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THE AMINOPEPTIDASE FROM HOG INTESTINAL BRUSH BORDER

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SUMMARY

The aminopeptidase bound to the brush border membrane of porcine jejunum and ileum has been purified. After treatment of the mucosa with Triton X-100, the enzyme was found to be still attached to very small membrane fragments (the aminopeptidase complex) from which it was liberated by trypsin. The free enzyme could be further purified to homogeneity by using classical techniques. The high molecular weight complex was shown to contain, besides aminopeptidase, at least one other protein inactive on the substrates of this enzyme.

The free aminopeptidase was observed to have a molecular weight of about 280 000, and to contain 23% sugars and 2 atoms of zinc per mole. Gel electrophoresis in sodium dodecyl sulfate showed 4 bands. One, however, is probably an artefact arising from trypsin treatment. The other 3 suggest the existence in aminopeptidase of 3 subunits with molecular weights of 130 000, 97 000, 49 000 and which were also obtained from the complex.

The aminopeptidase which has been purified accounts for all, or almost all, of the peptidase activity of the brush border membrane. It also accounts for all of the arylamidase activity, all of the activity against an heptapeptide substrate, about half the tripeptidase activity and a small but apparently significant part of the dipeptidase activity of the whole jejunal and ileal mucosa.

INTRODUCTION

The mucosal cells of the small intestine are known to contain a number of hydrolases which can be assumed to play an important role in the final stage of intraluminal digestion and in absorption[1,2]. Further information about the subcellular localization and properties of these enzymes will undoubtedly lead to a better understanding of their biological significance.

Several disaccharidases bound to the brush border membrane have already been identified and even partially or complely purified[3–5]. The situation is less clear, however, in the case of intestinal peptidase because of their relatively low specificity. For instance, the substrates Gly–Gly, Leu–Gly and Gly–Phe have been reported to

be hydrolyzed in several species by dipeptidases present in the soluble fraction of enterocytes[6–8], whereas 60% of the activity towards Phe–Gly and Phe–Ala has been found in the rat to be associated with the brush border membrane[8,9]. Moreover, at least 2 enzymes with tripeptidase activity, one soluble and the other membrane-bound, have been observed in enterocytes[10]. The relative proportions of these 2 activities, however, appear to depend on the method used to prepare the brush border.

In 1944, Smith *et al.*[11] partially purified from hog intestinal mucosa a soluble peptidase designated leucine aminopeptidase and characterized both by its activity on Leu–Gly, Leu–Gly_2 and leucinamide, and by its sensitivity towards Mn^{2+} and Mg^{2+}. These properties enabled its separation from a second soluble enzyme insensitive to Mn^{2+} and inactive towards Leu–Gly.

Like many other plasma membranes, the brush border membrane of the enterocytes shows activity towards leucine-β-naphthylamide. This "chromogenic" substrate was introduced by histologists as a substitute for leucinamide[12]. However, it is now clear that the 2 compounds are not always hydrolyzed by the same enzymes, with the consequence that enzymes acting on aminoacid-β-naphthylamides are often referred to as arylamidases[13–14]. In contrast, the characterization of this type of activity associated with the intestinal brush border membrane led other authors to postulate the existence in the membrane of an aminopeptidase, or even of a leucine aminopeptidase[15–16], that would be similar to the membrane-bound, Mn^{2+}- and Mg^{2+}-insensitive kidney aminopeptidase M[17]. Kidney aminopeptidase M has been observed to act on aminoacid-β-naphthylamides, aminoacid-p-nitroanilides and peptides.

It is probably of interest to point out here that another source of confusion in the field of intestinal peptidases is due to the fact that all the enzymes so far identified are metalloenzymes but the procedures employed until recently for the isolation of the brush border membrane involve the use of a strong metal-chelating agent, EDTA. In this respect, the situation can undoubtedly been improved by the use of a new method developed last year in our Laboratory[18] for the isolation, in the absence of EDTA, of the duodenal or jejunal brush border membrane in the form of closed vesicles sedimenting with the microsomal fraction of the mucosa. Taking advantage of the fractionation procedure finally leading to purified membrane vesicles, the subcellular localization of activities towards various substrates of dipeptidases, tripeptidases, aminopeptidases, arylamidases and endopeptidases were carefully ascertained. In hog jejunum, approximately 90% of the dipeptidase activity of the intestinal mucosa was found to be soluble, whereas the tripeptidase activity was equally divided between the cytoplasm and the brush border membrane. Arylamidase activity, as well as activity towards the heptapeptide Val–Ala_2–Lys–Ile–Val–Gly, was observed to be entirely localized in the membrane and to correspond to a unique aminopeptidase (EC 3.4.1-2), which turned out later to be responsible for all or almost all the proteolytic activity of the membrane. This aminopeptidase has been purified and some of its properties have been investigated.

