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IDENTIFICATION OF A MUCOSAL FORM OF ENTEROPEPTIDASE IN TRITON X-100 EXTRACTS OF PORCINE DUODENAL MUCOSA

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Summary

Porcine enteropeptidase (EC 3.4.21.9) purified from acetone powders of fresh duodenal fluid shows a molecular weight, as determined on Ultragel AcA-34, of 190 000.

Enteropeptidase has been solubilised from pig intestinal mucosa using 1% (v/v) Triton X-100. When Triton X-100 extracts of freeze-dried mucosa after partial fractionation on DEAE-cellulose were chromatographed on Sephadex G-200, the bulk of the activity eluted in the void volume rather than with an expected V_e/V_0 ratio of about 1.24 corresponding to a molecular weight of around 200 000. Gel filtration of aqueous mucosal extracts obtained in the absence of Triton X-100 showed two regions of enzymic activity in approximately equal proportions, one in the void volume, and the other with the expected V_e/V_0 ratio of 1.24, whereas the Triton X-100 extracts of the residue from the above extract showed the presence of only the macromolecular species of enteropeptidase. This species was excluded from Sepharose 4B. It was confirmed that aminopeptidase was also extracted by Triton X-100 in a molecular form which was excluded from Sepharose 4B.

The results suggest that Triton X-100 extracts enteropeptidase with a membrane component attached and in agreement with this it was found that proteolysis rapidly converted the macromolecular form to a stable smalle molecular species corresponding in size to that found in solution in the duodenal fluid. There was full recovery of the enzymic activity following this conversion. Papain and trypsin brought about an almost complete conversion to the smaller form of enteropeptidase whereas chymotrypsin, pancreatin and an intestinal peptidase preparation were only partially effective.

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It is concluded that membrane bound enzymes such as enteropeptidase and aminopeptidase are bound to the intestinal brush border membrane in a similar manner and are not actively secreted into the lumen but rather are largely released or solubilised by the combined action of the bile and pancreatic secretions.

Introduction

Enteropeptidase (EC 3.4.21.9) catalyses the critical step in the activation of the proenzymes of the pancreatic secretion: the proteolytic conversion of trypsinogen to the active trypsin [1,2]. It has been found both in association with the mucosa of the upper small intestine [3] and in solution in the intestinal lumen [1].

In the rat, enteropeptidase has been found in the apical part of the villi [3] and in the brush border membrane of the villous epithelial cells [4]. In the guinea pig, it has been found in the membrane fraction prepared from the brush borders by Tris disruption [5]. Also preparations of closed membrane vesicles from pig duodenal brush borders were found to have associated enteropeptidase activity and in the preparation of these vesicles there was no separation of the enteropeptidase from the aminopeptidase and alkaline phosphatase activities, two markers of the brush border membrane [6]. Thus these results suggest that enteropeptidase is a constituent of the duodenal brush border.

Enteropeptidase activity is also present in the intestinal contents [3] from which it has been purified [7,8]. Likewise aminopeptidase which is used as a marker of the intestinal brush border can also be detected in the lumen but to a much smaller extent than enteropeptidase [6]. The means whereby the membrane-bound enzymes are released into the lumen is still not fully understood although it is generally held that this release is the result of desquamation of the intestinal epithelial cells. Studies on the release of enteropeptidase have shown it can be solubilised with detergents including the bile salts [7,9—12] or with proteolytic enzymes [6,11—13]. This enzyme is released most rapidly with papain but it is also released with the pancreatic enzymes, trypsin and chymotrypsin in the rat [12] but apparently not in the pig [6]. On the other hand, aminopeptidase was not readily released under these conditions [6] suggesting a different mode of attachment of these two enzymes.

