Biochimica et Biophysica Acta, 309 (1973) 127–137
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BBA 66885

ON THE DISTRIBUTION OF ENTEROKINASE IN PORCINE INTESTINE AND ON ITS SUBCELLULAR LOCALIZATION

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(Received December 4th, 1972)

SUMMARY

Enterokinase, which is responsible for the *in vivo* activation of pancreatic zymogens, was shown in the pig to be exclusively synthetized by the duodenal mucosa. High amounts of the free enzyme were also encountered in the ileal content. By contrast, other intestinal enzymes, such as aminopeptidase and alkaline phosphatase, were found to be essentially synthetized by the mucosa of jejunum and ileum, thus demonstrating a specialization at the enzymatic level of the 3 characteristic regions of small intestine.

Enterokinase was not observed to separate to any detectable extent from aminopeptidase and alkaline phosphatase, 2 known markers of the brush border membrane, during a number of fractionation steps leading to apparently pure preparations of closed membrane vesicles from duodenal brush border. The enzyme was removed very fast from the vesicles by papain, but not by trypsin, chymotrypsin and subtilisin. These results confirm that enterokinase is attached to the external side of the duodenal brush border membrane.

INTRODUCTION

The fact that enterokinase (enteropeptidase, EC 3.4.4.8) is a key enzyme for digestion has recently been demonstrated by two independent findings: (a) the proteolytic zymogens of pancreas are not activated in the duodenal juice of children suffering from a congenital enterokinase deficiency[1,2] and (b) trypsinogen is a much better substrate for enterokinase than for trypsin (K_m, 6 times lower; k_{cat}, 2000 times higher) because of the absolute specificity[3] of the first enzyme for the polyaspartyl–lysine structure present in the N-terminal sequence of all trypsinogens so far known[4*]. Enterokinase is largely responsible for trypsinogen activation which, *via* the generated trypsin, controls the activation of the other pancreatic zymogens. The biological

* The sequence is $(Asp)_4$–Lys, except in lungfish trypsinogen[5] where it is $(Glu)_2$–Asp–Lys.

importance of this enzyme fully justifies a careful study of its localization in the intestine.

When the purification of porcine enterokinase was first undertaken in our laboratory, it was soon realized that, in contrast with earlier claims[6], the bulk of the enzyme was not in duodenal juice, but in the mucosa from which it could be extracted by sodium deoxycholate (Baratti, J., Maroux, S., Louvard, D. and Desnuelle, P., unpublished). Meanwhile, Nordström and Dalqvist[7] reported, using a cryostat cutting technique, that the specific activity of enterokinase was higher in the tips of the villi all along rat duodenum and gradually declined towards the crypts. They showed later[8] that an enterokinase activity could be measured in the brush borders arising from duodenal microvilli during rat mucosa homogenization in 5 mM EDTA according to Miller and Crane[9]. A further step forward was made when Holmes and Lobley[10] announced in a short note that enterokinase was associated with the membrane fraction of the brush borders which could be separated after dissociation by 1 M Tris[11].

Enterokinase was also reported by Nordström[12] to be removed faster from the brush borders than known markers of the membrane such as aminopeptidase, alkaline phosphatase and the disaccharidases[12]. Enterokinase was also observed to be solubilized by trypsin and chymotrypsin, whereas the other enzymes were not. These observations were interpreted as suggesting that enterokinase is superficially located in the brush border membrane in a probably mosaic-like pattern[12].

The main purpose of this paper is to show that enterokinase is exclusively biosynthetized in the pig by duodenal mucosa and to see to what extent the recently described preparation of closed brush border vesicles from porcine duodenum in the absence of EDTA[13] may contribute to a better understanding of the subcellular localization of the enzyme.

METHODS

Determination of enzyme activities

Enterokinase was determined as previously described[3] through its activating effect on bovine trypsinogen (Worthington, once crystallized). The resulting trypsin was measured titrimetrically with the substrate *N*-α-benzoyl-L-arginine ethyl ester or *N*-α-tosyl-L-arginine methyl ester. Aminopeptidase and alkaline phosphatase activities were evaluated spectrophotometrically with the aid of the substrates L-alanine *p*-nitroanilide[14] and *p*-nitrophenylphosphate[13], respectively.

