Hoppe-Seyler's Z. Physiol. Chem.
Bd. 361, S. 1401–1412, September 1980

Endopeptidase of the Brush Border Membrane of Rat Enterocyte

Separation from Aminopeptidase and Partial Characterization

Petr KOCNA, Přemysl FRIČ
2nd Research Division of Gastroenterology, Charles University, Faculty of General Medicine, Praha

Jan SLABÝ
Department of Internal Medicine, Policlinic of Charles University, Faculty of General Medicine, Praha

Evžen KASAFÍREK
Research Institute for Pharmacy and Biochemistry, Praha

(Received 17 November 1979/9 May 1980)

Summary: The brush border of the enterocytes of the rat was isolated by the method of differential centrifugation with $CaCl_2$ according to Schmitz. This material was solubilized with papain, trypsin and Triton X-100. The greatest amount of membrane enzymes was released to the supernatant (105000 × *g*) with the use of Triton X-100.

The tritonized supernatant was treated in the next step by papain, bromelain, ficin and trypsin (individually or in combinations). After simultaneous proteolysis with papain and bromelain a partial separation of the aminopeptidase from the endopeptidase by Sephadex G-200 chromatography was observed. These two enzyme activities were distinctly separated by isoelectric focusing at pH 4–6.

Two enzymatically active bands (R_F 0.13 and 0.24) in the aminopeptidase fraction and one single active band (R_F 0.16) in the endopeptidase fraction using polyacrylamide gel electrophoresis were found. Co-migrating proteins to all of these activities were detected.

Endopeptidase activity splits 3-carboxypropionyl-trialanin-4-nitroanilide ($SucAla_3NAp$) in the position P_2–P_1. Liberated aminoacyl-NAp may be further split to generate chromogenic 4-nitroaniline through aminopeptidase activity.

Endopeptidase of the brush border of the rat enterocytes is characterized by the following properties:

1) molecular mass 130000 ± 15000 dalton;
2) K_m value (substrate: $SucAla_3NAp$) 1.1×10^{-3} M;
3) pI 5.23;
4) pH optimum 8.5;
5) 50% activity remains after 15 min of preincubation at 50 °C;
6) activity is strongly inhibited by EDTA, *p*-chloromercuribenzoate, $Mn^{2\oplus}$ and $Co^{2\oplus}$.

Enzymes:
Alkaline phosphatase, orthophosphoric monoester phosphohydrolase (alkaline optimum) (EC 3.1.3.1);
Aminopeptidase (microsomal), α-aminoacyl-peptide hydrolase (microsomal) (EC 3.4.11.2);
Bromelain (EC 3.4.22.4);
Ficin (EC 3.4.22.3);
Papain (EC 3.4.22.2);
Sucrose α-D-glucohydrolase, sucrose α-D-glucohydrolase (also named sucrase) (EC 3.2.1.48);
Trypsin (EC 3.4.21.4).

Abbreviations:
Suc, 3-carboxypropionyl; Ac, acetyl; Z, benzylocycarbonyl; NAp, 4-nitroanilide; αNA, α-naphthylamide; βNA, β-naphthylamide; OEt, ethyl ester; Me_2SO, dimethylsulphoxide; iPr_2P-F, diisopropyl fluorophosphate; TEMED, *N,N,N',N'*-tetramethylenediamine; all amino acids had the L-configuration; Pl (i.e. Pl_1, Pl_2 ...), pellet; Sp (i.e. Sp_1, Sp_2...), supernatant.

0018-4888/80/0361-1401$02.00

Endopeptidase der Bürstensaum-Membran von Rattenterozyten; Abtrennung von Aminopeptidase und partielle Charakterisierung

Zusammenfassung: Der Bürstensaum der Enterozyten des Rattendünndarms wurde nach der Methode der Differential-Zentrifugation mit $CaCl_2$ nach Schmitz isoliert. Das so gewonnene Material wurde mit Papain, Trypsin und Triton X-100 solubilisiert. Die Hauptmenge der Membranenzyme wurde bei Verwendung von Triton X-100 in den Überstand (105000 × *g*) freigesetzt.

Der tritonisierte Überstand wurde sodann der Einwirkung von Papain, Bromelin, Ficin und Trypsin ausgesetzt, und zwar sowohl allein als auch in Kombinationen. Nach gleichzeitiger Proteolyse mit Papain und Bromelin erzielten wir durch Chromatographie an Sephadex G-200 eine partielle Trennung der Aminopeptidase von der Endopeptidase. Beide Enzymaktivitäten wurden sodann durch isoelektrische Fokussierung bei pH 4–6 abgetrennt.

Mittels Polyacrylamid-Gelelektrophorese wurden in der Aminopeptidasefraktion zwei enzymatisch aktive Streifen (R_F 0.13 und 0.24) und in der Endopeptidasefraktion ein aktiver Streifen (R_F 0.16) gefunden. Zu sämtlichen Aktivitäten haben wir auch mitwandernde Proteine nachgewiesen.

Die Endopeptidase spaltet 3-Carboxypropionyltrialanin-4-nitranilid ($SucAla_3NAp$) in Stellung $P_2–P_1$. Das freigesetzte Aminoacyl-NAp kann durch Aminopeptidase unter Bildung von chromogenem 4-Nitranilin weitergespalten werden.

Die Endopeptidase des Bürstensaums von Ratten-Enterozyten wird durch folgende Parameter charakterisiert:

1) Molekulargewicht = 130000 ± 15000;
2) K_m-Wert (für das Substrat $SucAla_3NAp$) = 1.1×10^{-3} M;
3) pI = 5.23;
4) das pH-Optimum = 8.5;
5) 50% der Aktivität bleiben nach 15 min Vorinkubation bei 50 °C erhalten;
6) die Aktivität wird durch EDTA, *p*-Chlormercuribenzoat, $Mn^{2\oplus}$ und $Co^{2\oplus}$ stark gehemmt.

Key words: Endopeptidase: brush border: rat small intestine.

In the brush border of the rat enterocyte different dipeptidases[1,2], oligopeptidases[3], amino-oligopeptidases[4,5] and other aminopeptidases[6,7] were described. With respect to the method of solubilization, two forms of these peptidases may be separated: the trypsin form and the triton form. The latter is composed of the hydrophilic head piece (projecting outside from the external surface of the membrane) and the hydrophobic chain (anchoring the enzyme into the membrane structure). The trypsin form includes the head piece only. Both forms differ in various physicochemical characteristics, e.g. molecular mass, electrophoretic mobility, etc.[8–11]. The additional cytoplasmic fragment has been identified in intestinal aminopeptidase[8,9]. In previous papers we presented a $SucAla_3NAp$-splitting activity found in the homogenates of rat[12] and human [13] intestinal mucosa. When using an analogous substrate $SucAla_3\alpha NA$ in a frozen microtome section of the human small intestine, we localized a chromogenic product in the brush border of enterocyte.