It has recently been shown that aminopeptidase is extracted by Triton X-100 from the intestinal mucosa as a macromolecular species which is excluded from Sepharose 4B [14]. This species can be converted to the smaller molecular weight species found in the intestinal lumen by digestion with papain or trypsin with the release of a non-enzymatic protein which binds the catalytic unit to the membrane [15]. In this report it is shown that porcine enteropeptidase is similarly extracted by Triton X-100 in a macromolecular form which can be rapidly converted by proteolysis to the smaller molecular weight form found in the lumen. It would appear that enteropeptidase and the other brush border enzymes are bound to the intestinal epithelial membrane in a similar manner and that the release of these enzymes into the intestinal lumen is largely a consequence of the combined action of the bile and pancreatic secretions.

Materials and Methods

Triton X-100 was a B.D.H. product. Papain (2X crystallised), trypsin (2X crystallised), chymotrypsin (3X crystallised) and pancreatin were purchased from Sigma while hog intestinal peptidase was a Schwarz-Mann product.

Fresh pig duodena were obtained from the abbatoirs and immediately packed in ice. The mucosa was separated from the underlying muscular material by scraping with a glass microscope slide using slight pressure and was then dispersed in an equal volume of distilled water. This material was freeze-dried and the light brown material was stored at 4°C. Under these conditions the enzymic activity was stable over many months storage.

Enteropeptidase activity was determined as previously described [16] using bovine trypsinogen as substrate but with one modification. After the trypsinogen activation was stopped by the addition of 0.55 M HCl the assay mixtures were incubated at 50°C for 10 min. This step ensured the complete inactivation of the enteropeptidase, traces of which may otherwise carry over to the trypsin determination causing an exponential release of *p*-nitroaniline from the substrate benzoylarginine-*p*-nitroanilide [8]. Aminopeptidase activity was determined using leucine-*p*-nitroanilide [8].

Digestion of the high-molecular-weight species was carried out at 22°C for 1 h using 2% (w/w) of proteolytic enzyme. Digestion with trypsin, chymotrypsin, pancreatin and intestinal peptidase was carried out at pH 8.0 in the presence of 0.05 M $CaCl_2$ and the reaction was stopped by adjusting the pH to 6.0. Papain digestion was at pH 6.0 in the presence of 2.5 mM cysteine and 0.05 mM dithiothreitol with final pH adjustment to 7.5. The digests were stored at −20°C until required for gel filtration.

Results

In earlier studies in this laboratory, the starting material for the preparation of enteropeptidase was acetone powders of fresh pig duodenal fluid. Highly purified preparations of the fluid enzyme were obtained having a molecular weight as determined by gel filtration on Ultragel AcA-34 (LKB Produkter) of 190 000. This value is in agreement with that reported by Baratti et al. [7].

Apart from being present in the soluble form in the duodenal fluid, enteropeptidase is also found membrane-bound in association with the duodenal microvilli [3]. Therefore the isolation of the mucosal activity was attempted. The isolation procedure of Baratti et al. [7] was followed but early troubles were encountered because the solubilising agent used, sodium deoxycholate, is sparingly soluble below pH 7.0 with the result that if sodium deoxycholate is not scrupulously removed prior to the initial pH adjustment to pH 6.0, a copious precipitate forms and considerable enzymic activity is lost. Experience showed that removal of sodium deoxycholate by dialysis was difficult, time-consuming and non-reproducible. Triton X-100 was subsequently found to be a far more suitable detergent because it was soluble in aqueous buffers over a wide pH range and it was non-ionic and so did not have to be removed prior to ion-exchange chromatography. Extraction of mucosal powder with 1% (v/v) Triton X-100 resulted in a three-fold increased recovery of enteropeptidase

activity (Table I) and this result is comparable to that achieved with 2.5% (w/v) sodium deoxycholate [7].

