneity was found in pig and monkey small intestine (Sjöström et al 1973, Das and Radhakrishnan 1972, 1973) indicating considerable species differences.

The cytosol of the epithelial cells has been shown to contain most of the dipeptidase activity associated with the mucosa with the remainder associated with the brush border membrane. It is not clear to date whether brush border membrane activity towards dipeptides is due to adsorbed cytosol dipeptidases or to a distinct group of dipeptidases associated with the brush border membrane proper. Brush border activity towards dipeptides may also be due to a general aminopeptidase (Maroux et al 1973).

The subcellular distribution of tri- and oligopeptidases is still uncertain but it appears that many tripeptidases have a dual location - brush border and cytosol - while others are predominantly in the brush border membrane. Kim et al (1972) observed a different zymogram pattern between brush border and soluble fractions of rat small intestine while Donlon and Fottrell (1972) reported that none of the multiple forms of peptidase in guinea pig were specific to a subcellular fraction. These different findings are further evidence that there is probably some variation in the intestinal peptidases from different species. Oligopeptidase activity seems to be confined to the brush border membranes (Peters 1973, Kim et al 1974).

Relatively little is known about peptidases - particularly brush border membrane peptidases - of the human intestinal mucosa.

The aim of the work in this thesis was to measure brush border membrane peptidases from human small intestinal mucosa and to determine the number of enzymes involved in peptide hydrolysis.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Intestinal tissue

Mucosa samples from the human small intestine were obtained during surgery. Duodenal mucosa samples were obtained from patients undergoing vagotomy and pyloroplasty or gastrectomy. Jejunal samples were obtained from patients undergoing pancreatectomy. The tissue was washed in ice-cold saline (0.9%) immediately after removal, sealed in parafilm and frozen on dry-ice. The samples were stored at -20^{o} in a deep-freeze cabinet until used.

2.2 Purification of human intestinal brush border membranes

The materials and the methodology used for the purification of intestinal brush border membranes are described in Chapter 3.

2.3 Protein estimation

The estimation of protein was performed by the method of Lowry et al (1951). To decrease the amount of material used in the assay, it was scaled down. To 50µl of protein sample were added 50µl 1N sodium hydroxide solution and the mixture was allowed to stand at room temperature for 30 minutes to solubilise all the protein. 1ml of alkaline copper reagent was then added, mixed and allowed to stand for a further 20 minutes at room temperature. 100µl Folin-Ciocalteu's reagent (diluted 1:2 with distilled water) were then added with immediate mixing. Colour development was complete after 45 minutes at room temperature and the extinction was measured at 750nm in a Cecil 'CE 272' spectrophotometer (Cecil Instruments Ltd, Cambridge, England) using a 1ml glass cuvette of 1cm pathlength. A standard curve was obtained using bovine serum albumin (Sigma, London) at concentrations of 100-1000µg/ml.

2.4 Estimation of glucose by glucose oxidase

The method described by Lloyd and Whelan (1969) was employed for the estimation of glucose. This method utilises the inhibitory effect of Tris (Tris-hydroxymethyl aminomethane) on α,1,4-glucosidase, an activity often associated with commercial preparations of glucose oxidase as a contaminant.

a) Glucose oxidase reagent:

The following were dissolved in 100ml of Tris-glycerol-phosphate buffer (36.3g Tris, 50g $NaH_2PO_4 \cdot H_2O$, 400ml glycerol made up to 1000ml with water, pH 7.0), glucose oxidase (Böhringer, Cat No 15424 EGAC) 30mg, horseradish peroxidase (Böhringer, Cat No 15302 EPAB) 3mg, o-dianisidine dihydrochloride (Sigma Chemical Co Ltd, London) 10mg. To minimise the handling of solid o-dianisidine a stock solution of 10mg/ml was prepared, of which 1ml was added to 100ml of reagent. The reagent has a shelf life of several weeks when stored at 4°.

b) Procedure:

A scaled down method was used as follows: to 40µl of test solution containing 0-5µmoles/ml of glucose, 400µl glucose oxidase reagent were added, mixed well and incubated for 30 minutes at 37°. To stop the reaction and simultaneously develop the pink colour, 800µl 5N hydrochloric acid were added. After mixing, the extinction at 525nm was measured using a 1ml glass cuvette of 1cm pathlength in a Cecil 'CE 272' spectrophotometer. A standard graph was obtained with 40µl of glucose standard solutions containing 0-5µmoles/ml of glucose. Over this range the concentration of glucose showed a linear relationship with extinction at 525nm.

2.5 Assays for subcellular enzyme markers

Here follows a list of enzymes assayed and the methods employed for the determination of their activity. The activities are expressed as units per mg protein. 1 unit is defined as the hydrolysis of 1µmole of substrate hydrolysed $hour^{-1}$.

2.5.1 Glucose-6-phosphatase (EC 3.1.3.9)

The activity of glucose-6-phosphatase was determined by the hydrolysis of glucose-6-phosphate measured by the liberation of glucose with the glucose oxidase reagent (Lloyd and Whelan 1969). As Hübscher and West (1965) noted, glucose-6-phosphate may also be hydrolysed by acid and alkaline phosphatases. For this reason, the buffer used in the assay (0.1M sodium malate, pH 6.0) contained 4mM EDTA (an inhibitor of alkaline phosphatase) and 2mM sodium fluoride (an inhibitor of acid phosphatase). Under these conditions the hydrolysis of glucose-6-phosphate proceeded linearly with time (up to 20 minutes at 37°) and protein concentration.

a) Procedure:

20µl of enzyme preparation were incubated with 60µl of buffer and 60µl 66.5mM glucose-6-phosphate (Sigma, London) for 20 minutes at 37°. After termination of the reaction by boiling for 2-5 minutes, the liberated glucose was estimated as described in Chapter 2.4, using glucose oxidase reagent. Suitable enzyme and substrate blanks were always performed.

2.5.2 Determination of sucrase activity

The activity of sucrase (EC 3.2.1.20) was measured by the rate

of production of the hydrolysis product glucose, from the substrate sucrose. Glucose was estimated as described in Chapter 2.4 using glucose oxidase reagent.

a) Procedure:

The enzyme preparation (20µl) was incubated with 56mM sucrose in 0.1M sodium malate buffer, pH 6.0 (20µl) for 30-60 minutes at 37°. The addition of 400µl of glucose oxidase reagent stopped the reaction of sucrase. Blanks containing water in place of enzyme were always performed.

2.5.3 Hydrolase activities against 4-methylumbelliferone derivatives

The use of 4-methylumbelliferone derivatives for the assay of glycosidases and phosphatases is now widespread. 4-methylumbelliferone fluoresces strongly in ultra-violet light at pH 10-11 while its conjugates show little or no fluorescence. It was Mead, Smith and Williams (1955) who introduced a method for the fluorimetric assay of β-glucuronidase, the method consisting of the enzymic hydrolysis of 4-methylumbelliferyl-glucuronide followed by the fluorimetric estimation of the liberated 4-methylumbelliferone. Robinson (1956) synthesised 4-methylumbelliferyl-β-glucoside and used it in the study of β-glucosidases from a variety of sources. Leaback and Walker (1961) published a method for the fluorimetric assay of N-acetyl-β-glucosaminidase. A method for assaying alkaline phosphatase using 4-methylumbelliferylphosphate was described by Fernley and Walker (1965). All these workers found these assay methods to be extremely sensitive as well as easy to use. The reactions were found to be linear in time (up to 60 minutes) and $^1/_v$ against $^1/_s$ plots were linear too and in agreement with those

obtained with other substrates.

a) Procedure: (Peters et al 1972)

Stock solutions of the 4-methylumbelliferyl substrates (Koch-Light Laboratories Ltd, Colnbrook, Bucks, England) of 15mM were prepared in ethanol (95%) and stored at 4°. 1.5mM solutions of the substrates were prepared immediately before use in the appropriate buffer containing 0.1% Triton X-100 (scintillation grade). The reaction mixture differed to that used by Peters et al (1972) and contained 20µl enzyme sample (suitably diluted), 100µl substrate (final concentration 0.15mM) and 880µl of the appropriate buffer and was incubated for 15 minutes at 37°. The reaction was stopped by the addition of 2ml 50mM NaOH-glycine buffer, pH 10.4 containing 5mM EDTA. The liberated 4-methylumbelliferone was estimated in a Perkin-Elmer fluorescence spectrophotometer (model 1000) (Perkin-Elmer Corp, Norwalk, Cam.) using a 3ml cell of 1cm pathlength. The excitation wavelength was 365nm, the emission wavelength 450nm. Blanks in which the enzyme was added after the stopping buffer were always performed. Standard: 100µl of 4-methylumbelliferone in 900µl of the appropriate buffer and 2ml NaOH-glycine (final concentration 1.5µM). 1 unit of activity = hydrolysis of 1µmole of substrate $hour^{-1}$ at 37°. A number of enzymes were assayed using this method:

1) α-glucosidase (EC 3.2.1.20)

Substrate: 4-methylumbelliferyl-α-glucopyranoside 1.5mM.

Buffer: Sodiumphosphate 0.1M, pH 6.1 containing 0.1% Triton X-100.

2) N-acetyl-β-glucosaminidase (EC 3.2.1.30)

Substrate: 4-methylumbelliferyl-2-deoxy-2-acetamido-β-gluco-pyranoside.

Buffer: 0.1M sodiumacetate, pH 5.0 containing 0.1% Triton X-100.

3) β-glucuronidase (EC 3.2.1.31)

Substrate: 4-methylumbelliferyl-β-D-glucuronide trihydrate.

Buffer: 0.1M sodiumacetate, pH 3.8 containing 0.1% Triton X-100.

4) Alkaline phosphatase (EC 3.1.3.1)

Substrate: 4-methylumbelliferyl-phosphate.

Buffer: 0.1M sodiumborate, 10mM $MgCl_2$, pH 9.2 containing 0.1% Triton X-100.

2.5.4 Lactate dehydrogenase (EC 1.1.1.27)

Lactate dehydrogenase catalyses the reaction:

$$CH_3COCOO^- + NADH_2 \longrightarrow CH_3CHOHCOO^- + NAD$$

pyruvate lactate

Two methods have been used to determine its activity. One, spectrophotometric, measured the decrease in extinction at 340nm due to the decrease in the level of $NADH_2$. At this wavelength $NADH_2$ has a strong absorption peak while the oxidised form has very little absorption (Wroblenski and LaDue 1955). The second method, which was fluorimetric, measured the amount of NAD produced after destroying the remaining amount of $NADH_2$ (Lowry et al 1957).

1) Spectrophotometric method (Wroblenski and LaDue 1955)

a) Reagents:

0.1M sodiumphosphate buffer, pH 7.5, $NADH_2$ (2.5mg/ml), sodiumpyruvate (2.5mg/ml).

b) Procedure:

50µl enzyme sample, 50µl $NADH_2$ and 850µl buffer were measured into a 1ml spectrophotometer cuvette (1cm pathlength) and pre-incubated in the constant temperature cell holder of the spectrometer (Cecil '272') for 5 minutes. 50µl sodiumpyruvate were then rapidly added and after mixing, the decrease in extinction at 340nm measured, using a recorder (Servoscribe RE 541.2).

1 unit of enzyme activity = change in extinction of 0.001 per minute.

2) Fluorimetric method (Lowry et al 1957)

Although the spectrophotometric method has proved to be of great value, the fluorimetric method was far more sensitive. Determinations of NAD as low as 10^{-8}M could be made accurately, which corresponds to only a thousandth of that required with the spectrophotometric method at 340nm.

a) Reagents:

0.1M sodiumphosphate buffer, pH 7.5, 0.4M hydrochloric acid, 10M sodium hydroxide, 50mM sodiumpyruvate, 50mM $NADH_2$. A cocktail was prepared containing 5mM pyruvate and 5mM $NADH_2$ in phosphate buffer.

b) Procedure:

20µl enzyme samples were incubated with 20µl cocktail at 37° for 15 minutes. The samples were made 0.2M in HCl for at least 30 seconds to destroy any residual NADH. This was achieved by the addition of 40µl 0.4M HCl. 120µl 10M NaOH were then added to give a concentration of NaOH of 6M and the samples incubated for 30 minutes at 37°. It was then diluted 5-fold by the addition of 2.3ml distilled water and the fluorescence measured in a Perkin-

Elmer fluorimeter (model 1000). Excitation was at 365nm, emission at 470nm.

Standards and blanks were always provided which were given identical treatment.

2.5.5 Succinate dehydrogenase

Succinate dehydrogenase activity, as measured by reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium hydrochloride (INT), was determined by the method of Pennington (1961) as modified by Porteous and Clark (1965).

a) Reagents:

50mM potassium phosphate buffer, pH 7.4, containing 0.1% INT, 50mM sodium succinate, 25mM sucrose and 2mM EDTA; 10% trichloroacetic acid; ethylacetate.

b) Procedure:

0.1ml of enzyme sample was added to 0.4ml of buffer reagent and left on ice for 30 minutes before incubation at 37^{o} for 15 minutes. The reaction was stopped by the addition of 0.5ml of 10% trichloroacetic acid and the formazan extracted with 2ml of ethylacetate. The extinction of the ethylacetate extract was measured at 490nm using a spectrophotometer (Cecil model '272'). Suitable substrate and enzyme blanks were treated the same way.

2.6 Estimation of peptide hydrolase activity

Peptide hydrolase activity was measured using di-, tri- and oligopeptide substrates as well as 2-naphthylamide substrates.

2.6.1 Activity against peptides

Hydrolase activity against di-, tri- and oligopeptides was measured using the method of Donlon and Fottrell (1971) employing L-amino acid oxidase, horseradish peroxidase and o-dianisidine. The principle of the method is as follows:

$$\text{Peptide} + H_2O \xrightarrow{\text{peptidase}} \text{amino acid}$$

$$\text{amino acid} + O_2 \xrightarrow[\text{oxidase}]{\text{amino acid}} \text{keto acid} + H_2O_2 + NH_3$$

$$H_2O_2 + \text{o-dianisidine} \xrightarrow{\text{peroxidase}} H_2O + \text{oxidised o-dianisidine (orange colour)}$$

The orange colour of oxidised o-dianisidine was measured spectrophotometrically at 460nm and the amount of liberated amino acid determined from a standard graph of the appropriate amino acid. 1 unit of activity is defined as the hydrolysis of 1μmole of substrate minute^{-1} at 37°. Specific activity is units/mg protein.

a) Reagents:

Sodium borate buffers (0.15M), pH 7.3 and 8.0 were used for the assay. Substrates were 10mM in borate buffer pH 8.0. o-dianisidine was prepared by the addition of 0.5ml of a stock solution (61.5mg/5ml water) to 70ml of borate buffer, pH 7.3. 4mg of L-amino acid oxidase and 8mg of peroxidase were dissolved in

10ml of water.

b) Procedure used for the determination of activity in subcellular fractions:

25µl of test solution (enzyme), 50µl borate buffer, pH 8.0 and 75µl peptide substrate were incubated for 10 minutes at 37°. To stop the reaction the tubes were transferred to a 75° water bath and incubated for a further 5 minutes. 100µl of incubation mixture were pipetted into a 1ml glass spectrophotometer cuvette(1cm path-length) containing 700µl o-dianisidine-borate buffer, pH 7.3. The mixture was preincubated for 1 minute in the constant temperature cell-holder of a spectrophotometer (Cecil '272') before the addition of 200µl of L-amino acid oxidase/peroxidase solution. The mixture was agitated quickly with a plastic stirrer and the increase in extinction at 460nm followed on a recorder (Servoscribe 15, RE 541.20) for 1 minute. Standard graphs were prepared using the appropriate L-amino acids at concentrations of 1 to 5µmoles/ml (L-leucine) and 0.1 to 1µmoles/ml (L-phenylalanine and L-tyrosine). The standard solutions, ie. 25µl were added to 50µl buffer and 75µl substrate and processed together with the test samples. Peptide hydrolase activity could therefore be determined directly from the standard graphs. All tests were duplicated and substrate and enzyme blanks were always performed.

c) Procedure for the determination of activity in polyacrylamide gel slices:

50µl of gel slice supernatant and 50µl of buffered peptide substrate (10mM in 0.15M sodium borate buffer, pH 8.0) were incubated at 37° in an incubator overnight. The tubes were tightly stoppered

to avoid evaporation of the small amount of sample. After incubation 300µl of o-dianisidine-borate buffer, pH 7.3, and 100µl L-amino acid oxidase/peroxidase solution were added, mixed and incubated for a further 5 minutes at 37°. The reaction was stopped by the addition of 500 µl of 5N hydrochloric acid which caused the simultaneous development of a pink colour which was measured spectrophotometrically at 525nm using a 1ml glass cuvette of 1cm pathlength in a Cecil '272' spectrophotometer.

2.6.2 Activity against 2-naphthylamides (Arylamidases)

Aminopeptidase M, aminopeptidase A, dipeptidylpeptidase IV and γ-glutamyltransferase were all assayed fluorimetrically by a scaled down method described by Peters et al (1972). The enzymes were incubated with the appropriate aminoacyl-2-naphthylamide and the release of 2-naphthylamine measured after stopping the reaction.

a) Procedure used for the determination of activity in subcellular fractions:

20µl of suitably diluted enzyme sample were incubated with 100µl substrate (0.2mM) and 880µl buffer for 15 minutes at 37°. The reaction was stopped by the addition of 2ml ice-cold 50mM NaOH-glycine buffer, pH 10.4 containing 5mM EDTA. The release of 2-naphthylamine was measuring using a Perkin-Elmer Fluorescence spectrophotometer (model 1000).

The excitation wavelength was 340nm and the emission wavelength 410nm. Suitable enzyme and substrate blank assays were performed. Standardisation was achieved by mixing 100µl of 2-naphthylamine working solution with 900µl assay buffer and 2ml NaOH-glycine buffer

and measuring the fluorescence as described above. The amount of 2-naphthylamine in the cuvette was equivalent to the amount in a cuvette where 10% of the substrate had been hydrolysed.

b) Procedure used for the determination of activity in polyacrylamide gel slices:

50µl of gel slice supernatant, 100µl of substrate (0.2mM) and 350µl of buffer were incubated at 37° in an incubator overnight. The tubes were tightly stoppered to avoid evaporation. After incubation, the reaction was stopped by the addition of 2ml ice-cold 50mM NaOH-glycine buffer, pH 10.4, containing 5mM EDTA. The release of 2-naphthylamine was measured as described above.

Substrates and buffers:

1) Aminopeptidase M (EC 3.4.11.2)

Substrate: L-leucine-2-naphthylamide.

Buffer: 0.1M sodiumphosphate, pH 7.3 containing 0.1% Triton X-100.

2) Aminopeptidase A (EC 3.4.11.7)

Substrate: L-α-glutamic acid-2-naphthylamide.

Buffer: 0.1M sodiumphosphate, pH 7.3 containing 0.1% Triton X-100.

3) Dipeptidyl peptidase IV (EC 3.4.14.-)

Substrate: Glycyl-L-proline-2-naphthylamide.

Buffer: 0.1M sodiumphosphate, pH 7.3 containing 0.1% Triton X-100.

4) γ-glutamyltransferase (γ-glutamyltranspeptidase) (EC 2.3.2.2)

Substrate: L-γ-glutamic acid-2-naphthylamide.

Buffer: 0.2M Tris-HCl, pH 8.5, 3mM glycyl-glycine.

Stock solutions of 20mM were prepared in ethanol. These were found to be stable for several weeks at 4°. A dilution of 1:100 on the day of the assay, gave the working solution of 0.2mM. Standard

stock solution of 2-naphthylamine was 2mM in ethanol. A dilution of 1:100 gave the working solution of 20μM. L-leucine-2-naphthylamide was obtained from Sigma, London; L-α-glutamic acid-2-naphthylamide, L-γ-glutamic acid-2-naphthylamide and glycyl-proline-2-naphthylamide were supplied by Bachem (Feinchemikalien AG, CH-4416 Bubendorf, Switzerland).

2.7 Preparation of polyacrylamide gels

2.7.1 Gels for SDS-polyacrylamide disc gel electrophoresis

SDS-polyacrylamide disc gel electrophoresis was performed using a multiphasic buffer system calculated from theory by Jovin et al (1971) and modified for SDS by Neville (1971). According to Hjerten (1962) upper and lower gels were 3.2x6.25 and 11.1x0.9 respectively. The first numeral (T) denotes the total weight of monomer (acrylamide N,N'-methylenebisacrylamide) per 100ml of solvent, and the second numeral (C) denotes the amount of bisacrylamide expressed as a percentage (w/w) of the total amount of monomer.

a) Reagents:

Upper reservoir buffer, pH 8.64, 0.04M boric acid, 0.041M Tris, 0.1% SDS.

Upper gel buffer, pH 6.1. 0.0267M sulphuric acid, 0.0541M Tris, (running pH 8.64).

Lower gel buffer, pH 9.18, 0.0305M hydrochloric acid, 0.4244M Tris (running pH 9.50).

Lower reservoir buffer, same as lower gel buffer.

Catalysts:

For upper gel: 0.3% N,N,N',N'-tetramethylethylenediamine (TEMED). 0.2% ammonium persulphate in upper gel buffer.

For lower gel: 0.3% TEMED, 0.1% ammonium persulphate in lower gel buffer.

For upper gel solution: 6g acrylamide, 0.4g bisacrylamide in 100ml of upper gel buffer.

For lower gel solution: 22g acrylamide, 0.2g bisacrylamide in 100ml of lower gel buffer.

Preparation of gels

After de-aeration of the solutions, one part of lower gel solution was mixed with one part of the appropriate catalyst solution and poured into precision bore glass tubes which were stoppered at one end, to within 2.5cm from the top. The tubes were held in an upright position. A small amount of water (1cm) was layered onto the surface of the gel solution to ensure a straight surface. After polymerisation (approx. 20 minutes) the water was removed with a pasteur pipette, the top of the gel rinsed once with upper gel solution and then the upper gel solution (1 part upper gel solution and 1 part catalyst solution) layered on top of the lower gel (1.5cm). A small amount of water (1cm) was layered on top and polymerisation allowed to proceed (approximately 20 minutes). The final gels had a dimension of 7.5x0.5cm.

2.7.2 Gels for polyacrylamide disc gel electrophoresis

Disc gel electrophoresis in the absence of SDS was essentially performed by the method of Ornstein and Davis (1964) using a discontinuous buffer system. The following description is based on the original description of Davis (1969), with the exception that both lower and upper gels were polymerised chemically using ammonium persulphate.

The following solutions were prepared and could be stored in the dark at 4^{o} for several weeks:

a) 48ml of N HCl, 36.6g of Tris, 0.23ml of N,N,N',N'-tetramethylethylenediamine and water to 100ml, pH 8.9.

b) 48ml of N HCl, 5.98g of Tris, 0.46ml of N,N,N',N'-tetra methylethylenediamine and water to 100ml, pH 6.7.

c) 28.0g acrylamide, 0.735g N,N'-methylenebisacrylamide and water to 100ml.

d) 10.0g acrylamide, 2.5g N,N'-methylenebisacrylamide and water to 100ml.

The following solutions were used to prepare the gels and were made up fresh on the day the gels were prepared:

1. Lower gel solution A: one part of a) above was mixed with two parts of c) and one part of water.

2. Lower gel solution B: 0.1% ammonium persulphate in water.

3. Upper gel solution A: One part of b) above was mixed with two parts of solution d) and one part of water.

4. Upper gel solution B: 0.1% ammonium persulphate in water.

The buffer used in the electrode compartments was 6.0g of Tris and 28.8g of glycine in 1000ml of water, pH 8.3.

Preparation of gels

After degassing, equal amounts of lower gel solution A and lower gel solution B were mixed and applied to 10x0.5cm precision glass tubes to within 2.5cm of the top. The solution in each tube was layered over carefully with 0.5cm of water so that a discrete boundary was formed between the gel solution and the water. Gelling was allowed to proceed for a period of 45 minutes after which time, the water layer was carefully removed with a pasteur pipette and the top of the

gel rinsed with upper gel solution A.

After degassing, equal amounts of upper gel solutions A and B were mixed and a 1cm layer added to the top of each gel and again covered with a water layer. When polymerisation of the upper gel was complete, the gels were ready for use. The dimension of the gels was 7.5x0.5cm.

2.7.3 Gels for polyacrylamide gel isoelectric focusing (slabs)

Thin layer gel electrofocusing in polyacrylamide gels was carried out by the method of Karlson et al (1973) with a pH gradient in the range of 3.5-9.5.

a) Reagents:

A. Acrylamide solution 29.1% (w/v) in distilled water.

B. N,N'-methylenebisacrylamide solution 0.9% (w/v) in distilled water.

C. Riboflavin solution 0.004% (w/v).

All stock solutions were kept in dark bottles at 4^{o} and used within two weeks after preparation.

Preparation of gels

The following solutions were mixed in a 100ml vacuum flask:

10.0ml A

10.0ml B

36.6ml distilled water, in which 7.5g sucrose was dissolved.

3.0ml Ampholine, pH 3.5-10.

After mixing the solutions thoroughly, they were de-aerated by aspirating for a few minutes. Finally, 0.4ml of C was added and mixed in by rotating the flask, avoiding shaking.

Moulding the gel slab

The set-up for moulding the polyacrylamide gels consisted of a glass plate (125x260x3mm), a thinner glass plate (125x260 x1mm), a silicone gasket of 1mm thickness and another glass plate (125x260x3mm) clamped together with bulldog clips. The mounted set for gel moulding was put in an upright position and the gel solution added at a corner with a syringe. The mould was filled completely and formation of air bubbles avoided. The gasket was closed at the corner and the last clamp put over. Polymerisation was allowed to proceed for a few hours in front of two Phillips TL 20W/08 daylight fluorescence tubes. After polymerisation the clamps were removed and the set of glass plates with the gel stored in a horizontal position in the fridge (usually overnight). On the day of the focusing experiment, the upper glass plate (125x260x3mm) was removed with the aid of a spatula and any unpolymerised acrylamide along the edges removed with a soft filter paper. The gel was now ready for use.

All chemicals for polyacrylamide gels were obtained from BDH Chemicals (Poole, Dorset) except riboflavin which was obtained from Sigma, London. Ampholine was obtained from LKB, South Croydon, Surrey, England.

2.8 Staining of polyacrylamide gels for protein

2.8.1 Protein staining after electrophoresis

The method of Weber and Osborn (1969) using Coomassie Brilliant Blue R250 was used for protein staining in polyacrylamide gels.

Procedure

Immediately after electrophoresis was completed, the gels were extruded from the glass tubes and placed in staining solution (1.25g Coomassie Brilliant Blue in 454ml 50% methanol, 46ml glacial acetic acid). Staining time was 2 hours. The gels were then rinsed with water before placing in destaining solution (75ml glacial acetic acid, 50ml methanol, 875ml water). The staining solution was changed after 1 hour and destaining allowed to proceed overnight.

2.8.2 Protein staining after isoelectric focusing

After completion of focusing experiment, the gels were placed in fixing solution for 1 hour (150mg methanol, 350ml distilled water, 17.25g sulphosalicylic acid, 57.5g trichloroacetic acid). This solution precipitates the proteins and lets the ampholine diffuse out of the gel. The plate was then placed in destaining solution (500ml ethanol, 160ml acetic acid, diluted to 2 litres with distilled water) for 5 minutes. Staining was performed for 10 minutes at 60° in staining solution (0.115g Coomassie Brilliant Blue R250 in 100ml destaining solution). The gel plate was then destained with several changes of destaining solution until clear of excess stain.

CHAPTER THREE

PURIFICATION OF HUMAN INTESTINAL BRUSH BORDER MEMBRANES

3.1 Introduction

The study of peptide hydrolases associated with the intestinal brush border membrane necessitated the purification of these membranes to avoid contamination by hydrolases from other cell loci, in particular lysosomes and the cytosol. In 1973 Schmitz *et al* published a method for the preparation of brush border membranes from human small intestine and their method was used as a starting point for the present study.

The method of Schmitz *et al* (1973) was based on the sequential use of differential centrifugation and disruption of partially purified brush border membranes by Tris followed by centrifugation in a glycerol gradient. A 1% homogenate was made in 50mM mannitol-2mM Tris-HCl buffer, pH 7.1 at 4^{o} using a Waring blender or a conical grinding tube. The homogenate was filtered through nylon mesh (40μm pore size). Solid calcium chloride was added to the filtrate to a final concentration of 10mM to aggregate microsomes which were then sedimented by centrifugation at 2000xg during 10 minutes. The clear supernatant (designated S_1) was centrifuged at 20,000xg for 20 minutes to yield a small pellet (designated P_2) containing the brush border fragments. Fraction P_2 was treated with Tris and layered onto a glycerol gradient. The gradient was centrifuged for 15 minutes at 63,000xg after which three to four bands were recovered in four fractions. The microvillous membranes were recovered in the largest band which was located in the centre of the gradient (designated fraction F_{II}). Figure 3.1 shows a schematic representation of this purification procedure.