MATERIALS AND METHODS

Substrates

L-Aminoacid-β-naphthylamides, L-alanine-p-nitroanilide and the peptides Gly_2,

Leu–Gly, Gly_3 and Ala–Gly_2 were Cyclo chemical products. The heptapeptide Val–Ala_2–Lys–Ile–Val–Gly was a gift from Drs Svarda and Bricas.

Total protein and sugar estimation

Total proteins were estimated spectrophotometrically at 280 nm using the extinction coefficient ($E\ {}^{1\%}_{1\,cm}$) of purified aminopeptidase (15.6). Neutral sugars, amino sugars and sialic acids were evaluated, respectively, by the phenol–sulfuric acid method[19], the Elson–Morgan technique as modified by Montreuil and Spick[20], and by reaction with thiobarbituric acid[21].

Enzyme activities

Activity towards amino acid-β-naphthylamides was measured colorimetrically after diazotation of the resulting naphthylamine[22]. The activity towards L-alanine-*p*-nitroanilide (AAN) was evaluated according to Roncari and Zuber[23], except that the Tris was replaced by a 50 mM phosphate buffer at pH 7.0. The molar extinction coefficient of the liberated *p*-nitroaniline was taken as 8800 $M^{-1} \cdot cm^{-1}$ (ref. 24). For the determination of activities towards peptides, 200 μl of a 30 mM peptide solution in 50 mM phosphate buffer pH 7.0 were incubated with enzyme at 37 °C. Samples (10 μl) were withdrawn and diluted with 1 ml of a 0.2 M sodium citrate buffer at pH 2.2. The nature and proportions of the liberated amino acid(s) were ascertained on 200 μl of the diluted sample with the aid of a Beckman analyzer Model 120 C equipped with an expanded scale. When only glycine and alanine were present, the column was eluted with a 0.2 M citrate buffer at pH 3.2, while standard conditions were used when a complete analysis was required. All enzyme activities (units) were expressed as nmoles of substrate hydrolyzed per min.

Polyacrylamide gel electrophoresis

Electrophoresis in the absence of sodium dodecyl sulfate was carried out in a Canalco apparatus using the techniques recommended by the manufacturer. In some assays, the position of the aminopeptidase band was made visible by the yellow color developing during incubation of the gel with 10 ml of a 1.5 mM alanine-*p*-nitroanilide solution in the phosphate buffer. In the presence of sodium dodecyl sulfate, the technique of Weber and Osborn[25] was employed.

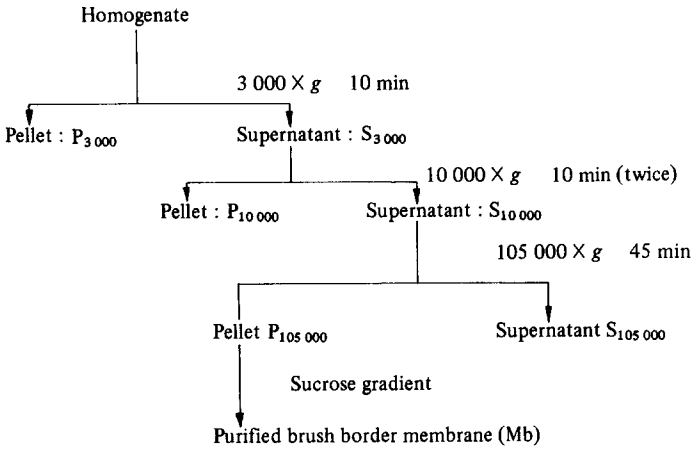
Ultracentrifugation

Preparative high speed centrifugations were carried out in a Beckman Ultracentrifuge Model L2-65 B with an angle rotor Model 50 Ti. The *g* values quoted are calculated for the top of the tubes. Sedimentation coefficient and molecular weight determinations by the sedimentation equilibrium technique of Yphantis were performed with a Spinco Beckman Model E analytical ultracentrifuge.

RESULTS

Subcellular distribution of peptidase activities in hog jejunal mucosa

The procedure already described[18] for the preparation from hog duodenum and jejunum of the brush border membrane in the form of closed vesicles, is summarized in Scheme I. This procedure essentially consists of the separation of the microsomal



Scheme 1. Flow diagram for the purification of brush border membrane originating from porcine jejunal mucosa. For experimental details, see ref. 18.

fraction of the mucosa by a series of centrifugations at 3000, 10 000 and 105 000 × *g* under conditions inducing the aggregation and, consequently, the early removal of endoplasmic reticulum. The pellet finally obtained by a 45-min centrifugation at 105 000 × *g* is further purified by a centrifugation through a discontinuous sucrose gradient to give highly purified brush border membrane preparations.