$SucAla_3NAp$ has already been described before as a substrate for pancreatic elastase[14–17]. It is neither split by trypsin nor by chymotrypsin but it is split by elastase in the position $P_1–P_1'$[17]. We have postulated, on the basis of our previous kinetic evaluations of the splitting of substrates $(Ala)_n$-NAp and $Suc(Ala)_n$-NAp by homogenate of the small intestine mucosa, that substrates of the type $Suc(Ala)_n$-NAp are split in the first reaction by endopeptidase activity in the position $P_2–P_1$ and in the following reaction the liberated aminoacyl-NAp is split by aminopeptidase in the position $P_1–P_1'$[13].

In this study the separation of endopeptidase free of microsomal aminopeptidase (EC 3.4.11.2) and its nearer characterization are presented. Both

activities correlate significantly in the duodenum, jejunum and ileum[12] and up to now the existence of a single structural protein was presumed[13].

Material and Methods

Substrates for this study were prepared as described[17]. Bromelain and papain were purchased from Merck, trypsin from Boehringer Mannheim GmbH and ficin from Koch-Light. All other chemicals were of reagent grade and comercially available.

Isolation of the brush border

Wistar rats (320–350 g, males) were fasted for 24 h and then killed by decapitation. The jejunum was freed from the ligament of Treitz, longitudinally dissected and washed in cold isotonic salt solution. The intestinal mucosa was obtained by scraping. The method of differential centrifugation with $CaCl_2$ according to Schmitz [18] was used for brush border isolation. The homogenate (1%) in 50mM mannitol, 2mM Tris/HCl (pH 7.1) was obtained by homogenization 2 x 30 s (UNIPAN 309, 250 V) in ice-water cooling mixture. In modification according to Andria[19] the concentration of the solid $CaCl_2$ was increased to 20mM and the final centrifugation was extended to 60 min (20000 x *g*: MSE Superspeed 65).

Solubilization of the brush border

The final pellet Pl_2 (20000 x *g*) was resuspended in the buffer solution and solubilized by papain 0.4 g/*l*, trypsin 0.44 g/*l* and Triton X-100 10 g/*l* in the same way as is described for human material[13]. After incubation each sample was centrifuged for 60 min at 4 °C and 105000 x *g* (MSE Superspeed 65).

Proteolytic cleavage

Supernatant Sp_3 obtained after centrifugation at 105000 x *g* was incubated for 60 min at 37 °C in 75mM phosphate buffer solution pH 6.8 in the presence of papain 0.4 g/*l*, ficin 1.0 g/*l*, bromelain 1.5 g/*l* or trypsin 0.44 g/*l* individually or in combinations. Cysteine hydrochloride was added as activator to papain, ficin and bromelain to a final concentration of 0.2 g/*l*. Centrifugation of the proteolytic enzyme-treated supernatant Sp_3 at 4 °C and 20000 x *g* for 30 min (Janetzki K 24) yielded the final supernatant Sp_4.

Gel filtration chromatography

Samples of 4 m*l* in 75mM phosphate buffer solution were applied at the top of the column K 16/100 (Pharmacia) on Sephadex G-200. Flow was maintained at 10 m*l* per h at 4 °C by a peristaltic pump (LKB Varioperpex 12000). 5-m*l* fractions were collected after out-flow through a UV-unit in a collector.

Isoelectric focusing[20]

5-m*l* samples of supernatant Sp_4 (20000 x *g*) were applied in the middle of column LKB 8101 – 110 m*l* in a sucrose gradient in the presence of 1% (w/v) ampholyte pH 4–6 (LKB). The separation on the column went on for 14 h, under water cooling at 11 °C, initially at 600 V to a maximum of 3 W and terminally at 1200 V to a maximum of 2.5 W. After the focusing run the column was emptied in 30-drop fractions, which were collected in the Ultrorac 7000 (LKB) after outflow through UV-unit Uvicord II (LKB). The pH of the fractions was immediately measured at room temperature on a pH-meter PHM 62 (Radiometer).

Molecular mass determination

For the estimation of molecular mass we established $s^0_{20,w}$ by ultracentrifugation analysis on MSE Superspeed 65. A sample (0.2 m*l* of fraction 60 after the focusing) was dialyzed overnight against 250 m*l* isotonic saline solution at 4 °C. This material was centrifugated for 90 min at 40000 rpm and 4 °C in sucrose gradient 5%–20%. The material after centrifugation was divided by means of density gradient fractionator (ISCO) into 20 fractions and the protein was detected on UA-5 absorbance monitor (ISCO). As a standard we used 0.5 mg of ferritin (Calbiochem) and the values of $s^0_{20,w}$ and molecular mass, were calculated from the Svedberg equation[21].

Polyacrylamide gel electrophoresis

The gel (6 x 0.5 cm) contained 7.5% acrylamide, 0.2% bis(acrylamido)methane in a Tris/HCl buffer solution of pH 8.9 (1M HCl 4.8 m*l*; Tris 3.63 g; TEMED 0.023 m*l*; distilled water filled up to 80 m*l*) and 0.4 g ammonium persulphate/*l*. The electrophoresis was run for 1.5 h at 4 mA per gel slab and 4 °C in a Tris-glycine buffer solution of pH 8.3. The enzymatic activity was detected by means of substrate $SucAla_3NAp$ in a medium of the following composition: 5 m*l* Tris/HCl buffer solution pH 8.0; 2.5mM substrate (resolved in Me_2SO) and 0.1 m*l* of isolated aminopeptidase. Protein staining was performed by the method according to Blakesley[22] with Coomassie Brilliant Blue G-250 (Serva).

Enzyme assays

Alkaline phosphatase was determined by the Lachema-kit (substrate: 10mM 4-nitrophenylphosphate) according to Bessey and Lowry[23]. In the isolated brush border this activity was detected by simultaneous azocopulation: 1 m*l* of the pellet Pl_2 (20000 x *g*) resuspended in the initial buffer solution was incubated for 30 min at room temperature with 1 m*l* of the staining medium

(0.22 mg β-naphthylphosphate and 0.5 mg Fast Blue BB in 0.08M borate buffer solution pH 9.0).

Sucrose glucohydrolase was assayed according to Dahlqvist[24] as modified by Mališ[25] using 56mM sucrose as substrate.

Aminopeptidase was determined by a chromogenic method using AlaNAp 2.5mM as substrate[12,13].