Preparation of duodenal vesicles

Closed vesicles known to contain the brush border membrane, the fuzzy coat normally covering the external side of this membrane and a fibrous material perhaps related to the cross filaments of the microvilli were prepared from porcine duodenum mucosa by a recently described technique[13]. This technique includes mucosa homogenization in 10 mM Tris–HCl buffer (pH 7.3) containing 10 mM $MgCl_2$, 1 mM $CaCl_2$ and 0.25 M sucrose, removal of nuclei, mitochondria, and endoplasmic reticulum by a succession of low- and high-speed centrifugations, and finally a centrifugation in a discontinuous sucrose gradient. The vesicles thus obtained were sedimented and the pellet was resuspended in a small volume of 10 mM Tris–HCl buffer (pH 7.3) con-

taining 10 mM $MgCl_2$ and 150 mM NaCl (Buffer A) at a concentration of 10 mg proteins per ml.

Further purification of the duodenal vesicles

The procedure outlined above was observed to lead to essentially pure preparations of jejunal brush border vesicles. But, duodenal vesicles were revealed by analysis to contain small amounts of several contaminants which should be eliminated by a last centrifugation through a linear sucrose gradient from 22 to 48% in Buffer A. The gradient was prepared with the aid of an Isco gradient former in 17-ml tubes of the small Spinco–Beckman swinging bucket rotor. The above mentioned vesicle suspension was layered at the top of the gradient and the tube was spun for 18 h at 22 000 rev./min. Fractions (1 ml) were collected with an Isco density gradient fractionator into small test tubes plunged in ice. The vesicle-containing fractions were pooled and diluted. The vesicles were collected by a 30-min centrifugation at 105 000 $\times$ *g*.

Enzymatic digestion of the vesicles

For papain, the Worthington 39 mg/ml suspension was diluted 10-fold by a 50 mM sodium phosphate buffer at pH 6.2 containing 1 mM cysteine and 0.03 mM dithiothreitol, and it was activated by a 1-h incubation at 4 °C. A papain unit was defined as the amount of enzyme hydrolyzing 1 μmole of *N*-α-benzoyl-L-arginine ethyl ester per min at 25 °C and pH 6.2 (substrate concentration, 25 mM). The activated enzyme solution (0.3 ml) containing 0.8 unit was added to 1.2 ml of the purified vesicles suspended in the reactivation buffer (2.8 mg of protein). Aliquots (0.3 ml) were periodically removed to follow kinetically the enzyme liberation due to the progressive digestion of the vesicles by papain at 37 °C. For this purpose, digestion was stopped by diluting the aliquot to 1 ml with a 50 mM phosphate buffer at pH 7.3 and cooling down to 4 °C. The free enzyme fraction was measured after filtration of the suspension through 0.33 μm Millipore filters.

Digestions with trypsin, chymotrypsin and subtilisin were carried out at 37 °C for 1 or 2 h. The vesicles were suspended (1.9 mg protein per ml) in a 10 mM Tris–HCl buffer (pH 7.3) containing 0.15 M NaCl and 10 mM $MgCl_2$. The suspensions were incubated with an amount of enzyme corresponding to 5% (trypsin and chymotrypsin) or 10% (subtilisin) of the protein weight and the assays were pursued as indicated above.

Fractionation of porcine intestine

Small intestines were removed from fasted pigs immediately after the death of the animals in the slaughterhouse and they were cut into the 3 anatomically and functionally distinct regions: duodenum, jejunum and ileum. The first two were found to be empty whereas the third was filled by 160–820 ml of a liquid which was collected. The pieces were washed by 0.9% saline (50 ml for duodenum; 400 ml for jejunum and ileum). The resulting liquids were designated as the washings. The mucosa itself was extruded from each piece by passage through closely adjusted rubber rollers, and homogenized with twice its weight of 0.9% saline for 5 s in a Waring blendor. The liquid found in the ileum was designated as ileal content. The fractions thus obtained were centrifuged at 105 000 $\times$ *g* for 30 min. The enzymatic

TABLE I

DISTRIBUTION OF ENTEROKINASE, AMINOPEPTIDASE AND ALKALINE PHOSPHATASE ALONG PORCINE SMALL INTESTINE

The figures in the table indicate the activities found in each fraction in percentage of the total. They are average values calculated from several independent assays. The figures in parentheses given for enterokinase indicate the extreme values.