Because of differences in equipment and reagents, it is sometimes

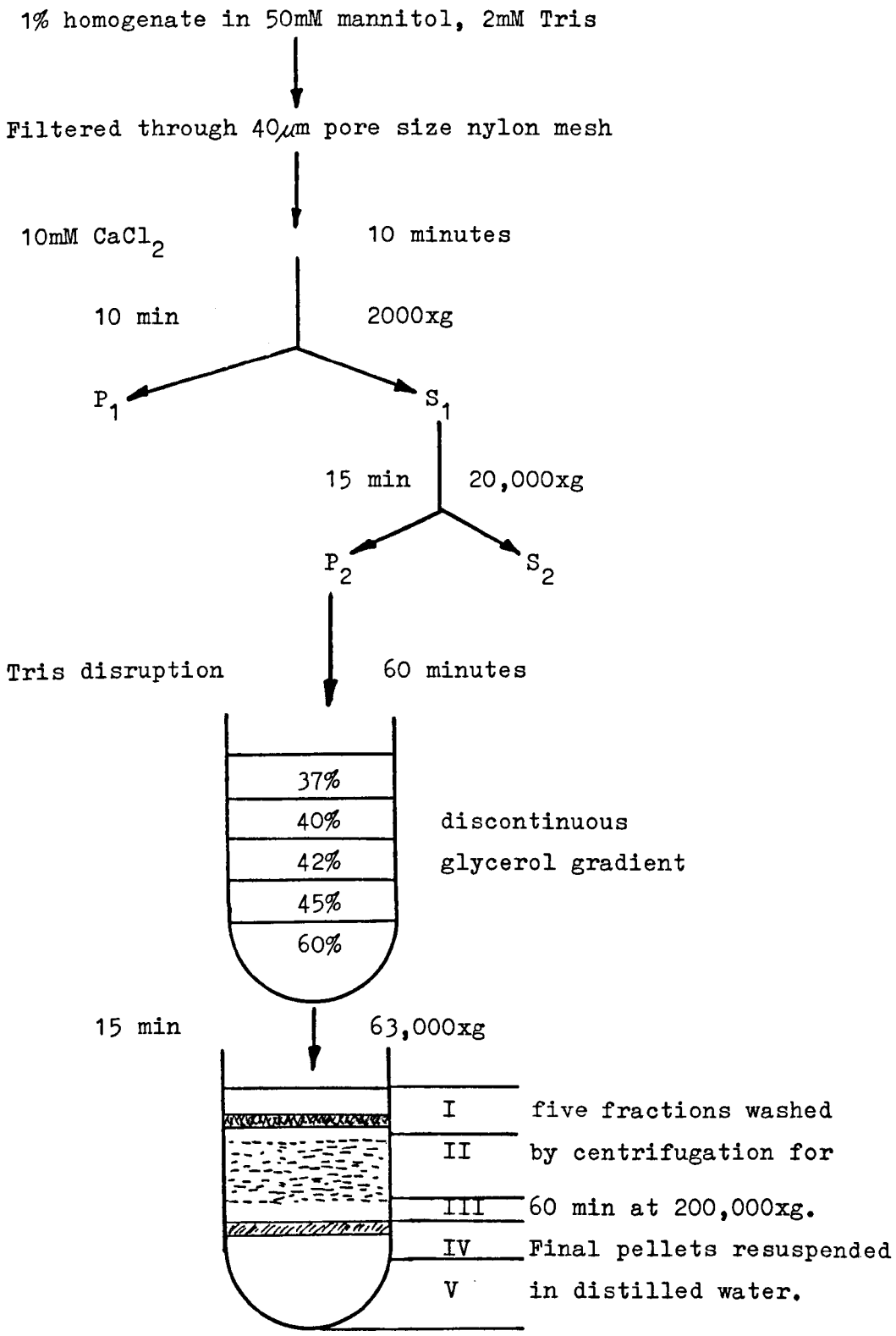


Figure 3.1 Schematic representation of the purification procedure of human brush border membranes (Schmitz et al 1973)

difficult to copy a published method satisfactorily in one's own laboratory. For this reason several fractionations were performed following the Schmitz method as closely as possible. The following Sections in this Chapter show the results obtained with the method of Schmitz et al (1973) and how this method was subsequently modified to yield the procedure used routinely for the work on peptide hydrolases of the brush border membrane.

3.2 Purification of human intestinal brush border membranes using the method of Schmitz et al (1973)

3.2.1 Intestinal samples

The nature and source of intestinal samples used for this study have been dealt with in Chapter 2.

3.2.2 Preparation of brush border fragments (P_2 fraction)

The still frozen mucosa was carefully dissected from the underlying muscle tissue.

A 1% homogenate was made in 50mM mannitol-2mM Tris-HCl buffer, pH 7.1 at 4° using a "Virtis" homogeniser (Virtis Research Equipment, Gardiner, New York, USA) at medium speed for 60 seconds. All following operations were performed at 4°. The homogenate was filtered through nylon mesh (with a pore size of 60μm) (Henry Simons Ltd, Cheadle, England). Solid calcium chloride was added to a final concentration of 10mM and the homogenate left stirring slowly for 30 minutes. During that time an increase in turbidity of the homogenate was observed. The calcium chloride-treated homogenate was then centrifuged at 2000xg for 10 minutes (MSE 18, 8x50ml rotor, 4200 rpm) to yield a heavy pellet (fraction P_1), which was resuspended in 50mM mannitol-2mM Tris for further analysis, and a clear supernatant (fraction S_1). The supernatant was centrifuged at 20,000xg for 15 minutes (MSE 18, 8x50ml rotor, 13,000rpm) to yield a small pellet (fraction P_2) containing the brush border fragments. Fraction P_2 was resuspended in mannitol/Tris buffer and the final supernatant (fraction S_2) was kept for analysis.

3.2.3 Preparation of microvillous membrane fraction (Fraction F_{II})

Tris was used to disrupt brush border fragments into microvillous membranes and core material. Freshly made up 1.6M Tris (pH adjusted to 7.1 with HCl) was added to fraction P_2 to yield a final Tris concentration of 0.8M and the Tris pellet mixture stirred for 1 hour. The mixture was then layered on top of a 37, 40, 42, 45, 60% glycerol gradient which was 0.05M in magnesium chloride and centrifuged for 15 minutes at 63,000xg (MSE 50, 3x25ml swing out rotor, 24,000 rpm). Several bands could be seen by the Tyndall effect and five fractions of 4ml were recovered from the top of the tube, transferred to 10ml MSE tubes and diluted to 10ml. After mixing, the fractions were spun down at 100,000xg for 1 hour (MSE 50, 10x10ml Angle rotor, 40,000 rpm). The final pellets were resuspended in mannitol-Tris-HCl buffer and kept frozen for analysis.

3.2.4 Assays

Protein estimation employed the method of Lowry et al (1951).

Sucrase, α-glucosidase and alkaline phosphatase were used as microvillous membrane markers. Succinate dehydrogenase was used as a mitochondrial marker. As a microsomal marker, glucose-6-phosphatase was used. β-glucuronidase and N-acetyl-β-glucosaminidase were chosen as lysosomal markers and LDH as a marker for the cytosol.

3.2.5 Results

Two fractionations were performed using the Schmitz method, the results of which are summarised in Tables 3.1 and 3.2. The

results are in terms of specific activity (u/mg protein), % recovery and increase in specific activity (degree of purification compared with the homogenate) of the various marker enzymes.

Fraction	Prot.	Sucrase			Glucose-6-phosphatase			β-Glucuronidase			Lactate dehydrogenase		
	%	s.a.	incr. s.a.	%	s.a.	incr. s.a.	%	s.a.	incr. s.a.	%	s.a.	incr. s.a.	%
H	100	8.1	1	100	0.44	1	100	0.25	1	100	454	1	100
P_1	3	42.1	5.2	15.9	1.7	3.9	11.6	0.11	0.44	1.4	63.2	0.14	0.42
P_2	6.5	49	6	39.7	1.4	3.2	20.9	0.061	0.24	1.6	31.4	0.07	0.45
S_2	86.5	2.9	0.4	31	0.3	0.7	58.9	0.21	0.84	72	424	0.93	81
1	0.9	56.4	7	12.5	4.1	9.3	8.4	0.03	0.13	0.12	343	0.76	1.36
2	0.2	75.8	9.4	4	14.2	32.3	6.8	0.04	0.16	0.035	485	1.07	0.45
3	0.1	25	3.1	0.79	11	25	3.2	0.02	0.09	0.011	600	1.32	0.23
4	0.04	21.4	2.6	0.24	22.8	51.8	2.3	0.04	0.15	0.007	571	1.26	0.11
5	0.06	8	0.98	0.13	14	31.8	2.05	0.06	0.22	0.014	400	0.88	0.11
	98.7			104.3			114			89.2			84.1

Table 3.1 Human brush border fractionation (II)

Fraction	Prot.	α-Glucosidase			Glucose-6-Phosphatase			N-acetyl-β-glucosaminidase			Lactate-dehydrogenase			Succinate dehydrogenase		
	%	s.a.	incr. s.a.	%	s.a.	incr. s.a.	%	s.a.	incr. s.a.	%	s.a.	incr. s.a.	%	s.a.	incr. s.a.	%
H	100	1.23	1	100	0.32	1	100	0.3	1	100	2.3	1	100	1.4	1	100
P_1	21	1.24	1	21.1	0.62	1.9	40.8	0.06	0.2	4.1	0.7	0.3	6.9	1.2	0.9	15.3
P_2	3.7	4.4	3.6	13.2	2.6	8.1	30.2	0.2	0.7	2.6	1.6	0.7	2.6	1.9	1.4	5
S_2	53.1	0.8	0.7	34	0	0	0	0.51	1.7	87.1	0.3	0.13	64.3	1.1	0.8	40.3
1	0.42	1.95	1.6	0.67	3.35	10.5	4.44	0.06	0.19	0.08	0.86	0.37	0.16			
2	0.17	5.28	4.3	0.71	5.67	17.7	2.96	0.143	0.48	0.08	2.57	1.1	0.19			
3	0.14	5.92	4.8	0.69	7.88	24.6	3.57	0.165	0.55	0.08	2.88	1.3	0.18			
4	0.064	6.9	5.6	0.36	13.5	42.2	2.7	0.296	0.99	0.06	4.22	1.8	0.12			
5	0.13	4.4	3.6	0.46	8.75	27.3	2.96	0.193	0.64	0.07	4.1	1.8	0.2			
	78.8			71.2			87.6			94.2			74.7			60.6

Table 3.2. Human brush border fractionation (IV)

3.2.6 Conclusions

The method of Schmitz *et al* has been applied to several fractionations of human small bowel mucosa. Tables 3.1 and 3.2 show the results of two such fractionations. Some variation in the data from different preparations has been observed. Neither the yield nor the purity of brush border membranes reported by Schmitz *et al* as determined by the marker enzyme sucrase or α-glucosidase were achieved in these experiments. In their final fraction (F_{II}), they recovered 23% of the total sucrase activity with an increase in specific activity of 26-fold over the homogenate. The maximum increase in specific activity of sucrase obtained in this laboratory, following the same method as closely as possible, was 9.4-fold with a total recovery of only 40%. The brush border fractions were found to be highly contaminated by the microsomal marker glucose-6-phosphatase. The mitochondrial marker succinate dehydrogenase could not be detected in significant amounts in the glycerol gradient fractions. The cytosol and lysosomal markers, lactate dehydrogenase and β-glucuronidase or N-acetyl-β-glucose-aminidase respectively, although recovered mainly in the soluble fraction (S_2) were still present in the gradient fractions. It was therefore decided to examine the effect of each step in the preparation procedure (ie. homogenisation, first and second centrifugation and Tris-disruption) on the distribution and purification of the marker enzymes used.

3.3 Validity of fluorimetric α-glucosidase assay

For the assay of α-glucosidase, methylumbelliferyl-α-D-glucopyranoside was used as a substrate. The assay was performed at pH 6.1. As this fluorimetric substrate can be hydrolysed by lysosomal α-glucosidase and as the assay pH used was relatively low at pH 6.1, it was decided that the validity of such an assay had to be tested. To do this a comparative experiment was performed using the fraction from a previous preparation. The fractions were assayed for α-glucosidase using methylumbelliferyl-α-D-glucopyranoside at pH 6.1 and for sucrase using sucrose at pH 6. The details of the assay procedures are given in Chapter 2.

The results are shown in Table 3.3.

3.3.1 Conclusion

The specific activity of α-glucosidase was found to be approximately half that of sucrase. The distribution of activity among the fractions was not significantly different. It was therefore concluded that the fluorimetric assay for α-glucosidase was a valid one and for all subsequent experiments this method was used.

Fraction	α- glucosidase			sucrase		
	% (H=100%)	% (Total=100%)	s.a.	% (H=100%)	% (Total=100%)	s.a.
H	100		0.389	100		0.39
P_1	9.4	17.6	0.336	6.1	11.1	0.22
P_2	27.1	50.6	2.05 (x 5.3)	30.3	54.7	2.29 (x 5.9)
S_2	17	31.8	0.104	19	34.2	0.115
Total	53.5	100		55.4	100	

Table 3.3 Comparison of α-glucosidase and sucrase activity in the fractions.

3.4 The effect of homogenisation time on the distribution of brush border membrane and microsomal marker enzymes in a preparation of brush border fragments (P_2)

One of the most critical steps in the fractionation procedure was undoubtedly the homogenisation of the tissue. It was not possible to follow the description of that step by Schmitz et al as the homogeniser available differed from that used by these workers. The optimal conditions for the homogenisation of the tissue using the available homogeniser (Virtis) therefore had to be determined.

3.4.1 Method

440mg of duodenal mucosa were used in a total volume of mannitol-Tris buffer of 44ml. Homogenisation was carried out as follows using the "Virtis" at setting 4 in a 50ml flask with two blades attached at right angles to each other:

1) 15 seconds

   A 10ml sample was taken for analysis H_1

2) 15 seconds ie. total 30 seconds

   A 10ml sample was taken for analysis H_2

3) 15 seconds ie. total 45 seconds

   A 10ml sample was taken for analysis H_3

4) 15 seconds ie. total 60 seconds

   A 10ml sample was taken for analysis H_4

Each homogenate was then treated as described in Section 3.2.2.

3.4.2 Results

The results of this experiment are shown in Table 3.4.

%H % recovery (H = 100%)

%T % recovery (Total recovered = 100%)

Increase in s.a. in P_2

Homogenisation time.	Fraction	Protein		α-glucosidase			alkaline phosphatase			glucose-6-phosphatase		
		% H	% T	% H	% T	s.a.	% H	% T	s.a.	% H	% T	s.a.
15 s	H	100		100		0.53	100		1.4	100		4.5
	P_1	12.2	14	13.5	19.1	0.58	22.5	38.1	2.55	11.9	13	4.4
	P_2	6.1	7	35.9	50.4	3.05	24	41.5	5.4	5.4	5.8	3.9
	S_2	69	79	21.6	30.5	0.16	12	20.4	0.24	74.5	81.2	4.85
		87.3	100	71	100	[5.8]	58.9	100	[4.2]	91.8	100	
30 s	H	100		100		0.56	100		1.41	100		3.38
	P_1	11.5	13.5	8.2	20.4	0.4	16.6	31.7	2.03	13.3	12.7	3.92
	P_2	4.9	5.8	20.7	51.5	2.36	17.5	33.5	5.0	6.3	6	3.7
	S_2	69	80.7	11.3	28.1	0.09	18.2	34.8	0.37	85.7	81.3	4.21
		85.4	100	40.2	100	[4.2]	52.3	100	[3.6]	105	100	
45 s	H	100		100		0.39	100		1.53	100		3.33
	P_1	10.9	13.7	9.4	17.6	0.34	13.2	32.8	1.85	12.9	13.2	3.94
	P_2	5.1	6.4	27.1	50.6	2.1	17	42.2	5.08	5.7	5.8	3.7
	S_2	63.7	79.9	17	31.8	0.1	10.1	25	0.24	78.9	81.1	4.12
		79.7	100	53.5	100	[5.3]	41.3	100	[3.3]	97.5	100	
60 s	H	100		100		0.48	100		1.4	100		3.05
	P_1	10.9	13.5	5.8	12.9	0.26	13.4	23.5	1.72	12.2	11.7	3.7
	P_2	5.5	6.7	21.8	48.3	1.93	20.8	36.6	5.33	6.2	5.5	3.5
	S_2	64.7	79.8	17.5	38.8	0.13	22.7	39.9	0.49	93.6	82.8	4.42
		81.1	100	45.1	100	[4.0]	62.3	100	[3.8]	112	100	

Table 3.4. Effect of homogenisation on the distribution of marker enzymes.

3.4.3 Conclusions

From the results in Table 3.4, it was apparent that the recovery of brush border and microsomal markers in the P_2 fraction did not appreciably vary with increased homogenisation time. There seems to have been a tendency of increased activity in the S_2 fraction with both markers as the time of homogenisation was increased. This could have been due to either the membrane being disrupted into smaller fragments or to the removal of the actual enzymes from the membrane. Centrifugation of S_2 at 100,000xg for an hour might have helped to investigate this but was not carried out at this stage.

3.5 The effect of time and speed of the first centrifugation step on the distribution of brush border membrane and microsomal marker enzymes in a preparation of brush border fragments (P_2)

3.5.1 Method

A 1% homogenate of duodenal mucosa was prepared (homogenisation 30 seconds, medium speed) and filtered. After calcium chloride treatment the homogenate was divided into four equal portions of 10ml and each centrifuged as follows:

1) 2000xg, 10 min
2) 2000xg, 20 min (MSE super minor, 3400 rpm)
3) 3000xg, 10 min (MSE 18, 8x50ml rotor, 5000 rpm)
4) 4200xg, 10 min (MSE 18, 8x50ml rotor, 6000 rpm)

The supernatants were poured off and centrifuged at 20,000xg for 15 minutes in the MSE 18. The pellets were resuspended in 50mM mannitol-2mM Tris.

3.5.2 Assays

The fractions were assayed for protein, alkaline phosphatase, α-glucosidase and glucose-6-phosphatase.

3.5.3 Results

The results of this experiment are summarised in Table 3.5.

%H % recovery (H = 100%)

%T % recovery (Total recovered = 100%)

increase in s.a. in P_2

centrifugation	Fraction	Protein		α-glucosidase			alkaline phosphatase			glucose-6-phosphatase		
		% H	% T	% H	% T	s.a.	% H	% T	s.a.	% H	% T	s.a.
2000 x g, 10 min	H	100		100		0.97	100		3.7	100		0.42
	P_1	20.9	26.3	16.9	30.5	0.77	14.4	31.1	2.52	24.3	36.3	0.49
	P_2	2.1	2.7	18.9	34.2	8.58	12.7	27.3	22.2	4.3	6.4	0.88
	S_2	56.3	71	19.5	35.3	0.33	19.3	41.6	1.25	38.4	57.3	0.29
		79.3	100	55.3	100	[9]	46.4	100	[6]	67	100	[2]
2000 x g, 20 min	H	100		100		0.95	100		3.7	100		0.42
	P_1	28.7	32	18.2	28.6	0.6	17	30.2	2.16	38.5	45.5	0.57
	P_2	2.2	2.5	22.7	38.5	10.6	18	32	30.2	7.7	9.2	1.5
	S_2	58.7	65.5	20.8	32.9	0.34	21.2	37.8	1.32	38.4	45.3	0.28
		89.6	100	61.7	100	[11]	56.2	100	[8]	84.6	100	[3.6]
3000 x g, 10 min	H	100		100		0.95	100		3.7	100		0.42
	P_1	22.6	28.4	19.9	43.5	0.83	18.3	43.4	2.95	37	31.8	0.7
	P_2	0.74	0.9	8.9	17.5	10.3	6.4	15.3	31.8	2.1	1.9	1.22
	S_2	56.3	70.7	17.9	39	0.3	12.4	41.3	1.13	77.1	66.3	0.58
		79.6	100	46.7	100	[11]	42.1	100	[8.6]	116	100	[2.9]
4200 x g, 10 min	H	100		100		0.95	100		3.7	100		0.42
	P_1	17.4	23.4	23.2	47.2	1.26	16.3	41.9	3.42	24.3	31.3	0.59
	P_2	0.61	0.8	9.4	19.1	14.6	6.95	17.9	41.7	2.1	2.7	1.49
	S_2	56.3	75.8	16.5	33.6	0.28	15.7	40.2	1.06	51.3	66	0.39
		74.3	100	49.1	100	[15.4]	39	100	[11]	77.7	100	[3.6]

Table 3.5. Effect of the first centrifugation step on the distribution of brush border and microsome marker enzymes.

3.5.4 Conclusions

Increasing the time of centrifugation at 2000xg from 10 to 20 minutes caused an increase in recovery of protein and enzyme markers in P_2. When the centrifugation speed was increased however, a fall in the recovery of protein and enzyme markers in P_2 and S_2 was observed. The specific activity in P_2 of both brush border membrane and microsomal markers was increased with faster centrifugation speeds. A high purification at the P_2 stage was of course desirable but was accompanied by a sharp fall in recovery of brush border marker. At 2000xg for 20 minutes, both recovery of brush border marker and specific activity were increased and it is for this reason that in all future fractionations, these conditions were applied for the first centrifugation step.

3.6 Sedimentability of brush border membrane and microsomal enzyme markers recovered in fraction S_2

Appreciable amounts of brush border enzyme marker were recovered in the S_2 fraction. This experiment was designed to establish if any of this activity could be sedimented by increasing the time and speed of the second centrifugation run.

3.6.1 Method

The four S_2 fractions from the previous experiment were pooled and then divided into two equal portions. The two portions were centrifuged as follows:

1) 25,000xg for 15 min
2) 25,000xg for 30 min

(MSE 18, 8x50ml rotor, 15,000 rpm)

The supernatants were poured off and the pellets resuspended in mannitol-Tris buffer.

3.6.2 Assays

The original supernatant (S_2), the resuspended pellets and the final supernatants were assayed for α-glucosidase and glucose-6-phosphatase activity.

3.6.3 Results

Table 3.6 shows the data obtained in this experiment.

Centrifugation conditions	Recovered Fractions	α-glucosidase			glucose-6-phosphatase		
		units	recovery S_2 = 100%	recovery of total	units	recovery S_2 = 100%	recovery of total
25,000 x g 15 min	S_2	1.6	100		1.77	100	
	pellet	0.555	34.7	30.8	0.28	15.8	14
	supern^t.	1.25	78.1	69.2	1.72	97	86
			113	100		113	100
25,000 x g 30 min	S_2	1.89	100		1.19	100	
	pellet	0.625	33.1	44.2	0.38	32.2	-
	supern^t.	0.79	41.8	55.8	-	-	-
			74.9	100		-	-

Table 3.6 Sedimentability of brush border and microsome markers from S_2.

3.6.4 Conclusions

This experiment has clearly shown that a large portion of α-glucosidase activity recovered in the S_2 fraction can be sedimented by increasing time and speed of the second centrifugation step. This suggested that S_2-α-glucosidase activity was, to a large extent, particulate in nature. The activity remaining in the supernatant after increased centrifugation at a longer time might have been due to free enzyme ie. enzyme which had been separated from the membrane during homogenisation. It appeared that glucose-6-phosphatase activity too was sedimented but apparently to a lesser degree. It was felt, therefore, that increasing the second centrifugation step to 25,000xg for 30 minutes was justified to give this extra recovery of α-glucosidase in the P_2 fraction. In all subsequent experiments these were the conditions applied for the second centrifugation step.

3.7 Effect of Tris-disruption of brush border fragments (P_2) on the distribution of brush border membrane and other markers in the glycerol gradient

3.7.1 Procedure

The tissue used in this experiment was duodenal mucosa.

a) Preparation of brush border fragments:

With the experience gained from the previous experiments the preparation of P_2 fraction was modified as follows:

1) Homogenisation was carried out with the "Virtis" homogeniser fitted with two blades at right angles to each other using a 50ml flask.

2) The first centrifugation step was at 2500xg for 20 minutes.

3) The second centrifugation step was at 25,000xg for 30 minutes.

b) Preparation of microvillous membranes:

To investigate the effect of Tris-disruption the P_2 fraction was split into two equal portions and processed as follows:

1) Addition of 1.6M Tris (pH 7.1) to give a final Tris concentration of 0.8M.

2) Addition of mannitol-Tris buffer in place of Tris.

The two preparations were then left on ice for one hour with occasional agitation before being layered on top of a 37, 40, 42, 45, 60% (v/v) glycerol gradient. The conditions for the remaining steps in the preparation were identical to those described in Section 3.2.2.

3.7.2 Assays

The fractions were assayed for α-glucosidase, glucose-6-phosphatase, N-acetyl-β-glucosaminidase, lactate dehydrogenase and

succinate dehydrogenase and protein as described earlier.

3.7.3 Results

The data obtained with the various marker enzymes are summarised in Table 3.7.

Fraction		Protein	α-glucosidase			glucose-6-phosphatase			N-acetyl-β-glucosaminidase			lactate dehydrogenase			succinate dehydrogenase		
			s.a.	incr. s.a.	%H	s.a.	incr. s.a.	%H	s.a.	incr. s.a.	%H	s.a.	incr. s.a.	%H	s.a.	incr. s.a.	%H
H		100	6.3	1	100	0.6	1	100	4.5	1	100	10.7	1	100	0.78	1	100
P_1		44.2	5.8	0.9	40.8	0.23	0.38	17.2	0.32	0.07	3.1	0.02	0.002	0.09	0.66	0.77	32.9
P_2		3.4	97.6	15.5	52.1	0.87	1.45	4.8	0.77	0.17	0.6	0.03	0.003	0.01	0.19	0.24	0.62
S_2		60.7	2.1	0.33	20.2	0.3	0.5	29.9	4.5	1	60.5	18.7	1.75	106	1.1	1.4	60.8
1	Tris-disruption	0.757	109.2	17.3	6.2	5.4	9	3.16	0	0	0	2.3					
2		0.593	37	5.9	1.64	6.2	10.3	2.84	0.59	0.13	0.04	0					
3		0.296	221	35.6	4.9	8	13.3	1.85	2.2	0.99	0.07	0.2		traces			traces
4		0.46	86.1	13.7	2.96	5.8	9.7	2.08	0.54	0.12	0.03	0.8					
5		0.46	40.2	6.4	1.58	4	6.7	1.64	0	0	0	0					
P		2.24	14.1	2.2	2.36	0.75	1.25	1.31	0.26	0.06	0.061	0					
1	no Tris-disruption	0.526	110	17.5	4.34	4.5	7.5	1.85	0.55	0.12	0.03	2.6					
2		0.46	257	40.7	8.84	6.1	10.8	2.18	1.6	0.35	0.07	2					
3		0.526	220	35	8.64	4.8	8	1.95	0.48	0.11	0.03	0.75					
4		-	-	-	2.29	-	-	1.64	0	0	0	0		traces			traces
5		0.09	177	28	1.31	19.1	33	1.53	0	0	0	8.7					
P		2.38	11.1	1.8	1.96	0.59	0.98	1.09	0.25	0.06	0.06	0.17					

Table 3.7 *Effect of Tris disruption.*

3.7.4 Conclusions

It was found with this experiment that Tris-disruption of the brush border membrane fragments (P_2) had little effect on the distribution of glucose-6-phosphatase through the glycerol gradient (% recovery). There was a slight tendency for the specific activity of glucose-6-phosphatase to be higher in the gradient fractions when Tris-disrupted P_2 was applied. Omitting Tris-disruption of P_2 resulted in an increased specific activity of α-glucosidase through the gradient with a high proportion of the activity contained in fractions 2 and 3. Over 17% of the total activity of α-glucosidase was recovered in these two fractionsaccompanied by an increase in specific activity of 35-40 times that of the original homogenate. It was decided to omit Tris-disruption in future experiments.

3.8 Modified method for the purification of human small intestinal brush border membranes

The procedure described here was used for all subsequent preparations of purified brush border membranes.

3.8.1 Method

a) Preparation of brush border fragments (P_2):

A 1% homogenate was prepared from intestinal mucosa using the "Virtis" homogeniser fitted with two blades at right angles to each other. Homogenisation was at medium speed for 30 seconds. The homogenate was filtered through nylon mesh (pore size 60μm) followed by the addition of solid calcium chloride to a final concentration of 10mM. The calcium chloride-treated homogenate was left on ice for 30 minutes, stirred at very low speed with a magnetic stirrer. After that time the homogenate showed increased turbidity and was centrifuged at 2000xg for 20 minutes (MSE 18, 8x50ml rotor, 4200 rpm). The pellets (fraction P_1) were resuspended in mannitol-Tris buffer and stored for analysis (deep-freeze). The supernatant (fraction S_1) was centrifuged at 25,000xg for 30 minutes (MSE 18, 8x50ml rotor, 15,000 rpm). The pellets from this spin (P_2) were resuspended in a small amount of mannitol-Tris buffer and the supernatant stored frozen for analysis.

b) Preparation of brush border membranes (BB):

The P_2 fraction was layered on top of a 37, 40, 42, 45, 60% (v/v) glycerol gradient 0.05M in magnesium chloride and centrifuged at 63,000xg for 15 minutes (MSE 50, 3x25ml swing-out rotor, 24,000 rpm). Five fractions were recovered with a syringe from the top of

the gradient and diluted to 10ml with mannitol-Tris buffer. The diluted fractions were then centrifuged for an hour at 100,000xg (MSE 50, 10x10ml angle rotor, 40,000 rpm). The supernatants were poured off and the pellets resuspended in 0.5 to 1.0ml of mannitol-Tris buffer and kept frozen for analysis. Figure 3.2 shows a schematic representation of this procedure. The fractions were assayed for protein, α-glucosidase, N-acetyl-β-glucosaminidase and lactate dehydrogenase.

3.8.2 Results

Table 3.8 shows a summary of data obtained from several different preparations.

The electronmicrophotograph is of the final brush border membrane pellet recovered from the glycerol gradient.