Activities of the jejunal mucosa towards several peptidase substrates have now been measured after each centrifugation in both pellet and supernatant. The results of this investigation are given in Table I where they are set out as percentages of the total activity submitted to centrifugation at each step. Recovery was always better than 80%, as would be expected if only mechanical losses had occurred during the handling of the fractions. The enzyme distribution observed after the first centrifugation at 3000 × *g* cannot be considered as significant because of the mucus which considerably increases the volume of the corresponding pellet (35–45% of the initial

TABLE I

ACTIVITIES TOWARDS VARIOUS SUBSTRATES IN FRACTIONS DERIVED FROM PORCINE JEJUNAL MUCOSA

The separation of the fractions is described in Scheme I. The figures indicate for each substrate the distribution in % of activity between pellet and supernatant after each centrifugation. These figures are the averages of 3 assays, except for the heptapeptide and *p*-nitrophenylacetate (one assay only).

	Alanine-p-nitro-anilide	*Leucine-β-naphthyl-amide*	*Lysine-β-naphthyl-amide*	*Hepta-peptide*	Gly_3	*Ala–* Gly_2	Gly_2	*Leu–Gly*	*p-Nitro-phenyl-acetate*
$S_{3\,000}$	57	54	53	–	58	63	59	67	30
$P_{3\,000}$	43	46	47	–	42	37	41	33	70
$S_{10\,000}$	67	62	69	–	76	87	90	89	65
$P_{10\,000}$	33	38	31	–	24	13	10	11	35
$S_{105\,000}$	8	9	9	16	45	68	80	83	90
$P_{105\,000}$	92	91	91	84	54	32	20	17	10

homogenate volume). But, further fractionations at 10 000 and 105 000 × *g* clearly show that the bulk of the activity towards the dipeptidase substrates (Gly_2 and Leu-Gly) remains in the soluble fraction, whereas the activity towards the tripeptidase substrates (Gly_3 and Ala–Gly_2) is partitioned between the soluble fraction and the membranous material sedimenting at 105 000 × *g*. Practically all the arylamidase activity and the activity towards the heptapeptide are also found to be bound to the membrane.

Table II, in which the activities are calculated by reference to that with alanine-*p*-nitroanilide taken as a conventional standard (see later), extends to more purified fractions the results presented in Table I. The dipeptidase activity is seen to be significantly lowered (but not completely removed) by the last gradient centrifugation leading to the preparation of the vesicles. But, the other activities towards tripeptides, aminoacid-β-naphthylamides (except perhaps lysine-β-naphthylamide) and the hexapeptidase of the 105 000 × *g* pellet are entirely present in the purified membrane. This finding suggests that these activities are the property of an enzyme (or enzymes) firmly bound to the brush border membrane. Results listed in the last column of Table II (purified aminopeptidase) will be discussed later.

It is also noteworthy that only alanine was released after a 1-h incubation of Ala–Gly_2 by the membranous material (105 000 × *g* pellet or vesicles). Traces of glycine were also detected in the cases where dipeptidase activities were present (S_{3000},

TABLE II

RELATIVE ACTIVITIES OF THE FRACTION 105 000 P OF PURIFIED BRUSH BORDER MEMBRANE AND OF THE FREE AMINOPEPTIDASE TOWARDS VARIOUS SUBSTRATES AND TOWARDS ALANINE-*p*-NITROANILIDE

Ratios	*105 000 × g pellet*	*Purified membrane fraction*	*Purified amino-peptidase*
Leucine-β-naphthylamide / Alanine-*p*-nitroanilide	0.82	0.72	0.73
Lysine-β-naphthylamide / Alanine-*p*-nitroanilide	0.35	0.19*	0.15*
Gly_3 / Alanine-*p*-nitroanilide	2.23	2.32	2.22
Ala–Gly_2 / Alanine-*p*-nitroanilide	3.58	3.23	3.20
Heptapeptide / Alanine-*p*-nitroanilide	0.36	0.46	0.39
Gly_2 / Alanine-*p*-nitroanilide	0.73–1.8**	0.3–0.6**	0.2–0.3**
Leu–Gly / Alanine-*p*-nitroanilide	0.6–1.0**	0.4–1.0**	0.1–0.2**

* If confirmed, this low value may indicate that an enzyme acting on naphthylamides of basic aminoacids exists in a membrane of the mucosal cell different from the brush border.

** Results obtained with the dipeptides Gly_2 and Leu–Gly were variable and extreme values rather than average values have been recorded here.

$S_{10\,000}$, $S_{105\,000}$). With the heptapeptide, only valine and lesser amounts of alanine were released in 3 h by the 105 000 × *g* pellet. The conclusion is that the peptidase activity associated with the brush border membrane is of the aminopeptidase type. No endopeptidase activity capable of hydrolyzing the heptapeptide was ever detected in any of the fractions mentioned in Scheme I. If present, this activity would have induced the further degradation of the chain by the di- and tripeptidases.

Purification of the membrane-bound aminopeptidase

By analogy with the experience gained with kidney aminopeptidase M[17], the intestinal enzyme bound to the brush border membrane was purified using alanine-*p*-nitroanilide as the substrate. This enzyme turned out later to be responsible for all, or nearly all, the peptidase activity of the membrane.