Enzyme	*Number of assays*	*Mucosa*			*Washings*			*Content*			*Total*
		Bound	*Free*	*Total*	*Bound*	*Free*	*Total*	*Bound*	*Free*	*Total*	
Duodenum											
Enterokinase	5	38 (27–68)	13 (6–35)	51 (40–77)	0	6 (0–15)	6 (0–15)	—	—	—	57 (40–77)
Aminopeptidase	2	0.9	0	0.9	—	—	—	—	—	—	1
Alkaline phosphatase	2	2.8	0	2.8	—	—	—	—	—	—	3
Jejunum											
Enterokinase	2	0	0	0	0	0	0	—	—	—	0
Aminopeptidase	2	18.5	0.5	19	14	1	15	—	—	—	34
Alkaline phosphatase	2	14.3	0.7	15	7.8	0.2	8	—	—	—	23
Ileum											
Enterokinase	5	0	0	0	0	4	4	0	39 (19–55)	39 (19–56)	43 (23–60)
Aminopeptidase	2	31	3	34	6.4	1.6	8	11	12	23	65
Alkaline phosphatase	2	37.5	1.5	39	4.7	0.3	5	19	11	30	74

activities measured in the sediment were considered as belonging to molecules bound to intact membranes, membrane sheets or other structures. The activities found in the supernatant were the property of isolated or "free" molecules.

Preparation of pancreas extracts

Porcine pancreas, used here as a source of trypsin activity, were also removed at the slaughterhouse, freed from visible adipose and connective tissue and homogenized for 1 min in a Waring blendor with 4 times its weight of 5 mM Tris–HCl buffer (pH 7.9) containing 40 mM NaCl and 50 mM $CaCl_2$. The homogenate was filtered through gauze and centrifuged at 40 000 × *g* for 30 min. An aliquot was activated by an 18-h incubation at 4 °C with a trace of pure enterokinase.

RESULTS

Enterokinase distribution along porcine intestine

Table I and Fig. 1 indicate the level of free and bound enterokinase, aminopeptidase and alkaline phosphatase in porcine duodenum, jejunum and ileum. Several independent assays were carried out in each case and the results were averaged. As already mentioned in the Method section, free- and bound-enzyme activities were separated by a 30-min centrifugation at 105 000 × *g*. The free fraction was recovered in the supernatant and the bound fraction in the sediment. Trypsin levels were considered as characteristic of an enzyme directly entering the intestine in the free form.

The significance of the results presented in Table I and Fig. 1 depends on the stability of the enzymes under the conditions existing in the intestine. During a 19-h incubation of the ileal content at 37 °C, bound aminopeptidase and alkaline phosphatase were stable and very slowly solubilized. Free alkaline phosphatase and enterokinase were also stable for 19 h. However, free aminopeptidase was almost

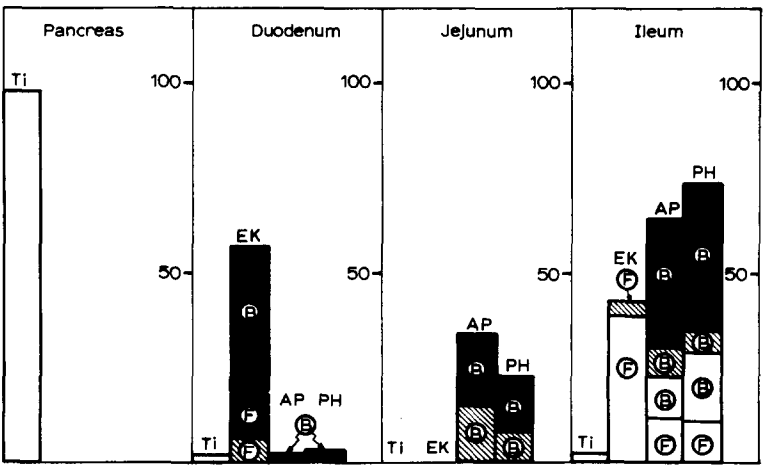

Fig. 1. Free and bound enzymes in porcine duodenum, jejunum and ileum. Ti, active trypsin; EK, enterokinase; AP, aminopeptidase; PH, alkaline phosphatase. The letters B and F in circles designate the bound and free enzyme fractions, respectively. Black, hatched and white regions indicate the activities (expressed in per cent of the total) present in the membrane fraction, the washings and the ileal content, respectively.