FRACTION	PROTEIN		α-GLUCOSIDASE (BRUSH BORDER MARKER)			N-ACETYL-β-D-GLUCOSAMINIDASE (LYSOSOME MARKER)			LACTATE DEHYDROGENASE (CYTOSOL MARKER)		
	mg/ml	%	Specific activity ×	Increase in specific activity	Recovery %	Specific activity	Increase in specific activity	Recovery %	Specific activity	Increase in specific activity	Recovery %
	[9]	[9]*	[5]	[5]	[5]	[4]	[4]	[4]	[5]	[5]	[5]
HOMOGENATE (H)	0.761 (0.26-1.12)	100	41.8 (29.1 - 60.2)	1	100	9.2 (4.83 - 14.5)	1	100	15.3 (13.3 - 19.2)	1	100
LOW SPEED PELLET (P1)	4.13 (1.6 - 8.9)	39.5[xx] (14 - 61) †	48.1 (34 - 69.5)	1.1 (0.9 - 1.26)	47.5 (28.1 - 50.5)	1.66 (1.1 - 3.4)	0.22 (0.12 - 0.31)	8.5 (4.1 - 12.6)	2.4 (0.12 - 3.17)	0.17 (0.01 - 0.5)	11.1 (1.2 - 34.9)
HIGH SPEED (S_2) SUPERNATANT	0.46 (0.21 - 0.675)	60.8 (46.1 - 78)	4.5 (0.95 - 9.12)	0.14 (0.08 - 0.176)	6.3 (1.9 - 11.9)	13.4 (6.2 - 19.5)	1.46 (1.3 - 1.7)	86.2 (79.2 - 95)	19.2 (12.2 - 22.9)	1.26 (0.76 - 1.76)	87.1 (39.6 - 148)
I	0.482 (0.225 - 1.01)	0.195 (0.1 - 0.297)	846 (436 - 1349)	21 (10.7 - 31.6)	3.56 (2.14 - 6.7)	4.76 (3.4 - 5.5)	0.46 (0.37 - 0.51)	0.11 (0.072 - 0.14)	3.4 (1.17 - 7.4)	0.2 (0.087 - 0.38)	0.298 (0.02 - 0.8)
II/III (BRUSH BORDER MEMBRANE)	0.305 (0.09 - 0.78)	0.372 (0.19 - 0.64)	956 (504 - 1734)	23.6 (9.6 - 28.8)	8.62 (5.3 - 14.6)	1.21 (0 - 2,57)	0.21 (0.145 - 0.3)	0.064 (0 - 0.12)	2.95 (0 - 5.9)	0.224 (0.15 - 0.31)	0.46 (0 - 1.24)
IV	0.22 (0.03 - 0.66)	0.078 (0.02 - 0.18)	785 (287 - 1824)	18.4 (8.6 - 30.8)	1.26 (0.27 - 2.33)	0.96 (0.29 - 1.5)	0.085 (0.04 - 0.14)	0.013 (0.006 - 0.03)	3.4 (2.7 - 4.21)	0.218 (0.17 - 0.294)	0.105 (0.02 - 0.22)
V	0.37 (0.03 - 1.12)	0.124 (0.02 - 0.3)	730 (317 - 1498)	17.7 (8.6 - 32.9)	2.22 (0.19 - 4.51)	1.97 (0.18 - 3.63)	0.157 (0.03 - 0.25)	0.021 (0.008 - 0.03)	3.61 (2.87 - 4.1)	0.23 (0.21 - 0.28)	0.29 (0.02 - 0.85)
TOTAL RECOVERY		101.1			64.4			95.3			99.4

Table 3.8 Purification of brush border membranes from human small intestine.

3.9 Discussion

The purity and yield of brush border membranes reported by Schmitz et al (1973) could not be achieved using that method in this laboratory. The method was therefore examined step by step and subsequently modified for routine use in this laboratory. The time of the first centrifugation was increased from 10 to 20 minutes resulting in a P_2 fraction of greater purity with respect to brush border markers without loss of yield. The yield was further improved in the P_2 fraction by increasing time and speed of the second centrifugation. These modifications brought about an increased recovery in P_2 of glucose-6-phosphatase which was used as a marker for endoplasmic reticulum. Forstner et al (1968) also reported a parallel increase in specific activity of glucose-6-phosphatase and invertase and questioned the validity of this marker enzyme. They observed that in the homogenate, glucose-6-phosphatase was inhibited 50% by glucose, whereas in purified brush border membranes no inhibition occurred and concluded that brush border activity was due to non-specific phosphatases. The assay procedure used in the present study included inhibitors of alkaline and acid phosphatase as suggested by Hübscher and West (1965) (see Chapter 2.5.1) but the effectiveness of these inhibitors was not tested. It is therefore possible that some of the activity against glucose-6-phosphate is not due to glucose-6-phosphatase.

Tris-disruption of P_2 prior to glycerol gradient centrifugation was omitted after the observation that recovery and purity of brush border membranes from the glycerol gradient fractions was higher when P_2 was not disrupted with Tris. Around 4% of the total glucose-

6-phosphatase activity was still associated with the final brush border membrane fraction, succinate dehydrogenase activity was not measurable and only traces of lactate dehydrogenase and N-acetylglucosaminidase were recovered in that fraction. Tris is a known inhibitor of some enzymes and the omission of Tris treatment of P_2 was preferred for this reason too, as selective inhibition of some peptide hydrolases might have distorted sub-cellular distribution results.

For routine purposes the fractionations were later assayed for α-glucosidase, N-acetylglucosaminidase and lactate dehydrogenase as well as protein. It was particularly important that the final brush border membrane fraction should be free of contamination from cytosol and lysosomes. This aim was achieved by the final fractionation procedure used (Figure 3.2). The recovery of brush border marker in the final fraction was somewhat lower than that obtained by Schmitz et al (1973) but the purity, although similar overall, was substantially higher in many fractionations.

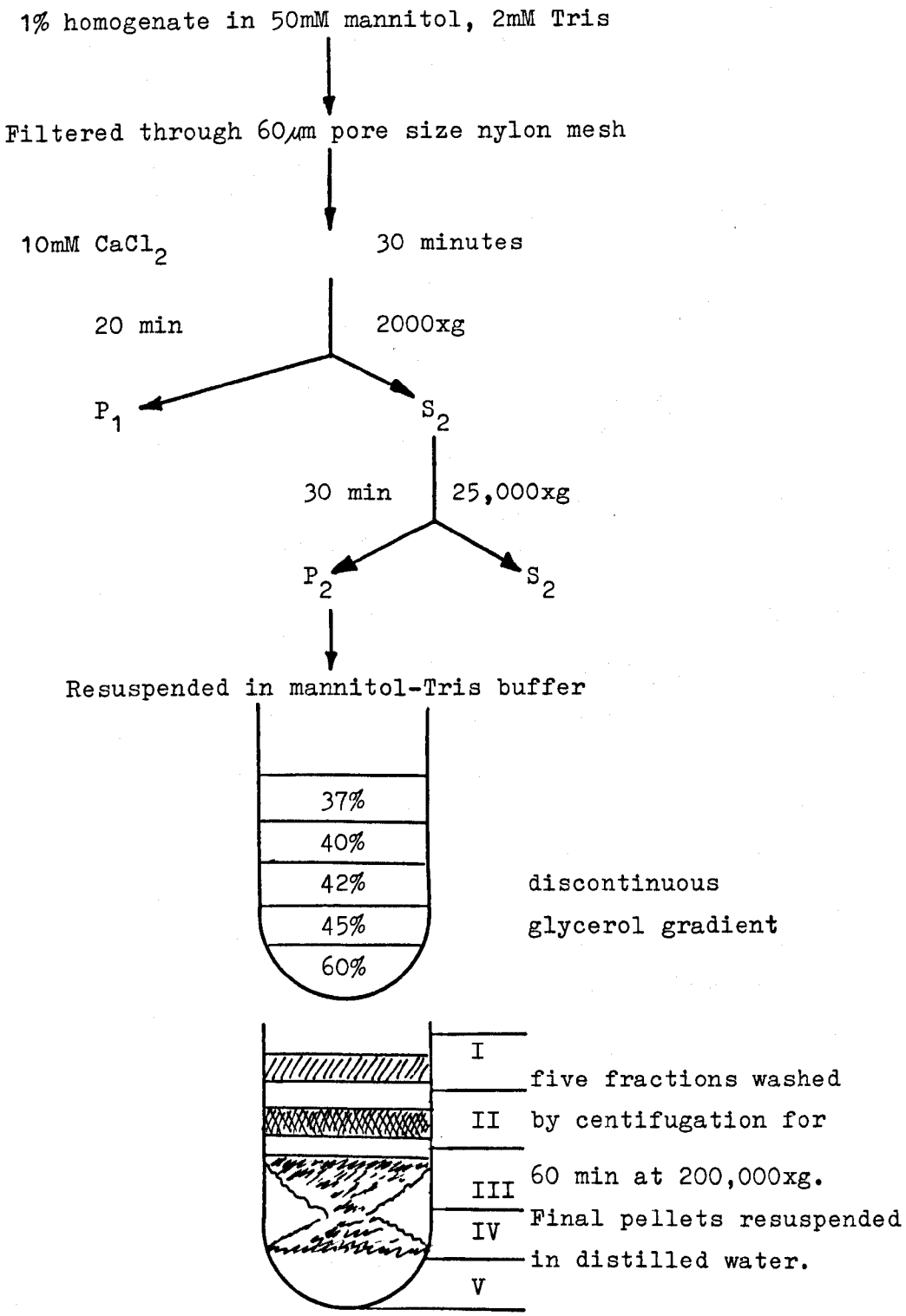


Figure 3.2 Schematic representation of the purification procedure of human brush border membranes as used in the investigations for this thesis

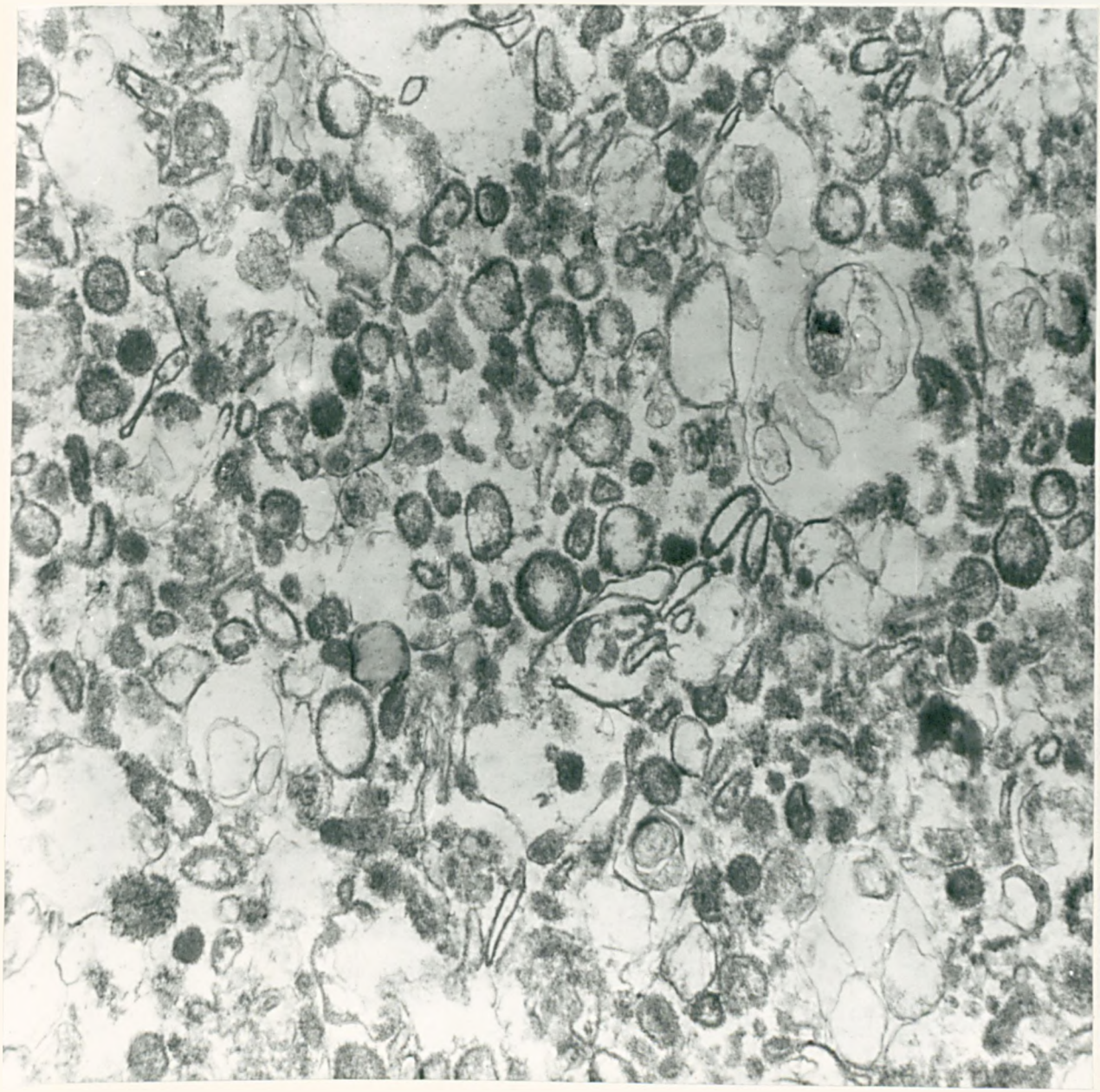


Figure 3.3 Electron micrograph of the purified brush border membrane fraction.

Magnification : x7,800 (Neg), x48,000 (total).

(Kindly performed by the Biochemistry Department, University of Leeds)

CHAPTER FOUR

PEPTIDE HYDROLASE ACTIVITIES IN HUMAN INTESTINAL BRUSH BORDER MEMBRANES AND SOLUBLE FRACTIONS

4.1 Introduction

The aim of the investigations described in this Chapter was primarily to study the peptide hydrolases associated with the brush border membrane of the human small intestine using a selection of peptides, and naphthylamides as substrates. In addition, the peptide hydrolase activity in the soluble fraction towards the same selection of substrates was investigated.

Peptide hydrolase activity towards di-, tri- and oligopeptides was measured using the method of Donlon and Fottrell (1971) employing L-amino acid oxidase as described in Chapter 2, Section 6.1. The method is a simple and accurate procedure for measuring peptide hydrolase activity in intestinal mucosa. The activity of L-amino acid oxidase varies depending on the amino acid used. Some amino acids, including L-leucine, L-tyrosine, L-phenylalanine and L-methionine, show a high reactivity with the enzyme while others such as glycine, L-alanine, L-glutamic acid and L-proline are not active as substrates (Auricchio et al 1971, Fujita et al 1972, Sterchi and Woodley 1976, unpublished). When measuring amino-peptidase activity the choice of substrates is therefore most important. Clearly, peptides in which only the N-terminal amino acid reacts with L-amino acid oxidase are ideal. Where both amino acids in a dipeptide react with L-amino acid oxidase a correction factor has to be introduced. In the case of dipeptides made up of one reactive and one non-reactive amino acid, the release of 1 µmole of amino acid corresponds to the hydrolysis of 1 µmole of substrate. In dipeptides made up of two identical reactive amino acids, the hydrolysis of 1 µmole of substrates corresponds to the release of 2 µmoles of amino acid. Dipeptides made up of two different reactive

amino acids were not used in this study as comparison of data from such substrates is difficult. With respect to oligopeptides, those with only the N-terminal amino acid reactive with L-amino acid are again ideal. Others, for example leucyl-leucyl-leucine and tyrosyl-tyrosyl-tyrosine have been used in this work but the specific activities obtained with these substrates were the same as those obtained with leucyl-glycyl-glycine and tyrosyl-glycyl-glycine respectively (Sterchi and Woodley 1976, unpublished). It was therefore decided that, provided the incubation time was limited to 10 minutes, these substrates would be very useful in the study of intestinal peptide hydrolases.

The homogenate (H), the low speed pellet fraction (P_1), the soluble fraction (S_2) and the purified brush border membrane fraction (BB) obtained by the routine fractionation procedure of human intestinal mucosa were all assayed for peptide hydrolase activity. By comparing the activities in the different fractions it was hoped to obtain a pattern of subcellular localisation of these activities.

4.2 Peptide hydrolase activity against di-, tri- and oligopeptides and 2-naphthylamides

The following substrates were used to assay peptide hydrolase activity.

Dipeptides

L-leucyl-L-leucine	Leu-Leu
L-leucyl-glycine	Leu-Gly
L-phenylalanyl-glycine	Phe-Gly
L-tyrosyl-glycine	Tyr-Gly
L-tyrosyl-L-tyrosine	Tyr-Tyr

Tripeptides

L-leucyl-L-leucyl-L-leucine	Leu-Leu-Leu
L-leucyl-glycyl-glycine	Leu-Gly-Gly
L-phenylalanyl-glycyl-glycine	Phe-Gly-Gly
L-tyrosyl-glycyl-glycine	Tyr-Gly-Gly
L-tyrosyl-L-tyrosyl-L-tyrosine	Tyr-Tyr-Tyr

Oligopeptides

L-phenylalanyl-glycyl-glycyl-L-phenylalanine	Phe-Gly-Gly-Phe
poly-L-leucine Type I (mol wt. 3000-15,000)	poly-Leu I
Poly-L-leucine Type II (mol wt. 15,000-50,000)	poly-Leu II

2-naphthylamides

L-leucine-2-naphthylamide	Leu-2-Nap
L-α-glutamic acid-2-naphthylamide	α-Glu-2-Nap
L-γ-glutamic acid-2-naphthylamide	γ-Glu-2-Nap
Glycyl-L-proline-2-naphthylamide	Gly-Pro-2-Nap

All di-, tri- and oligopeptides were obtained from Sigma Chemical Co Ltd, London; naphthylamides were from Bachem, Switzerland.

4.2.1 Results

Six different fractionations of human small intestinal mucosa were performed. All the tissue samples were obtained at surgery and included three duodenal and three jejunal mucosa specimens.

The homogenate (H), the purified brush border membrane fraction (BB), the soluble fraction (S_2) and the low speed pellet fraction (P_1) were assayed for peptide hydrolase activity. The results obtained with these different fractionations are shown in Tables 4.1 to 4.6. All activities are expressed as specific activities, s.a. (μmoles substrate hydrolysed min^{-1} mg protein), the enrichment as the increase in specific activity over that in the homogenate and the recovery as the percentage from the homogenate (ie. H = 100%). The total recovery was not calculated because not all the fractions have been assayed.

Substrate	Homogenate (H)			Low speed Pellet (P_1)			High speed supernatant (S_2)			Brush border fraction (BB)		
	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%
Leu-Leu	1.3	1	100	0.136	0.1	5.03	0.69	0.53	28	1.65	1.27	0.16
Leu-Gly	0.23	1	100	-	-	-	0.087	0.38	19.8	0.625	2.72	1.9
Gly-Leu	0.57	1	100	0.039	0.068	3.24	0.3	0.52	27.6	0.46	0.8	0.14
Phe-Gly	0.109	1	100	0.022	0.2	9.85	0.022	0.2	10.4	0.485	4.45	0.72
Tyr-Gly	0.061	1	100	0.017	0.28	13.7	0	0	0	0.36	5.89	0.96
Tyr-Tyr	0.165	1	100	0.028	0.17	8.27	0	0	0	0.63	3.81	0.62
Leu-Leu-Leu	0.518	1	100	0.168	0.324	15.6	0.049	0.095	4.97	3.28	6.3	0.76
Leu-Gly-Gly	0.096	1	100	-	-	-	0.009	0.094	5.15	2.23	4.64	0.67
Phe-Gly-Gly	0.137	1	100	0.7	0.5	25.5	0	0	0	1.73	12.7	2.1
Tyr-Gly-Gly	0.137	1	100	0.031	0.23	10.7	0	0	0	0.944	6.89	1.12
Tyr-Tyr-Tyr	0.113	1	100	0.032	0.283	13.6	0	0	0	0.613	5.42	0.885
α-Gly-2-Nap	0.048	1	100	0.054	1.1	53.4	-	-	16.9	0.75	15.6	1.3
γ-Glu-2-Nap	0.041	1	100	0.031	0.76	35.7	0.001	0.024	1.1	0.65	15.6	1.98
Gly-Pro-2-Nap	0.16	1	100	0.11	0.7	31.2	-	-	3.6	2.75	17.2	1.4
α-Glucosidase	0.66	1	100	0.51	0.8	47.1	0.10	0.2	11.8	11.7	17.8	3.7

Table 4.1. Fractionation VII (Jejunum)

Substrate	Homogenate (H)			Low speed Pellet (P_1)			High speed supernatant (S_2)			Brush border fraction (BB)		
	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%
Leu-Leu	0.343	1	100	-	-	-	0.7	2.04	117	10.0	29.2	6.1
Leu-Gly	0.107	1	100	-	-	-	0.36	3.36	193	1.18	11.0	2.3
Phe-Gly	0.028	1	100	-	-	-	0.036	1.29	73.7	0.608	21.7	4.51
Tyr-Gly	0.026	1	100	-	-	-	0.011	0.42	25.8	0.91	35	7.4
Tyr-Tyr	0.035	1	100	-	-	-	0.103	2.9	170	1.1	31.4	6.5
Leu-Leu-Leu	0.139	1	100	-	-	-	0.047	0.34	19.5	3.83	27.6	5.76
Leu-Gly-Gly	0.032	1	100	-	-	-	0.059	1.84	105	0.665	20.8	4.3
Phe-Gly-Gly	0.037	1	100	-	-	-	0.033	0.89	51.4	1.09	29.5	6.1
Tyr-Gly-Gly	0.058	1	100	-	-	-	0.023	0.4	22.8	2.44	42	8.7
Tyr-Tyr-Tyr	0.037	1	100	-	-	-	0	0	0	1.29	34.9	7.2
Phe-Gly-Gly--Phe	0.023	1	100	0.026	1.13	44.7	0	0	0	0.417	18.1	3.7
α-Glu-2-Nap	0.0242	1	100	0.04	1.65	67.3	0.003	0.12	7.9	0.45	18.6	5.13
γ-Glu-2-Nap	0.1	1	100	0.182	1.82	44.1	0.13	1.3	45.2	1.55	15.5	2.58
Gly-Pro-2-Nap	0.0141	1	100	0.036	2.6	51.8	0.01	0.71	20.2	0.617	43.8	5.98
α-Glucosidase	29.1	1	100	36.6	1.26	50.5	6	0.21	11.9	852	28.7	6.6

Table 4.2. Fractionation VIII (Duodenum)

Substrate	Homogenate (H)			Low speed pellet (P_1)			High speed supernatant (S_2)			Brush border fraction (BB)		
	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%
Leu-Leu	2.09	1	100	-	-	-	1.72	0.82	38	4.94	2.36	0.63
Leu-Gly	1.03	1	100	-	-	-	1.7	1.65	76.3	2.41	2.34	0.63
Phe-Gly	0.142	1	100	-	-	-	0.155	1.09	50.6	1.23	8.66	2.34
Tyr-Gly	0.11	1	100	0.075	0.68	32.8	0.016	0.15	6.72	0.808	7.35	1.97
Tyr-Tyr	0.246	1	100	0.202	0.824	39.5	0.041	0.167	7.66	1.4	5.7	1.53
Leu-Leu-Leu	1.36	1	100	-	-	-	0.236	0.17	8.03	1.56	1.15	0.31
Leu-Gly-Gly	0.593	1	100	-	-	-	0.236	0.398	18.4	2.73	4.6	1.23
Phe-Gly-Gly	0.238	1	100	-	-	-	0.175	0.735	33.9	2.55	10.7	2.86
Tyr-Gly-Gly	0.179	1	100	0.146	0.82	39.2	0.002	0.01	0.59	0.732	4.1	1.09
Tyr-Tyr-Tyr	0.182	1	100	0.29	1.59	77	0.061	0.34	15.5	1.72	9.45	2.53
Phe-Gly-Gly--Phe	0.235	1	100	0.253	1.07	51.8	0.057	0.24	11.2	2.21	9.4	2.52
Leu-2-Nap	0.186	1	100	0.084	0.45	21.9	0.0145	0.08	3.6	1.77	9.5	2.55
α-Glu-2-Nap	0.017	1	100	0.02	1.2	57.4	0.0073	0.43	19.8	0.192	11.3	3
γ-Glu-2-Nap	0.0151	1	100	0.022	1.5	71.3	0.025	1.7	77.4	0.162	10.9	2.9
Gly-Pro-2-Nap	0.104	1	100	0.093	0.89	43	0.104	1	45.9	1.17	11.25	3
α-glucosidase	52.7	1	100	52.4	0.9	47.9	4	0.08	3.5	504	9.6	6.1

Table 4.3. Fractionation IX (Jejunum)

Substrate	Homogenate (H)			Low speed pellet (P_1)			High speed supernatant (S_2)			Brush border fraction (BB)		
	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%
Leu-Leu	1.94	1	100	0.4	0.21	6.94	1.42	0.73	45.8	3.82	2.02	0.57
Phe-Gly	0.144	1	100	0.074	0.514	17.3	0.093	0.646	40.6	1.22	8.47	2.4
Leu-Leu-Leu	0.77	1	100	0.48	0.623	20.8	0.128	0.166	10.4	13.3	17.3	5
Phe-Gly-Gly	0.297	1	100	0.015	0.05	1.7	0.153	0.515	32.3	3.43	11.5	3.32
Leu-2-Nap	0.216	1	100	0.234	1.08	36.5	0.056	0.26	16.3	-	-	-
α-Glu-2-Nap	0.018	1	100	0.028	1.52	50.9	0.007	0.35	22	0.45	18.6	5.13
γ-Glu-2-Nap	0.0159	1	100	0.0025	0.159	5.3	0.0046	0.289	18.1	0.287	18.1	5.2
Gly-Pro-2-Nap	0.176	1	100	0.32	1.82	60.9	0.167	0.94	59.2	3.39	19.3	5.5
α-Glucosidase	60.2	1	100	69.5	1.15	38.7	9.12	0.151	9.45	1734	28.8	14.6

Table 4.4. Fractionation X (Duodenum)

Substrate	Homogenate (H)			Low speed pellet (P_1)			High speed supernatant (S_2)			Brush border fraction (BB)		
	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%
Leu-Leu	0.343	1	100	-	-	-	0	0	0	2.38	6.94	0.6
Phe-Gly	0.02	1	100	-	-	-	0	0	0	0.592	29.6	2.56
Leu-Leu-Leu	0.465	1	100	0.085	0.183	5.5	0	0	0	11.9	25.6	2.2
Phe-Gly-Gly	0.151	1	100	0.315	2.09	63.2	0.18	1.19	90	5.36	35.5	3.0
Phe-Gly-Gly--Phe	0.079	1	100	0.034	0.43	13	0	0	0	1.91	24.2	2.1
Leu-2-Nap	0.12	1	100	0.23	1.9	56.7	0.01	0.008	5.8	3.3	27.5	2.3
α-Glu-2-Nap	0.02	1	100	0.017	0.85	0.16	0.005	0.25	2.73	0.27	13.5	0.19
γ-Glu-2-Nap	0.175	1	100	0.053	0.3	9.18	0.023	0.13	9.59	1.17	6.7	0.52
Gly-Pro-2-Nap	0.28	1	100	0.052	0.19	5.6	0.27	0.96	71.7	0.7	2.5	0.21
α-Glucosidase	36.8	1	100	34	0.95	28.1	0.95	0.179	1.9	863	23.5	5.3

Table 4.5. Fractionation XI (Duodenum)

Substrate	Homogenate (H)			Low speed pellet (P_1)			High speed supernatant (S_2)			Brush border fraction (BB)		
	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%	s.a.	increase s.a.	%
Leu-Leu	1.17	1	100	0	0	0	0.193	0.164	9.7	0	0	0
Gly-Phe	0.527	1	100	0.373	0.71	33.3	0.616	1.17	69.2	0.45	0.85	0.6
Phe-Pro	2.05	1	100	0	0	0	0.674	0.33	19.4	0	0	0
Phe-Gly-Gly--Phe	1.76	1	100	1.04	0.59	27.8	0	0	0	28.1	16	11

Table 4.6. Fractionation XIII (Jejunum)

It was observed that the homogenates of all fractionations contained peptide hydrolase activity against the substrates tested but there was considerable variation in the level of activity. The brush border fraction from all fractionations contained peptide hydrolase activity against the substrates tested. Considerable variation was observed in the activity of the soluble fraction with activities against some peptides unmeasurable in some of the fractions. Peptide hydrolase activity appeared to be somewhat higher in jejunal fractions than in duodenal fractions. There was, however, no clear indication that substrate specificities from the two locations were different.

Table 4.7 shows a summary of peptide hydrolase activities in the homogenates, the soluble and the brush border membrane fractions from all six fractionations.

* Figures in square brackets = Number of fractionations.

** Mean specific activity

† Figures in parenthesis = range.

SUBSTRATE	HOMOGENATE specific activity	SOLUBLE (S_2) specific activity	BRUSH BORDER specific activity
L-leucyl-L-leucine [6]*	0.6**(0.17 -1.05)†	0.4 (0.- 0.86)	1.9 (0.- 5.0)
L-leucyl-Glycine [3]	0.456(0.107-1.03)	0.716(0.087-1.7)	1.41 (0.625-2.41)
L-Phenylalanyl-Glycine [5]	0.089(0.02 -0.144)	0.061(0 - 0.155)	0.83 (0.485-1.23)
L-Tyrosyl-Glycine [3]	0.066(0.026-0.11)	0.009(0-0.016)	0.693(0.36-0.9)
L-Tyrosyl-L-Tyrosine [3]	0.0743(0.0175-0.123)	0.0237(0-0.05)	0.522(0.315-0.7)
L-leucyl-L-leucyl-L-leucine [6]	0.66 (0.139-1.36)	0.077(0 - 0.236)	6.28 (1.56 -13.3)
L-leucyl-Glycyl-Glycine [3]	0.24(0.032-0.593)	0.101(0.009-0.236)	1.88 (0.665-2.73)
L-Phenylalanyl Glycyl-Glycine [5]	0.172(0.037-0.29)	0.108(0-0.18)	2.83 (1.09-5.36)
L-Tyrosyl-Glycyl-Glycine [3]	0.125(0.058-0.179)	0.008(0-0.023)	1.37 (0.944-2.44)
L-Tyrosyl-L-Tyrosyl-L-Tyrosine [3]	0.111(0.037-0.182)	0.02 (0-0.061)	1.21 (0.613-1.72)
L-Phenylalanyl-Glycyl-Glycyl-L-Phenylalanine [4]	0.52 (0.023-1.76)	0.014(0 - 0.057)	8.15 (0.42 -28.1)
L-Leucyl-2-Naphthylamide [3]	0.174(0.12 -0.216)	0.027(0.01 -0.056)	4.1 (1.7 - 7.12)
α-Glutamyl-2-Naphthylamide [5]	0.025(0.017-0.048)	0.0045(0 -0.0073)	0.38 (0.192-0.75)
γ-Glutamyl-2-Naphthylamide [5]	0.069(0.0151-0.175)	0.037 (0.001-0.13)	0.76 (0.287-I.55)
Glycyl-L-Prolyl-2-Naphthylamide [5]	0.147(0.0141-0.28)	0.138 (0.01 -0.27)	1.73 (0.617-3.39)

Table 4.7 Peptide Hydrolase Activities in Fractions

4.3 Enrichment of peptide hydrolases in the brush border membrane

The degree of enrichment of any enzyme activity in the membrane fraction expressed as an increase in specific activity over the homogenate, reflects the distribution of location of that activity between brush border membrane and soluble fraction. Ideally, the criterion that a peptide hydrolase is located exclusively in the brush border membrane should be that it is enriched to the same extent as the brush border marker α-glucosidase. Where the enrichment of a peptide hydrolase in the brush border fraction does not reach that of α-glucosidase, a dual location in both brush border and soluble fraction is indicated. Hydrolase activities against the substrates tested were present in both brush border membrane and the soluble fraction. The soluble fraction contains all enzymes normally located in the cytosol of the enterocytes and most of the lysosomal activity due to the disruption of lysosomes during the homogenisation of the tissue. The pH optimum of lysosomal enzymes is well in the acid region of the pH scale. The pH at which peptide hydrolases were assayed, however, was 8.0 and it was therefore unlikely that any possible lysosomal peptidase activity was detected.