The mucosal powder (25 g) was extracted with 500 ml 1% Triton X-100 in 0.01 M Tris/Cl^-, pH 8.0, with stirring for 45 min at 0°C. The pH was then adjusted to pH 5.0 with HCl and the extract was centrifuged at 23 000 × *g* for 40 min at 4°C. The supernatant was recovered and the pH readjusted to pH 6.0 with NaOH. The enzyme solution was ultrafiltered using an XM 50 Amicon membrane with concomittant dialysis against 0.02 M Tris/acetate/0.05 M NaCl/0.2% Triton X-100, pH 6.0. The extract was then fractionated on a 17 × 6-cm DEAE-Cellulose (Whatman DE 23) column equilibrated with the dialysis buffer. After washing the enzyme was eluted with a linear salt gradient to 0.4 M NaCl in the equilibrating buffer. The most active fractions were pooled, concentrated by ultrafiltration and the enzyme solution was applied to a 5 × 80-cm Sephadex G-200 column equilibrated with 0.02 M Tris/acetate/0.5 M NaCl/0.1 mM $CaCl_2$, pH 6.0. Enteropeptidase and aminopeptidase emerged from the column in a broad peak where the bulk of both activities were eluted in the void volume rather than with the expected V_e/V_0 ratio of about 1.24 corresponding to a molecular weight of around 200 000 similar to the fluid enzymes (Fig. 1). The possibility that the mucosal form of enteropeptidase was in fact a larger molecule than the fluid counterpart was indicated when mucosal extracts which had been stored for several months at −20°C were filtered on Sephadex G-200 (Fig. 2). Two regions of enteropeptidase activity were clearly resolved, one being eluted in the void volume as before but the other showed a V_e/V_0 ratio of 1.23. It therefore appeared that mucosal extracts contained principally a large molecular weight form of enteropeptidase (and aminopeptidase) and that this could slowly revert to a smaller species with aging. This conversion was subsequently achieved by proteolysis when eluates from the Sephadex G-200 column were incubated with papain and then rechromatographed (Fig. 3). The elution profile showed an almost total conversion of enteropeptidase to the smaller form although with the aminopeptidase the conversion was not as complete. There was no loss of activity of either enzyme. In the case of amino-

TABLE I

EXTRACTION OF ENTEROPEPTIDASE ACTIVITY FROM FREEZE-DRIED PIG MUCOSA

A. Freeze-dried mucosal powder (5 g) was extracted with 120 ml 0.01 M Tris/Cl^-, pH 8.0, at 0°C with stirring (the pH was maintained at 8.0 with NaOH). After 1 h, the pH was adjusted to 5.3 with HCl and the extract was centrifuged for 30 min at 23 000 × *g*. The pH was brought to 7.0 with NaOH. B. The pellet from A was further extracted with 100 ml 0.01 M Tris/Cl^-, pH 8.0, containing 1% (v/v) Triton X-100 and processed as in A. C. Same as A except that the extracting buffer contained 1% (v/v) Triton X-100. D. Same as C except for 0.5% (v/v) Triton X-100. E. Mucosal powder (5 g) was extracted with 120 ml 0.01 M Tris/Cl^-, pH 8.0, containing 2.5% (w/v) sodium deoxycholate at 0°C for 1 h. The extract was centrifuged at 23 000 × *g* for 30 min and the sodium deoxycholate was removed.

Additions to Extraction Buffer	Volume (ml)	Total Activity (units)
A. None	99	7.43
B. Pellet from A/1% (v/v) Triton X 100	108	13.32
C. 1% (v/v) Triton X 100	103	20.31
D. 0.5% (v/v) Triton X 100	102	18.53
E. 2.5% (w/v) deoxycholate	110	18.39