completely inactivated during the same period. Therefore, the proportions of the free enzyme shown in Fig. 1 and Table I to be present in ileal content are probably too low. A special assay was performed for trypsin. A known amount of the crystalline enzyme was added to a sample of the ileal content and the mixture was incubated at 37 °C as above. After 19 h, 65% of the initial activity was still measurable. Moreover, the liquid in the ileum was observed to contain substantial quantities of bile salts which, according to Hadorn *et al.*[15], may activate enterokinase. In fact, the activation of enterokinase by addition of a sample of the ileal content was found to be 2.3-fold for the bound form and 1.8-fold for the free form. The results were accordingly corrected.

When all these observations are taken into account, the results in Table I and Fig. 1 show that enterokinase is present in the duodenal membrane where it is predominantly in the bound form and in the ileal content where it is exclusively in the free form. By contrast, bound aminopeptidase and alkaline phosphatase are encountered in jejunum and ileum. These enzymes are partly free in ileum.

Subcellular distribution

As already mentioned in the Introduction, the procedure recently worked out for the purification of duodenal and jejunal brush border vesicles was monitored by the determination in all fractions of 2 membrane markers, aminopeptidase and alkaline phosphatase. In 9 independent assays, enterokinase activity was also measured with the results listed in Table II.

The 3 enzymes were also evaluated in the fractions resulting from a centrifugation of the duodenal vesicles in a linear sucrose gradient (see Methods). Their distribution is indicated in Fig. 2.

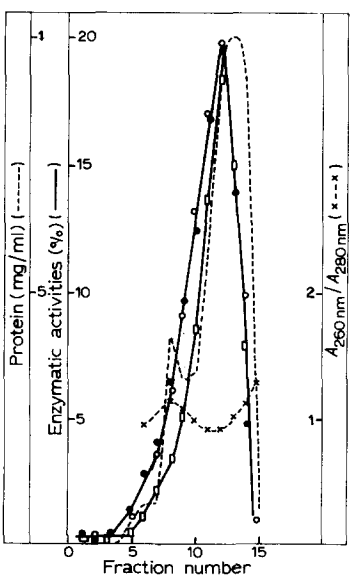

Fig. 2. Enzyme distribution after centrifugation of crude vesicle preparations in a linear sucrose gradient. Ordinates on the left indicate the enzymatic activities in per cent of the total submitted to centrifugation. ●—●, enterokinase; ○—○, alkaline phosphatase; □—□, aminopeptidase. The $A_{260\,nm}/A_{280\,nm}$ values plotted on the right designate the absorbance ratio of the fractions at 260 and 280 nm.

TABLE II

ENTEROKINASE DISTRIBUTION DURING THE PURIFICATION OF DUODENAL BRUSH BORDER VESICLES[13]

Yields are not cumulative, but given after each step. The figures in parentheses indicated the purification effect attained after each step for each enzyme (basis: 1.0 in the crude homogenate). All the figures are average values derived from 9 independent assays.

Purification step	*Yields (units %)*			*Specific activity (units per mg protein)*		
	Enterokinase	*Alkaline phosphatase*	*Aminopeptidase*	*Enterokinase*	*Alkaline phosphatase*	*Aminopeptidase*
Crude homogenate	100	100	100	3.6 (1.0)	83 (1.0)	61 (1.0)
Supernatant S1 (600 × *g*; 10 min)	68	60	63	6.0 (1.7)	142 (1.7)	107 (1.7)
Supernatant S_2 (10 000 and 12 000 × *g*; 10 min)	86	91	84	7.0 (2.0)	165 (2.0)	127 (2.1)
Pellet P_1 (105 000 × *g*; 30 min)	87	102	73	18 (5.0)	400 (4.7)	237 (3.9)
Supernatant S_4 (10 000 × *g*; 5 min)	68	56	69	20 (5.5)	457 (5.5)	306 (5.0)
Fraction II (after gradient centrifugation)	75	71	75	34 (9.7)	888 (10.7)	552 (9.0)