Endopeptidase was assayed by means of $SucAla_3NAp$ cleavage either by an indirect chromogenic method[12,13] or by a direct method after resolution from aminopeptidase. The method includes the shaking out of hydrophobic AlaNAp into ethyl acetate and the estimation of 4-nitroaniline concentration after alkaline hydrolysis of the hydrophobic fraction. Incubation was performed in a total volume of 1.5 ml containing: substrate 2.5mM (resolved in Me_2SO), 0.2 ml of the enzyme fraction and 0.1M Tris/HCl buffer solution pH 8.0. Incubation was carried out for 90 min at 37 °C. Immediatelly after incubation 1.5 ml of ethyl acetate was added, shaken for 30 s and 0.5 ml of the upper fraction was slowly mixed with 2.0 ml of 5M NaOH in 50% ethanol. The concentration of liberated 4-nitroaniline was determined spectrophotometrically at 405 nm (Unicam SP-800). The endopeptidase activity is expressed as μmol of alanin-4-nitroanilide liberated/min:

Activity per volume

$$[\text{mU/ml}] = A_{405} \times \frac{F_1 \times F_2 \times v_1 \times 1000}{\epsilon_{405} \times t \times v_2 \times d}$$

F_1 ... 1.18
extraction factor ($F_1 = \frac{D+1}{D}$);

D ... 5.49
coefficient of partition;

F_2 ... 5.0
dilution factor (dilution from 0.5 ml to 2.5 ml);

v_1 ... 1.5
volume of reaction mixture [ml];

v_2 ... 0.2
volume of the enzyme fraction [ml];

t ... 90
time of incubation [min];

d ... 1.0
light path through the cuvette [cm];

ϵ_{405} ... 9.35
molar extinction coefficient of NAp × 10^{-3} [$mol^{-1} \times l$].

Protein determination

Protein determination was carried out by the method of Lowry[26] with phenol Folin reagent or according to Sedmak[27] using Coomassie Brilliant Blue G-250. After electrofocusing the protein was assayed by determining the absorbance at 280 nm in flow-through quartz cell (Uvicord II, LKB).

Semiquantitative assay of hydrolytic products

The substrate $SucAla_3NAp$ (2.5mM) was incubated in Tris/HCl buffer solution for 120 min at 37 °C with each enzyme fraction after Sephadex G-200 chromatography or after electro-focusing. The chromatographic analyses of these products were carried out on a thin-layer silica gel (Silufol, Kavalier) in the system butanol/acetic acid/water 4 : 1 : 1 (v/v/v) and the substances were detected with ninhydrin (Calbiochem) and *p*-dimethylaminobenzaldehyde (after reduction with $SnCl_2$)[17].

Determination of pH optimum, K_m value, thermostability and effects of inhibitors

Incubation was performed in a total volume of 1.5 ml containing substrate 2.5mM (resolved in Me_2SO), enzyme (0.2 ml of enzyme fraction) and 0.1M Tris/HCl buffer solution of pH 8.0. Incubation was carried out for 90 min at 37 °C in a water bath.

For determination of the K_m value the concentration of the substrate was changed in the range from 0.039 to 5.0mM, and the final value was estimated from the linear plot K_m against V according to Eisenthal[28].

Thermostability was determined by means of 15 min preincubation of the enzyme fraction in the range of 30 °C to 70 °C.

For pH optimum determination the pH of the Tris/HCl buffer solution was changed in the range of pH 6.0 to 9.0 with 0.2 intervals.

The effects of bivalent ions and organic inhibitors were studied in the final concentration of 1mM each: EDTA, *p*-chloromercuribenzoate, iPr_2P-F, β-mercaptoethanol, $Mn^{2\oplus}$, $Co^{2\oplus}$, $Zn^{2\oplus}$, $Mg^{2\oplus}$ or $Ca^{2\oplus}$.

Results

Isolation and solubilization of the brush border

The presence of the brush border in the pellet Pl_2 was verified by means of a histochemical reaction of alkaline phosphatase. Activities of the investigated membrane-bound enzymes in the order of isolation steps (Fig. 1) are demonstrated in Table 1. The specific activity of endopeptidase increased from 15.1 mU/mg in the initial homogenate to 69.5 mU/mg in the final pellet Pl_2, i.e. more than 4 times. The highest activities of the brush border enzymes were detected after solubilization in the supernatant Sp_3 when Triton X-100 was used. In

this way 78%–89% of the specific activities were released (Tab. 2). Gel filtration of tritonized supernatant Sp_3 on Sephadex G-200 by elution resulted in one common peak (11th fraction) of alkaline phosphatase, aminopeptidase and endopeptidase (Fig. 2).

Separation of endopeptidase from aminopeptidase

The tritonized supernatant Sp_3 was subsequently digested by various proteases (papain, bromelain, ficin and trypsin) and the separation of enzyme activities was followed on Sephadex G-200 gel chromatography. Before this treatment all activities were found in Fract. 11 (Fig. 2). The most effective proteolysis was accomplished with papain. Its use was followed by splitting of the Triton complex and by separation of an aminopeptidase activity with lower molecular weight found in Fract. 15 (Fig. 3). The highest activity of endopeptidase was present, however, with the second maximum of aminopeptidase activity in Fract. 12. Simultaneous proteolysis with papain and

Table 1. Activities of membrane-bound enzymes in steps of isolation procedure.

Steps are described in Methods and Fig. 1. H_1 is the 1% initial homogenate of the mucosa, Sp_1 and Pl_2 were steps of differential centrifugation. Activities were expressed as specific activities related to total protein (mU/mg).

Spec. act. [mU/mg]	H_1	Sp_1 (2000 × g)	Pl_2 (20000 × g)
Endopeptidase	15.1	31.5	69.5
Aminopeptidase	112	192	499
Alk. phosphatase	679	543	1875
Sucrase	0.36	0.66	1.65