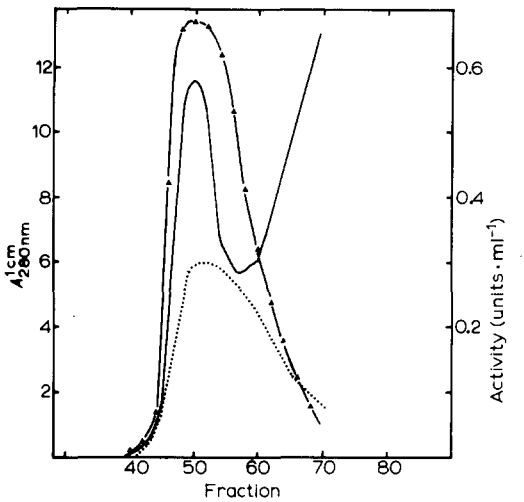

Fig. 1. Sephadex G-200 elution profile of enteropeptidase and aminopeptidase preparations isolated from pig duodenal mucosa. Freeze-dried mucosa (25 g) was extracted in the presence of 1% (v/v) Triton X-100 as detailed in the text. After partial fractionation on DEAE-cellulose, the extract was concentrated to 60 ml by ultrafiltration and applied to a 5 × 80-cm Sephadex G-200 column equilibrated with 0.02 M Tris/acetate/0.5 M NaCl/0.1 mM $CaCl_2$, pH 6.0. Elution was achieved with the equilibrating buffer at flow rate of 50 ml per h. Fraction volume was 12 ml. A^{1cm}_{280nm}, ———; enteropeptidase, ... ; aminopeptidase ▲———▲.

peptidase, these results confirmed the earlier report of Maroux et al. [14] and suggested that Triton X-100 could also extract a mucosal form of enteropeptidase which was larger than the equivalent fluid enzyme and which constituted the bulk of the enzyme in fresh detergent extracts of duodenal mucosa.

To test this, mucosal powder was extracted as described above except no Triton X-100 was added and the extract was chromatographed on a 5 × 80-cm

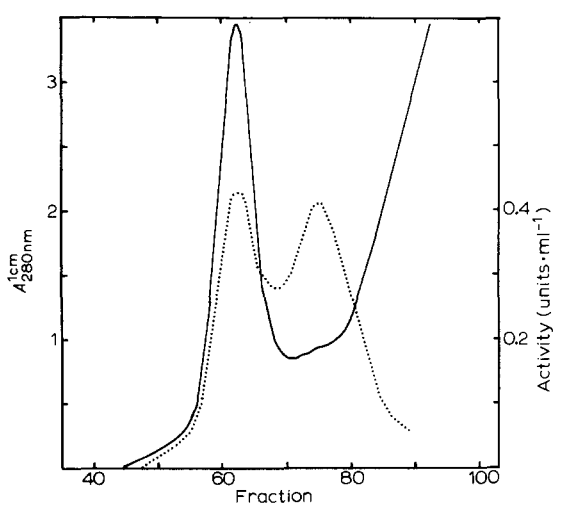

Fig. 2. Mucosal extracts prepared and fractionated on DEAE-cellulose as in Fig. 1, were stored at −20°C for several months prior to gel filtration on Sephadex G-200. Conditions were as detailed for Fig. 1 except that fraction volume was 9 ml. A^{1cm}_{280nm}, ———; enteropeptidase, ...