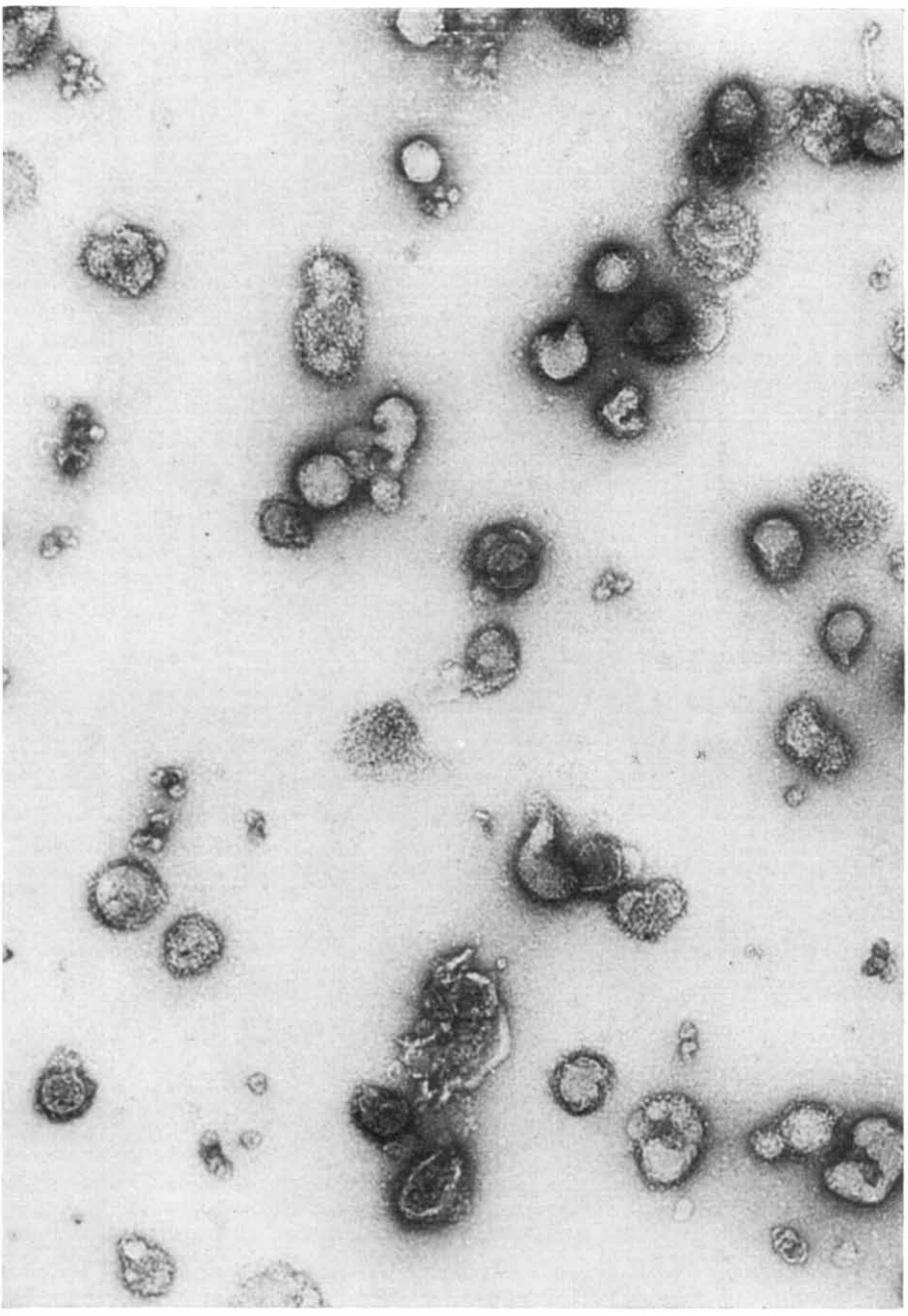

Fig. 3. Typical electron microscope picture of the vesicles in Fraction 11 of the graph presented in Fig. 2. Negative staining was obtained by treatment with uranyl acetate. Magnification × 60 000.