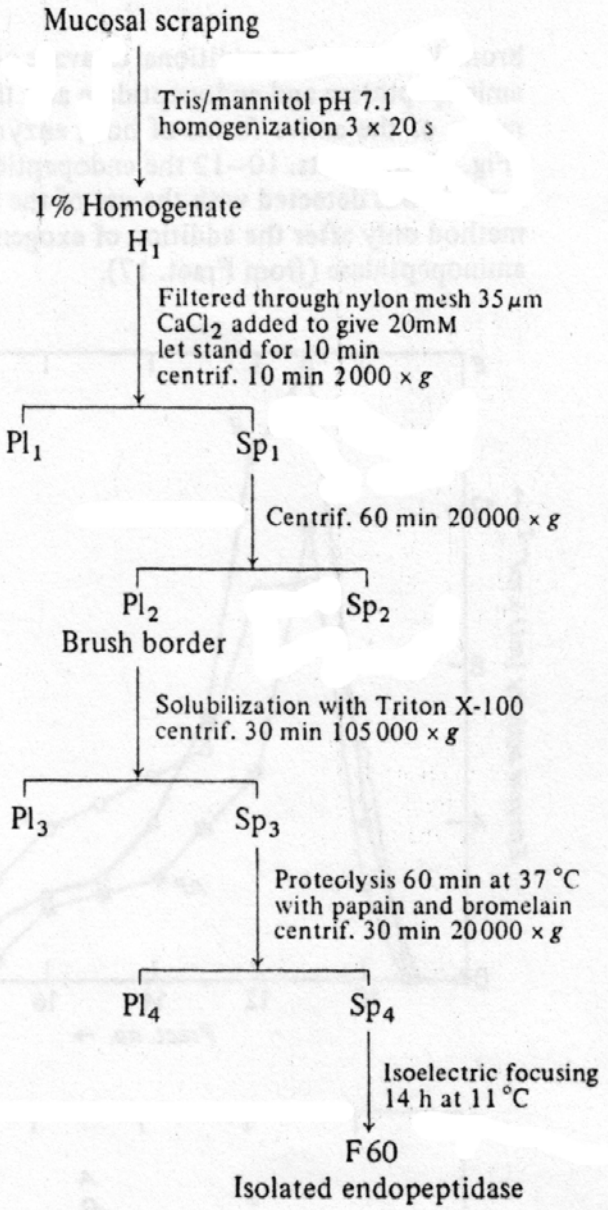

Fig. 1. Schematic representation of the isolation procedure of the brush border endopeptidase from rat enterocytes as described in Methods.

Table 2. Solubilization of brush border enzymes.

Brush border was isolated as described in Methods, treated by papain, trypsin or Triton X-100 and centrifuged for 30 min at 105000 × g. Sp_3 and Pl_3 were supernatant and pellet, respectively. The results are expressed in percentage of distribution.

Activity [%]	Papain		Trypsin		Triton X-100	
	Sp_3	Pl_3	Sp_3	Pl_3	Sp_3	Pl_3
Endopeptidase	47	53	11	89	89	11
Aminopeptidase	81	19	35	65	82	18
Alk. phosphatase	89	11	35	65	78	22

bromelain caused an additional cleavage of both aminopeptidase and endopeptidase and the separation of the active forms of both enzymes (Fig. 4). In Fracts. 10–12 the endopeptidase activity was detected with the use of the indirect method only after the addition of exogenous aminopeptidase (from Fract. 17).

By means of thin-layer chromatography on Silufol (Fig. 5) in Fracts. 11 and 12 alanin-4-nitroanilide was detected, which suggests the presence of the endopeptidase free from aminopeptidase. The latter activity does not appear before Fract. 13, where its presence is demonstrated through 4-nitroaniline spots.

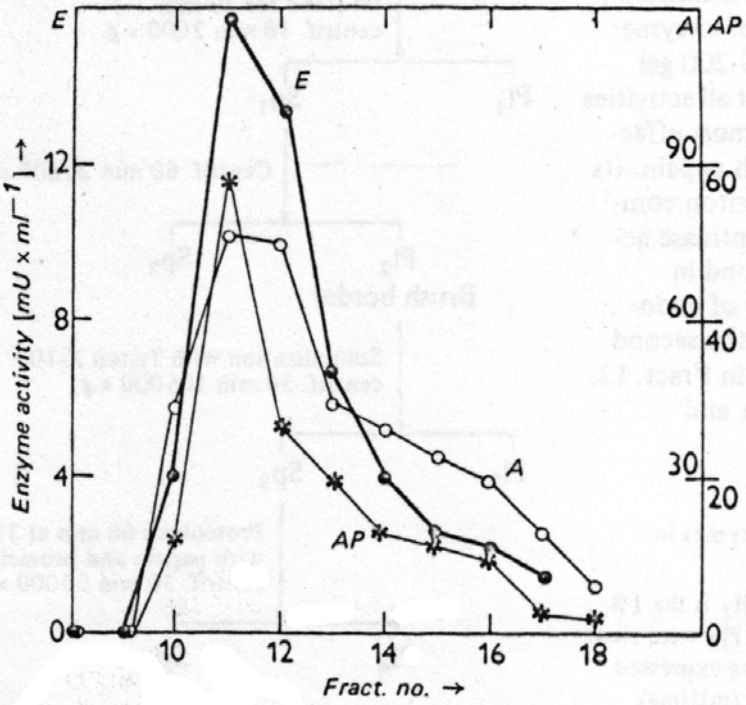

Fig. 2. Distribution of membrane-bound enzymes of the tritonized supernatant Sp_3 (105 000 × *g*) after Sephadex G-200 chromatography.

The fractions were assayed for endopeptidase (E), aminopeptidase (A) and alkaline phosphatase (AP). The activities are expressed as mU/ml.

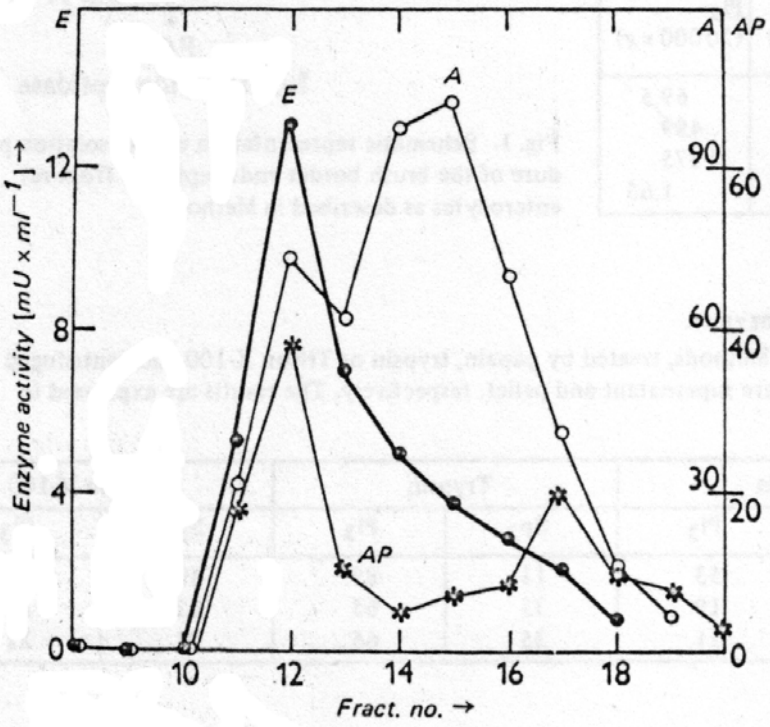

Fig. 3. Partial separation of the aminopeptidase (A) and alkaline phosphatase (AP) from the endopeptidase (E) after papain digestion.

The samples were centrifugated for 30 min at 20000 × *g* and applied on Sephadex G-200 column.

Isoelectric focusing

The isoelectric points (pI) of the aminopeptidase (Fracts. 35–45) and of the endopeptidase (Fracts. 55–65) are 4.73 and 5.23, respectively (Fig. 6). Both activities were detected by thin-layer chromatography on Silufol and the endopeptidase activity was estimated also by the direct method described in this paper.