4.3.1 Results

Table 4.8 shows the enrichment of various peptide hydrolase activities in the purified brush border membrane fraction. It further shows the recovery of activities in the brush border and the soluble fractions.

In the purified brush border fraction a wide spectrum of

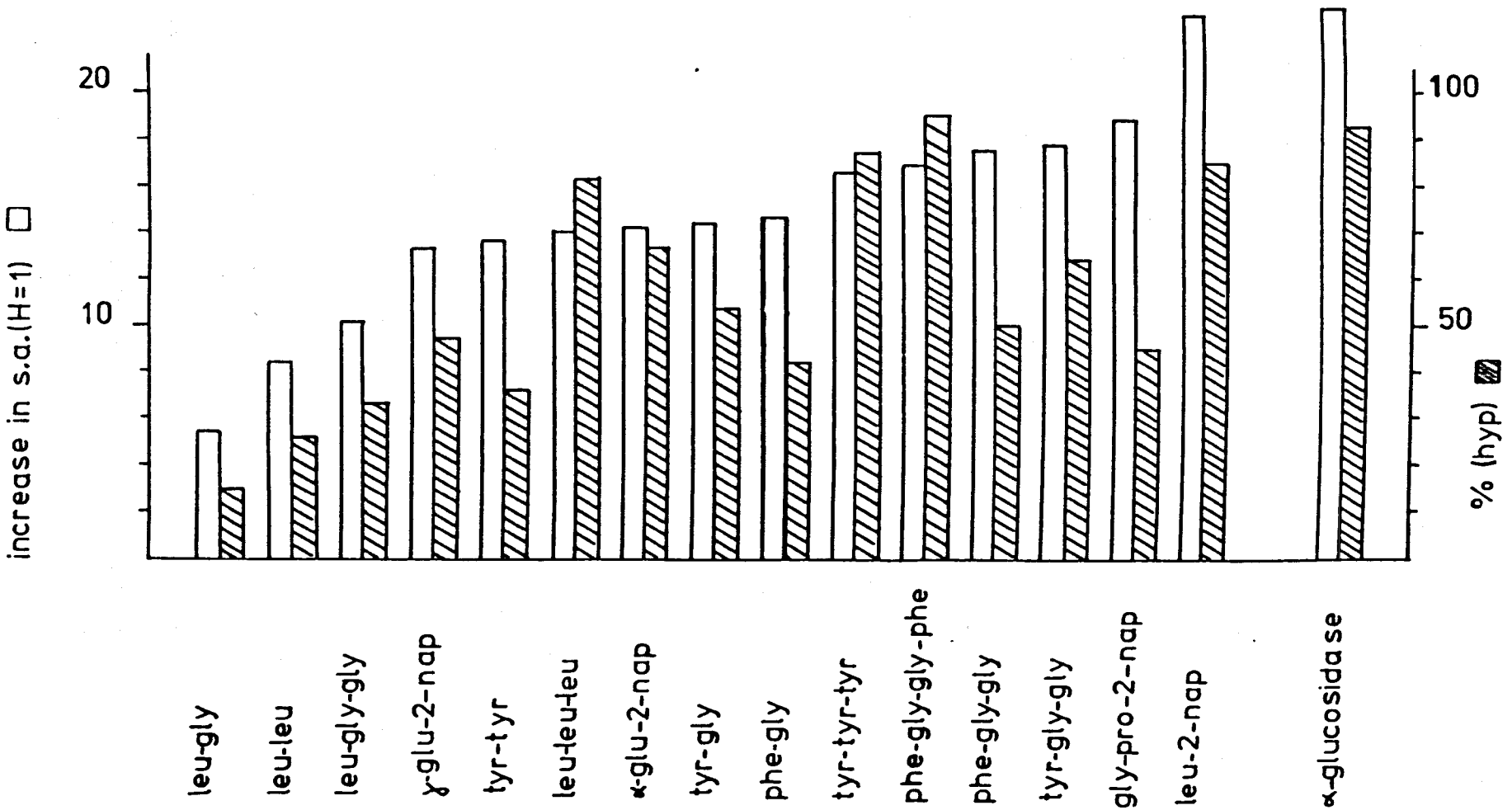
activities was observed with leucyl-leucine and leucyl-glycine-hydrolase at one end (enrichment of 8.35 and 5.35 respectively) and leucine-2-naphthylamide-hydrolase at the other end of the spectrum (enrichment of 23.3). Leucine-2-naphthylamide-hydrolase showed a very similar distribution and enrichment to α-glucosidase (enrichment of 23.6) and might therefore be assumed to be exclusively in the brush border membrane. In general tri- and oligopeptidase activities showed a greater enrichment in the brush border membrane than dipeptidase activities. Hydrolases against peptides with an aromatic amino acid at the N-terminal end showed greater enrichment than those with aliphatic N-terminal amino acids. This was found with both di- and tripeptide hydrolases.

Figure 4.1 shows the enrichment as well as the estimated % recovery in the brush border fraction of peptide hydrolase activities towards the substrates used.

SUBSTRATE	BRUSH BORDER MEMBRANE		SOLUBLE (S_2)
	INCREASE IN SPECIFIC ACTIVITY (H = 1)	RECOVERY % (H = 100%)	RECOVERY % (H = 100%
L-Leucyl-L-Leucine [6]*	8.35** (1.27-29.2)	1.34(0.00-6.1)	39.8 (0.00-117)
L-Leucyl-Glycine [3]	5.35(2.34-11)	1.6 (0.63-2.3)	96.4(19.8 -193)
L-Phenylalanyl-Glycine [5]	14.6 (4.45-29.6)	2.51(0.72-4.51)	35.1 (0.00-73.7)
L-Tyrosyl-Glycine [3]	14.3 (5.9 - 35)	1.23(0.73-1.99)	10.8 (0.00-25.8)
L-Tyrosyl-L-Tyrosine [3]	13.6 (3.8 -31.4)	2.88(0.62-6.5)	59.2 (0.00-170)
L-Leucyl-L-Leucyl-L-Leucine [6]	13.9 (1.15-27.6)	2.95(0.31-5.76)	7.15(0.00-19.5)
L-Leucyl-Glycyl-Glycine [3]	10.0 (4.6 -20.8)	2.1 (0.69-4.3)	42.9 (5.15-105)
L-Phenylalanyl-Glycyl -Glycine [6]	17.6 (5.6 -35.5)	3.55(2.1 -6.1)	36 (0.00-90)
L-Tyrosyl-Glycyl- -Glycine [3]	17.7 (4.1 -42)	1.39(1.09-1.95)	7.8 (0.00-22.8)
L-Tyrosyl-L-Tyrosyl- -L-Tyrosine [3]	16.6 (5.4 -34.9)	3.54(0.89-7.2)	5.17(0-15.5)
L-Phenylalanyl-Glycyl- -Glycyl-L-Phenylalanine [4]	16.9 (9.4 -24.2)	4.83(2.1 -11)	2.8 (0.00-11.2)
L-Leucyl-2-Naphthyl-amide [3]	23.3 (9.5-33)	4.8 (2.3 -9.5)	8.6 (3.6 -16.3)
α-Glutamyl-2-Naphthylamide [5]	14.2(11.3-18.6)	2.6 (0.19-5.13)	13.9 (2.73-22)
α-Glutamyl-2-Naphthylamide [5]	13.4(6.7 -18.1)	2.64(0.52-5.2)	30.3 (1.1 -77.4)
Glycyl-Prolyl-2-Naphthylamide [5]	18.8(2.5 -43.8)	3.22(0.21-5.98)	40.1 (3.6 -71.7)
α-Glucosidase [5]	23.6(9.6-28.8)	8.62(5.3 -14.6)	6.3 (1.9 -11.9)

Table 4.8 Purification and recovery of peptide hydrolase in brush border fractions.

Figure 4.1: Enrichment and Recovery of Peptide hydrolase activities in Brush Border membranes



4.4 Distribution of peptide hydrolase activities between purified brush border membrane and soluble fractions

In order to determine the subcellular distribution of an enzyme an analytical approach to the fractionation is used as described by De Duve et al (1962). All fractions obtained are assayed for subcellular marker enzymes and the enzyme whose subcellular distribution is to be determined. By comparing the distribution of the unknown enzyme in the fractions with that of the marker enzymes for subcellular organelles, the localisation of that enzyme within the cell may be assessed.

The fractionation method used in this investigation was however, preparative with respect to brush border membranes.

When developing the purification method for brush border membranes, emphasis was put on quality rather than quantity. This resulted in a relatively low yield of brush border membranes (8.62% of α-glucosidase activity) of high purity (enrichment of α-glucosidase 23.6). Approximately half of the α-glucosidase activity was lost in the low speed pellet (P_1) and up to 10% in the remaining fractions from the glycerol gradient. The recovery of peptide hydrolase activity too was consequently found to be low in the brush border membrane fraction.

In order to shed more light on the question of intracellular distribution of the various activities between brush border and soluble fractions, the recoveries in the two fractions for each substrate were recalculated as the percentage of α-glucosidase and lactate dehydrogenase. For this calculation to be of any validity it had to be assumed that α-glucosidase was located exclusively in

the brush border membrane (ie. 8.62% = 100%) and lactate dehydrogenase exclusively in the soluble fraction (ie. 94.7% = 100%). For each substrate the values of %/α-glucosidase in the brush border fraction and %/LDH in the soluble fraction were calculated and when added together gave a hypothetical total recovery (% Total hyp) in those two fractions. A hypothetical distribution in % (% hyp) was in turn calculated from the % Total (hyp). It was assumed that any brush border peptidase was lost in P_1 and other glycerol gradient fractions to the same degree as was α-glucosidase.

4.4.1 Results

The results are shown in Table 4.9.

It appears from these results that all activities were present in both soluble and brush border membrane fractions. With the exception of tyrosyl-glycine hydrolase activity, all activities against dipeptides were predominantly located in the soluble fraction. With the exception of leucyl-glycyl-glycine hydrolase activity, over 50% of tripeptide hydrolase activity was found in the brush border membrane fraction. Recovery of activities against leucyl-leucyl-leucine, tyrosyl-tyrosyl-tyrosine, leucine-2-naphthylamide and phenylalanyl-glycyl-glycine approached that of α-glucosidase in the brush border and they might therefore be assumed to be predominantly brush border in origin.

It is emphasised that these figures are merely hypothetical and can only be used to give an indication of the subcellular distribution of these peptide hydrolases in the mucosa of human small intestine.

Substrate	Brush Border		Soluble		Total %/α-gluc. + %/LDH	Distribution	
	%/Hom	%/ α-gluc	%/Hom	%/LDH		BB %	Soluble %
Leu-Leu	1.34	15.5	39.8	45.7	61.2	25.3	74.7
Leu-Gly	1.6	18.6	96.4	110.7	129	14.4	85.6
Phe-Gly	2.51	29.1	35.1	40.3	69.4	41.9	58.1
Tyr-Gly	1.23	14.3	10.8	12.4	26.7	53.5	46.5
Tyr-Tyr	2.88	33.4	59.2	68	92.6	36.1	63.9
Leu-Leu-Leu	2.95	34.2	7.15	8.2	42.4	80.7	19.3
Leu-Gly-Gly	2.1	24.4	42.9	49.3	73.7	33.1	66.9
Phe-Gly-Gly	3.55	41.2	36	41.3	82.5	49.9	50.1
Tyr-Gly-Gly	1.39	16.1	7.8	9	25.1	64.1	35.9
Tyr-Tyr-Tyr	3.54	41.1	5.18	5.9	47	87	13
Phe-Gly-Gly--Phe	4.83	56	2.8	3.2	59.2	94.6	5.4
Insulin β-chain	-	-	-	-	-	-	-
Leu-2-Nap	4.8	55.7	8.6	9.9	65.6	84.9	15.1
α-Glu-2-Nap	2.6	30.2	13.9	15.3	45.5	66.4	33.6
γ-Glu-2-Nap	2.64	30.6	30.3	34.8	65.4	46.8	53.2
Gly-Pro-2-Nap	3.22	37.6	40.3	46.3	84.2	44.7	55.3
α-Glucosidase	8.62	100	6.3	7.2	107.2	93.3	6.7
LDH	0.46	5.3	87.1	100	105.3	5.3	94.7

4. 9 Distribution of peptide hydrolase activity between brush border membranes and soluble fraction.

4.5 Discussion

The results in this Chapter established that homogenates of human small intestinal mucosa are capable of hydrolysing a selection of peptide and naphthylamide substrates. There was considerable variation in the level of activity towards the same substrates when different homogenates were used. This could have been due to a number of factors, for example, the nutritional state of the individual from whom the tissue was obtained. In addition, most of the duodenal samples were obtained from gastric ulcer patients undergoing surgery for this complaint. These samples were taken from the proximal part of the duodenum ie. next to the pyloric sphincter. Although these samples looked normal macroscopically, it is possible that proximal peptidase activity in the duodenum might differ depending on the severity of the ulceration. Hyperproduction of gastric acid is likely to result in a change of the pH environment of the proximal duodenum adversity affecting more labile enzymes. This could also account for the observed tendency of lower peptidase activity in duodenal compared to jejunal mucosa.

Considerable variation in activity has also been observed between different substrates (see Table 4.7). Generally, activity against peptides (both di- and tripeptides) containing aliphatic N-terminal amino acids eg. leucine, was higher than activity against peptides with aromatic N-terminal amino acids. One exception was the tetrapeptide Phe-Gly-Gly-Phe which had a similar level of activity to the peptides with an aliphatic N-terminal.

An attempt was made to determine the distribution of peptide hydrolases between the purified brush border and the soluble fraction.

There are two parameters by which the subcellular location of enzyme may be assessed; these are the extent by which a particular activity is enriched in a specific subcellular fraction, usually expressed as an increase in specific activity of that enzyme in the fraction over that of the homogenate, and the recovery, expressed either as a percentage of the homogenate or of the total activity recovered in all fractions. By comparing enrichment factor and distribution of an enzyme with unknown location with known subcellular marker enzymes, the probable subcellular location of that enzyme may be determined. For a peptide hydrolase activity to be located exclusively in the brush border membrane, enrichment in that fraction should ideally be the same as for the brush border marker α-glucosidase. Peptide hydrolase activities towards all substrates tested were found to be enriched in the brush border fraction indicating that this fraction contained enzymes capable of hydrolysing all these substrates. Only leucine-2-naphthylamidase, an activity associated with aminopeptidase M, reached the same enrichment factor (23.3) as α-glucosidase (23.6). This enzyme has been reported to be located exclusively in the brush border membrane and has been used as a marker for brush border membranes (Peters 1972). A group of peptide hydrolases followed with activity towards tri- and tetra-peptides containing aromatic N-terminal amino acids which had an enrichment factor in the brush border membrane of over 16. This group also included activity towards glycyl-proline-2-naphthylamide, ie. dipeptidyl peptidase IV. Another group of peptidases with activity towards aliphatic tripeptides, aromatic dipeptides and α-glutamic acid-2-naphthylamide had an enrichment factor in the brush border fraction of between 13 and 15 and a further group with activity

towards aliphatic di- and tripeptides, an enrichment of less than 10.

The evaluation of the recovery data for the activity towards the various substrates tested was more difficult. The fractionation procedure used for intestinal mucosa was developed as a preparative method for brush border membranes of high purity with the result that recovery in the final fraction was rather low. This also led to low recoveries of peptide hydrolase activities making it difficult to assess their subcellular distribution by the usual method. For this reason the recovery of activity towards each substrate in the brush border and cytosol fraction was expressed as a percentage of α-glucosidase and lactate dehydrogenase respectively. The sum of these two percentages yielded a hypothetical total recovery in these two fractions from which the distribution could be calculated. These calculations have shown that the tetrapeptidase activity towards phenylalanyl-glycyl-glycyl-phenylalanine had an almost identical distribution (94.6% BB, 5.4% S_2) to α-glucosidase, the brush border marker (93.3% BB, 6.7% S_2). 50-87% of activity towards tripeptides appeared to be brush border in origin with the exception of leucyl-glycyl-glycine peptidase which appeared to have only a 33% brush border location. Leucine-2-naphthylamidase had an apparent distribution of 85% BB to 15% S_2 ie. was mainly brush border in origin. This group also included α-glutamic acid-2-naphthylamide hydrolase with a distribution of 66.4% BB to 33.6% S_2. Dipeptidase activity appeared to be mainly located to the cytosol with 58-88% found in the soluble fraction, one exception being tyrosyl-glycine-hydrolase with a distribution of 53.5% BB to 46.5% S_2. γ-glutamic acid and glycyl-proline-2-naphthylamide hydrolases were found to have

a distribution of around 50% BB to 50% S_2.

In conclusion, it appears that the brush border membrane probably possesses all the tetrapeptidase activity and most of the tripeptidase and leucine-2-naphthylamidase activity of the intestinal mucosa. The brush border also contains considerable amounts of dipeptidase activity, particularly towards aromatic substrates. Most of the dipeptidase activity towards aliphatic substrates seems to be located in the cytosol of the epithelial cells.

CHAPTER FIVE

SEPARATION OF SDS-SOLUBILISED PEPTIDE HYDROLASES FROM HUMAN INTESTINAL BRUSH BORDER MEMBRANES BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

5.1 Introduction

From experimental data obtained in the investigations described in Chapter 4, it was concluded that the brush border membrane from human small intestine contained peptide hydrolase activities towards a considerable number of peptide and 2-naphthylamide substrates. It was not however, possible to draw any conclusions about the number of enzymes which might be involved in the hydrolysis of these different substrates. The hydrolase activity observed against the peptide and naphthylamide substrates studied, could be due to one enzyme or enzyme complex with a broad substrate specificity or it could be the result of several different enzymes.

One approach to determine the number of enzymes involved in the terminal digestion of peptides, is to disrupt the brush border membrane, to separate the protein components and to identify the enzymes. The techniques for the extraction and analysis of membrane lipids are well worked out and are mainly based on the use of organic solvents. These have also been used to extract proteins, but the resultant irreversible aggregation and denaturation of many membrane proteins limit their use. Partial and selective protein solubilisation can be obtained by a number of methods involving chelating agents, manipulation of ionic strength or pH and protein perturbants (eg. urea, guanidine). However, these procedures do not lead to solubilisation of the proteins which are more strongly bound to the lipid matrix of the membrane. For such proteins the use of detergents and proteolytic enzymes appears to provide generally useful extraction methods.

In this Chapter, the solubilisation of brush border peptide

hydrolases by sodium dodecyl sulphate (SDS) and their subsequent separation by polyacrylamide gel electrophoresis is investigated. Sodium dodecyl sulphate is an anionic detergent and has been used extensively for the solubilisation of membrane proteins (eg. Pitt-Rivers and Impiombato 1968, Reynolds and Tanford 1970a&b). The binding of detergents to proteins has been found to occur to discrete high affinity binding sites and is a function of the free detergent concentration. When the concentration of anionic and cationic detergents is increased to above that required to saturate these binding sites, binding to other sites occurs. This binding is cooperative and is accompanied by a conformational change of the protein molecule, in which presumably, many previously buried hydrophobic groups become exposed. For sodium dodecyl sulphate the cooperative mode of association is common to virtually all proteins and the maximum amount bound to protein is the same for most of them (Pitt-Rivers and Impiombato 1968, Reynolds and Tanford 1970a). Multi-chain proteins (if reduced by mercaptoethanol) are usually dissociated into their constituent polypeptide subunits during cooperative binding. Two types of SDS-polypeptide complexes are obtained:

a) at free detergent concentrations of 0.5 to 0.8mM, 0.4g of sodium dodecyl sulphate is bound to 1g of protein;

b) at detergent concentrations higher than 0.8mM, 1.4g sodium dodecyl sulphate are bound to 1g of protein.

The SDS-polypeptide complexes form extended rod-like structures with a length roughly proportional to the molecular weight of the polypeptide (Reynolds and Tanford 1970b). This is the basis of the use of SDS-polyacrylamide gel electrophoresis to estimate the molecular

weights of reduced proteins (Shapiro et al 1967, Weber and Osborn 1969).

Most membrane proteins have been found to be denatured by sodium dodecyl sulphate at concentrations of 1% as used for SDS-polyacrylamide gel electrophoresis. There are however, some membrane proteins which have been found to be resistant to denaturation by this detergent, eg. neuraminidase of influenza virus (Laver 1963) and alkaline phosphatase in liver cell plasma membrane (Sohn and Marinetti 1974). Recent research by Critchley et al (1975) on the solubilisation of brush border membranes from hamster small intestine and the fractionation of some of the components has also shown that 90% of the protein could be solubilised in 0.25% (w/v) sodium dodecyl sulphate without total loss of enzyme activities.

5.2 Solubilisation of brush border membranes with sodium dodecyl sulphate (SDS)

5.2.1 Method

0.5ml of SDS (10-times concentrated) was added to 4.5ml aliquots of P_2 fraction to give final concentrations of 0.1% (detergent:protein ratio 0.1), 0.25% (detergent:protein ratio 0.25) and 0.5% (detergent:protein ratio 0.5). To a further aliquot of 4.5ml of P_2 0.5ml of distilled water was added. Immediately after the addition of SDS, the tubes were agitated to mix the contents and small samples removed for the purpose of assay. After leaving the tubes at room temperature for 30 minutes, they were centrifuged at 100,000xg for 1 hour (MSE SS50, 10x10ml angle rotor). After centrifugation, the supernatants were decanted and the pellets resuspended in 5ml of distilled water, except the pellet from the tube containing 0.5% SDS which was resuspended in 2ml of distilled water. The samples taken prior to centrifugation, the supernatants and the resuspended pellets were assayed for protein and leucine-2-naphthylamidase. The tube containing 0.25% SDS was also assayed for peptide hydrolase activity towards leucyl-leucyl-leucine.

5.2.2 Results and conclusions

The results obtained are shown in Tables 5.1 and 5.2. SDS has proved to be a very good solubilising agent for brush border membranes, 0.25% SDS solubilising 86.7% and 0.5% solubilising 92.8% of the protein. Unfortunately, SDS strongly inhibited peptide hydrolase activities. After solubilisation with 0.25% SDS, only about 15% of the peptide hydrolase activity towards leucyl-leucyl-

leucine and only about 8% of hydrolase activity towards leucyl-2-naphthylamide remained. After high speed centrifugation, 67% of the remaining leucyl-leucyl-leucine-hydrolysing activity was recovered in the supernatant. No activity towards leucyl-2-naphthylamide was detected in the supernatant. The relatively high value of leucyl-leucyl-leucine-hydrolysing activity in the pellet may have been due to dilution of SDS during resuspension of the pellet and thus partial reversal of the inhibitory effect of SDS.

Although peptidase activities were strongly inhibited by SDS, it was felt that the use of this detergent at low concentrations for the solubilisation of brush border peptidases followed by their separation by polyacrylamide gel electrophoresis in the presence of SDS, could still give valuable information about these enzymes. Obviously a quantitative estimation of brush border peptidases by such a method was out of the question, but the determination of the number of enzymes involved was still possible, provided some of the activity survived the treatment with SDS.

Treatment	Fraction	Total mg	% Recovery (P_2 = 100)	% of total recovered
	P_2	52.5	100	
Control	Pellet	35	66.7	75.7
	Supernatant	11.25	21.4	24.3
	Total		88.1	100
	P_2	45	100	
0.1% SDS	Pellet	17.5	38.9	35
	Supernatant	32.5	72.2	65
	Total		111.1	100
	P_2	45	100	
0.25% SDS	Pellet	5	11.1	13.3
	Supernatant	32.5	72.2	86.7
	Total		83.3	100
	P_2	42.5	100	
0.5% SDS	Pellet	3	7.1	7.2
	Supernatant	38.75	91.2	92.8
	Total		98.3	100

Table 5.1 Solubilisation of protein from Brush Border Membranes by SDS.

Substrate	Treatment	Fraction	Enzyme Units	% Recovery	% Solubilisation
Leu-leu-leu	Control	P_2	0.705	100	
		Pellet	0.645	91.8	
		Supernatant	0.055	7.8	7.8
	0.25% SDS	P_2	0.105	14.8	
		Pellet	(0.82)		
		Supernatant	0.07	9.5	66.7
Leu-2-nap	Control	P_2	1.235	100	
		Pellet	1.30	105	
		Supernatant	0.03	2.4	2.4
	0.25% SDS	P_2	0.100	8.1	
		Pellet	(0.095)		
		Supernatant	0	0	

Table 5.2 Solubilisation of peptide hydrolase activities from Brush Border membranes by SDS.

5.3 Separation of SDS-solubilised peptide hydrolase activities from human intestinal brush border membranes by SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using a multiphasic buffer system calculated from theory by Jovin et al (1971) and modified for SDS by Neville (1971). The preparation of the gels for electrophoresis is described in Chapter 2, Section 7.1.

5.3.1 Experimental Procedure

a) Solubilisation and electrophoresis:

Brush border membranes were solubilised with SDS at a final concentration of 0.25% (detergent:protein ratio 0.25). This was achieved by the addition of concentrated (x10) SDS solution to the brush border membrane fraction to give the correct final SDS concentration. After 30 minutes at room temperature the SDS-treated brush border fraction was centrifuged at 100,000xg for 1 hour to remove any remaining insolubles. To the decanted supernatant, one drop of bromophenol blue was added and prior to layering the sample on top of a polyacrylamide gel (16x0.5cm) a few crystals of sucrose were added and dissolved. Electrophoresis was performed in a Shandon disc gel electrophoresis apparatus (Model : Analytical) at a constant current of 1.5mA per tube until the bromophenol blue marker dye reached to within 1cm from the bottom of the gel. 50-100 µg of protein were usually applied to each gel. Duplicate gels were run of each sample, one of which was used for protein staining (see Chapter 2.8) and the other for the determination of peptide hydrolase activity.

b) Determination of peptide hydrolase activity in polyacrylamide gels after electrophoresis:

Immediately after the completion of electrophoresis, the gel was extruded from the glass tube and frozen on dry-ice. Using a slicer made of razor blades mounted in a comb arrangement, the gel was sliced into 1mm sections while still frozen. The sections were added to small tubes containing 1ml of 0.15M sodium borate buffer, pH 8.0 (ice cold) and left on ice for several hours to allow proteins in the sections to diffuse out into the buffer. After mixing, the slice supernatants were used to determine peptide hydrolase activity as described in Chapter 2.6.1.

5.3.2 Results

Tables 5.3 and 5.4 show the peptide hydrolase activities in gel slices obtained after SDS polyacrylamide gel electrophoresis of purified brush border membranes solubilised with 0.25% SDS. The results in Table 5.3 were obtained with purified brush border membranes from duodenal mucosa (Prep VIII), those in Table 5.4 with purified brush border membranes from jejunal mucosa (Prep IX). Activity against leucyl-leucine, leucyl-leucyl-leucine, glycyl-leucine, leucyl-glycine, leucyl-glycyl-glycine, phenylalanyl-glycine, phenylalanyl-glycyl-glycine and tyrosyl-tyrosyl-tyrosine were measured and expressed in arbitrary units (extinction at 525nm). No activity was observed against the tripeptides phenylalanyl-glycyl-glycine and tyrosyl-tyrosyl-tyrosine and activity against leucyl-glycyl-glycine was low in both experiments. Activity against the remaining di- and tripeptides was recovered in two distinct peaks. The first peak exhibited activity against the tripeptides leucyl-

leucyl-leucine and leucyl-glycyl-glycine as well as the dipeptide leucyl-leucine and the second peak exhibited only activity against the dipeptides (leucyl-leucine, leucyl-glycine, glycyl-leucine and phenylalanyl-glycine). Although the maximum of the dipeptide hydrolase peak was quite distinct from that of the tripeptide hydrolase peak, there was considerable overlap of dipeptide hydrolase activity into the tripeptide hydrolase peak and it was not possible to exclude the possibility that the tripeptide hydrolase peak in fact, had considerable dipeptide hydrolase activity (see Figures 5.1 and 5.2).