Table II and Fig. 2 demonstrate that none of the steps used for the fractionation induced any detectable separation of enterokinase from alkaline phosphatase and aminopeptidase. As shown by Fig. 3, the vesicle preparations in which the final activity determinations were carried out, appear to be free of any visible contaminant. Therefore, all these data strongly support the assumption that enterokinase is indeed a constituant of the duodenal brush border membrane.

To return now to Fig. 2, the crude duodenal vesicles prepared by our previous technique[13] are seen to contain a previously mentioned material, strongly absorbing at 260 nm and devoid of any membrane marker activity. This material is seen in Fig. 2 to split during centrifugation into a lighter and a heavier fractions situated on both sides of the vesicle peak. These fractions probably correspond to differently aggregated endoplasmic reticulum.

A last information about enterokinase localization inside the brush border membrane was derived from digestion assays of the vesicles with 4 proteolytic enzymes: papain, trypsin, chymotrypsin and subtilisin. The rate of enterokinase, aminopeptidase and alkaline phosphatase removal during digestion by papain is shown in Fig. 4. About $\frac{3}{4}$ of the total aminopeptidase activity is seen to be liberated very fast while the last $\frac{1}{4}$ remains tenaciously attached to the vesicles. Enterokinase

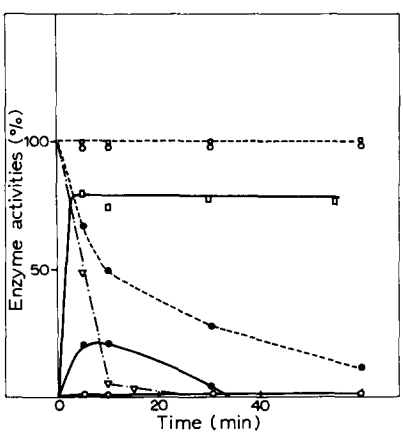

Fig. 4. Solubilization of enterokinase , alkaline phosphatase and aminopeptidase during digestion of the duodenal vesicles by papain. □---□ and ○---○, total (free + bound) aminopeptidase and alkaline phosphatase. □—□ and ○—○, progress of aminopeptidase and alkaline phosphatase liberation during digestion. ●---●, total enterokinase. ●—●, liberated enterokinase remaining at time t. ▽--▽ are related to special assays in which pure enterokinase was incubated with papain under the conditions of the vesicle digestion. This latter curve indicates that free enterokinase is still more labile towards papain than the bound form.

is also rapidly solubilized by papain. But, the rate in this case cannot be exactly evaluated because of the unexpected lability of the enzyme in the presence of papain. Free enterokinase is still more labile towards papain than the bound form.

Digestion of the vesicles by trypsin, chymotrypsin or subtilisin was not observed to induce any solubilization of enterokinase, aminopeptidase and alkaline phosphatase. These latter were not inactivated during incubation.

DISCUSSION

The data presented above appear to shed some light on the distribution and subcellular localization in porcine intestine of enterokinase as well as of intestinal aminopeptidase and alkaline phosphatase. Table I and Fig. 1 show that membrane-bound enterokinase is almost exclusively present in duodenal mucosa. No trace of the enzyme has ever been characterized in jejunal and ileal mucosa. This observation clearly designates duodenal mucosa as the unique site of enterokinase biosynthesis in the pig. The smaller part of the enzyme found in the free form in the mucosa most probably arises from membrane disruption during homogenization. Enterokinase can be expected to be generated by intestine in the vicinity of the outlet of the Wirsung duct since pancreatic zymogens have been shown to be fully activated in normal human duodenal juice[16]. It has also been reported to be a duodenal enzyme in the guinea pig[10], but to be present in all regions of the small intestine in the rat[8]. However, the bound and free forms of the enzyme were not separated in these studies. Only the localization of the bound form is significant for biosynthesis.