Recoveries of enzyme activity at each stage of the final purification scheme described are recorded in Table III.

(a) Solubilization. Hog small intestines were collected at the slaughterhouse immediately after the death of the animals. Duodenum was discarded and the remainder (jejunum + ileum) was emptied and transported on ice to the laboratory. The mucosa was extruded at once by passage of the intestine through rubber rollers and the resulting slurry was stored at −20 °C until use. All further operations were performed at + 4 °C. Samples of this frozen slurry were cut into pieces, allowed to thaw and gently stirred for 30 min in twice their weight of tensioactive solutions at various concentrations. The solubilization effect was derived from the amount of activity remaining in the supernatant after a centrifugation at 105 000 × *g* for 1 h. According to this criterion, Tween 20 and 80 (concentrations of up to 2%) exerted no effect. Deoxycholate (up to 2.5%) caused a 25% inhibition of the activity towards alanine-*p*-nitroanilide and solubilized only 10% of the remainder. Much better results were obtained with Triton X-100 at concentrations higher than 1%, which were

TABLE III

FLOW SHEAT OF THE PURIFICATION OF INTESTINAL AMINOPEPTIDASE

Step	*Specific activity (units/mg protein)*	*Purification factor*	*Yield (%)*
Homogenate	–	–	100
First treatment with Triton X-100	–	–	100
First pH 6 supernatant	1600	1.0	70
Ammonium sulfate precipitation (0.3–0.6 saturation)	2700	1.7	60
Second treatment with Triton X-100	–	–	60
Second ammonium sulfate precipitation* (0.3–0.6 saturation)	–	–	60
Tryptic treatment	–	–	60
Supernatant from 0.6 saturated ammonium sulfate solution	6200	3.9	40
Dialysis (2 days)	–	–	30
DEAE-cellulose chromatography	13 000	8	20
Sephadex G-200 filtration	29 000	18	15

* The complex may be isolated from this fraction by filtration through Sepharose 4B (see text).

observed to solubilize rapidly 80–90% of the activity without any detectable inhibition. In preparative runs, the frozen mucosa was homogenized by magnetic stirring in twice its weight of water, and Triton X-100 was added dropwise to give a final concentration of 1% (by vol.). After 2 h of stirring, the suspension was dialyzed for 24 h to remove most of the detergent, then adjusted to pH 6.0 and centrifuged at 12 000 × *g* for 30 min.

(b) The preparation of a high molecular weight form of aminopeptidase: the "aminopeptidase complex". The activity towards alanine-*p*-nitroanilide of the pH 6 supernatant described above was recovered in the fraction precipitating in ammonium sulfate between 30 and 60% saturation. The precipitate was taken up in a minimal volume of water and dialyzed. The resulting solution was strongly active. The activity was observed to be irreversibly bound by DEAE-cellulose, and to be totally excluded from Sephadex G-200 and to fail to penetrate the 7.5% gels used for electrophoresis. These facts suggested that the corresponding enzyme was soluble in the sense that it did not sediment at 105 000 × *g* but that it was still attached to very small fragments of membrane and was consequently included in a high molecular weight complex. Fig. 1A illustrates the elution profile resulting from filtration of the above solution