Molecular mass estimation

The molecular mass was calculated on the basis of R_F values of endopeptidase and ferritin standard (Fig. 7) after centrifugation of dialyzed fraction F 60. The molecular mass of endopeptidase is 130000 ± 15000 dalton. This deviation was derived from the minimal discriminative capacity of the UA-5 monitor. The sedimentation coefficient $s^0_{20,w}$ is 7.2 S.

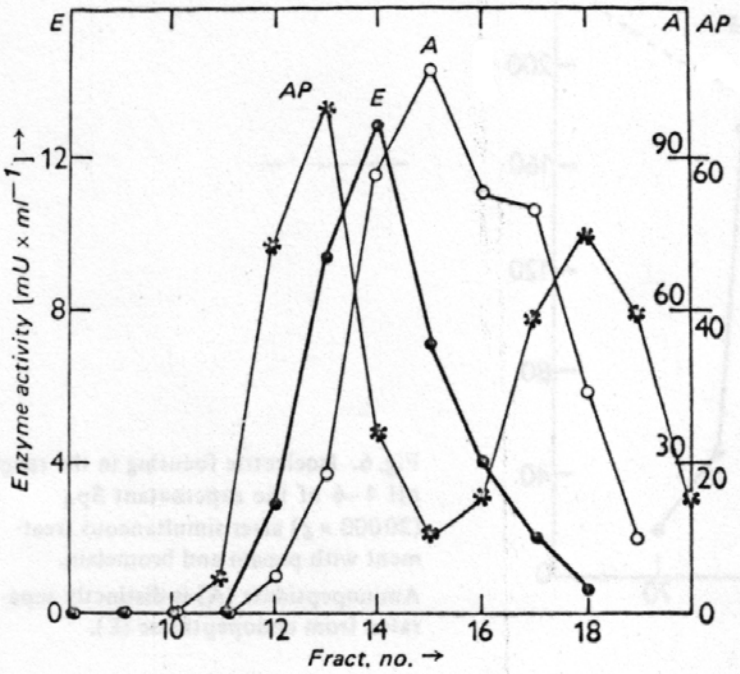

Fig. 4. Separation of brush border enzymes on Sephadex G-200.

The sample was treated simultaneously by papain and bromelain, centrifuged for 30 min at 20000 × *g* and applied on the column. Activities of endopeptidase (E), aminopeptidase (A) and alkaline phosphatase (AP) are expressed as mU/ml.

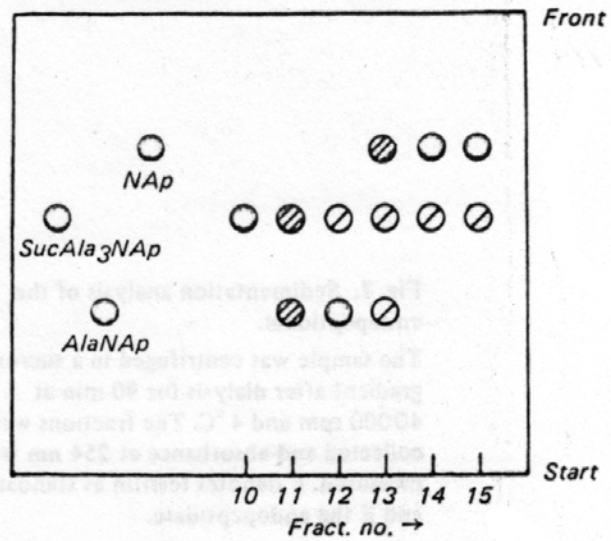

Fig. 5. Thin-layer-chromatographic analysis of the hydrolytic products of $SucAla_3NAp$ on Silufol.

The Fracts. 10–15 after Sephadex G-200 chromatography were incubated for 90 min at 37 °C with the substrate. The 2.5 mM standards of substrate, AlaNAp and NAp are on the left side and the samples on the right. The colour intensity was demonstrated as follows: ● > ◉ > ◌.

Polyacrylamide gel electrophoresis

Two enzymatically active bands (R_F 0.13 and 0.24) in the aminopeptidase fraction F 40 and single active band-splitting $SucAla_3NAp$ (R_F 0.16) in the endopeptidase fraction F 60 were found (Fig. 8). Co-migrating proteins were detected for all these bands.

Determination of pH optimum, K_m value, thermostability and the inhibitor effects on endopeptidase activity

Using $SucAla_3NAp$ as a substrate the K_m value was estimated as 1.1×10^{-3} M (Fig. 9). The activity decreased after preincubation for 15 min to 50% of the initial activity at 50 °C (thermostabil-

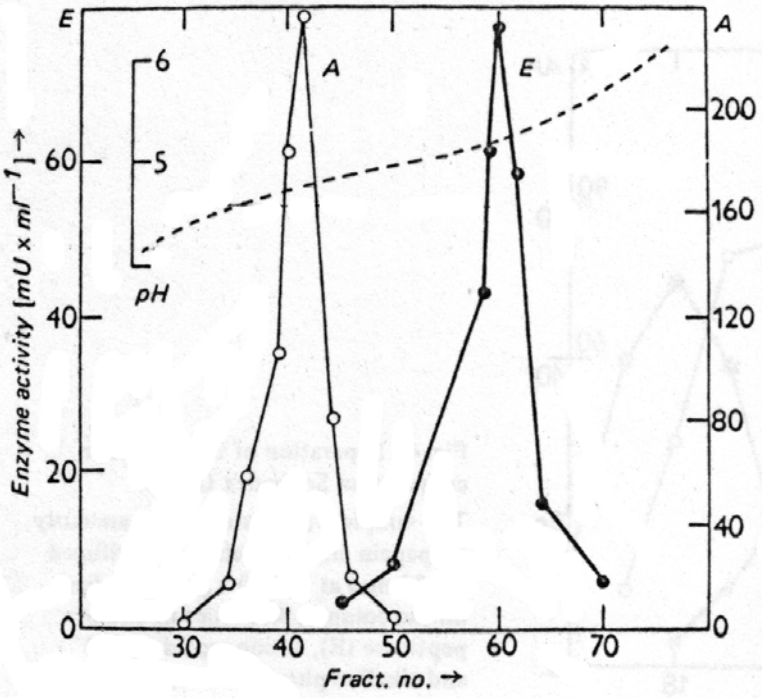

Fig. 6. Isoelectric focusing in the range pH 4–6 of the supernatant Sp_4 (20000 × *g*) after simultaneous treatment with papain and bromelain. Aminopeptidase (A) is distinctly separated from endopeptidase (E).