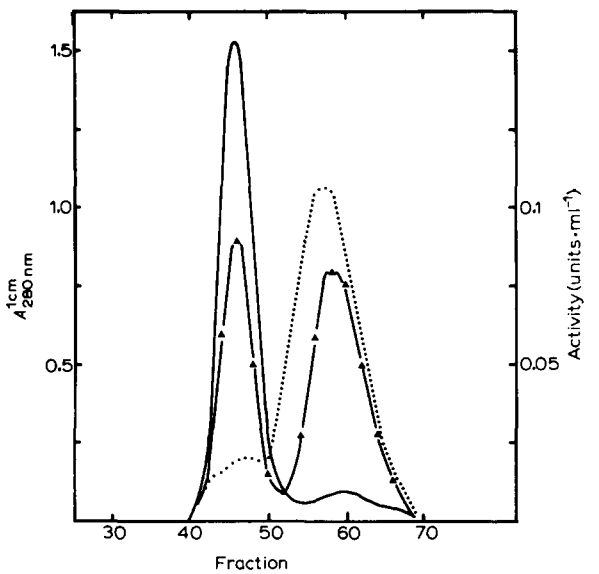

Fig. 3. Papain digestion of the macromolecular forms of enteropeptidase and aminopeptidase. Eluant from Sephadex G-200 gel filtration (Fig. 1) was concentrated to 30 ml by ultrafiltration and then incubated with 2% (w/w of protein) papain at 22°C for 1 h at pH 6.0 in the presence of 2.5 mM cysteine and 0.05 mM dithiothreitol. The pH was then adjusted to 7.5 and the solution applied to a 5 × 80-cm Sephadex G-200 column as detailed in Fig. 1. A^{1cm}_{280nm}, ———; enteropeptidase, ... ; aminopeptidase ▲———▲.

Sephadex G-200 column equilibrated with 0.02 M Tris/acetate/0.5 M NaCl/ 0.1 mM $CaCl_2$, pH 6.0. The elution profile (Fig. 4A) showed two regions of enteropeptidase activity in approximately equal amounts with the first appearing in the void volume and the second having a V_e/V_0 ratio of 1.23. The elution profile of intestinal aminopeptidase was similar with two peaks of enzyme activity. The residue from the aqueous extract above was then further extracted with 1% Triton X-100 in the extraction medium and Sephadex G-200 gel filtration of this extract showed that only the macromolecular forms of enteropeptidase and aminopeptidase were present as predicted (Fig. 4B). The bulk of the enteropeptidase and aminopeptidase activities in the detergent extract were completely excluded from Sepharose 4B (Fig. 5). Therefore the mucosal form of both enteropeptidase and aminopeptidase as extracted by Triton X-100 would appear to be much larger than their counterparts in the lumen.

Treatment of the macro-molecular species with 5 mM mercaptoethanol in the presence of 0.05 mM dithiothreitol or with 0.5 M sodium thiocyanate did not break down the mucosal species although higher concentrations of these reagents tended to destroy enzymic activity. However it was shown earlier that incubation of the macromolecular species with papain rapidly converted both enteropeptidase and aminopeptidase to low molecular weight forms with no loss of enzymic activity (Fig. 3). The enteropeptidase after papain digestion was found to have a V_e/V_0 ratio of 1.24 and a molecular weight of 190 000 in agreement with that determined for the purified fluid enzyme. Other proteo-

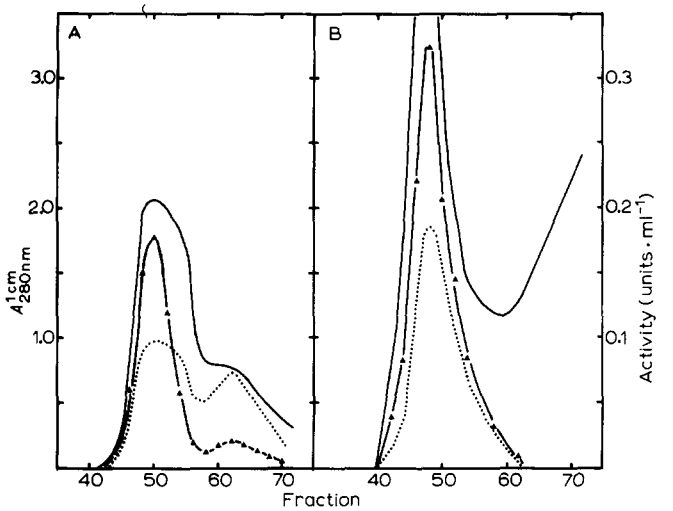

Fig. 4. Sephadex G-200 gel filtration of mucosal extracts obtained in the absence (A) and presence (B) of 1% (v/v) Triton X-100. A. Freeze-dried mucosa (5 g) was extracted for 45 min at 0°C with 120 ml 0.01 M Tris/Cl^-, pH 8.0. The pH was adjusted to pH 5.3 and the insoluble material was removed by centrifuging at 23 000 × *g* for 20 min at 4°C. The supernatant was adjusted to pH 6.0, concentrated to 20 ml, and applied to a 5 × 80-cm Sephadex G-200 column as detailed for Fig. 1. B. The pellet from the above extract was further extracted for 45 min at 0°C with 100 ml 0.01 M Tris/Cl^-, pH 8.0, containing 1% (v/v) Triton X-100. Triton X-100 was removed by dialysis and the extract was processed as in A above. A^{1cm}_{280nm}, ———; enteropeptidase, ...; aminopeptidase, ▲———▲.