Moreover, enterokinase was found to be very low in the jejunum of the fasted pig, but to accumulate for a still unknown reason in ileum content where it was entirely free. The proportions of free enterokinase in ileum are seen in Fig. 1 and Table I to be distinctly higher than those of the other enzymes. This fact cannot be interpreted as suggesting the existence for enterokinase of a special secretory mechanism, since free aminopeptidase has been shown to be labile in the intestine and alkaline phosphatase to be strongly attached to the membrane (see later). By contrast, the observation made in the course of this work that bound enterokinase is still more effective for trypsinogen activation than the free form (K_m = 25 and 70μM, respectively) is probably significant in this respect. It proves that solubilization is not an absolute prerequisite for enterokinase to activate trypsinogen very fast.

An additional remark is that our observations related to trypsin give no support to the assumption that enterokinase flow along the intestine should be synchronized with pancreatic secretion. This important point, however, cannot be settled by a limited number of assays uniformly carried out with fasted pigs.

As far as the two other enzymes are concerned, it is appropriate at first to stress that porcine intestinal mucosa as a whole contains much more aminopeptidase and alkaline phosphatase than enterokinase. The representation in percentage adopted in Fig. 1 and Table I may be misleading in this respect. Moreover, in sharp contrast with enterokinase, they are low in duodenal mucosa, but very high in jejunal and ileal mucosa by which they are visibly synthesized. This unequal distribution clearly shows that the enzymatic equipment of the 3 morphologically distinct regions of small intestine may be quite different in accordance with their function.

Some conclusions related to the subcellular localization of enterokinase have already been formulated in Results. No detectable separation between enterokinase on one hand, aminopeptidase and alkaline phosphatase on the other has ever been observed in the course of a number of fractionation steps leading to highly purified duodenal brush border membrane vesicles. Aminopeptidase and alkaline phosphatase are known to be two of the most characteristic markers of this membrane. The vesicles were prepared in the absence of metal-chelating EDTA so that enzyme determinations in the fractions caused no problem and quantitative balance-sheets

or diagrams could be established which, like those reproduced in Table II and Fig. 2 definitely proved that enterokinase is a constituant of the duodenal brush border.

Finally, the fact that our observations during digestion of the vesicles markedly differ from those previously reported by Nordström[12] after digestion of rat brush borders is noteworthy. Enterokinase was invariably observed to be solubilized very fast. But, it could not be confirmed here that this solubilization was indeed faster than that of aminopeptidase, because of the unexpected lability of free and perhaps bound porcine enterokinase towards papain. This first discrepancy may arise from a species difference, porcine enterokinase having an essential, papain-sensitive bond which would not exist in the rat enzyme. Another and more important finding was that alkaline phosphatase was not removed at all from the vesicles by papain and that trypsin, chymotrypsin and subtilisin digestions had no detectable effect on the 3 enzymes. Alkaline phosphatase was observed by Nordström[12] to be solubilized from the brush borders by papain, although at a slower rate than for the other enzymes. Moreover, an appreciable liberation of enterokinase by trypsin and chymotrypsin was reported by Nordström who attributed to this process a physiological role for the passage of the enzyme into the intestinal lumen.

The mode of attachment of a number of intestinal enzymes to the brush border membrane and the mechanism by which they are freed under the influence of proteolytic digestion are still poorly understood. The removal of an enzyme from a membrane is generally interpreted in terms of accessibility or location of the enzyme on the external side of the membrane. However, the accessibility towards an enzyme of any bond in any macromolecule or plurimolecular system does not depend merely on the position of the bond, but also on the nature of the adjacent structure which may, or may not, correspond to the specificity of the enzyme. In addition, the above concept requires that the external side of the membrane is unambiguously defined in the investigated material. In this respect, it is probably noteworthy that the vesicles employed in the present work have been prepared by a mild treatment avoiding the use of EDTA and that they can be seen under the electron microscope (Fig. 3) to be closed with their outside corresponding to the external side of the membrane characterized by the fuzzy coat[13]. Brush borders are open structures in which the internal side of the membrane, although less accessible than the external side, may be attacked by enzymes.

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