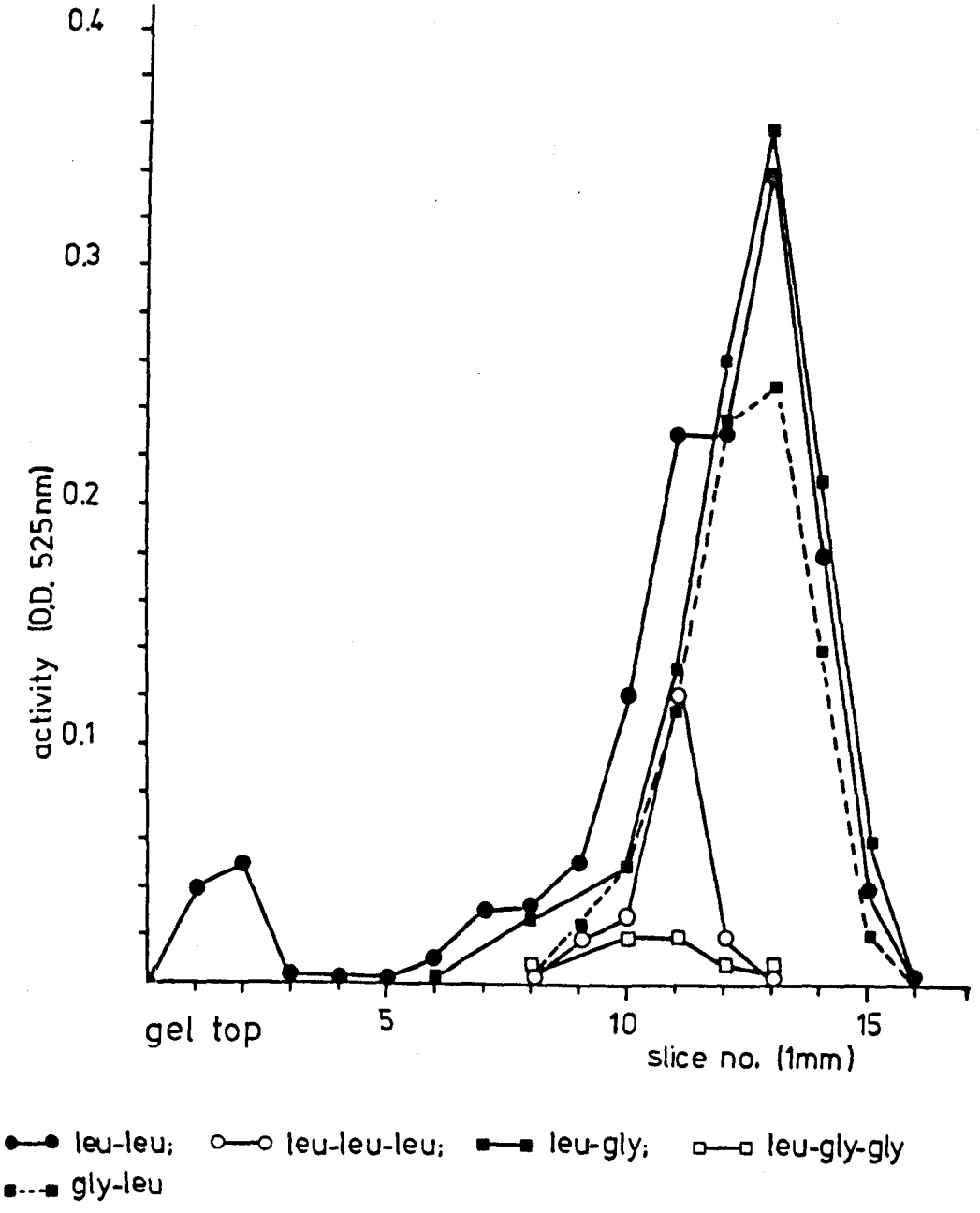
Arylamidase activity could not be measured in SDS-gels as this activity was totally abolished by the SDS-treatment.

Gel slice No. (1 mm)	extinction at 525 nm (arbitrary units)				
	leu-leu	leu-leu-leu	gly-leu	leu-gly	leu-gly-gly
1	0.04	0			
2	0.05	0	0	0.005	0.01
3	0	0			
4	0	0	0	0.005	0.005
5	0	0			
6	0.01	0	0	0.005	0.005
7	0.03	0			
8	0.03	0	0.2	0.03	0.005
9	0.05	0.02			
10	0.12	0.03	0.05	0.05	0.02
11	0.23	0.12	0.115	0.13	0.02
12	0.23	0.02	0.23	0.26	0.01
13	0.34	0	0.25	0.36	0
14	0.18	0	0.14	0.21	0.01
15	0.04	0	0.02	0.06	0.005
16	0	0	0	0.005	0
17	0	0		0	
18	0	0	0	0	0
19	0	0			
20	0	0	0	0	0

Table 5.3 Peptide hydrolase activities in gel slices (1 mm) after SDS electrophoresis; prep. VIII (duodenum).

Figure 5.1: Peptide hydrolase activities in gel slices (SDS)

Prep. VIII (duodenum)



Gel slice No. (1 mm)	extinction at 525 nm (arbitrary units)			
	leu-leu	leu-leu-leu	leu-gly	leu-gly-gly
1	0.04	0.02	0.05	0.03
2	.09	.05	.09	.03
3	.09	.05	.10	.03
4	.06	.04	.08	.04
5	.07	.06	.12	.04
6	.09	.07	.12	.05
7	.20	.20	.16	.06
8	.57	.65	.30	.17
9	.34	.33	.36	.08
10	.41	.09	.45	.04
11	.74	.06	.85	-
12	.69	.05	.83	.04
13	.64	.12	.75	-
14	.47	.05	.65	.03
15	.21	.05	.38	-
16	.05	.03	.10	.03
17	.01	.01	.02	-
18	0	0	.005	.03
19	0	0	0	-
20	0	0	0	.03

Table 5.4 Peptide hydrolase activities in gel slices (1 mm) after SDS electrophoresis; prep IX (jejunum)

Figure 5.2: Peptide hydrolase activities in gel slices (SDS)

Prep.IX (jejunum)

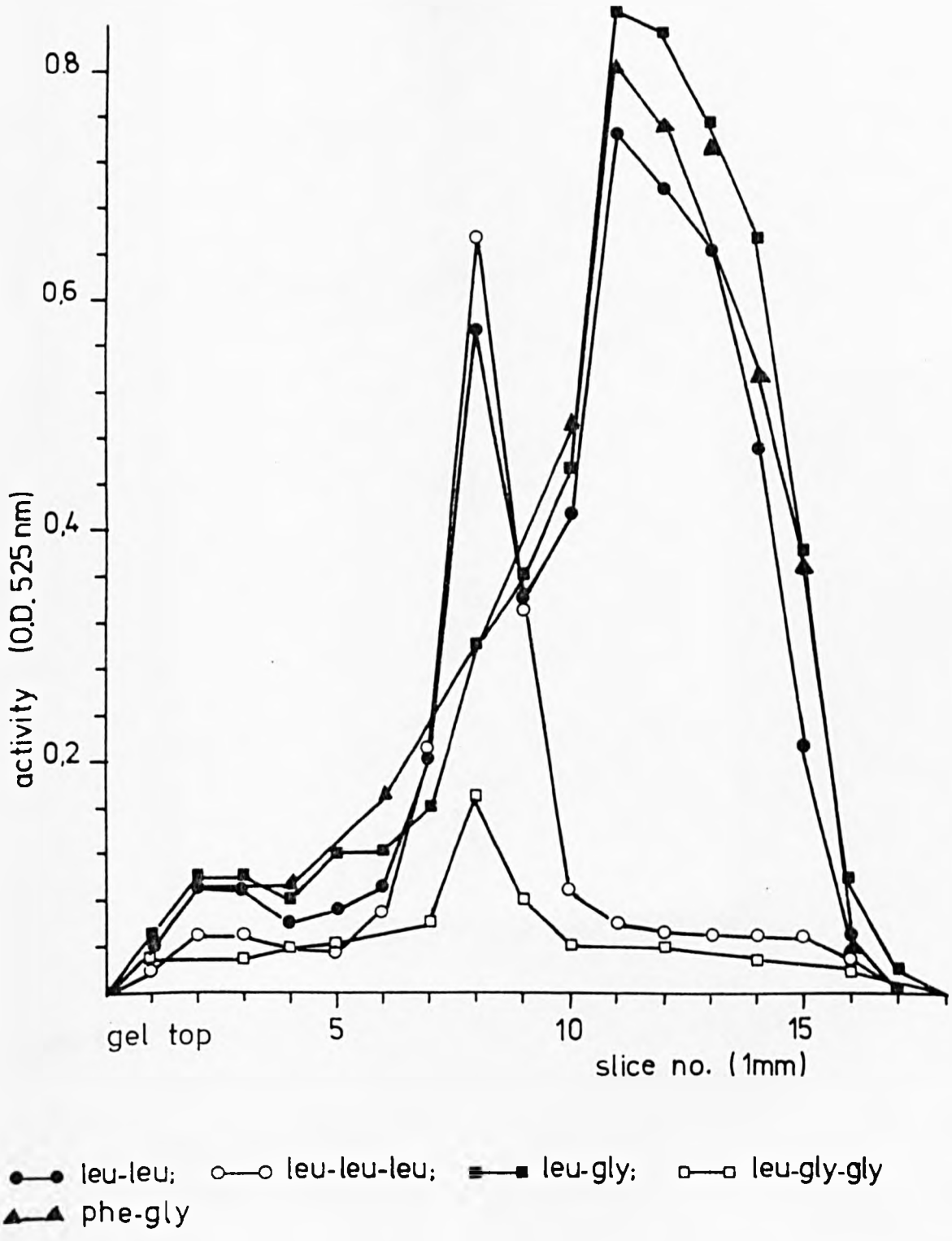


Figure 5.3 Protein pattern in polyacrylamide gel of SDS-solubilised brush border membranes after SDS-electrophoresis. Protein staining was with Coomassie Brilliant Blue R250.

5.4 Determination of substrate specificity of the two peptide hydrolase peaks obtained by SDS-polyacrylamide gel electrophoresis

The question of substrate specificity could not be resolved by the experiments described in Section 3 of this Chapter. The aim of the investigation in this section was to establish whether or not the tripeptide hydrolase peak (peak 1) also possessed dipeptide hydrolase activity and whether or not the dipeptide hydrolase peak (peak 2) possessed any activity against tripeptides.

For this experiment, gel slice supernatants 8 (peak 1) and 11 (peak 2), obtained after SDS-polyacrylamide gel electrophoresis of purified brush border membranes from Prep IX (jejunum), were used (see Figure 5.2). Both peaks were tested for activity against leucyl-leucine, leucyl-leucyl-leucine and leucyl-glycyl-glycine.

5.4.1 Experimental Procedure

100µl of gel slice supernatant and 100µl substrate (10mM peptide in borate buffer, pH 8.0) were incubated for up to 48 hours at 37° in tightly sealed tubes in an incubator. Samples of 20µl were taken at 0, 24 and 48 hours and applied to a silica thin layer chromatography plate, together with the appropriate standards. Chromatography was performed using a solvent system consisting of 60% n-butanol, 15% glacial acetic acid and 25% water and took approximately three hours to run. After chromatography was complete, the solvent front was marked and the plate dried in an oven or with a hairdryer. To visualise the spots, the plate was sprayed with ninhydrin (200mg/100ml acetone) and returned to a hot oven (100°)

for the development of the colour. The distance of the spots from the origin was measured and their Rf values determined.

5.4.2 Results

Tables 5.5 and 5.6a and 5.6b show the results obtained in this experiment. Peak 1 showed activity towards leucyl-leucyl-leucine, leucyl-glycyl-glycine and also leucyl-leucine. The hydrolysis products observed from the substrate leucyl-glycyl-glycine were leucine and glycyl-glycine. The absence of free glycine after 48 hours of incubation indicated that glycyl-glycine was not further hydrolysed. Leucyl-leucine however, was readily hydrolysed by peak 1. Peak 2 showed no activity towards the tripeptides but hydrolysed the dipeptides readily.

Standards	Rf values of standards	Rf values of spots on TLC plate					
		Peak 1			Peak 2		
		0 hours	24 hours	48 hours	0 hours	24 hours	48 hours
leu-gly-gly	0.197	0.197	0.197	0.197	0.197	0.197	0.197
leu-gly	0.299	-	-	-	-	-	-
gly-gly	0.080	-	0.080	0.080	-	-	-
leucine	0.336	-	0.336	0.336	-	-	-
glycine	0.117	-	-	-	-	-	-

Table 5.5. Hydrolysis of leu-gly-gly by peptide hydrolase activities from peaks 1 and 2.

Standards	Rf values of standards	Rf values of spots on TLC plate					
		Peak 1			Peak 2		
		0 hours	24 hours	48 hours	0 hours	24 hours	48 hours
leu-leu-leu	0.616	0.616	0.616	0.616	0.616	0.616	0.616
leu-leu	0.536	-	0.536	0.536	-	-	-
leucine	0.336	-	0.336	0.336	-	-	-

Table 5.6a Hydrolysis of leu-leu-leu by peptide hydrolase activities from peaks 1 and 2.

leu-leu	0.536	0.536	0.536	0.536	0.536	0.536	0.536
leucine	0.336	-	0.336	0.336	-	-	0.336

Table 5.6b Hydrolysis of leu-leu by peptide hydrolase activities from peaks 1 and 2.

5.5 Discussion

Brush border proteins were solubilised with sodium dodecyl sulphate. 0.5% sodium dodecyl sulphate solubilised around 93% of the protein from brush border membranes. The inhibition of peptide hydrolase activity however, rendered the use of this detergent at concentrations of 0.5% and higher useless. At a sodium dodecyl sulphate concentration of 0.25% (detergent:protein ratio 0.25), solubilisation of protein from the brush border membrane was only slightly less (around 87%) and some peptide hydrolase activity was preserved. This activity was limited to that towards di- and tripeptides containing leucine, while arylamidase activity was totally abolished even at lower concentrations of sodium dodecyl sulphate.

SDS-polyacrylamide gel electrophoresis of SDS-solubilised brush border proteins, a method which separates proteins according to their molecular weight, yielded two distinct peaks of peptide hydrolase activity. Both peaks were located in the top portion of the gel indicating the involvement of high molecular proteins. It was not possible to determine the molecular weights of these components because to do this satisfactorily, they have to be boiled in the presence of high concentrations of sodium dodecyl sulphate. Peak 1 was found to possess activity towards leucyl-leucyl-leucine, leucyl-glycyl-glycine and leucyl-leucine while peak 2 exhibited activity towards leucyl-leucine, leucyl-glycine, glycyl-leucine and phenylalanyl-glycine only. No activity was observed in either peaks towards tripeptides with aromatic amino acids or 2-naphthylamide substrates. Although the activity in peak 1 was capable of splitting leucyl-leucine into its constituent amino acids, glycyl-glycine

produced by the initial hydrolysis of leucyl-glycyl-glycine was not broken down any further.

In conclusion then, two leucyl-peptide hydrolases have been isolated by polyacrylamide gel electrophoresis after solubilisation with sodium dodecyl sulphate. Both were amino peptidases as determined by the hydrolysis products, one (peak 1) preferring to hydrolyse tripeptides, the other being specific for dipeptides. The two activities appear to be different to those hydrolysing 2-naphthyalmides and peptides containing aromatic amino acids as activity towards these latter substrates is totally abolished by sodium dodecyl sulphate. One of the brush border peptidases assayed by the use of leucine-2-naphthylamide is aminopeptidase M. This enzyme, which has been localised to the brush border membrane of both kidney and intestine (see Chapter 1, Section 4(d)), must therefore be a distinct enzyme to the two peptidases separated on SDS gels.

The abolition of some of the peptidase activities by SDS made an assessment of the number of brush border peptidases impossible. The results, however, suggest that more than two enzymes are involved.

Sodium dodecyl sulphate was thus of limited use in studying the number of brush border peptidases in the human small intestine.

CHAPTER SIX

SEPARATION OF TRITON X-100-SOLUBILISED PEPTIDE HYDROLASES FROM HUMAN INTESTINAL BRUSH BORDER MEMBRANES BY POLYACRYLAMIDE GEL ELECTROPHORESIS

6.1 Introduction

The use of sodium dodecyl sulphate for the solubilisation of peptide hydrolases from brush border membrane has proved to be of limited use due to the partial or complete inhibition of some of those enzyme activities. A milder detergent, Triton X-100, has been sucessfully used by several research groups to solubilise membrane proteins (eg. Simons et al 1973, Kirkpatrick et al 1974). In contrast to denaturing detergent such as sodium dodecyl sulphate, Triton X-100 appears to interact predominantly with those proteins which are bound to the membrane lipid by hydrophobic interactions. According to this view, the detergent molecules bind to the hydrophobic portion of the membrane protein in effect replacing the membrane lipid. Thus, the hydrophobic domain of the protein remains non-polar and the milieu around the hydrophobic portion polar throughout solubilisation. The protein-bound detergent therefore mimics the lipid environment of the membrane, the orientation of the protein molecule is preserved and so is its biological activity. Direct support for this scheme has been obtained from studies of the Semliki Forest virus membrane proteins (Utermann and Simons 1974).

In this Chapter the use of Triton X-100 for the solubilisation of brush border membrane protein hydrolases followed by the separation of these enzymes by polyacrylamide gel electrophoresis is discussed.

6.2 Solubilisation of brush border membranes with Triton X-100

6.2.1 Method

0.5ml of Triton X-100 (scintillation grade), 10 times concentrated, was added to 4.5ml of P_2 fraction (approximately 10mg protein per ml) to give final detergent concentrations of 0.1% (detergent:protein ratio 0.11); 0.2% (detergent:protein ratio 0.22); 0.5% (detergent:protein ratio 0.55) and 1.00% (detergent:protein ratio 1.1). To a further aliquot of 4.5ml of P_2 fraction, 0.5ml of distilled water was added. The contents of the tubes were mixed and left at room temperature for 30 minutes after which time, 50µl samples were removed for the purpose of assay. The tubes were then centrifuged at 100,000xg for 1 hour (MSE SS50, 10x10ml angle rotor), the resultant supernatants decanted and the pellets resuspended in 1ml of distilled water. The samples taken prior to centrifugation, the supernatants and the resuspended pellets, were then assayed for peptide hydrolase activity using leucyl-leucyl-leucine.

6.2.2 Results

Table 6.1 shows the results obtained in this experiment. Triton X-100 was found to be as effective as sodium dodecyl sulphate in solubilising peptide hydrolase activity. With a slightly higher concentration of Triton X-100, solubilisation better than that obtained with sodium dodecyl sulphate has been obtained, with over 70% of peptide hydrolase activity towards leucyl-leucyl-leucine being recovered in the supernatant at detergent concentrations of over 0.2% (detergent:protein ratio 0.22 and above). Triton X-100 did not cause any inhibition of the enzyme, on the contrary, at a

lower concentration, peptide hydrolase activity appeared to be increased.

Treatment	Fraction	Enzyme Units	% Recovery	% Solubilisation
Control	P_2	0.705	100	
	Pellet	0.645	91.5	
	Supernatant	0.055	7.8	7.8
0.1% Triton X-100	P_2	0.988	140	
	Pellet	0.524	74.3	
	Supernatant	0.695	98.5	70.3
0.2% Triton X-100	P_2	0.978	139	
	Pellet	0.39	55.3	
	Supernatant	0.707	100	72.4
0.5% Triton X-100	P_2	0.675	95.7	
	Pellet	0.22	31.2	
	Supernatant	0.51	72.3	75.6
1% Triton X-100	P_2	0.76	108	
	Pellet	0.24	34	
	Supernatant	0.545	77.3	71.7

Table 6.1 Solubilisation of peptide hydrolase activity (substrate leucyl-leucyl-leucine) from Brush Border membranes by Triton X-100.

6.3 Separation of Triton X-100 solubilised peptide hydrolase activities from human intestinal brush border membranes by polyacrylamide gel electrophoresis in the presence and absence of Triton X-100

Polyacrylamide gel electrophoresis was performed by the method of Davis and Ornstein (1961) using a multiphasic buffer system. The only modification was the addition to some gels of 0.1% Triton X-100. The preparation of the gels is described in Chapter 2.7.2.

6.3.1 Experimental procedure

a) Solubilisation and electrophoresis:

Brush border membranes were solubilised with Triton X-100 at a final concentration of 2%. At this concentration the detergent: protein ratio was about 20. Solubilisation was allowed to proceed at room temperature for 30 minutes after which time, the sample was centrifuged at 100,000xg for 1 hour to remove unsolubilised material. The supernatant was decanted into a clean tube, one drop of bromophenol blue was added and a few crystals of sucrose dissolved in the sample prior to layering it on top of polyacrylamide gels (7.5x0.5cm). Electrophoresis was performed at 2mA/tube until the marker dye reached to within 1cm of the bottom of the gel. 50-100µg of membrane protein were applied to each gel. Each sample was electrophoresed in duplicate under identical conditions, one gel being stained for protein, the second being used for the determination of peptide hydrolase activity after electrophoresis.

b) Determination of peptide hydrolase activity in polyacrylamide gels after electrophoresis:

The gel was extruded from the tube and frozen on dry-ice immediately after the completion of electrophoresis. The gel was then sliced into 1mm sections as described in Chapter 5.3 or into 0.5mm sections using a Mickle gel slicer (Mickle Laboratory Engineering Co, Gomshall, Surrey, England). The 1mm sections were transferred to small tubes containing 1ml of cold borate buffer pH 8.0, the 0.5mm sections to tubes containing 0.5ml of the same buffer. After allowing the proteins to diffuse out of the slices into the buffer solution, peptide hydrolase activity was determined as described in Chapter 2, Sections 6.1 and 6.2.

6.3.2 Results

Tables 6.2(a) and (b) and Figure 6.1 show the results of electrophoresis of Triton X-100-solubilised brush border in the absence of additional Triton X-100 in polyacrylamide gels or electrophoresis buffer solution. From the figures in Tables 6.2 (a) and (b), it is apparent that most of the peptide hydrolase activity was recovered in the first section of the gel indicating that most of the protein did not enter the gel. The high activities in slice No 1 are not included in Figure 6.1. Some protein did, however, enter the gel and a total of three peaks of peptide hydrolase activity were obtained, all located in the top portion of the gel. Peak 1 showed activity towards glycyl-proline-2-naphthylamide; peak 2 activity towards leucine-2-naphthylamide and peak 3 activity towards alanine-2-naphthylamide. Peak 3 also contained activity towards a number of di- and tripeptides studied containing leucine and phenylalanine. Activity in that peak was greater towards the tripeptides than similar dipeptides. No activity towards α-glutamic acid-2-naphthylamide was observed eventhough considerable activity was

shown to be contained in slice 1.

Tables 6.3 (a) and (b) and Figure 6.2 show the results obtained when 0.1% Triton X-100 was added to the gel and the electrophoresis buffer. In total four peaks were observed which contained peptide hydrolase activity. Peaks 1, 2 and 3 showed activity towards α-glutamic acid-2-naphthylamide, glycyl-proline-2-naphthylamide and γ-glutamic acid-2-naphthylamide respectively. Peak 4 contained activity towards leucine- and alanine-2-naphthylamide (not shown in diagram as activity was off scale) as well as a number of di-, tri- and tetrapeptides. Again, activity towards the tripeptides was in general higher than that towards dipeptides.

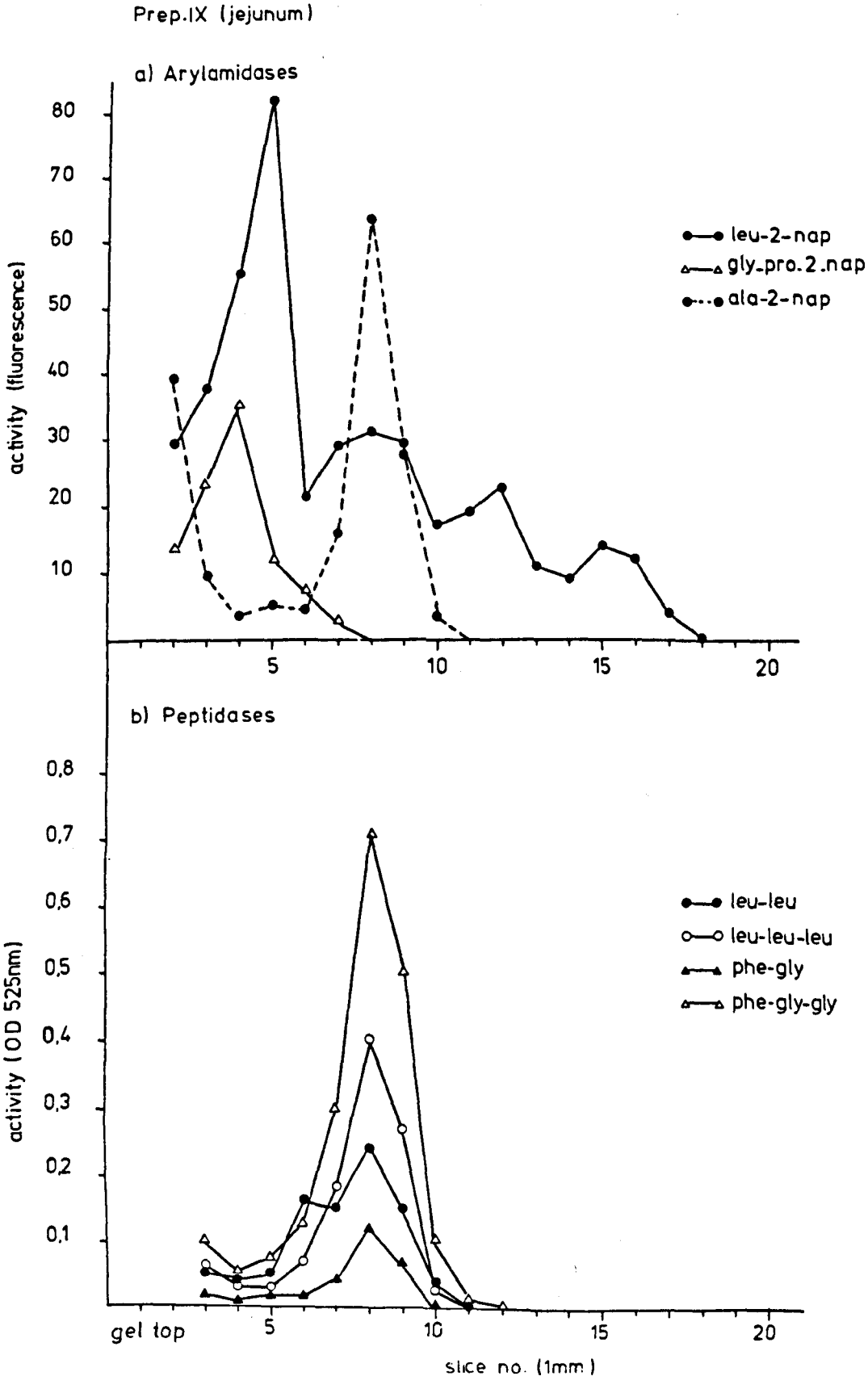
Gel slice No. (1 mm)	fluorescence (λEX. 340 nm, λEM. 410 nm)			
	Leu-2-nap	gly-pro-2-nap	ala-2-nap	α-glu-2-nap
1	146	123	195	52
2	29	14	39	2
3	38	24	10	0.5
4	55	35	3.5	1
5	82	12	5	1
6	21	7	4	0.5
7	29	3	16	-
8	31	4	63	-
9	29	4	28	-
10	17	7	13	1
11	19	7	1	0.5
12	23	7	-	-
13	11	5	-	-
14	9	5	-	-
15	14	4	-	-
16	12	7	-	-
17	4	2	-	-
18	8	7	-	-
19	8	0	-	-
20	7	2	-	-

Table 6.2a Arylamidase activities in gel slices (1 mm) after polyacrylamide gel electrophoresis; prep. IX (jejunum) solubilised with 2% Triton X-100.

Gel slice No. (1 mm)	extinction at 525 nm			
	leu-leu	leu-leu-leu	phe-gly	phe-gly-gly
1	0.84	0.84	0.79	0.89
2	-	0.15	0.10	0.20
3	0.05	0.05	0.02	0.10
4	0.04	0.03	0.01	0.06
5	0.05	0.03	0.02	0.08
6	0.16	0.07	0.02	0.13
7	0.15	0.18	0.04	0.30
8	0.24	0.40	0.12	0.71
9	0.15	0.27	0.07	0.51
10	0.04	0.05	-	0.11
11	-	-	-	0.02
12	-	-	-	-

Table 6.2b Peptide hydrolase activities in gel slices (1 mm) after polyacrylamide gel electrophoresis; prep IX (jejunum) solubilised with 2% Triton X-100.

Figure 6.1: Peptide hydrolase activities in gel slices (Triton X100)



Gel slice No. (0.5 mm)	fluorescence (λex. 340 nm, λem 410 nm)				
	leu-2-nap	ala-2-nap	gly-pro-2-nap	γ-glu-2-nap	α glu-2-nap
1	12	4	3	1	29
2	12	17.5	8	8	7
3	20	39	13	12	14
4	24	57.5	42	15	76
5	48	116	98	57	73
6	84	248	74	78	15
7	134	254	8	15	18
8	145	266	-	2	18
9	140	248	1	-	12
10	43.5	109	9	-	1
11	14	22	9	-	1
12	8	19	11	-	1
13	5	11	5	-	21
14	4	10	-	-	51
15	3.5	8		-	3
16	4	9	7	-	1
17	6	16		-	1
18	9.5	18	15	-	1
19	15	50		-	1
20	13	31	15	-	1
21	11	29		-	-
22	5	12	4	-	-
23	4	5	-	-	-
24	2	4	-	-	-
25	1		-	-	-
26	1	2	-	-	-
27	0.5		-	-	-
28	1.5	1.5	-	-	-
29	0.5		-	-	-
30	0.5	2	-	-	-

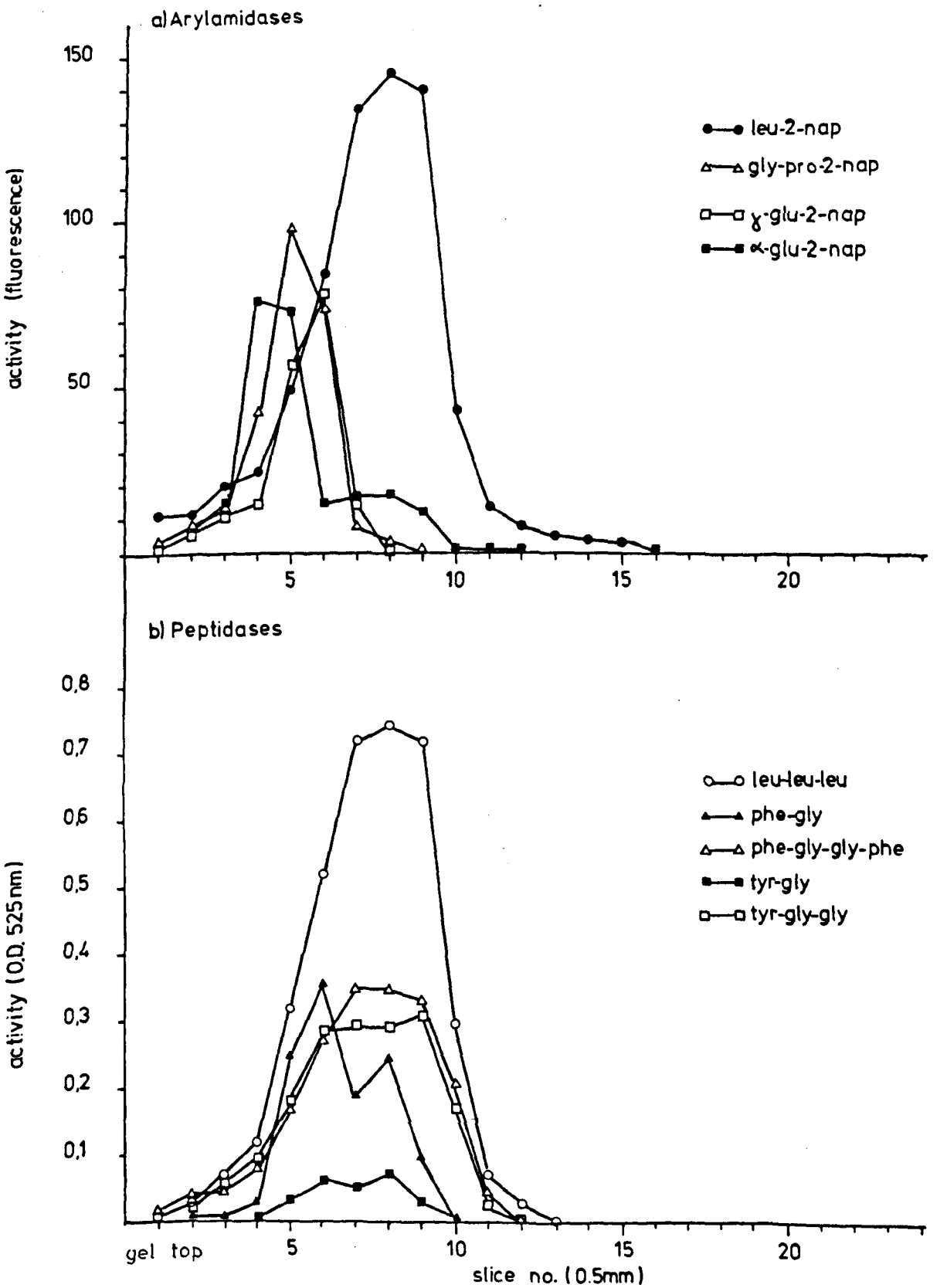
Table 6.3a Arylamidase activities in gel slices (0.5 mm) after polyacrylamide gel electrophoresis in the presence of 0.1% Triton X-100; prep X (duodenum) solublised with 2% Triton X-100.