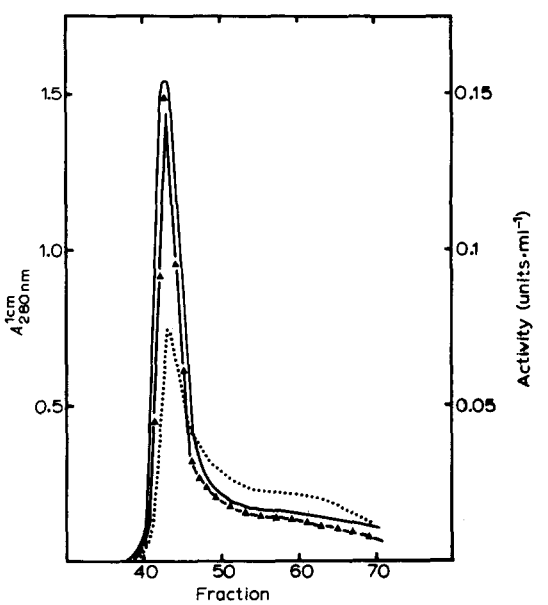

Fig. 5. Sepharose 4B gel filtration of the macromolecular form of enteropeptidase and aminopeptidase. Eluant from Fig. 4B containing the bulk of the enzymic activity was pooled and concentrated by ultrafiltration and an aliquot (10 ml) was applied to 5 × 80-cm Sepharose 4B column equilibrated with 0.02 M Tris/acetate/0.5 M NaCl/0.1 mM $CaCl_2$, pH 6.0. The column was developed with the equilibrating buffer at a flow rate of 50 ml per h. Fraction volume was 12 ml. A^{1cm}_{280nm}, ———; enteropeptidase, ...; aminopeptidase, ▲———▲.

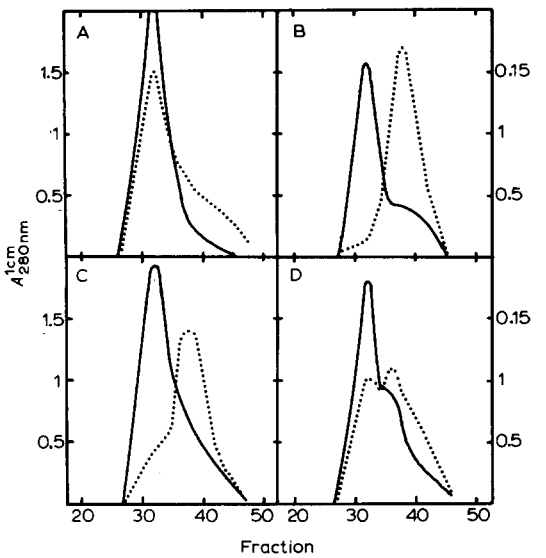

Fig. 6. Proteolytic digestion of the macromolecular form of enteropeptidase. Eluant from Sephadex G-200 gel filtration (Fig. 1) was concentrated to 30 ml by ultrafiltration. Aliquots (5 ml) were then incubated for 1 h at 22°C with various proteolytic enzymes (2% w/w of protein) as described in the text. The digests were applied to a 2.5 × 90-cm Sephadex G-200 column equilibrated with 0.02 M Tris/acetate/0.5 M NaCl/0.1 M $CaCl_2$, pH 6.0. Flow rate was 20 ml per h and fraction volume was 7.6 ml. A, Control incubation: no enzyme added; B, Papain; C, Trypsin; D, Pancreatin. A_{280nm}^{1cm}, ———; enteropeptidase, ...

lytic enzymes such as trypsin, chymotrypsin, pancreatin, and intestinal peptidase were also found to produce this same conversion of enteropeptidase to the low molecular weight species but with varying degrees of efficiency and again with no loss of enzymic activity (Fig. 6). Papain and trypsin were the most effective at this conversion while chymotrypsin and intestinal peptidase were the least effective.