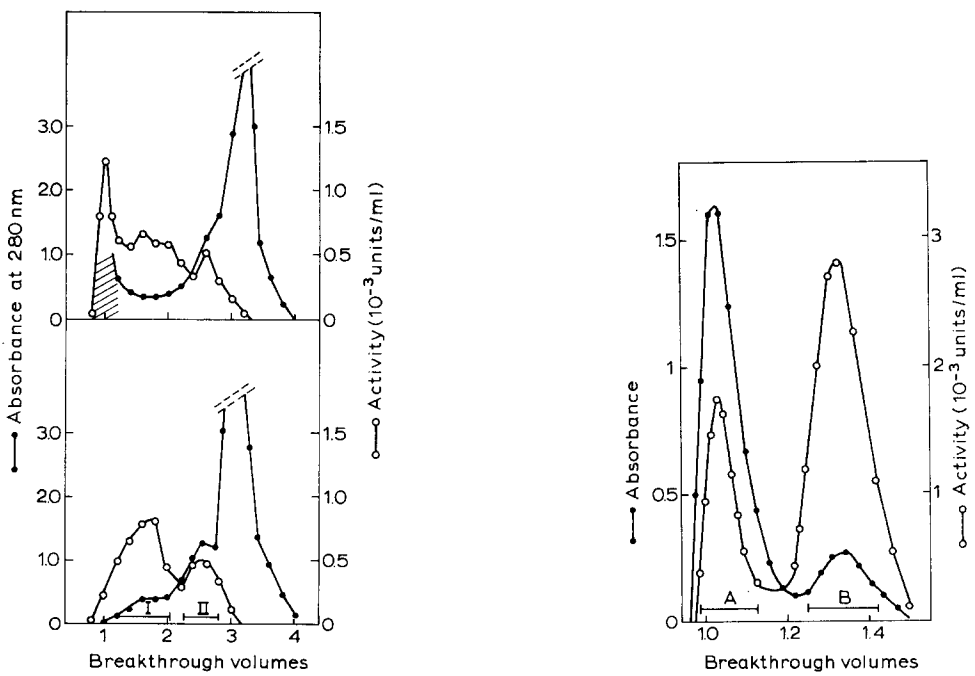


Fig. 1. Filtration through Sepharose 4B of the high molecular weight aminopeptidase-containing material solubilized from jejunal and ileal mucosa by Triton X-100. (A). Elution profile after one treatment with Triton X-100. Hatching indicates the emergence of turbid fractions. (B). Elution profile after a second treatment with Triton X-100. 500 mg of protein were loaded onto a Sepharose 4B column, 1.6 cm × 200 cm, equilibrated and eluted with a 10 mM Tris–HCl buffer (pH 8.0) containing 0.15 M NaCl.

Fig. 2. Filtration through Sephadex G-200 of the purified aminopeptidase complex after trypsin treatment. The complex was digested with trypsin under the conditions given in the text, and then loaded onto a Sephadex G-200 column, 1.6 cm × 200 cm, equilibrated and eluted with a 10 mM Tris–acetate buffer (pH 6) containing 0.5 M NaCl.

through Sepharose 4B. The activity emerges from the column as a broad peak, indicating that the size of the membrane fragments to which the enzyme is bound varies within very large limits.

Fig. 1B gives the result of a second treatment of the active fragment with 1% Triton for 18 h followed by ammonium sulfate precipitation and dialysis. The activity is now seen to be concentrated in only 2 peaks (Peaks I and II). The position of peak I shows that it is still composed of a high molecular weight material, from now on be designated "aminopeptidase complex". Peak II, with a variable area relative to Peak I, corresponds to free aminopeptidase (see later).

(c) Treatment of the aminopeptidase complex with trypsin: preparation of the free form of the enzyme. The dialyzed solution (20 mg/ml) of the preceeding complex was made 0.1 M in Tris–HCl (pH 8.0) and incubated for 1 h at 4 °C with 0.5 mg/ml crystalline trypsin. The activity towards alanine-*p*-nitroanilide was entirely preserved during the treatment. However, the elution profile reproduced in Fig. 2 shows that the complex has yielded 2 fractions separable on Sephadex G-200. The first (Peak A) was excluded from the gel, and accounted for 54% of the total proteins and 25% of the total aminopeptidase activity. The second (Peak B), which was obviously composed of a material of distinctly lower molecular weight, accounted for only 14% of the proteins but for 75% of the activity. The corresponding 6-fold specific activity increase (from 3000 units/mg protein in Peak A to 18 500 in Peak II) indicated that

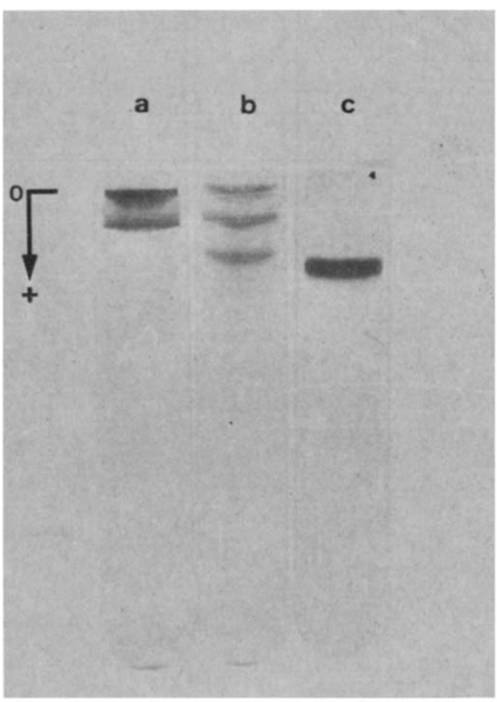


Fig. 3. Gel electrophoresis of the trypsinized complex (Tube (b)) and of Peaks A and B (Tubes (a) and (c)), previously separated on Sephadex G-200 (see Fig. 2). The horizontal bar marked O (origin) indicates the upper surface of the gels. Proteins were stained by aniline blue-black.

a large amount of a protein (or proteins) inactive towards alanine-*p*-nitroanilide has been separated from the enzyme proper during tryptic digestion of the complex.

The electrophoretic patterns of the trypsinized complex and of the separated Peaks A and B on polyacrylamide gel are reproduced in Fig. 3. It is clear that the complex has been dissociated by trypsin into a minimum of 3 electrophoretically distinct components. Two of these components are in Peak A and one in Peak B. The first band at the top of Tubes (a) and (b), which did not penetrate the gel and was active towards alanine-*p*-nitroanilide, could be assumed to correspond to the intact or slightly degraded complex which had escaped degradation by trypsin. The second band, visible a little below in Tubes (a) and (b), penetrated the gel and was inactive towards alanine-*p*-nitroanilide. Considering that the total activity of the complex towards this substrate was not decreased by the trypsin treatment, the material of the second band is very likely to be another constituent (or other constituents) of the complex, distinct from aminopeptidase, rather than an inactivated form. The nature, proportions and role of this constituent are still unknown.