Gel slice No. (0.5 mm)	extinction at 525 nm					
	leu-leu-leu	phe-gly	phe-gly-gly-phe	phe-tyr	tyr-gly	tyr-gly-gly
1	-	0.03	0.01	0.13	-	-
2	0.03	0.01	0.03	-	-	0.02
3	0.07	0.01	0.05	0.03	-	0.06
4	0.12	0.03	0.08	0.07	-	0.09
5	0.32	0.25	0.17	0.025	0.03	0.17
6	0.52	0.36	0.27	0.32	0.06	0.28
7	0.72	0.19	0.35	0.33	0.05	0.29
8	0.74	0.25	0.35	0.28	0.07	0.29
9	0.72	0.10	0.34	0.33	0.035	0.31
10	0.30	0.01	0.21	0.11	-	0.17
11	0.07	-	0.05	0.05	-	0.03
12	0.03	-	0.03	-	-	-

Table 6.3b Peptide hydrolase activities in gel slices (0.5 mm) after polyacrylamide gel electrophoresis in the presence of 0.1% Triton X-100; prep. X (duodenum) after solubilisation with 2% Triton X-100.

Figure 6.2: Peptide hydrolase activity in gel slices (Triton X100)

Prep. X (duodenum)



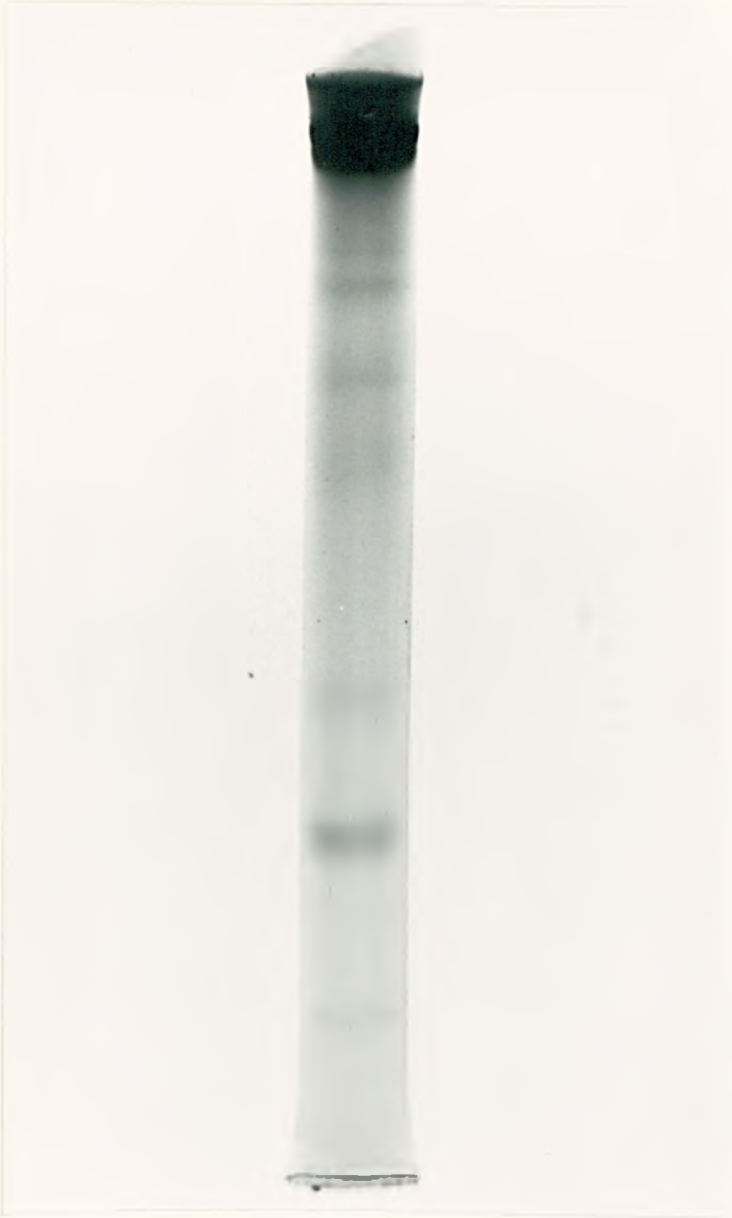


Figure 6.3 Protein pattern in polyacrylamide gels of Triton X-100-solubilised brush border membranes after electrophoresis in the presence of 0.1% Triton X-100. Protein staining was with Coomassie Brilliant Blue R250.

6.4 Discussion

Evidence from the work described in this Chapter has shown that Triton X-100 was as effective as sodium dodecyl sulphate in solubilising brush border membrane peptidases. At detergent concentrations of 0.5 and 1% (detergent:protein ratios 0.55 and 1.1 respectively) about 70% of peptide hydrolase activity was solubilised. Enzyme activities were slightly activated at lower detergent concentrations and no inhibition was observed at the higher concentrations. This fact more than made up for the slightly less efficient solubilisation.

Although peptidases could readily be solubilised by Triton X-100 used at the above detergent:protein ratios, separation by polyacrylamide gel electrophoresis could not be achieved to any degree of satisfaction. This was due to the precipitation of proteins on top of the polyacrylamide gel, making it impossible for them to enter the gel. To obtain satisfactory separation on polyacrylamide gels, a detergent:protein ratio of 20 was used for the solubilisation and 0.1% Triton X-100 was added to both gel and electrophoresis buffer. When Triton X-100 was omitted in the gels, most of the peptidase activity was still found in gel slice No 1, ie. at the top of the gel (see Tables 6.2 a and b and Figure 6.1). Some activity did enter the gel but this was a small amount compared to the activity remaining on top of the gel. When Triton X-100 was added to the gel, four peptidase peaks were obtained after electrophoresis. Peaks 1, 2 and 3 contained activity towards α-glutamic acid-2-naphthylamide, glycyl-proline-2-naphthylamide and γ-glutamic acid-2-naphthylamide respectively and could be identified therefore as aminopeptidase A (EC 3.4.11.7), dipeptidyl peptidase IV (EC 3.4.14.-) and γ-glutamyl-

transferase (EC 2.3.22). Peak 4 contained activity towards leucine-2-naphthylamide and alanine-2-naphthylamide, an activity associated with aminopeptidase M (EC 3.4.11.2). Peak 4 also contained activity towards di-, tri- and tetrapeptides containing both aliphatic and aromatic N-terminal amino acids. The di- and tripeptidase peaks obtained by SDS-polyacrylamide gel electrophoresis could not, therefore, be resolved in the Triton system. The peptides hydrolysed by peak 4 included some which were hydrolysed by one or both SDS peaks (leucyl-leucine, leucyl-leucyl-leucine and phenylalanyl-glycine) and others which were hydrolysed by neither SDS peaks (phenylalanyl-glycyl-glycine and tyrosyl-glycyl-glycine). Although aminopeptidase M has been shown to hydrolyse natural peptides as well as 2-naphthylamides, the activity towards the former type of substrates was found to be a great deal less than towards chromogenic substrates (Kenny 1977). This indicates that the activity in peak 4 was due to more than one enzyme. Triton appears to be very inefficient in breaking protein-protein interactions and it is therefore conceivable that the di- and tripeptidases are part of a multienzyme complex which is only resolved by SDS treatment.

CHAPTER SEVEN

SEPARATION OF PAPAIN-SOLUBILISED PEPTIDE HYDROLASES FROM HUMAN INTESTINAL BRUSH BORDER MEMBRANES BY POLYACRYLAMIDE GEL ELECTROPHORESIS

7.1 Introduction

The previous two chapters have dealt with the electrophoretic separation of peptide hydrolases solubilised with anionic and non-ionic detergents. The aim of the work in this Chapter was to study the release from the brush border of peptide hydrolases by papain and to separate the enzymes by polyacrylamide gel electrophoresis.

Papain is an endopeptidase derived from the papaya plant and belongs to the group of proteinases which depend on free sulphydryl groups for their activity. These enzymes are activated by the sulphydryl activators H_2S, cysteine etc. Papain has been used extensively by workers to study membrane bound proteins and presumably acts by cleaving the peptide bonds at the point of entry into the lipid matrix, thus releasing the polar portion of the bound protein molecule only and leaving the non-polar portion, the anchor, embedded in the lipid matrix of the membrane. Many membrane bound enzymes have been found to retain most of their activity after release by papain.

7.2 Solubilisation of brush border peptide hydrolases with papain

7.2.1 Materials and method

Papain, twice crystallised, was obtained from Sigma, London. The buffer used was 10mM sodium potassium phosphate pH 7.1, 2mM EDTA, 5mM cysteine. A working solution of papain of 0.5mg/ml of the above buffer was prepared and left at room temperature for 30 minutes to activate the papain prior to the experiment. P_2 fraction was resuspended in the above buffer to give a brush border protein concentration of 500μg/ml. For the solubilisation, 100μl of papain solution (50μg) were added to each of three tubes containing 1ml of resuspended P_2 fraction (500μg protein) and incubated at 37^{o} in a water bath fitted with a shaker for 15, 30 and 60 minutes respectively. To a fourth tube containing 1ml of P_2 fraction, 100μl of buffer were added to give a control. After incubation, 4ml of cold Na/K phosphate buffer were added, mixed, a 1ml sample taken and frozen immediately and the remaining sample centrifuged at 100,000xg for 1 hour. The supernatants were decanted and the pellets resuspended in 1ml 0.1M sodium phosphate buffer, pH 7.3. The samples were then assayed for peptide hydrolase activity using leucine-2-naphthylamide, glycyl-proline-2-naphthylamide and γ-glutamic acid-2-naphthylamide.

7.2.2 Results

The results of the solubilisation of peptide hydrolase activity towards leucine-2-naphthylamide, glycyl-proline-2-naphthylamide are shown in Tables 7.1, 7.2 and 7.3. Figure 7.1 is a summary of the results obtained with all three substrates. A large proportion of

the total enzyme activity was released from brush border membranes in the first fifteen minutes of incubation with papain. After 30 minutes, release of particular enzyme activity slowed down. After 60 minutes of incubation with papain, 85% and 93% of the total recovered activity against leucine-2-naphthylamide and glycyl-proline-2-naphthylamide respectively and 76% of the total recovered activity against γ-glutamic acid-2-naphthylamide were found in the high speed supernatant. Activity against leucine-2-naphthylamide was destroyed somewhat after 60 minutes of incubation with papain.

Incubation time (min)	Fraction	Enzyme units	% Recovery	% Solubilisation	% Solubilisation of total recovered.
	P_2	119	100		
0	Pellet	34	28		
	Supernatant	0	0	0	0
	P_2	136	114		
15	Pellet	25	21		
	Supernatant	60	54	54	70
	P_2	95	80		
30	Pellet	16	13.5		
	Supernatant	84	70.6	89	84
	P_2	69	58		
60	Pellet	3	2.52		
	Supernatant	42	35.3	61	93

Table 7.1 Solubilisation of peptide hydrolase activity towards leucine-2-naphthylamide from Brush Border membranes by treatment with papain.

% Solubilisation = % Recovery in Supernatant (P_2 = 100%)

% Solubilisation of total recovered = % Recovery in Supernatant (Pellet + Supernatant = 100%)

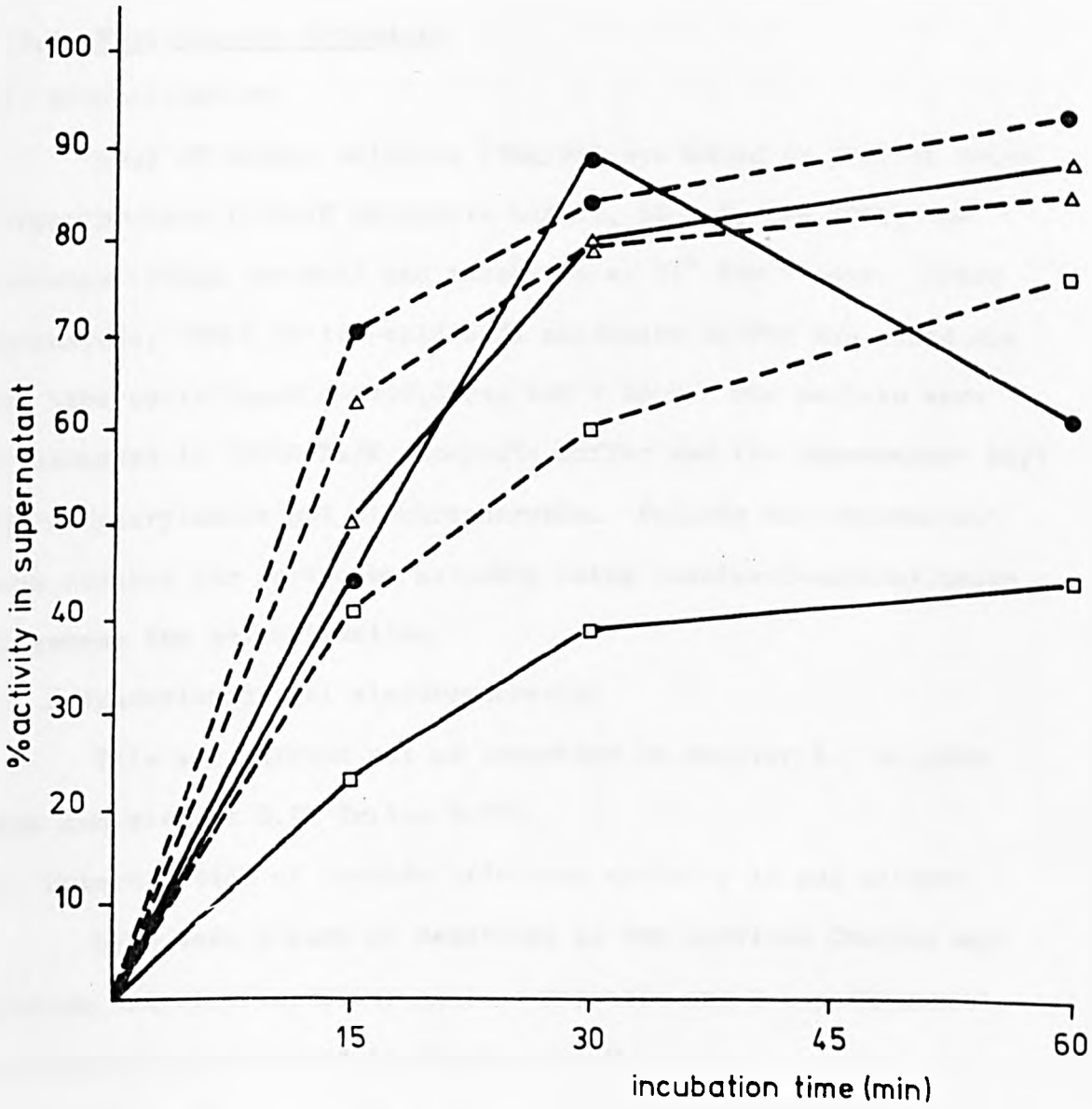
Incubation time (min)	Fraction	Enzyme units	% Recovery	% Solubilis-ation	% Solubilis-ation of total recovered.
	P_2	143	100		
0	Pellet	70	49		
	Supernatant	6.5	5	5	8
	P_2	161	113		
15	Pellet	48	33.6		
	Supernatant	81	56.7	50	63
	P_2	158	111		
30	Pellet	33	23.1		
	Supernatant	126	88.2	80	79
	P_2	146	102		
60	Pellet	22	15.4		
	Supernatant	129	90.2	88	85

Table 7.2 Solubilisation of peptide hydrolase activity towards glycyl-proline-2-naphthylamide from Brush Border membranes by treatment with papain.

Incubation time (min)	Fraction	Enzyme units	% Recovery	% Solubilisation	% Solubilisation of total recovered
0	P_2	450	100		
	Pellet	256	57		
	Supernatant	0	0	0	0
15	P_2	395	88		
	Pellet	127	28.7		
	Supernatant	90	20	23	41
30	P_2	285	63.3		
	Pellet	72	16		
	Supernatant	110	24.5	39	60
60	P_2	240	53.4		
	Pellet	33	7.35		
	Supernatant	105	23.3	44	76

Table 7.3 Solubilisation of peptide hydrolase activity towards γ-glutamic acid-2-naphthylamide from Brush Border membranes by treatment with papain.

Figure 7.1: Solubilisation of peptide hydrolases from brush border membranes by papain



●---● leu-2-nap; △---△ gly-pro-2-nap; □---□ γ-glu-2-nap
solid lines- % recovery (P_2 = 100%)
dotted lines-% of total recovered

7.3 Separation of papain solubilised peptide hydrolases from the brush border membrane of the human small intestine by polyacrylamide gel electrophoresis in the absence and the presence of Triton X-100

7.3.1 Experimental procedure

a) Solubilisation:

50µl of papain solution (1mg/ml) was added to 50µl of brush border protein in Na/K phosphate buffer, pH 7.1, 2mM EDTA, 5mM cysteine (250µg protein) and incubated at 37° for 1 hour. After incubation, 150µl of ice-cold Na/K phosphate buffer was added and the tube centrifuged at 100,000xg for 1 hour. The pellets were resuspended in 250µl Na/K phosphate buffer and the supernatant kept for polyacrylamide gel electrophoresis. Pellets and supernatant were assayed for peptidase activity using leucine-2-naphthylamide to assess the solubilisation.

b) Polyacrylamide gel electrophoresis:

This was carried out as described in Chapter 6.3 in gels with and without 0.1% Triton X-100.

c) Determination of peptide hydrolase activity in gel slices:

Gels were sliced as described in the previous Chapter and peptide hydrolase activity against peptides and 2-naphthylamide determined as described in Chapter 2.6.2.

7.3.2 Results

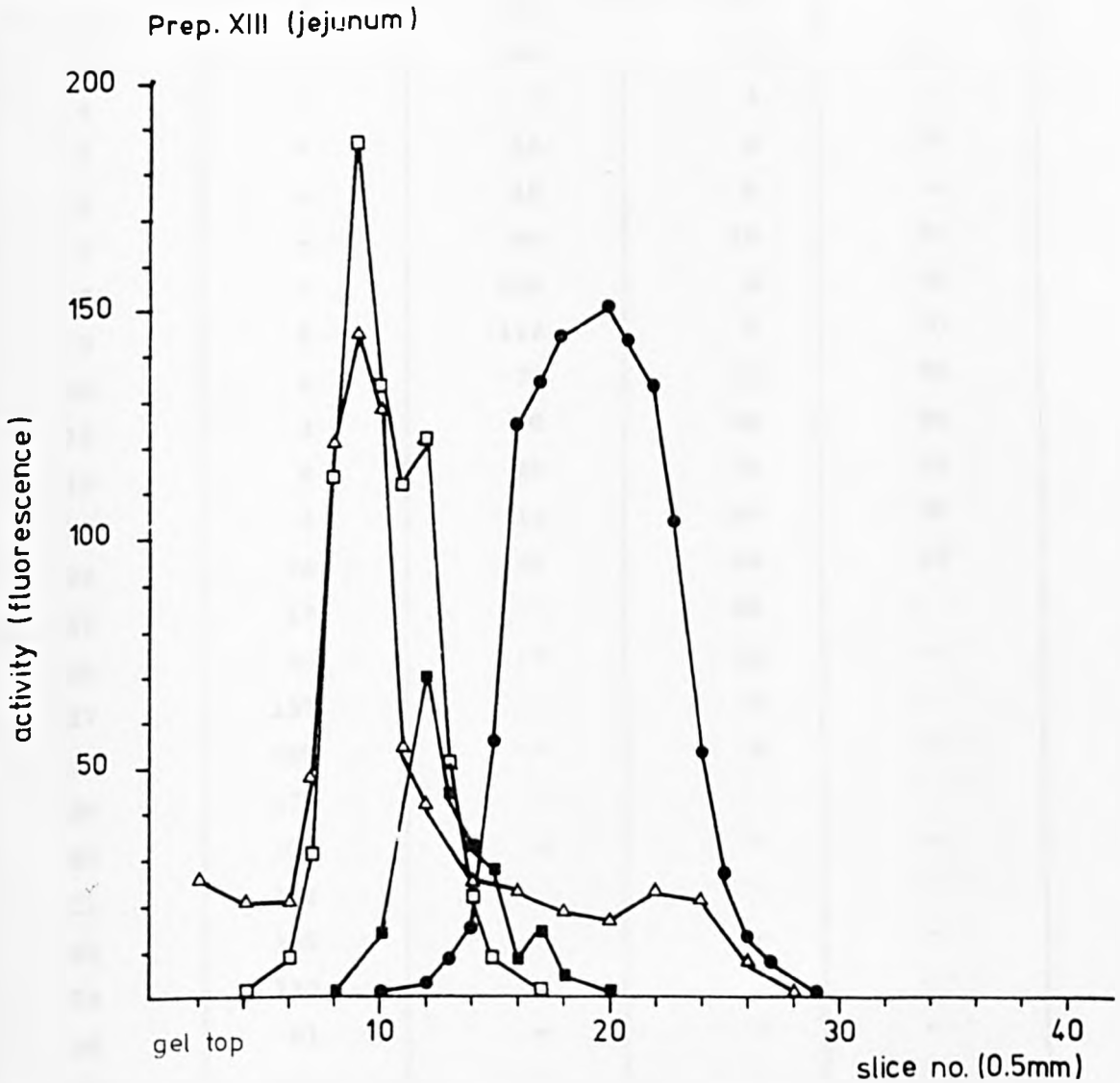
83% of peptide hydrolase activity towards leucine-2-naphthylamide have been recovered in the high speed supernatant after treatment of the brush border membrane with papain. Table 7.4 and Figure 7.2

show the peptidase activity towards 2-naphthylamide substrates recovered in gel slices when Triton X-100 was not included in the gels. Some resolution of peptide hydrolase activity has been achieved. There is a distinct peak containing activity towards leucine-2-naphthylamide. The activities towards glycyl-proline-2-naphthylamide, α-glutamic acid-2-naphthylamide and γ-glutamic acid-2-naphthylamide were recovered in the same gel slices and could not, therefore, be clearly resolved. When 0.1% Triton X-100 was added to the gels, four distinct peaks of peptide activity were observed. Peaks 1, 2 and 3 contained activity towards glycyl-proline-, α-glutamic acid-, and γ-glutamic acid-2-naphthylamide and peak 4 contained activity towards leucine-2-naphthylamide as well as di- and tripeptides. These results are shown in Tables 7.5 a and b and in Figure 7.3.

Gel slice No. (0.5 mm)	fluorescence (λEX. 340 nm, λEM. 410 nm)			
	leu-2-nap	gly-pro-2-nap	α-glu-2-nap	γ-glu-2-nap
1	-	0	3	-
2	-	27	2	1
3	-	13	-	-
4	-	21	-	1
5	-	3	4	3
6	-	20	1	9
7	-	49	1	32
8	2	121	1	113
9	1	145	3	186
10	1	128	13	132
11	1	53	19	112
12	3	42	70	121
13	8	25	44	50
14	15	25	33	22
15	55	18	28	9
16	124	24	8	12
17	133	18	15	1
18	143	19	4	2
19	144	15	8	1
20	150	18	7	-
21	142	16	4	-
22	133	23	2	-
23	103	15		-
24	53	21	-	-
25	27			
26	12	8	-	-
27	8			
28	8	-	-	-
29	1			
30	-	-	-	-

Table 7.4 Arylamidase activities in gel slices (0.5 mm) after polyacrylamide gel electrophoresis; prep. XIII (jejunum), solubilised with papain.

Figure 7.2: Peptide hydrolase activity in gel slices (Papain)



Gel slices No. (0.5 mm)	fluorescence (λEX. 340 nm, λEM. 410 nm)			
	leu-2-nap	gly-pro-2-nap	α-glu-2-nap	γ-glu-2-nap
1	1	-	12	20
2	1	10	8	-
3	-	26	8	10
4	-	7	4	-
5	-	15	4	10
6	-	15	6	-
7	-	84	18	20
8	-	108	4	40
9	1	112	6	70
10	2	70	16	90
11	3	30	38	80
12	4	20	36	70
13	4	12	60	30
14	14	10	68	10
15	17		26	
16	80	7	18	-
17	137		8	
18	159	-	4	-
19	173			
20	169	-	-	-
21	172			
22	165	-	-	-
23	130			
24	91	-	-	-
25	41			
26	7	-	-	-
27	1			
28	-	-	-	-
29	-			
30	-	-	-	-

Table 7.5a Arylamidase activities in gel slices (0.5 mm) after polyacrylamide gel electrophoresis*; prep. XIII (jejunum), solubilised with papain.

*in the presence of 0.1% Triton X-100

Gel slice No. (0.5 mm)	extinction (525 nm)			
	gly-leu	leu-leu-leu	tyr-gly	tyr-gly-gly
1	0.05	-	-	-
2		-	-	-
3	0.06	-	-	-
4		-	-	-
5	0.06	-	-	-
6		-	-	-
7	0.06	-	-	-
8		-	-	-
9	0.05	-	-	-
10		-	-	-
11	0.06	0.01	-	-
12		0.01	-	-
13	0.07	0.01	-	-
14	0.08	0.05	0.01	0.03
15	0.12	0.05		0.04
16	0.10	0.13	0.03	0.10
17	0.42	0.50	0.09	0.20
18	0.39	0.61	0.14	0.21
19	0.36	0.69	0.19	0.22
20	0.31	0.69	0.21	0.21
21	0.15	0.69	0.22	0.20
22	0.13	0.41	0.10	0.18
23	0.07	0.19	0.05	0.13
24	0.06	0.22	0.08	0.16
25	-	0.09	0.03	0.07
26	-	-	0.01	-
27	-	-	-	-
28	-	-	-	-
29	-	-	-	-
30	-	-	-	-

Table 7.5b Peptide hydrolase activities in gel slices (0.5 mm) after polyacrylamide gel electrophoresis in the presence of 0.1% Triton X-100; prep XIII (jejunum), solubilised with papain.

Figure 7.3 : Peptide hydrolase activities in gel slices (Papain)
Prep. XIII. (jejunum)

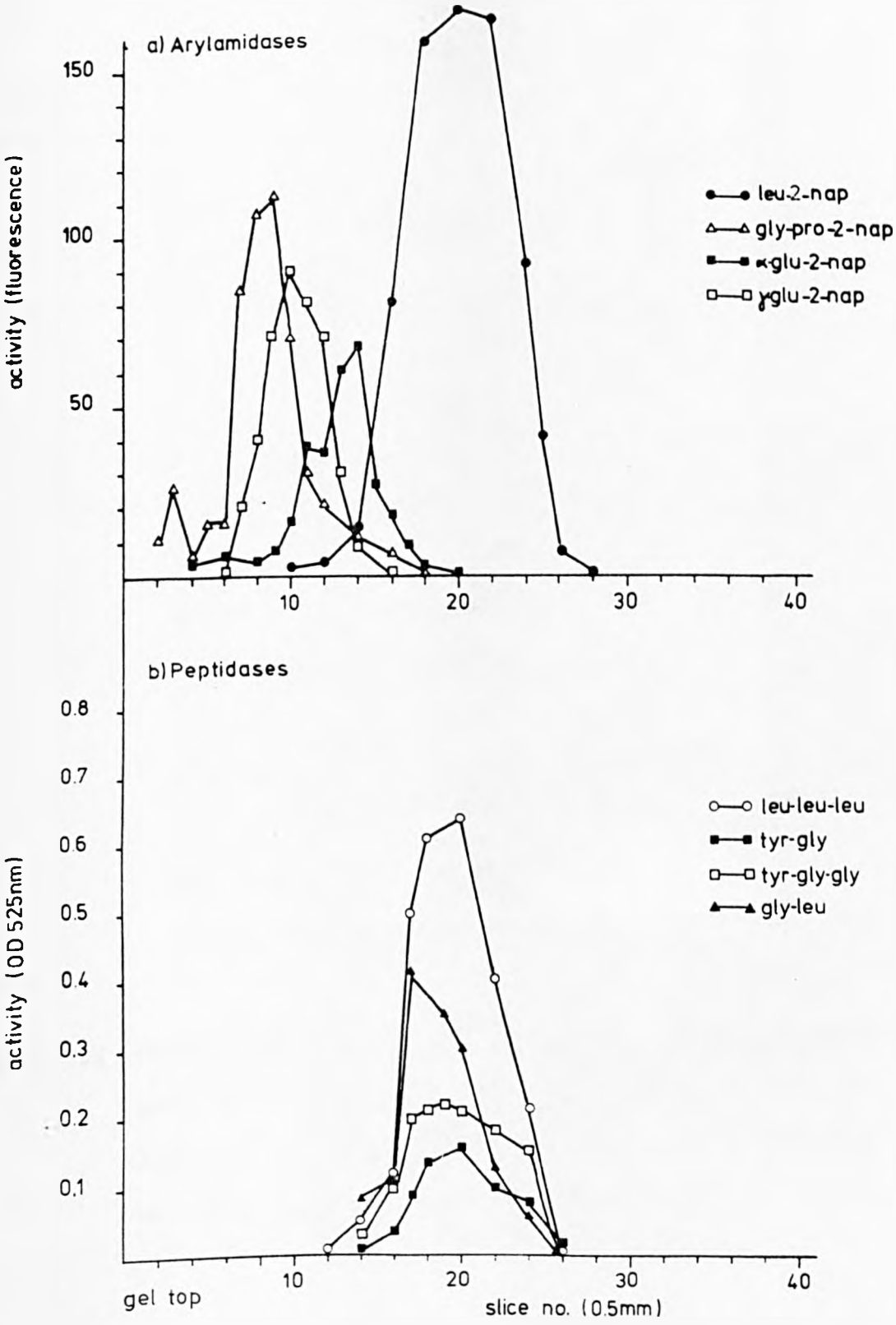


Figure 7.4 Protein pattern in polyacrylamide gels of brush border proteins released by papain after electrophoresis in the presence of 0.1% Triton X-100. Protein staining was with Coomassie Brilliant Blue R250.