Discussion

Use of detergents in the extraction medium results in a three-fold increase in enteropeptidase activity (Table I). In the case of sodium deoxycholate, if this is not scrupulously removed, essentially all of the extra enzymic activity solubilised by the detergent is lost in the copious precipitate which forms when the pH of the extract is lowered. Even after exhaustive dialysis losses still occurred at this stage again probably the result of coprecipitation. With Triton X-100, removal is not necessary and adjustment of the pH to 5.0 is recommended to remove much unwanted protein. The presence of Triton X-100 apparently ensures the continued solubility of the mucosal species.

When the extract was chromatographed on DEAE-cellulose in the absence of Triton X-100, the elution of the enteropeptidase occurred in a very broad band with little resolution and disappointing recoveries. The addition of 0.2% Triton X-100 to the column buffer improved the resolution and recovery considerably even though elution still occurred over a large volume. Thus the

absence of Triton X-100 in this ion exchange step also accounted for further losses of the large molecular species so that at this stage in the procedure of Baratti et al. [7] despite the almost certain extraction of the large molecular species by sodium deoxycholate as indicated in Table I the enteropeptidase remaining is almost solely the fluid or small molecular weight species. Subsequent gel filtration did not require the presence of Triton X-100. Attempts at further purifying this form beyond this stage have been unsuccessful.

Papain was the most effective enzyme at releasing the soluble enzyme (Fig. 6) in agreement with earlier studies on rat and pig membrane preparations [6, 12]. However no enteropeptidase (or aminopeptidase) was destroyed by papain in contrast to the losses in enteropeptidase activity reported by Louvard et al. [6] following the incubation of pig duodenal membrane vesicles with papain. This was probably due to different experimental conditions; in their study the incubation was at 37°C and the papain to protein ratio was 1 to 2.4 by weight whereas in this study the digestion was at 22°C with a ratio of 1 to 50 by weight.

Trypsin was almost as effective as papain in releasing the soluble form of enteropeptidase (Fig. 6). In fact the absence of the large molecular form in the sodium deoxycholate extracts of Baratti et al. [7] may have resulted in part from the action of trypsin which was present in their extracts over the prolonged period of dialysis required for the removal of sodium deoxycholate from their initial extracts. In this study, negligible free trypsin was detected in the extracts of the freeze-dried mucosal powder and dialysis was effected rapidly by ultrafiltration on an XM 50 Amicon membrane which had the additional benefit of allowing the removal of any tryptic (or chymotryptic) activity that may have been present.

Louvard et al. [6] were unable to detect any release by trypsin of enteropeptidase (or aminopeptidase) from their pig duodenal membrane vesicles in contrast to its effectiveness with the preparations in this study and also in earlier reports with rat brush borders [12] and the detergent form of hog intestinal aminopeptidase [14,15]. Even pancreatin, a crude pancreatic preparation, was capable of effecting a considerable conversion of the mucosal species to the smaller form over the 1-h incubation (Fig. 6). The use of EDTA in the preparation of the rat brush borders and Triton X-100 in this study could have altered the membrane in such a way as to allow tryptic release of the enteropeptidase. However it must be remembered that the lumenal fluid which normally bathes the intestinal brush borders is rich in detergent in the form of the bile salts. Thus the release of enteropeptidase and aminopeptidase from the intestinal membrane by the combined action of detergent and proteolytic digestion as shown in this study could simulate the in vivo solubilisation of the membrane-bound enzymes.