The last, fastest migrating band given by tryptic hydrolysis of the complex was absent in Peak A but represented by the single band in the electrophoregram of Peak B (Tube c in Fig. 3). The fact that the material of Peak B yields a single band by electrophoresis suggests an advanced state of purity. This band was strongly active and, therefore, was considered to contain the free form of aminopeptidase.

(d) Final purification of aminopeptidase. The tryptic digest of mucosa after Triton X-100 treatments and ammonium sulfate precipitations was adjusted to pH 6.0 and brought to 60% saturation with ammonium sulfate: the free aminopeptidase was not precipitated. The supernatant was dialyzed, adjusted to 10 mM Tris–HCl (pH 8.0) and loaded onto a DEAE-cellulose column equilibrated with a 10 mM Tris-HCl buffer (pH 8.0) containing 50 mM NaCl. After a preliminary washing of the column with this buffer, the aminopeptidase activity was eluted by a linear NaCl concentration gradient from 0.05 to 0.3 M. Fig. 4 shows that, under these conditions, aminopeptidase emerged as a nearly symmetrical peak between 0.115 and 0.140 M NaCl.

The last step of the purification was a filtration of the material under the active

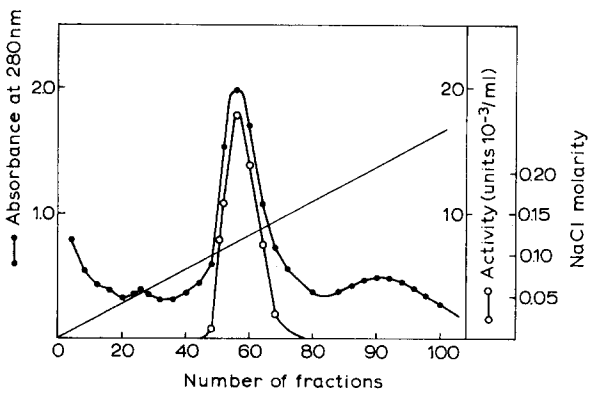


Fig. 4. Chromatography of aminopeptidase on DEAE-cellulose at pH 8.0. A 6.3 cm × 10 cm column was used for a preparation derived from 500 g of mucosa. Volume of fractions, 14 ml. Other conditions are described in text. ———, indicates the NaCl gradient.

peak in Fig. 4 through a Sephadex G-200 column in a 10 mM Tris–acetate buffer (pH 6.0) containing 0.5 M NaCl. The final product was dialyzed and lyophilized. Table III summarizes the scheme outlined above for the identification of the complex and for the purification of the free form of aminopeptidase from intestinal brush border. The overall purification calculated for the free enzyme from the first pH 6 supernatant was 18-fold, with a yield of 15%. Starting from 500 g of wet mucosa, 80 mg of enzyme were obtained.

Preparation of free aminopeptidase without treatment with trypsin

Some batches of mucosa, handled according to the above procedure, yielded an unstable complex dissociating spontaneously during the second Triton X-100 treatment. In this case, Peak II in Fig. 1B, which contains the free enzyme, was largely predominant and the aminopeptidase could be further purified by proceeding directly to the Step d designed "supernatant from 0.6 saturated ammonium sulfate solution" in Table III.

A second technique avoiding trypsin was to use papaïn, which is known to solubilize directly 80% of the aminopeptidase activity from the brush border[27] and even from our membrane preparation[26]. Starting from the 105 000 $\times$ g supernatant of papain-treated vesicles, purified aminopeptidase was obtained by applying the general scheme outlined above to Step d. Chromatography on DEAE-cellulose could be omitted in this case because of the much higher purity of the starting material.

The aminopeptidase preparations from these two sources behaved, during the fractionation steps and during gel electrophoresis, exactly as the enzyme released by tryptic attack.

Molecular parameters of the free enzyme

As already pointed out before, the final product gave a single band on polyacrylamide gel electrophoresis. When a 0.5% solution in a 10 mM Tris–HCl buffer (pH 8.0) containing 50 mM NaCl was centrifuged at 20 °C and 60 040 rev./min, a single peak was visible (Fig. 5) showing a minor contaminant of lower molecular weight amounting to less than 5% of the main constituant. The sedimentation coefficient $s_{20,w}$ was 9.95 S.