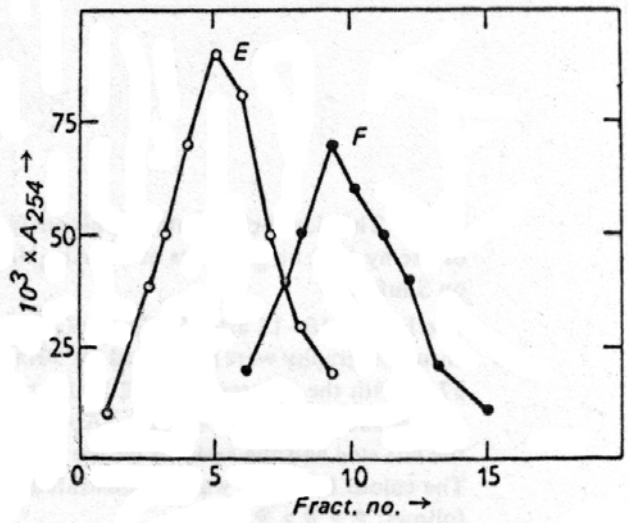

Fig. 7. Sedimentation analysis of the endopeptidase.

The sample was centrifuged in a sucrose gradient after dialysis for 90 min at 40000 rpm and 4 °C. The fractions were collected and absorbance at 254 nm was measured. F denotes ferritin as standard and E the endopeptidase.

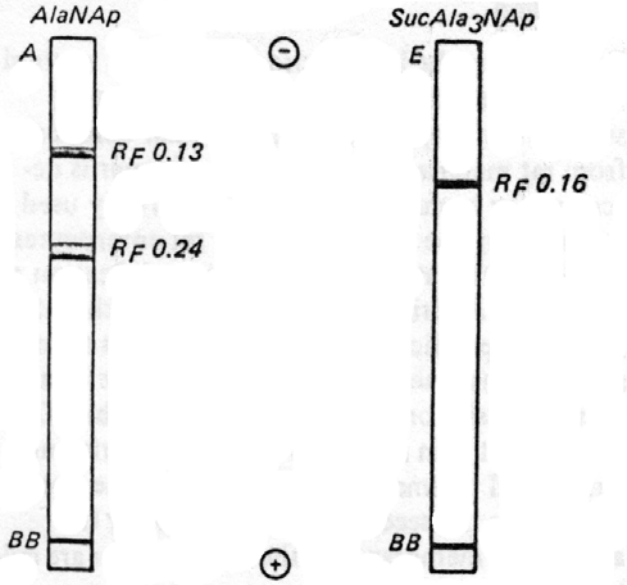

Fig. 8. Polyacrylamide gel electrophoresis of the isolated endopeptidase (E: fraction F 60) and aminopeptidase (A: fraction F 40).

Gels were prepared as described in Methods. Bromphenol blue (BB) was used as indicator in a concentration of 4×10^{-4} g/l.

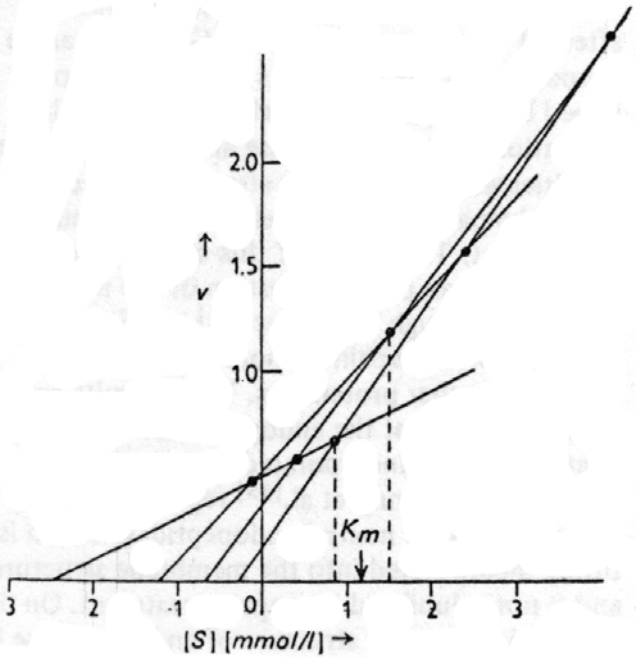

Fig. 9. Linear plot of substrate concentration ([S]) against reaction velocity (v) according to Eisenthal[28].

As substrate $SucAla_3NAp$ was used. The value of K_m was calculated as a median and was found to be 1.1mM.

ity), (Fig. 10). The pH optimum determined in 0.1M Tris/HCl buffer solution is 8.5 (Fig. 11). The inhibition effect of bivalent metal ions decreased in the following order: $Mn^{2\oplus}$, $Co^{2\oplus}$, $Zn^{2\oplus}$, $Mg^{2\oplus}$, and $Ca^{2\oplus}$ slightly stimulates the activity (Fig. 12).

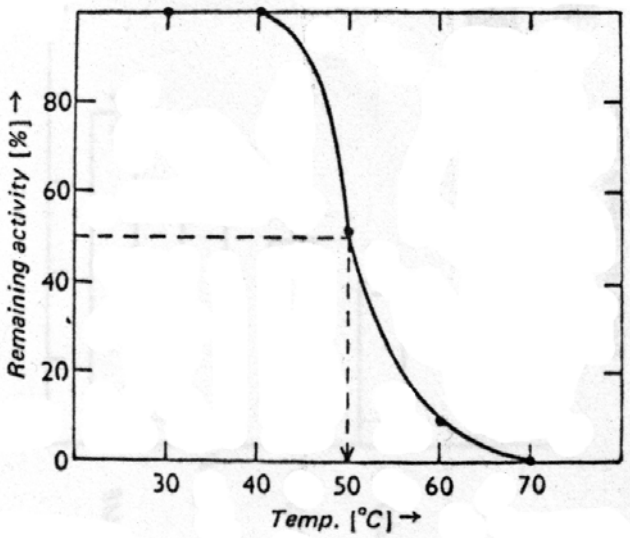

Fig. 10. Thermostability of the brush border endopeptidase.

The sample (Fract. F 60) was preincubated for 15 min at a of 30 °C to 70 °C. The results are expressed in percentage of residual activity.

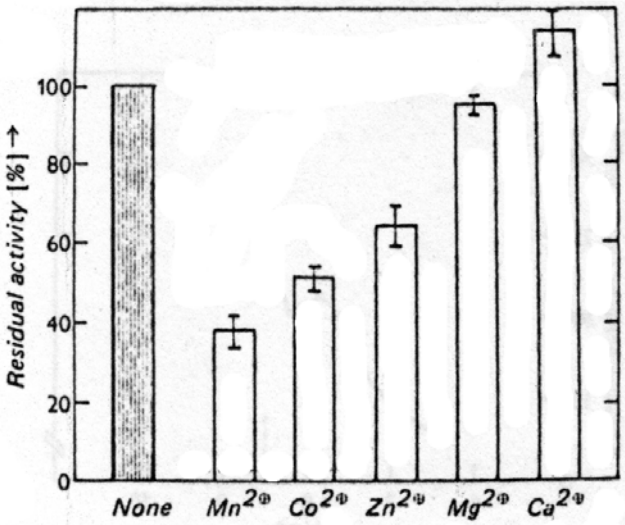

Fig. 11. Effect of bivalent metal ions of manganese, cobalt, zinc, magnesium and calcium on the endopeptidase activity.