Gotze et al. [10] in a study involving perfusion of rat small intestine as well as the study of Nordström [11] have confirmed the involvement of both the bile and pancreatic secretions in the release or solubilisation of membrane-bound enzymes. Both cholecystokinin and to a lesser extent secretin stimulated the release of the intestinal enzymes, enteropeptidase, alkaline phosphatase and sucrase, into the perfusing fluid and in both cases this release was greatly enhanced by the presence of bile in the lumen. In contrast to cholecystokinin,

secretin stimulation only showed release of these when the common bile and pancreatic duct was patent suggesting that cholecystokinin had an additional direct effect on the intestinal membrane. This could be attributed to the increased motility of the gut following cholecystokinin stimulation enhancing the rate of shedding of the villous epithelial cells. However because of the rapid response shown to hormonal stimulation desquamation is hardly likely to be the sole or perhaps even the main mechanism of release of enzymes into the lumen. In addition to desquamation there is presumbaly a continual leaching of certain membrane components by a combination of detergent and proteolytic attack and this is maximised following pancreatic and bile secretion. This is in accord with the finding that the turnover rate in vitro of brush border disaccharidases was higher than the turnover rate of the epithelial cells themselves [17]. At this stage, it is unknown whether the enteropeptidase released during the perfusion studies of Gotze et al. [10] and Nordström [11] is in the macromolecular or smaller-molecular-weight form although the latter form would predominate when pancreatic enzymes are present.

Intestinal aminopeptidase which was also extracted by Triton X-100 as a macromolecular species excluded from Sepharose 4B [14] was apparently extracted as a somewhat smaller species by the detergent Emulphogen BC720. This was claimed to have similar gel filtration characteristics and therefore a similar molecular weight to the soluble or trypsin form of the enzyme [15] although the possible effect of 1% Emulphogen BC 720 on the gel filtration medium was disregarded. This detergent form of aminopeptidase when treated with trypsin yielded as a major end product a non-enzymatic, hydrophobic peptide of 9000 daltons. Maroux and Louvard [15] proposed that this digestion product was derived from a polypeptide which served to bind the catalytic unit to the membrane but which because of its hydrophobic nature caused the detergent form of aminopeptidase to aggregate. Thus the actual molecular size of the detergent form of aminopeptidase remains in doubt. Likewise the size of the macromolecular form of enteropeptidase is in doubt since the nature of this species, its size and degree of aggregation probably vary according to the detergent used for extraction. This raises the question as to what constitutes the mucosal form of enteropeptidase and other brush border enzymes. Also, the ready susceptibility of the macromolecular species to proteolysis means that the presence of the pancreatic enzymes should be monitored especially when extractions are prolonged. However, by analogy with aminopeptidase it is likely that the macromolecular species of enteropeptidase in the Triton X-100 extracts is in fact smaller than its exclusion from Sepharose 4B suggests although details of its composition must await further investigation. It would appear from these results that enteropeptidase is bound to the intestinal brush border membrane in a similar manner to aminopeptidase (and other brush border enzymes).

Enteropeptidase (and aminopeptidase) is equally active when membrane-bound or in solution in the lumen as indicated by the constancy of activity following proteolysis of the macromolecular species. Thus release of the intestinal enzymes from the membrane to the lumen must be of little consequence in determining the overall level of enzymic activity available for digestion and in the case of enteropeptidase trypsinogen activation would occur both in the

lumen and at the microvilli membrane surface so that release of the enzyme into the intestinal fluid would have no influence in controlling the activation of the pancreatic proenzymes. Furthermore, it would appear that enteropeptidase is bound to the brush border membrane in a similar manner to aminopeptidase and other brush border membranes and that these enzymes are not actively secreted into the lumen but rather are released or solubilised from the brush border membrane and shedded membrane fragments by the combined action of the bile and pancreatic secretions.

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