As for enterokinase[28], the presence of a bulky sugar part in the aminopeptidase molecule could be expected to lower the partial specific volume of the molecule below the value calculated from amino acid analysis. This parameter was directly measured with the aid of a microdensitometer (Model 120-C, A. Parr, Austria) and was found not to exceed 0.693 ml/g in the 10 mM buffer used for centrifugation assays. Molecular

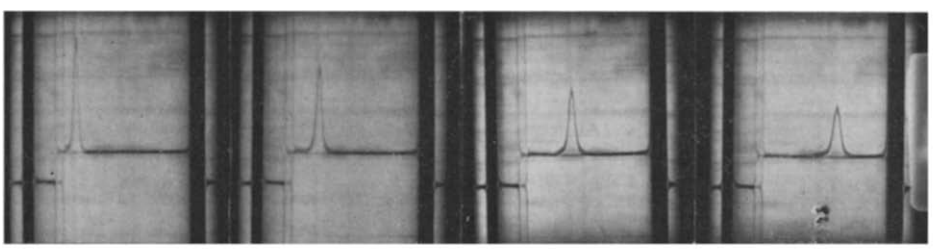


Fig. 5. Sedimentation pattern of aminopeptidase. Photographs (left to right) were taken 16, 20, 24, 28 and 32 min after reaching maximal rotor velocity.

weight determinations were subsequently undertaken on 0.8 mg/ml enzyme solutions by the equilibrium method of Yphantis. Two independent assays gave values of 248 000 and 240 000.

Aminopeptidase is a glycoprotein containing (by weight) 77% amino acids, 15% neutral sugars, 8% amino sugars and 0.3% sialic acids. Using an Eel atomic absorption spectrometer, 1.74 atoms of zinc were found per 245 000 g of protein.

Existence of subunits

Patterns obtained by gel electrophoresis, in the presence of sodium dodecyl sulfate, of the aminopeptidase complex and of the free enzyme resulting from trypsinization are reproduced in Fig. 6. In both cases the material was reduced and carboxymethylated prior to electrophoresis. The pattern of the free enzyme was not modified by this reducing treatment, indicating that the aminopeptidase was devoid of chains linked by disulfide bridges. The unreduced complex gave poorly reproducible results.

The free aminopeptidase is seen in Fig. 6 to yield 4 bands separable by electrophoresis and corresponding to molecular weights of approximately 129 000; 98 000; 91 000 and 49 500. In the pattern corresponding to the complex, 3 bands lead to values (130 000, 96 000 and 48 000) so close to those mentioned above that they are

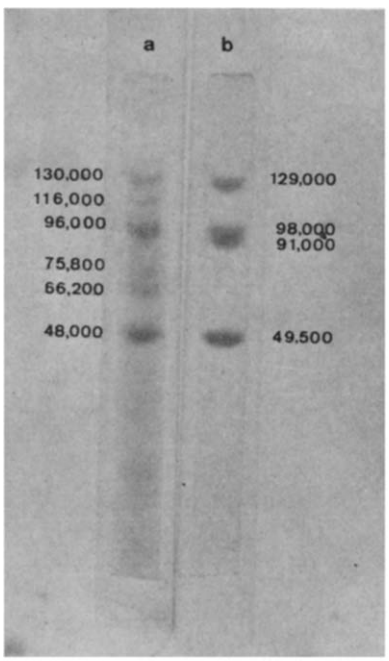


Fig. 6. Gel electrophoresis, in the presence of sodium dodecyl sulfate, of reduced and carboxymethylated aminopeptidase complex (Tube (a)) and free enzyme (Tube (b)). In both cases, the derivative was incubated with 1% sodium dodecyl sulfate for 18 h at room temperature and run on polyacrylamide gels containing 0.1% sodium dodecyl sulfate. The following reduced and carboxymethylated proteins were used for the calibration of the gel: bovine trypsinogen (molecular weight, 24 000); porcine pepsin (34 000); ovalbumin (43 000) and bovine serum albumin (69 000). Figures on the left and on the right of the tube indicate the estimated molecular weight of each subunit.

likely to correspond to the same material. Three more bands (116 000, 76 000 and 66 000) are also visible in the pattern given by the complex.

Treatment by trypsin, used for obtaining free aminopeptidase, may be expected to give rise to artefact fragments not present in the native enzyme. To check this point, free aminopeptidase samples prepared by the 2 other methods described earlier (spontaneous dissociation of the complex and solubilization by papain) were separated by electrophoresis under the same conditions and gave exactly the same pattern composed of 4 bands.

Activity of the pure enzyme towards various substrates

Considering that purification was exclusively monitored by assays using alanine-*p*-nitroanilide as the substrate, it was of interest to delineate more clearly the activity pattern of the pure enzyme towards other compounds. The figures listed in the last column of Table II show that practically all the peptidase activities of the 105 000 $\times$ *g* pellet and in the purified membrane vesicles can be ascribed to the aminopeptidase that has been purified. The case of dipeptidase activity is still unclear in this respect. There may exist in the brush border membrane a dipeptidase distinct from aminopeptidase.