The incubation mixture contained besides substrate and buffer solution 1 mmol of the respective ions/l. The activities are expressed as percentage of remaining activity.

The endopeptidase activity is further strongly inhibited by EDTA and *p*-chloromercuribenzoate and slightly inhibited by iPr_2P-F and β-mercaptoethanol (Fig. 13).

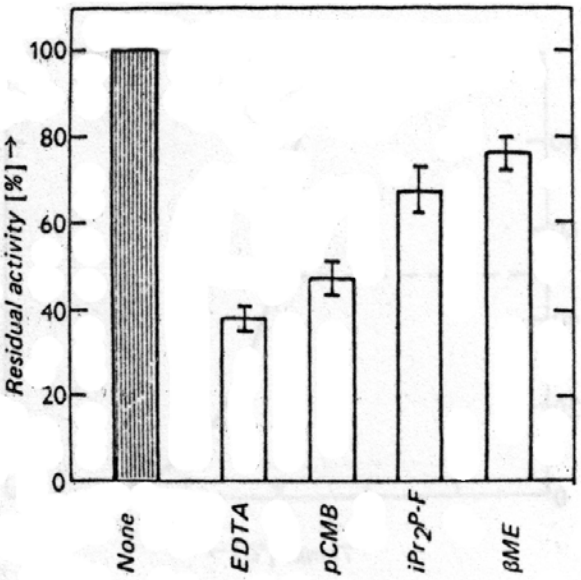

Fig. 12. Effect of some inhibitors on the brush border endopeptidase.
One of the inhibitors [EDTA, *p*-chlormercuribenzoate (pCMB); diisopropylfluorophosphate (iPr_2P-F) and β-mercaptoethanol (βME)] was added to the incubation mixture at a concentration of 1 mM. The results are expressed as percentage of the remaining activity.

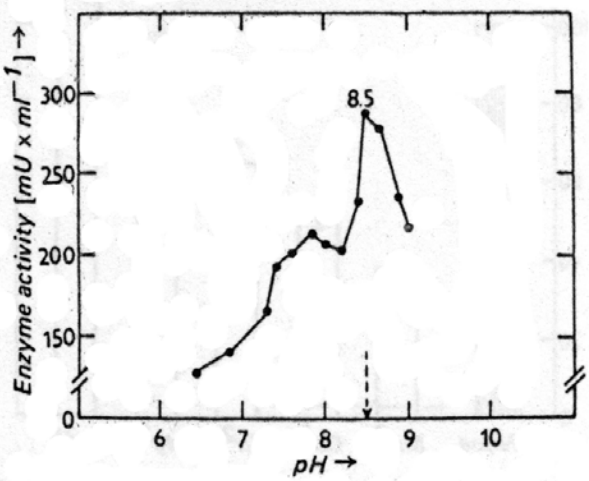

Fig. 13. The pH optimum of the brush border endopeptidase from the rat enterocytes.
The buffer solution (0.1 M Tris/HCl) in the incubation mixture was changed in the range of pH 6.0–9.0. The activity was expressed as mU/ml.

Discussion

For isolating the brush border Schmitz's method [18] was used, zonal centrifugation not being suitable for preparation of subcellular components from rat material[29]. Though the former is described for human material, it was already used for isolating the brush border of rat enterocytes [30,31]. In this study we used the modification according to Andria[19]. The method of Schmitz is based on specific binding of calcium ions to microsomal membranes[32] and in this way the separation of brush border membranes is possible. During the first spin at 2000 × *g* mitochondria and basolateral plasma membranes sink to the bottom and during the second spin at 20000 × *g* the aggregated membranes of the brush border are collected as the pellet Pl_2.

Trypsin and papain only release a small amount of endopeptidase from the brush border of enterocytes as compared to the action of Triton X-100. Marshall et al.[33] during their studies of solubilization procedures for membrane enzymes also found Triton X-100 most effective. As in such solubilized material the activities in question were present together in one common fraction after gel filtration on Sephadex G-200, it can be supposed that the fragments of membrane obtained by this method are relatively large. The separation of aminopeptidase and endopeptidase activities was achieved only after simultaneous digestion by papain and bromelain of the tritonized supernatant. The effect of this combination was better than papain digestion of the material Sp_3 alone, though Maestracci[34] and Slabý et al.[13] reported papain as more effective compared with other proteinases. These results enable us to presume that the endopeptidase activity is relatively firmly incorporated into the brush border structure. Kenny et al.[35] reported similar behavior of renal neutral endopeptidase which is firmly incorporated into the membrane structure and is not solubilized by papain treatment. On the assumption that the structure of endopeptidase is similar to other membrane enzymes[8,9], it may be suggested that only the head piece (protease form) of endopeptidase has been obtained with the use of our isolation procedure. On the other hand, the native enzyme (triton form) cannot be properly separated from aminopeptidase[13].

The endopeptidase of the brush border of rat enterocytes splits the bond in the position P_2-P_1 [13,36] as follows:

$$\text{SucAla}_3\text{NAp} \xrightarrow{\text{Endopeptidase}} \text{SucAla}_2 + \text{AlaNAp}$$

$$\text{AlaNAp} \xrightarrow{\text{Aminopeptidase}} \text{Ala} + \text{NAp}.$$

The detection of the hydrolytic products of substrate $\text{SucAla}_3\text{NAp}$ on Silufol confirms the procedure shown above. The endopeptidase did not split the substrates amino acid-4-nitroanilide as opposed to amino-oligopeptidases as reported by Gray et al.[4,5] and other intestinal peptidases as reported Kim et al.[1,2] and Louvard et al.[7,37,38] that hydrolyse the analogous substrate Leu-βNA very well. The pattern of splitting of the $(\text{Ala})_n$-NAp and $\text{Suc}(\text{Ala})_n$-NAp series of substrates[13] reveals that it is not possible to classify the endopeptidase as "aminopeptidase with additional endopeptidase activity". These peptidases also have a much higher molecular mass while the endopeptidase described in this paper has a molecular mass of 130000 ± 15000 dalton estimated by sedimentation analysis ($s^0_{20,w}$ is 7.2 S).