7.4 Discussion

Papain has been found to be very effective in releasing peptide hydrolases from brush border membranes. After 60 minutes of incubation of purified brush border membranes with papain, 93% of aminopeptidase M (substrate:leucine-2-naphthylamide), 85% of diaminopeptidase IV (substrate:glycyl-proline-2-naphthylamide) and 76% of γ-glutamyltransferase (substrate:γ-glutamic acid-2-naphthylamide) were released from the membranes. The efficiency by which proteins are released from membranes by treatment with papain reflects their orientation in the lipid matrix. α-glutamyltransferase was released to a lesser extent from brush border membranes than the other enzymes investigated, indicating that this enzyme might be located deeper in the membrane. It has been postulated that γ-glutamyltransferase is involved in the transport of amino acids across cell membranes (Meister 1973), a function which would necessitate a location deep in the membrane or even a spanning of the whole width of that membrane. The relative ease on the other hand, by which aminopeptidase M and diaminopeptidase IV were released from the brush border membrane by the papain treatment suggests a more superficial location of these enzymes on the brush border.

Polyacrylamide gel electrophoresis of papain solubilised brush border membrane proteins yielded four visible bands on protein-staining. Four distinct peaks of peptide hydrolase activity were resolved in the region of the protein bands. Peaks 1, 2 and 3 have been identified as diaminopeptidase IV, γ-glutamyltransferase and aminopeptidase A respectively. The position in the gel of the aminopeptidase A (peak 3) in relation to diaminopeptidase IV and

γ-glutamyltransferase was different from that obtained with Triton X-100 solubilised enzymes using the same electrophoresis system (Chapter 6). There was a shift in the position of the aminopeptidase A peak down the gel and instead of being recovered in the first peak, the enzyme was found in peak 3. This was presumably due to the action of papain on this enzyme, resulting in an alteration of the net charge and/or molecular weight of the molecule and thus affecting its electrophoretic migration. Peak 4 contained the aminopeptidase M activity, as well as activity towards di- and tripeptides. The question whether this activity was due to more than one enzyme could not be resolved by electrophoresis of papain solubilised enzymes.

CHAPTER EIGHT

SEPARATION OF PAPAIN-SOLUBILISED PEPTIDE HYDROLASES FROM HUMAN INTESTINAL BRUSH BORDER MEMBRANES BY ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS

8.1 Introduction

In Chapters 5, 6 and 7 the separation of peptide hydrolases by two different systems of polyacrylamide gel electrophoresis has been discussed. In SDS-polyacrylamide gel electrophoresis (Chapter 5) separation was based on the molecular weight of the protein molecules while electrophoresis in the absence of sodium dodecyl sulphate (Chapters 6 and 7) depended both on molecular weight and net charge of the molecules. In this Chapter, separation of peptide hydrolases by isoelectric focusing in polyacrylamide gel slabs is discussed. This is essentially an equilibrium electrophoretic technique for the separation of amphoteric macromolecules such as proteins according to their isoelectric points in stable pH gradients. Stable pH gradients are produced by the electrolysis of carrier ampholytes in a suitable anticonvective medium. Initially developed as a preparative technique using sucrose gradients in columns as the anticonvective medium, isoelectric focusing has recently been adopted for use in polyacrylamide gels. In order to minimise molecular sieving in gel isoelectric focusing, gels which are highly cross-linked are used. These highly cross-linked gels allow the relatively unrestrictive passage of macromolecules. Small quantities of proteins have been separated in tubes containing polyacrylamide gel using an ordinary apparatus for disc electrophoresis. Although systems for gel isoelectric focusing have features in common with apparatus for gel electrophoresis, there are major differences in design that are crucial for reliable and reproducible results. For this investigation, an apparatus designed specifically for isoelectric focusing in thin layer polyacrylamide

gels has therefore been used. A thin layer technique offers many advantages over the tube technique. There is more efficient cooling of the gel and therefore a considerable shortening of the experimental time.

8.2 Separation of papain solubilised peptide hydrolases from the brush border membrane of the human small intestine by isoelectric focusing in polyacrylamide gels

8.2.1 Experimental procedure

a) Solubilisation:

Insoluble papain (Sigma Chemicals, London) was used for the solubilisation of brush border membrane peptidases. 5mg of insoluble papain were suspended in 2ml of distilled water in a small centrifuge tube and left to swell for 1 hour at room temperature. After swelling was complete, the tube was centrifuged at 1000xg for 10 minutes (MSE 4L, 2000 rpm) and the resulting supernatant discarded. The pellet was washed several times with 10mM Na/K phosphate buffer, pH 7.1 by centrifuging at 1000xg for 10 minutes, discarding the supernatant and resuspending the pellet in fresh buffer. For the last wash 10mM Na/K phosphate buffer, pH 7.1 containing 2mM EDTA and 5mM cysteine was used and the suspension left at room temperature for 30 minutes to activate the papain prior to centrifugation. The activated papain pellet was resuspended in 300μl 10mM Na/K phosphate buffer, pH 7.1, 2mM EDTA, 5mM cysteine containing 300-400μg brush border membrane protein and incubated at 37^{o} in a water bath for 1 hour. During incubation the tube containing the brush border papain mixture was shaken in a horizontal position at 50-60 cycles per minute. Immediately following incubation, the mixture was centrifuged at 100,000xg for 1 hour to remove the papain and remaining insoluble membrane material. The supernatant was used for the isoelectric focusing experiment.

b) Isoelectric focusing:

Isoelectric focusing was performed in the pH range 3.5 to 9.5 using a LKB 2117 Multiphor apparatus designed for analytical thin layer gel electrofocusing in polyacrylamide gels. The preparation of the gels used is described in Chapter 2.7.3. The gels contained 0.6% Triton X-100 to minimise the possibility of isoelectric precipitation of proteins. The samples were applied to the gel on pieces of Whatman 3MM filter papers (5x10mm). Five pieces of filter paper stacked on top of each other enabled the application of up to 150μl of sample solution. The electrode solutions used were 1M sodium hydroxide (cathode) and 1M phosphoric acid (anode). Electrofocusing was carried out across the width of the gel at a final power of 20 watt. The powerpack (Model No PPS 102, Stogate Technical Developments Ltd) was set at a constant voltage which had to be adjusted regularly for the first hour of focusing to make up for decreased current as the pH gradient was formed. During this period the power never exceeded 30 watt. Once the pH gradient was formed, the powerpack setting was retained at 1000 volts with a current of 20mA. The whole procedure of electrofocusing took 2 hours.

c) Determination of peptide hydrolase activity:

A strip of gel containing the focused brush border proteins was cut out using a thin perspex plate with a knife sharp edge along one side and lifted onto a perspex plate covered in aluminium foil. The perspex plate with gel strip mounted perfectly straight on it, was then transferred to an Igloo container with dry-ice to freeze the gel. The aluminium foil and the gel could then be lifted off the perspex plate and the aluminium foil peeled off the frozen gel. The gel was sliced into 1mm sections using the Mickle gel slicer

and the slices transferred to small tubes containing 0.5ml of cold sodium borate buffer, pH 8.0. After the proteins had been allowed to diffuse out into the buffer, the solution was used for the determination of peptide hydrolase activity as described in Chapter 2, Sections 6.1 and 6.2.

d) Determination of pH gradient:

The pH gradient in gels was determined directly with a surface pH electrode (Type LOT 403-30-M8, Ingold, Zürich, Switzerland) immediately at the end of the experiment. The isoelectric point of a particular band was determined from the pH gradient graph.

8.2.2 Results

The results obtained from two different preparations of brush border membranes are summarised in Tables 8.1 (a), (b) and Figure 8.1 (Preparation XIV) and Tables 8.2 (a), (b) and Figure 8.2 (Preparation XVI). The mucosa for both preparations was from duodenum. Isoelectric focusing of papain solubilised brush border proteins in polyacrylamide gels yielded 10 bands after staining with Coomassie Brilliant Blue. Two distinct peaks of peptide hydrolase activity towards leucine-2-naphthylamide and alanine-2-naphthylamide as well as di- and tripeptides were recovered in gel slices after isoelectric focusing. The isoelectric points of these peaks have been determined with Preparation XVI and found to be 4.5 for peak 1 and 6.8 for peak 3. Peak 1 also contained activity towards α-glutamic acid-2-naphthylamide in both preparations. The activity in peak 3 from Preparation XVI (Figure 8.2) was much lower than that obtained in the equivalent peak from Preparation XIV (Figure 8.1). From diagram b in Figure 8.1, it appears that peak 3

was far more active against tripeptides than similar dipeptides, while peak 1 showed no significant preference. Peak 1 obtained from Preparation XVI (Figure 8.2), however, showed a clear preference in activity towards tripeptides.

A third peak (peak 2) towards glycyl-proline-2-naphthylamide was obtained with Preparation XIV (Figure 8.1) which was close to peak 1. This substrate was not tested with gel slices from Preparation XVI.

No activity towards γ-glutamic acid-2-naphthylamide was observed in gel slices from either preparations. Figure 8.3 shows the pH gradient in the gel after focusing was complete.

Gel slice No. (1 mm)	fluorescence (λEX. 340 nm, λEM. 410 nm)			
	leu-2-nap	gly-pro-2-nap	α-glu-2-nap	γ-glu-2-nap
10	1	8	4	
11	3	7	8	
12	9	7	2	
13	39	-	14	
14	74	-	18	
15	82	6	26	
16	85	8	41	
17	85	8	66	
18	86	10	53	
19	76.5	15	31	
20	37	20	14	
21	22	25	4	
22	16	17	5	
23	16.5	18	5	
24	8	17	4	
25	6	11	2	
26	6	12	4	
27	7	6		No activity above blanks
28	7	4	5	
29	6	7		
30	6	7	-	
45	6	-	-	
46	4	-	-	
47	8	-	-	
48	15	-	-	
49	17	-	-	
50	17.5	-	-	
51	58.5	-	-	
52	67.5	-	-	
53	56	-	-	
54	19	-	-	
55	13	-	-	
56	13	-	-	
57	10	-	-	
58	10	-	-	
59	11	-	-	
60	10	-	-	

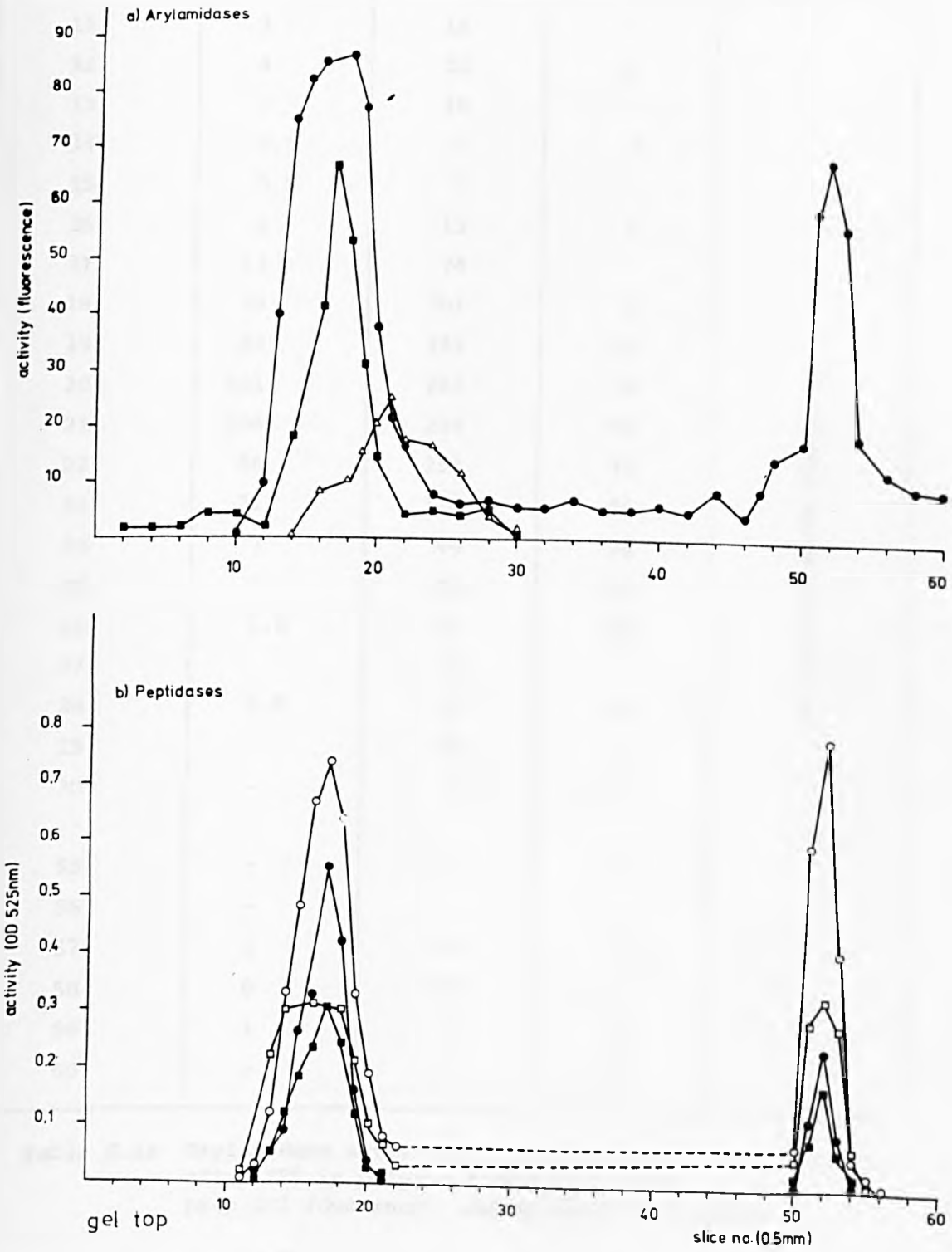
Table 8.1a Arylamidase activities in gel slices (1 mm) after IEF on polyacrylamide gel slabs prep. XIV (duodenum), solubilised with papain.

Gel slice No. (1 mm)	extinction at 525 nm			
	leu-gly	leu-gly-gly	tyr-gly	tyr-gly-gly
11	0.02	-	-	0.01
12	0.01	0.02	0.01	0.05
13	0.03	0.12	0.05	0.22
14	0.09	0.33	0.12	0.30
15	0.26	0.48	0.18	0.32
16	0.33	0.67	0.23	0.31
17	0.55	0.74	0.31	0.31
18	0.42	0.69	0.24	0.32
19	0.16	0.33	0.11	0.30
20	0.04	0.19	0.03	0.21
21	-	0.08	0.01	0.11
22	-	0.06	-	0.10
23	-	-	-	0.07
24	-	-	-	0.03
25	-	-	-	-
50	-	0.07	-	0.05
51	0.12	0.60	0.08	0.29
52	0.24	0.79	0.17	0.33
53	0.09	0.41	0.06	0.28
54	-	0.05	-	0.06
55	-	0.02	-	-

Table 8.1b Peptide hydrolase activities in gel slices (1 mm) after IEF on polyacrylamide gel slabs. prep XIV (duodenum), solubilised with papain.

Figure 8.1 : Peptide hydrolase activities in gel slices (IEF)

Prep. XIV (duodenum)



a) leu-2-nap, glu-2-nap, gly-pro-2-nap

b) leu-leu, leu-leu-leu, tyr-gly, tyr-gly-gly

Gel slice No. (1 mm)	fluorescence (λEX. 340 nm, λEM. 410 nm)			
	leu-2-nap	ala-2-nap	α-glu-2-nap	γ-glu-2-nap
11	3	16	-	
12	4	23	13	
13	2	10		
14	0.5	3	3	
15	0.5	5		
16	2	15	-	
17	13	74		
18	39	201	5	
19	84	253	15	
20	101	262	36	
21	106	269	69	
22	86	254	71	
23	21	128	55	
24	7	44	26	
25	3	23	12	
26	1.5	12	10	
27		6		
28	0.5	5	4	
29		4		
30	-	3	-	
55	-	-	-	
56	-	-	-	
57	1	3	-	
58	6	49	-	
59	1	1	-	
60	-	-	-	

No activity above blanks

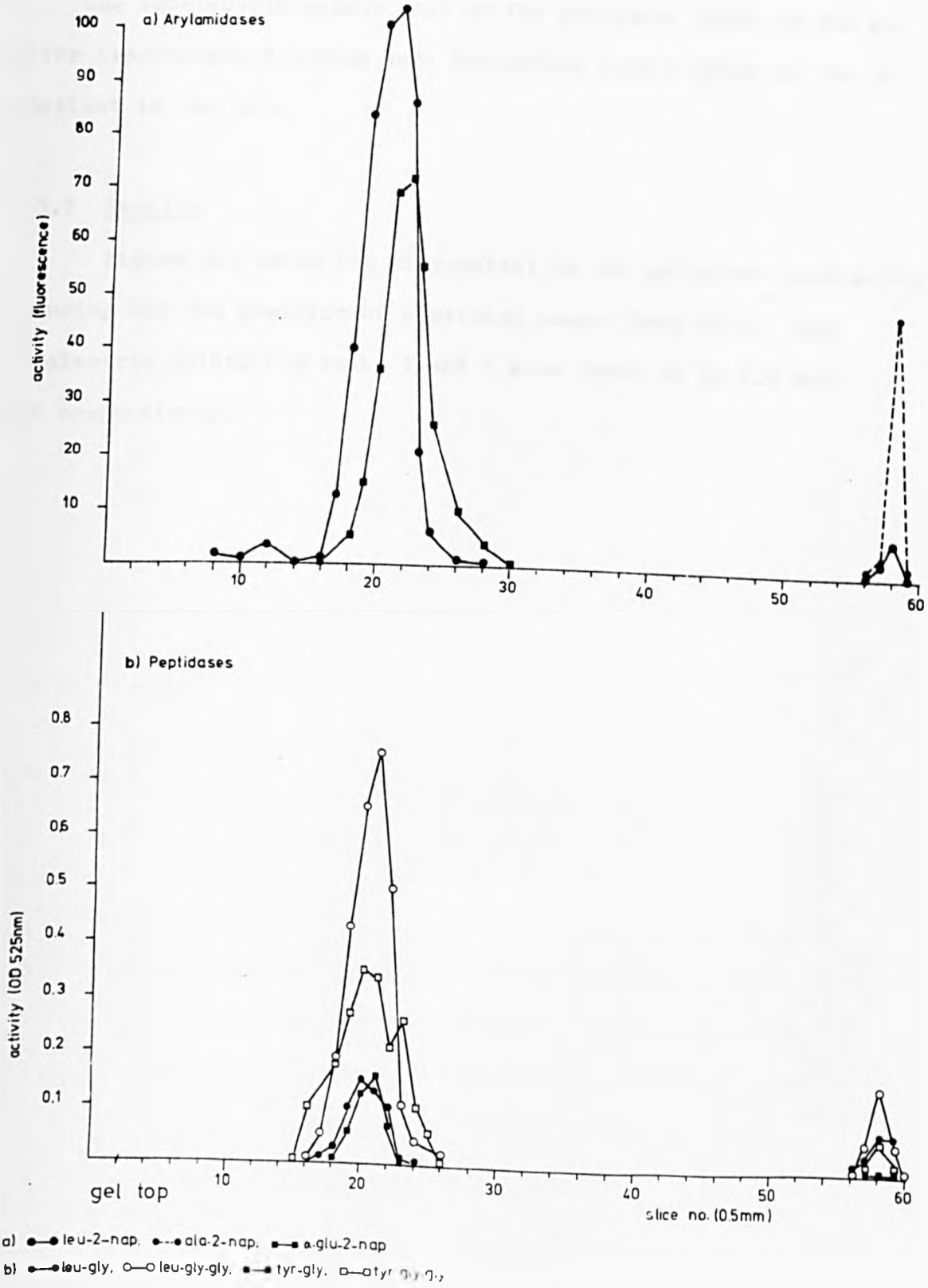
Table 8.2a Arylamidase activities in gel slices (1 mm) after IEF in polyacrylamide gel slabs. prep XVI (duodenum), solubilised with papain.

Gel slice No. (1 mm)	extinction at 525 nm			
	leu-gly	leu-gly-gly	tyr-gly	tyr-gly-gly
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	-	-	-	-
15	-	-	-	-
16	-	0.01	-	0.10
17	0.01	0.05	-	0.10
18	0.03	0.19	0.01	0.18
19	0.10	0.43	0.06	0.27
20	0.15	0.65	0.12	0.35
21	0.13	0.75	0.15	0.34
22	0.10	0.50	0.07	0.21
23	0.01	0.11	-	0.26
24	-	0.04	-	0.10
25	-	-	-	0.06
26	-	-	-	0.01
27	-	-	-	-
28	-	-	-	-
29	-	-	-	-
30	-	-	-	-
55	-	-	-	-
56	-	-	-	-
57	0.03	0.06	-	0.02
58	0.07	0.16	-	0.06
59	0.07	0.06	-	0.02
60	-	-	-	-

Table 8.2b Peptide hydrolase activities in gel slices (1 mm) after IEF as polyacrylamide gel slabs; prep XVI (duodenum) solubised with papain.

Figure 8.2: Peptide hydrolase activities in gel slices (IEF)

Prep xvi (duodenum)



8.3 Determination of isoelectric points

The isoelectric points (pI) of the peptidase bands in the gel after isoelectric focusing were determined from a graph of the pH gradient in the gel.

8.3.1 Results

Figure 8.3 shows the pH gradient in the gel after isoelectric focusing and the position of peptidase peaks (Prep XVI). The isoelectric points for peaks 1 and 3 were found to be 4.5 and 6.8 respectively.

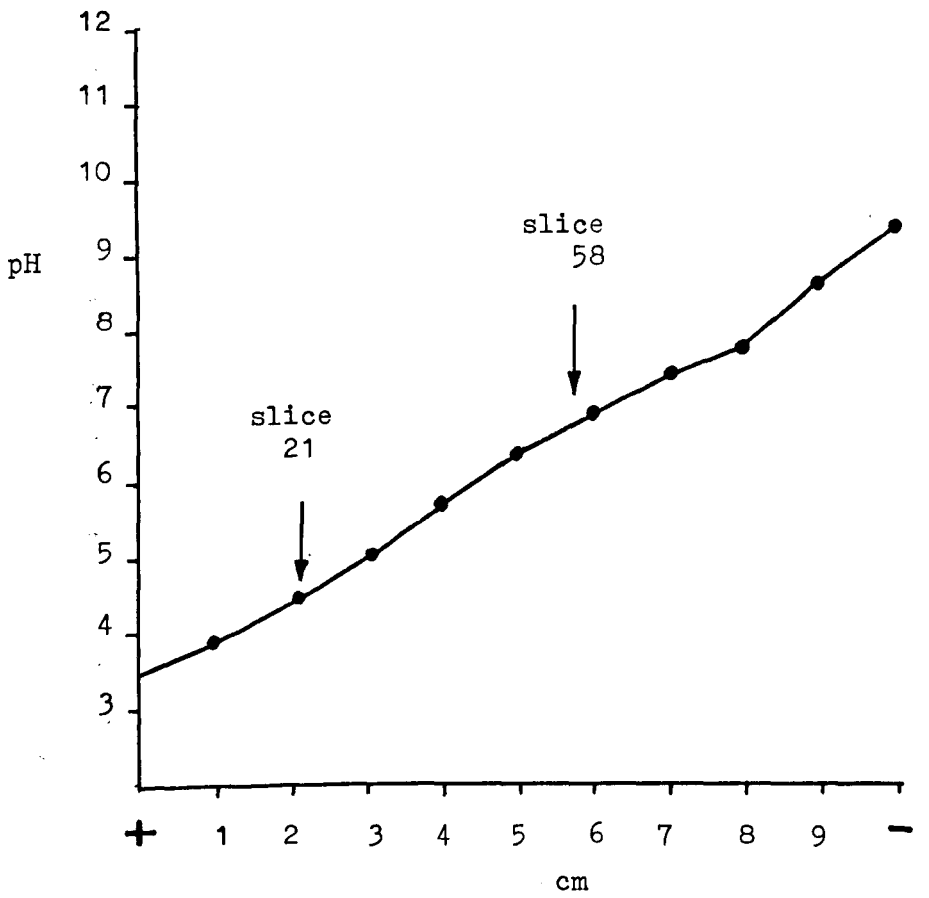


Figure 8.3. pH gradient in IEF gel immediately after the completion of the experiment. The positions of the gel slices with aminopeptidase M-like activity are indicated by the arrows. The pI of these bands can be read off the graph.

8.4 Effect of EDTA and Mg^{2+} ions on the peptide hydrolase activity from peaks 1 and 3

The effect of EDTA and Mg^{2+} ions on the activity of the two aminopeptidase M-like peaks were investigated using alanine-2-naphthylamide.

8.4.1 Experimental procedure

Peptide hydrolase activity towards alanine-2-naphthylamide was determined as described in Chapter 2.6.2. The assays were performed in quadruplicate with the buffer containing the following additions:

a) None

b) 5mM EDTA

c) 5mM $MgCl_2$

d) 5mM EDTA and 5mM $MgCl_2$

8.4.2 Results

Additions	% activity Peak 1	Peak 3
None	100	100
5mM EDTA	13.7	0
5mM $MgCl_2$	98.1	68
5mM EDTA, 5mM $MgCl_2$	12.9	0

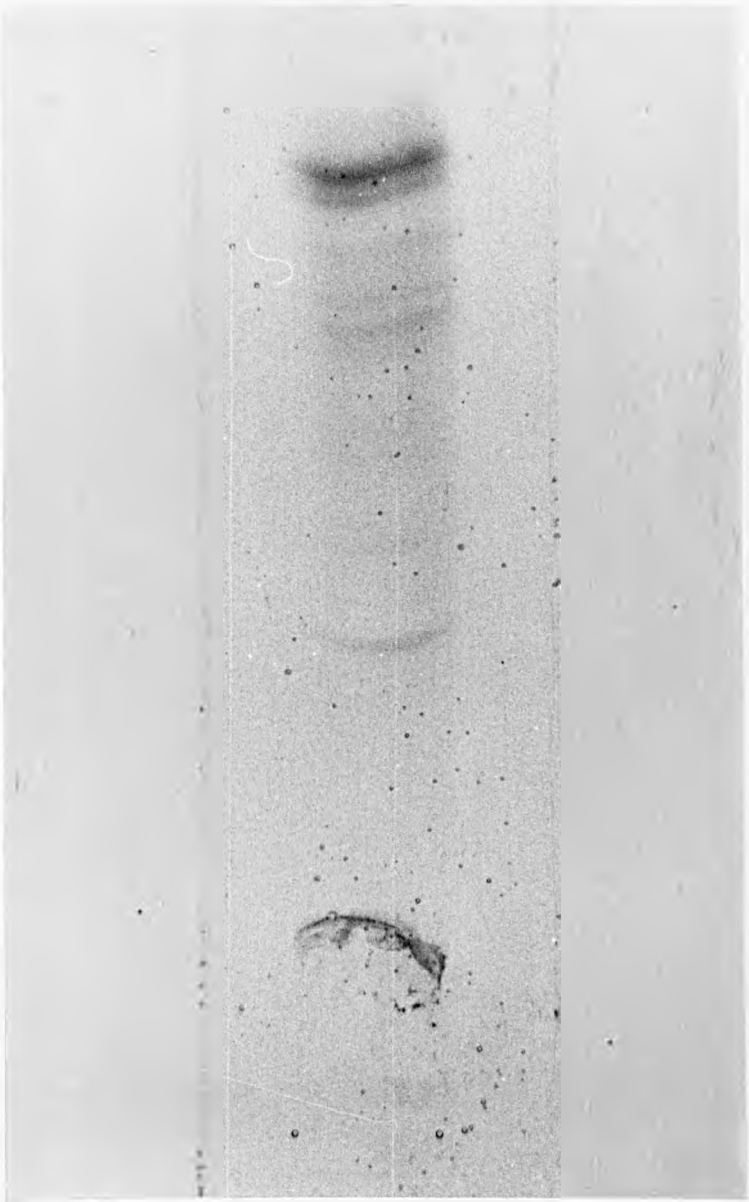


Figure 8.4 Protein pattern in polyacrylamide gels of brush border proteins released by papain after isoelectric focusing in the presence of 0.6% Triton X-100. Protein staining was with Coomassie Brilliant Blue R250.