DISCUSSION

The main purpose of the present report is to investigate the aminopeptidase bound to the brush border membrane of the jejunum and ileum in the pig. This enzyme is responsible for all the arylamidase activity, all the aminopeptidase activity towards a heptapeptide substrate, about half the tripeptidase activity and, finally, a small but apparently significant part of the dipeptidase activity of the mucosa. The remainder of the tripeptidase and all, or almost all, the remainder of the dipeptidase activity of this mucosa are soluble. It is noteworthy that this aminopeptidase accounts for practically all the peptidase activity of the membrane. Unlike enterokinase[28], the membrane-bound aminopeptidase is not released by bile salts nor by several other tensioactive compounds. However, it is converted by Triton X-100 into a form which is not sedimented at 105 000 $\times$ *g* for 45 min and which is designated the aminopeptidase complex. This complex has a very high molecular weight and can be shown to contain at least 2 proteins species, one active towards alanine-*p*-nitroanilide and the other inactive. It will be interesting in the near future to study this inactive species which may be assumed to be very close to the aminopeptidase in the protein pattern of the intact membrane. The complex is not dissociated by treatment with lipid solvants.

In some cases, the complex was observed to be unstable and to dissociate spontaneously upon filtration through Sepharose 4 B. In other cases, it was quite stable and dissociation only occurred, as for kidney aminopeptidase M[17], after trypsin digestion. The mechanism by which trypsin and other proteases release membrane-bound enzymes is still unclear. This release appears to be generally specific. For instance, trypsin can solubilize aminopeptidase from the Triton X-100-treated membrane (see above) but the same enzyme is not solubilized from membrane sheets obtained in the presence of EDTA[29] and from membrane vesicles obtained by a new technique[18] without EDTA. In this latter case, papain must be used in place of tryp-

sin[26,29]. If, as discussed later in more details, the brush border aminopeptidase is involved in amino acid transport, it would not be expected to be detached from the intact membrane by a digestive enzyme like trypsin, which is normally present in the lumen.

After dissociation of the complex by trypsin, aminopeptidase could be shown by gel electrophoresis in the presence of sodium dodecyl sulfate to be composed of 4 subunits with molecular weights of, respectively, 129 000, 98 000, 91 000 and 49 500. That the 91 000 subunit was not present in the native enzyme, and was consequently generated by trypsin from the 98 000 subunit, was suggested by the following two observations. In the first place, the 91 000 band was not visible in the electrophoretic pattern of the undissociated complex ,but 3 bands in the same pattern corresponded very closely to the other values (130 000, 96 000 and 48 000). The additional 116 000, 76 000 and 66 000 bands yielded by the complex may be assumed to arise from the other protein(s) of the complex. Moreover, the cumulated weights of the 4 subunits greatly exceeds the molecular weight of the entire enzyme (280 000). In contrast, the 3 bands given by both complex and free enzyme lead to a correct total value. Therefore, a provisional conclusion in this field is that intestinal aminopeptidase is composed of 3 subunits and that a small fragment is split off by trypsin from one of them during the solubilization step. The assumption that the brush border aminopeptidase actually contains not less than 3 subunits is supported by the observation according to which electrophoretic patterns showing 4 bands identical to that illustrated by Fig. 6 are obtained either after papain solubilization or after spontaneous dissociation of the complex. The bond (or bonds) responsible for the appearance of the 91 000 band should, therefore, be very labile.

Aminopeptidase differs from another brush border peptidase, enterokinase[30] by its specificity, its main localization in jejunum and ileum, its Zn^{2+} requirement and the presence of subunits instead of chains linked by disulfide bridges[28]. Common factors are a molecular weight in the 200 000–300 000 range and the presence in both enzymes of a large sugar part (40% of the total for enterokinase; 23% for aminopeptidase). Kidney aminopeptidase M, which like intestinal aminopeptidase is attached to an absorptive membrane, is also a zinc enzyme with a molecular weight of about 280 000[31]. It has been reported at first to contain not less than 10 subunits[32]. However, this number has later been reduced to 3, with molecular weights of 140 000, 100 000 and 60 000[33], as for the intestinal enzyme investigated here.

Finally, the intestinal aminopeptidase, which is very abundant in the brush border of jejunum and ileum (8% of the total proteins of the corresponding vesicles[18]) may be assumed to be involved in the transport of amino acids across the membrane. Intestinal absorption of amino acids has been known for a number of years to be more rapid when they are supplied as peptides than when they are given as free amino acids[34], even though only free amino acids appear in the bloodstream. Several hypotheses have been advanced to explain these observations, two of which are currently favored. According to some authors, small peptides would cross the brush border membrane and be subsequently hydrolyzed within the cell[34]. Others support the view that peptides are hydrolyzed at the brush border, the amino acids then passing directly into a membrane transport system apparently distinct from that involved in the absorption of free amino acids[2]. These two hypotheses are not incompatible as the second may apply to peptides above a certain size, the first being valid for shorter

peptides, especially dipeptides. Assays are presently undertaken in our laboratory to investigate these problems with the aid of the vesicles obtained from the jejunal brush border membrane.

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