8.5 Discussion

Isoelectric focusing in thin layer polyacrylamide gels was found to be a very useful technique for the separation of papain solubilised proteins from brush border membranes. The four protein bands obtained by the electrophoresis of papain solubilised brush border membrane proteins (Chapter 7) have been resolved into at least 10 distinct bands by gel electrofocusing. This suggests that the four bands obtained by electrophoresis were not homogeneous ie. they contained more than that number of proteins. This was a very interesting result particularly with respect to the peptide hydrolase activity exhibited by peak 4 recovered in gels after Triton or papain solubilisation. There was now a real possibility that this activity was due to more than one enzyme. This view was strengthened further by the discovery of two distinct peaks of aminopeptidase M-like activity in the focused gels. Peak 1 had an isoelectric point of around 4.5 and contained activity towards leucine- and alanine-2-naphthylamide as well as several di- and tripeptides. It also contained aminopeptidase A activity indicating that their isoelectric points must be very close together. A further peak (peak 2) was resolved which had dipeptidyl peptidase IV activity and which was very close to peak 1. Peak 3 was the other aminopeptidase M-like activity and had an isoelectric point of 6.8. The amount of activity recovered in peak 3 varied greatly with different preparations of papain solubilised brush border proteins. No clear explanation can be given for this, except that the activity of the papain preparations might have varied. Activity in the peaks appeared to be generally greater towards tripeptides than towards similar dipeptides. This does not

necessarily reflect the true substrate specificity of the native membrane enzymes because the determination of peptidase activity in the gel slices must essentially be regarded as a qualitative rather than a quantitative method. Limited studies on the effect of chelating agents have shown that the activity from peak 1 was inhibited by 86% and activity from peak 3 by 100% when treated with 5mM EDTA. Mg^{2+} had little effect on the activity from peak 1 and inhibited activity from peak 3 by 32%. Both enzymes are therefore metal dependent but the metal with which their activity is associated cannot at this point be identified.

Activity towards γ-glutamyl-2-naphthylamide (γ-glutamyl-transferase) could not be detected in gels after isoelectric focusing and it must be assumed that this enzyme is inactivated by this procedure.

CHAPTER NINE

GENERAL DISCUSSION

9.1 Purification of brush border membranes from human small intestine

A method has been developed for obtaining highly purified brush border membranes from human small intestine, using frozen tissue obtained during surgery. While the method was based on that of Schmitz et al (1973), there were a number of differences. The homogenisation procedure was devised for a small multispeed, changeable blade homogeniser which has the advantage of being usable with varying volumes of material and different size blades. The speeds of the centrifugation runs were increased after the demonstration that this improved the yield. Most importantly, the use of high concentrations of Tris to disrupt brush borders was found to be of no value and was omitted. This was desirable as peptidase enzymes may be sensitive to such high concentrations of Tris which would be disadvantageous in peptidase studies.

Although basically a preparative technique, the purification did not involve the use of buffers containing EDTA. The omission of EDTA was considered very important when studying peptide hydrolases in the fractions as many of these enzymes are inhibited by this chelating agent. The only other published method, apart from that of Schmitz et al (1973), describing the preparation of human brush border membranes (Welsh et al 1972), used EDTA and Tris disruption in the preparation. Many workers studying intestinal peptidases in animal systems have used EDTA-buffers in their fractionation methods (Rhodes et al 1967, Peters 1970, 1973, Auricchio et al 1972, Fujita et al 1972, Kim et al 1974). Some of the results obtained by these investigators, particularly negative ones, concerning metal-dependent peptidases might be invalidated as a direct result of EDTA-inhibition.

The final preparation of brush border membranes showed a 24-fold increase in α-glucosidase on average compared to the mucosal homogenate and contained only trace amounts of cytosol and lysosome marker activity. Some activity (approximately 3% of the homogenate) towards glucose-6-phosphate (ER marker) was retained in the brush border fraction but as discussed in Chapter 6, Section 9, this was possibly due to non-specific phosphatases of the brush border membrane. The purity of the human brush border membrane preparation, assessed enzymatically and by electron microscopy, compared very favourably with those from other species prepared by different methods (Miller and Crane 1961, Hübscher et al 1972). The purity of the final brush border preparation was considerably higher than that obtained by Welsh et al (1972) using human tissue. Compared to the method of Schmitz et al (1973), the method described in this thesis was quicker, resulting in pure brush border membranes which on average showed the same factor of purification as that of Schmitz et al (1973). With gained experience however, many of the preparations were found to have a much higher level of purity (with enrichment of α-glucosidase in the brush border membrane fraction reaching 50).

9.2 Subcellular localisation of intestinal peptide hydrolases

As far as is known, the work presented in this thesis is the first major investigation on peptidases of human small intestine, in comparison with relatively numerous studies on animals. The reason for this lack of human studies may be largely due to the difficulty encountered in the way suitable sources of tissue must be arranged and the cooperation of surgeons sought. The material obtained may be diseased and it is probable that there is much greater individual variation between patients than, say, members of a colony of laboratory animals of a single strain of species. Consequently, precise quantitatively based interpretations are difficult to obtain. The major difficulty, however, is in terms of quantities of material obtained. Surgical procedures involving the excision of large amounts of undamaged or undiseased small bowel are rare and so, tissue specimens obtained are small. This shortage of material limits the methods available for enzyme studies. Large scale preparations, enzyme separations, purifications and characterisations are not possible. Given also, that in studying peptidases any particular activity may be located in both brush border and cytosol, studies on brush border enzymes must start with a purified preparation of brush border membranes which means that quantities of starting material are even smaller. In this study the amount of whole tissue for each fractionation was usually only between one and two grams.

Although the preparation was essentially preparative, ie. to obtain purified brush border membranes, useful information was obtained on the subcellular localisation of the peptide hydrolases.

This investigation has shown that the mucosa of the human small intestine is capable of hydrolysing di- and oligopeptides containing aliphatic and aromatic amino acids, as well as amino acid-2-naphthylamide derivatives. The rate by which these substrates were hydrolysed depended on the N-terminal amino acid, peptides containing aliphatic N-terminal amino acids (eg. leucine) generally being hydrolysed at a faster rate than peptides with aromatic N-terminal amino acids (eg. phenylalanine).

On the basis of enrichment and distribution studies it was concluded that oligopeptides with four or more amino acids are hydrolysed by enzymes located exclusively in the brush border membrane. The estimated recovery in the brush border fraction of phe-gly-gly-phe-hydrolase, for example, was 95% (compared to 93% for α-glucosidase) and the increase in specific activity was around 17-times that of the homogenate (compared to approximately 24 for α-glucosidase). These findings are in agreement with those of Peters (1970) and Kim et al (1974) who found that the soluble fractions of small intestinal mucosa from guinea pig and rat respectively were not capable of hydrolysing peptides containing more than three amino acid residues. There appeared to be a limit to the size of oligopeptides which could be hydrolysed by brush border membrane fractions. Larger oligopeptides such as the poly-leucines used in this study are clearly resistant to brush border hydrolysis. Leucine-2-naphthylamide too was found to be predominantly located in the brush border membrane with an enrichment equal to that of α-glucosidase (brush border membrane marker) and an estimated distribution of 85% brush border and 15% soluble fraction. Activity towards this substrate is associated with aminopeptidase M, an enzyme which has been isolated

and purified from kidney and intestinal brush border membrane (eg. George and Kenny 1973, Maroux et al 1973). Maroux et al (1973) reported that the purified enzyme accounted for almost all of the peptidase and all of the arylamidase activity of the brush border membrane in the pig. Some of the activity towards leucine-2-naphthylamide recovered in the soluble fraction was possibly due to some of the enzyme having been removed during the fractionation procedure (eg. by homogenisation of the tissue), a possibility supported by the fact that a small amount (approximately 7%) of α-glucosidase was also recovered in the soluble fraction. The remainder of the soluble leucine-2-naphthylamidase activity was presumably due to the action of soluble enzymes such as cytosol leucinaminopeptidase, an enzyme which does hydrolyse 4-nitroanilides and presumably also 2-naphthylamides slowly (Wachsmuth 1966).

An estimated 33-87% of tripeptidase activity, depending on substrate, with an enrichment in the brush border of between ½ and $^{2}/_{3}$ of that of α-glucosidase were recovered in the brush border with the remainder in the soluble fraction. Recovery and enrichment in the brush border varied and appeared to be dependent on the N-terminal amino acid residue of the substrate. In general, activities towards tripeptides with an aromatic N-terminal sidechain (eg. phenylalanine and tyrosine) exhibited a higher recovery accompanied by greater enrichment in the brush border fraction than activities towards tripeptides with an aliphatic N-terminal side group (eg. leucine). Tyr-gly-gly-hydrolase thus had an enrichment of around 18 with an estimated brush border location of 65% while leu-gly-gly-hydrolase showed an enrichment of only 10 with a recovery in the brush border of only around 6%. A similar observation was made by Peters (1970) who found

that in the guinea pig, peptidase activities towards tripeptides with larger N-terminal residues showed a greater brush border location than those towards tripeptides with less bulky sidechains. It was therefore evident that both brush border membrane and cytosol contained enzymes capable of hydrolysing tripeptides but the question of whether these activities were due to distinctly different enzymes was not resolved. Studies on the heat-stability, electrophoretic mobilities, metal requirement and inhibition studies with p-chloromercuribenzoate (PCMB) have been carried out by other workers but there appear to be distinct species differences in the results. Kim et al (1974) have found distinct differences in these parameters between brush border and cytosol in the rat and Heizer et al (1972) reported differences in heat-stability and PCMB inhibition between the peptidases from rat brush border and cytosol. Donlon and Fottrell (1972) on the other hand, have found no consistent differences in electrophoretic mobility between guinea pig brush border and cytosol peptidases. These authors do not appear to have solubilised their peptidases from the particulate fractions prior to submitting them to starch gel electrophoresis. It is therefore more than likely that the activities they recovered in the gel after electrophoresis were due to soluble contaminant enzymes. The human brush border membrane in this study, was also shown to contain considerable activity towards α-glutamic acid-2-naphthylamide (aminopeptidase A), glycyl-proline-2-naphthylamide (dipeptidyl peptidase IV) and γ-glutamic acid-2-naphthylamide (γ-glutamyltransferase). Aminopeptidase A from rat kidney brush border has been purified and found to be free of contamination by aminopeptidase M (Glenner et al 1962). It is specific for 2-naphthylamides and peptides with a α-glutamic- or α-aspartic

Addendum: Page 130, after line 1.

Aminopeptidase A has been shown to be located in the rabbit intestinal brush border and has been separated from other brush border aminopeptidases (Andria et al 1976).

acid N-terminal.

Dipeptidyl peptidase IV, a serine peptidase, has been demonstrated in kidney microvillous membranes where it is enriched greatly (Booth and Kenny 1974). The enzyme has been purified from pig kidney (Kenny et al 1976) and is active towards a number of peptides and peptide-2-naphthylamides. A proline or alanine adjacent to the N-terminal residue is required for activity. The enzyme has also been shown to have endopeptidase activity but the specificity for proline residues is maintained.

γ-glutamyltransferase has been found in high activities in epithelial membranes of absorptive organs, in particular the kidney (Albert et al 1961) and the small intestine (Ross et al 1973, Greenberg et al 1967, Garvey et al 1976). The enzyme has been purified from the kidney of the pig (Orlowsky and Meister 1965, Leibach and Binkley, 1968) and the rat (Tate and Meister 1974). Maestracci et al (1975) estimated the molecular weight of γ-glutamyltransferase from human small intestine by SDS-polyacrylamide gel electrophoresis. Their molecular weight value of 80,000 compared well with that obtained by Leibach and Binkley (1968) but other workers reported molecular weights of up to 3.15×10^6 (Szewczuk and Connell 1965). According to Meister (1973) γ-glutamyltransferase functions as the membrane-bound transporter of amino acid across membranes. The enzyme catalyses the transfer of the γ-glutamyl group of glutathione (or other γ-glutamyl-containing peptides) to a free amino acid or small peptide. A recent investigation of rat intestinal γ-glutamyltransferase and the comparison of its location in relation to sites of amino acid absorption, have given support to the hypothesis that this enzyme is involved in the transport of amino acids in

mammalian cells (Garvey et al 1976).

An estimated 58-88% of dipeptide hydrolase activity was recovered in the soluble fraction in the present study indicating that the bulk of dipeptide hydrolase activity in the intestinal mucosa is located in the cytosol of the enterocytes. Several workers have reported recoveries of dipeptidase activity of over 90% in the soluble fraction of intestinal mucosa (Rhodes et al 1967, Heizer and Laster 1969, Rubino et al 1969, Peters 1970, Kim et al 1972, Das and Radhakrishnan 1973) and Peters (1975) suggested that dipeptidases had a localisation to the soluble entity of the cytosol and that the 10% or so of activity recovered in the brush border membrane fraction was due to contamination. The investigations for this thesis have clearly shown that the brush border membrane fraction contained considerable dipeptide hydrolase activity and that this activity was not due to cytosol contaminants. If the activity had been due to cytosol contaminants, one would have expected to recover some activity towards proline-containing dipeptides in the brush border fraction, an enzyme action associated very strongly with the cytosol. No such activity was, however, recovered in the brush border fraction. The recovery of dipeptide hydrolase activity in the brush border or the soluble fraction was again found to be dependent on the substrates used. Recovery of activity towards dipeptides containing aliphatic amino acids was consistently higher in the soluble fraction (about 86% for leucyl-glycine-hydrolase) than activity towards aromatic dipeptides (less than 50% for tyrosyl-glycine-hydrolase). There is no doubt from these data that the human brush border membrane does contain dipeptide hydrolase activity; however, this activity does not have to be due to a true dipeptidase, ie. an enzyme specific for di-

peptides, but could be the result of an aminopeptidase with a wide substrate specificity. Heizer and Laster (1972) have found differences in heat-stability and inhibition by PHMB (p-hydroxy-mercuribenzoate) between peptidase activities from the brush border and the soluble fraction of the rat. Using phenylalanyl-glycine and glycyl-phenylalanine they recovered 61% of the activity towards the former substrate but only 3% of the latter substrate in the brush border fraction. The brush border enzyme was not inhibited by PHMB and was more heat-stable. The authors concluded from these results that different enzymes are involved in the hydrolysis of dipeptides by cytoplasmic and brush border fractions of intestinal mucosa. There was no indication from these data, however, that the brush border activity was a true dipeptidase and so far no such brush border enzyme has been characterised.

Evidence from the investigations in this thesis suggests that human brush border membranes contain no peptidase capable of hydrolysing proline-containing dipeptides. All the activity towards these substrates was found in the soluble fraction. These findings are in agreement with other reports using rat tissue (Kim et al 1972).

Addendum: Page 133, after line 19.

Enzymes I and II have been further purified and shown to have molecular weights of 320,000 and 180,000 respectively (Gray and Santiago 1977). Kinetic studies on Enzyme II showed that it had broad specificity and that both affinity and hydrolytic rate were enhanced when a residue with a bulky side chain was at the NH_2 terminus (Kania et al 1977).

9.3 Peptide hydrolases of the intestinal brush border membrane

There is both direct and indirect evidence from studies on animal tissue that brush border membranes contain several peptide hydrolases. Kim et al (1972) recovered four bands with peptide hydrolase activity in starch gels using rat brush border membranes. No difference in substrate specificity was reported between the different bands. Using a brush border/nuclear fraction of guinea pig intestinal mucosa, Donlon and Fottrell (1972) reported five different peptide hydrolase bands in zymograms. As previously mentioned, these activities were more likely due to contamination by soluble enzymes. Fujita et al (1972) on the basis of kinetic and inhibition studies divided dipeptide substrates into three groups and concluded that the substrates from each group were hydrolysed by separate brush border peptidases. Wojnarowska and Gray (1975) separated three peptide hydrolases from rat brush borders by chromatography on Sephadex G-200, velocity gradient centrifugation and polyacrylamide gel electrophoresis. Designated peptidases I, II and III, they were found to have different substrate specificities. Enzymes I and II had oligopeptidase character with maximum specificity for tripeptides. Leucine-2-naphthylamide was also hydrolysed. Peptidase III in the first instance, hydrolysed dipeptides but was found to attack oligopeptides at much slower rates also. Shoaf et al (1976) have also reported the isolation - by means of chromatography (DEAE cellulose) and preparative polyacrylamide gel electrophoresis - and characterisation of three peptide hydrolases from rat brush borders. These authors too designated their enzymes peptidases I, II and III. Peptidase II was resolved by polyacrylamide gel electrophoresis under slightly different conditions into two proteins, IIa and IIb, with

identical substrate specificities. Peptidase III evidently is the same enzyme Wojnarowska and Gray (1975) isolated as their peptidase III. Both enzymes are most active with dipeptides, in particular, glycyl-leucine, leucyl-glycine and glycyl-phenylalanine but do not hydrolyse leucine-2-naphthylamide. There are, however, some discrepancies between peptidases I and II from the two groups. Peptidases I and II isolated by Wojnarowska and Gray (1975) were found to be active against leucine-2-naphthylamide and tripeptidases while peptidase I described by Shoaf et al (1976) only had marginal activity against these substrates compared to their peptidase II. These differences highlight the difficulty encountered when comparing substrate specificity of peptidases isolated by different means from brush border membranes which in turn were prepared by different methods. The generally overlapping substrate specificities of such peptidases makes characterisation of individual enzymes more difficult still. In order to resolve these problems, the isolated peptidases will have to be studied further with respect to their chemical, physical and biological properties.

Knowledge of peptidases in the brush border membrane from human small intestine is very limited. For obvious reasons, purification studies of human brush border peptidases on the scale used by Wojnarowska and Gray (1975) and Shoaf et al (1976) are impractical and investigations have to be limited to analytical methods. Dolly and Fottrell (1969) detected several bands of peptide hydrolase activity in zymogram studies using 12,000xg supernatants of whole human mucosal homogenates. Such studies are of limited use in determining the number of enzymes involved in peptide hydrolysis in the brush border membrane. Low speed supernatant preparations,

in addition to brush border membranes, contain cytosol and lysosome components some of which are more than capable of hydrolysing many peptides. In addition, due to overlapping substrate specificity of many peptidases, no conclusions about the number of enzymes present may be reached from the number of bands containing peptidase activity.

From the results presented in this thesis it is evident that the brush border membrane from human small intestine contains seven peptide hydrolases with aminopeptidase character. Evidence from this laboratory indicates that the human intestinal brush border membrane also contains endopeptidase activity (Sterchi and Woodley 1976). Activity towards B-chain of insulin was enriched in the brush border fractions to the same extent as α-glucosidase and was not inhibited by DFP, thus ruling out pancreatic contamination. Welsh et al (1972) also reported activity towards B-chain of insulin in human brush borders and Fujita et al (1972) found such activity in rat brush borders. Whether this endopeptidase activity is due to the action of one of the seven peptidases isolated or whether it is the result of a distinct enzyme was not resolved.

Dipeptidyl peptidase IV, aminopeptidase A, γ-glutamyltransferase and aminopeptidase M have clearly been separated as distinct enzymes by electrophoresis after solubilisation with Triton X-100 and papain (see Figures 6.2 and 7.3). Separation of enzymes in these systems was due to a combination of net charge and molecular weight of the protein molecules. Evidence from SDS gels, however, indicates that hydrolysis of some substrates which appear to have been due to aminopeptidase M in Triton gels after solubilisation with either Triton X-100 or papain, could be attributed to two enzymes distinct from the aminopeptidase M. These two peptidases must therefore have

identical electrophoretic mobilities in the Triton gel system as aminopeptidase M. The first of these two peptide hydrolases was found to hydrolyse aliphatic tripeptide and, to a lesser degree, dipeptides and is referred to as peptidase 1. The second enzyme was specific for dipeptide substrates and is designated peptidase 2. These results were confirmed by the substrate specificity study using thin layer chromatography (see Chapter 5, Section 4). Evidence from gel electrofocusing experiments further suggests the presence of an additional aminopeptidase M-like enzyme (with respect to substrate specificity) which shall be called aminopeptidase M'. Both aminopeptidase M and M' are inhibited by EDTA and also by Mg^{2+}. Inhibition of aminopeptidase M' by Mg^{2+} appeared to be stronger than that of aminopeptidase M. Table 9.1 is a summary of the human brush border peptide hydrolases found and their substrate specificities.

Table 9.1 Peptide hydrolases of the human intestinal brush border membrane

Enzyme	Substrates hydrolysed
Dipeptidyl peptidase IV	glycyl-proline-2-naphthylamide
Aminopeptidase A	α-glutamic acid-2-naphthylamide
γ-glutamyltransferase	γ-glutamic acid-2-naphthylamide
Aminopeptidase M	leucine- and alanine-2-naphthylamide
	di- and tripeptides
	oligopeptides.
Aminopeptidase M'	leucine- and alanine-2-naphthylamide
	di- and tripeptides
	oligopeptides (?)

Table 9.1 continued.

Enzyme	Substrates hydrolysed
Peptidase 1	leucyl-leucyl-leucine
	leucyl-glycyl-glycine
	leucyl-leucine
Peptidase 2	leucyl-leucine, leucyl-glycine
	glycyl-leucine
	phenylalanyl-glycine

Due to differences in species, tissue preparation and isolation and separation of the peptide hydrolases, comparison of the data presented in this thesis with those obtained by other workers is difficult. Neither Wojnarowska and Gray (1975), nor Shoaf et al (1976) assayed their enzymes with substrates for dipeptidyl peptidase IV, aminopeptidase A and γ-glutamyltransferase. Taking these enzymes into account, the rat brush border membrane might contain six or seven different peptide hydrolases in all. The presence in the brush border membrane from human small intestine of an apparently "true" dipeptidase is unique in that no such enzyme has been characterised in other species. Peptidases III isolated by Wojnarowska and Gray (1975) and Shoaf et al (1976) preferentially attack dipeptides but hydrolyse oligopeptides also. With respect to dipeptide substrates, these enzymes had very similar specificities to peptidase 2 described here and it is likely that they are similar enzymes. Aminopeptidases M and M' resemble peptidases I and II respectively as described by Wojnarowska and Gray (1975). Both enzymes have a broad substrate specificity for di- and tripeptides,

hydrolyse leucine-2-naphthylamide and are inhibited by EDTA. To conclude that aminopeptidase M and aminopeptidase M' in human brush borders correspond to peptidase I and II (Wojnarowska and Gray 1975) in rat brush borders would be somewhat premature as relatively little is known about their physico-chemical properties. The possible relationship of the peptide hydrolases of the human intestinal brush border membrane with those of the same tissue from other species can only be established by purifying these enzymes in greater quantities followed by detailed investigations of their physical, chemical and biological properties. This task might well prove impossible as large quantities of viable human tissue are not readily available. Some investigators have studied peptidases in human intestine by using autopsy material (Kim et al 1972) and while this offers opportunities to obtain greater quantities of tissue, the gut is the first tissue to be affected by autolytic processes after death and results obtained from such studies must be viewed with the appropriate reservations.

9.4 The role of brush border peptide hydrolases in the intestinal absorption of proteins

Studies on the uptake of dipeptides and tripeptides have shown that amino acids presented in peptide form are taken up faster than from equivalent mixtures of free amino acids (Craft et al 1968, Adibi 1968, Matthews et al 1974). In addition, competition for uptake between amino acids is avoided when peptides are presented (Cheng et al 1971). These findings are strong evidence for the existence of separate uptake mechanisms for dipeptides and amino acids, a hypothesis strengthened by the studies on peptide uptake in Hartnup and cystinurea patients (Asatoor et al 1970, 1972). Further investigations have shown that glycyl-sarcosine is accumulated in intestinal tissue and that this accumulation is abolished by anoxia, cyanide and DNP (Addison et al 1972) - strong evidence for an energy dependent active transport system for dipeptides. Few workers in the field would therefore deny that there is an active uptake mechanism for di- and possibly tripeptides in the gut which is different from that for free amino acids. The observations in sub-cellular fractionation studies, that 90% of dipeptidase activity is located in the cytosol (Heizer and Laster 1969, Das and Radhakrishnan 1973, Peters 1970) was taken as strong evidence by many investigators that uptake of intact dipeptides and possibly tripeptides, followed by intracellular hydrolysis by cytosol peptidases, was a major route by which protein degradation products are absorbed. Brush border peptide hydrolase activity seems to have either been dismissed as being insignificant and thus not involved in peptide hydrolysis, or, as being due to contamination by peptide hydrolases from the cytosol

(Peters 1970). Evidence presented in this thesis on humans, plus evidence obtained by other groups of workers using animals, leave no doubt however, that brush border membranes from human and animal small intestine contain several peptide hydrolases distinct from cytosol enzymes. The exact number of peptide hydrolases involved has not yet been clearly established but it can be concluded that brush border membranes are capable of hydrolysing a wide range of di-, tri- and oligopeptides as well as artificial substrates such as aminoacyl-2-naphthylamides. The role these peptide hydrolases play in the absorption ie. relationship with amino acid and peptide transport, is still open to speculation.

It appears that different peptides are dealt with by the brush border membrane in different ways. Heizer et al (1972) reported 61% of peptidase activity towards phenylalanyl-glycine but only 3% of activity towards glycyl-phenylalanine in the brush border membrane. Silk et al (1976) studied the relationship between mucosal hydrolysis and transport of these same two dipeptides and found that in the jejunum there was a relationship between rates of appearance of free phenylalanine in vivo and the rates of brush border hydrolysis. The appearance of free phenylalanine in the lumen was greater from phenylalanyl-glycine than from glycyl-phenylalanine. In vitro measurements of brush border peptidase activity revealed that activity towards phenylalanyl-glycine was in fact greater than activity towards glycyl-phenylalanine. The authors concluded that in jejunum, phenylalanyl-glycine tends to be hydrolysed on the surface of the enterocyte by brush border peptidases followed by uptake of free phenylalanine and glycine. In contrast, glycyl-phenylalanine is predominantly taken up into the enterocytes intact

by an active peptide transport system, followed by intracellular hydrolysis by cytosol peptidases. Three of the peptide hydrolases from human brush border membranes are capable of hydrolysing phenylalanyl-glycine, aminopeptidase M, aminopeptidase M' and peptidase 2 (dipeptidase). Subcellular distribution studies presented in this thesis have shown that peptidase activities towards peptides with aromatic N-terminal amino acid residues have a greater brush border location than activities towards peptides with aliphatic N-terminal amino acids. Similar data were published by Peters (1970) using guinea pig tissue. This indicates that the size of the sidechain of the N-terminal residue in a peptide might be important in determining whether this peptide is to be predominantly hydrolysed by brush border peptidases or transported into the enterocyte intact. This would explain why brush border peptidase activity towards some dipeptides appears to be greater than that towards less bulky tripeptides. This is not to say that this is the only criterion which determines what happens to a peptide at the brush border membrane level. There are peptides which do not fall into the category of bulky N-terminal peptides, that appear to be mainly transported into the enterocyte intact. The classic example of such a peptide is glycyl-proline which is not hydrolysed by brush border peptidases (Kim et al 1972) but which is transported by a sodium-dependent mechanism (Rubino et al 1971). With respect to this peptide it is interesting that the brush border membrane contains an enzyme - dipeptidyl peptidase IV - the hydrolysis products of which are proline dipeptides such as glycyl-proline. The role of this peptidase is not understood but perhaps it is closely associated with the transport system for glycyl-proline.

In conclusion it appears that after a protein-rich meal many dipeptides are taken up intact into the enterocyte by carrier-mediated transport systems followed by intracellular hydrolysis by cytosol peptidases. Other dipeptides are hydrolysed on the surface of the enterocytes by brush border peptidases releasing free amino acids into the lumen from where they are recaptured and transported across the membrane by specific amino acid carrier system. If the dipeptide transport system of more hydrolysis-resistant dipeptides should get saturated, they too may be hydrolysed by brush border peptidases. Some tripeptides are possibly taken up from the lumen into the enterocyte also, by either dipeptide transport systems as suggested by Sleisenger et al (1976), or by a specific tripeptide transport system. Most tripeptides appear however, to be hydrolysed by brush border peptidases, releasing free amino acids and dipeptides. The released amino acids are again taken up, while the dipeptides produced can either be taken up or further hydrolysed by either the same enzyme or by a different one (ie. a dipeptidase). Oligopeptides with four and more amino acid residues appear to need to be hydrolysed on the cell surface to tri- and dipeptides and amino acids. These hydrolysis products may then be treated like any other such peptide. Oligopeptides are not, however, taken up into the epithelial cells intact. The brush border peptide hydrolases demonstrated in the human small intestine by the work in this thesis could cope admirably with the hydrolysis of all types of peptides which might be presented to the brush border membrane of the intestinal epithelium.

9.5 Brush border peptide hydrolases and protein malabsorption

In comparison to the well-documented disaccharidase deficiencies (Jansen and Veeger 1965, Huang and Bayliss 1967) and enterokinase deficiency (Hadorn et al 1969), no disorders due to congenital deficiencies of brush border peptidases have been reported. It has been suggested that coeliac disease, a protein-induced malabsorption syndrome, was due to a deficiency of a specific mucosal peptidase (Frazer 1956). Some support for this hypothesis has come from the work of Cornell and Townley (1973) who found that a particular fraction of a peptic-tryptic digest of wheat gliadin (the toxic factor in coeliac disease) was digested to a lesser extent by intestinal mucosa from coeliac patients. The authors concluded that coeliac disease may thus be the result of a specific peptidase deficiency which results in mucosal damage when wheat protein is ingested. Although the concept that coeliac disease is due to such a peptidase specificity has gone out of fashion as it were over the past few years, no concrete evidence is to date available on the basis of which it could be dismissed as improbable. Of the seven peptide hydrolases demonstrated in the human brush borders in this thesis, there are three which are highly specific ie. aminopeptidase A, γ-glutamyltransferase and dipeptidyl peptidase IV. A deficiency in one of these enzymes might well produce a serious disturbance such as coeliac disease. The most interesting candidate for such a deficiency in the case of coeliac disease is dipeptidyl peptidase IV, an enzyme which splits off dipeptides of the type aminoacyl-proline from peptides. Gliadin, the wheat protein toxic to coeliac patients is known to contain a large proportion of proline and it is possible

that due to a dipeptidyl peptidase IV deficiency in these subjects, this protein is not completely hydrolysed with the result that a non-degradable peptide remains in the intestine which causes the lesion characteristic for the disease.

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