The "post-proline-cleaving enzyme" isolated recently from lamb kidney[39,40] differs basically in the mechanism of splitting, but the physico-chemical constants (Tab. 3) are similar to those of the brush border endopeptidase. The post-proline-cleaving enzyme cleaves the alanine peptides too, but tetraalanine is split more rapidly than ZAla_3, as reported by Yoshimoto et al.[41], while our enzyme practically does not split Ala_4NAp at all[13] and a substrate comparable to ZAla_3, i.e. AcAla_3NAp, used by the authors is hydrolysed optimally.

Table 3. Some enzymatic and physico-chemical properties of brush border endopeptidase from rat enterocytes.

Molecular mass, M_r	130000 ± 15000
Sedimentation coefficient $s^0_{20,w}$	7.2 S
Isoelectric point, pI	5.23
Electrophoretic mobility, R_F	0.16
Optimum pH	8.5
Thermostability	50 °C
Michaelis constant K_m ($\text{SucAla}_3\text{NAp}$)	1.1×10^{-3} M

The pH optimum value of the brush border endopeptidase is 8.5 and this value is near to the values described for other intestinal peptidases. The temperature at which 50% of activity remains (thermostability) is 50 °C, which is a little more than for the post-proline-cleaving enzyme and much less than described for other intestinal aminopeptidases.

EDTA and *p*-chloromercuribenzoate inhibit strongly the activity of endopeptidase already in very low concentrations and comparable concentrations of bivalent ions of manganese and cobalt have the same effect. These results suggest that the endopeptidase of the rat enterocytes' brush border is a metallo-enzyme. A rather broad series of inhibitors affecting endopeptidase activity and acting on various sites of the molecule is not specific for this enzyme and was described in other membrane-associated proteases as well[42].

The neutral endopeptidase as reported by Kerr et al.[43,44] differs from the above described enzyme in the localization in kidney tubules of the rabbit. It has a lower pH optimum (6.0), a smaller molecular mass (about 90000) and it is a Zn-dependent enzyme. Vannier et al.[45] even prove a similarity of the intestinal and renal aminopeptidases.

Literature

1 Kim, Y.S. & Brophy, E.J. (1976) *J. Biol. Chem.* 251, 3199–3205.
2 Kim, Y.S., Brophy, E.J. & Nicholson, J.A. (1976) *J. Biol. Chem.* 251, 3206–3212.
3 Wojnarowska, F. & Gray, G.M. (1975) *Biochim. Biophys. Acta* 403, 147–160.
4 Gray, G.M. & Santiago, N.A. (1977) *J. Biol. Chem.* 252, 4922–4928.
5 Kania, R.K., Santiago, N.A. & Gray, G.M. (1977) *J. Biol. Chem.* 252, 4929–4934.
6 Shoaf, C.R., Berko, M. & Heizer, W. (1976) *Biochim. Biophys. Acta* 445, 694–719.
7 Louvard, D., Maroux, S., Vannier, Ch. & Desnuelle, P. (1975) *Biochim. Biophys. Acta* 375, 236–248.
8 Louvard, D., Semeriva, M. & Maroux, S. (1976) *J. Biol. Chem.* 106, 1023–1035.
9 Maroux, S., Louvard, D., Vannier, P. & Semeriva, M. (1977) *Biochem. Soc. Trans.* 5, 520–527.
10 Pattus, F., Verger, R. & Desnuelle, P. (1976) *Biochem. Biophys. Res. Commun.* 69, 718–723.
11 Ugolev, A.M., Mityushova, N.M., Egorova, V.V., Gozite, I.K. & Koltushkina, G.G. (1979) *Gut* 20, 737–742.

12 Kocna, P. (1977) Abstr. XX. Faculty stud. sci. confer. Charles University, Faculty of General Medicine Prague (CSSR) pp. 1/7.
13 Slabý, J., Frič, P. & Kasafírek, E. (1978) *Acta Hepato-Gastroenterol.* 25, 295–302.
14 Frič, P., Slabý, J., Kasafírek, E. & Mališ, F. (1976) *Dtsch. Z. Verdau.* 26, 239–244.
15 Frič, P., Slabý, J., Kasafírek, E. & Mališ, F. (1975) *Clin. Chim. Acta* 63, 309–316.
16 Kasafírek, E., Frič, P. & Mališ, F. (1974) *FEBS Lett.* 40, 353–356.
17 Kasafírek, E., Frič, P., Slabý, J. & Mališ, F. (1976) *Eur. J. Biochem.* 69, 1–13.
18 Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B.K., Cerda, J.J. & Crane, R.K. (1973) *Biochim. Biophys. Acta* 323, 98–112.
19 Andria, G., Marzi, A. & Auricchio, S. (1976) *Biochim. Biophys. Acta* 419, 42–50.
20 Haglund, H. (1967) Sci. Tools (LKB Instrum. J.) 14, 17–23.
21 Martin, R.G. & Ames, B.N. (1961) *J. Biol. Chem.* 236, 1372–1379.
22 Blakesley, R.W. & Boezi, J.A (1977) *Analyt. Biochem.* 82, 580–582.
23 Bessey, O.A., Lowry, O.H. & Broch, M.J. (1946) *J. Biol. Chem.* 164, 321–329.
24 Dahlqvist, A. (1964) *Anal. Biochem.* 7, 18–25.
25 Mališ, F. (1968) *Sb. Lek.* 70, 321–329.
26 Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) *J. Biol. Chem.* 193, 265–275.
27 Sedmak, J.J. & Grossberg, S.E. (1977) *Anal. Biochem.* 79, 544–552.
28 Eisenthal, R. & Cornish-Bowden, A. (1974) *Biochem. J.* 139, 715–720.
29 Peters, T.J. (1976) *Clin. Sci. Mol. Med.* 51, 557–574.
30 Mazzacca, G., Musella, A., Andria, G., Agostino, L.G., Cimino, L. & Budillon, G. (1977) *Acta Hepato-Gastroenterol.* 24, 364–367.
31 Seetharam, B., Yeh, K.Y., Moog, F. & Alpers, D.H. (1977) *Biochim. Biophys. Acta* 470, 424–436.
32 Kamath, S.A., Kummeron, F.A. & Narayan, K.A. (1971) *FEBS Lett.* 17, 90–92.
33 Marshall, J.J., Sturgeon, C.M. & Whelan, W.J. (1977) *Anal. Biochem.* 82, 435–444.
34 Maestracci, D. (1976) *Biochim. Biophys. Acta* 433, 469–481.
35 Kenny, A.J., Booth, A.G. & MacNair, R.D.C. (1977) *Acta Biol. Med. Germ.* 36, 1575–1585.
36 Schechter, I. & Berger, A. (1968) *Biochim. Biophys. Res. Commun.* 32, 898–902.
37 Louvard, D., Maroux, S., Baratti, J., Desnuelle, P. & Mutaftschiev, S. (1973) *Biochim. Biophys. Acta* 291, 747–763.
38 Maroux, S. & Louvard, D. (1977) *Gastroenterol. Clin. Biol.* 1, 377–388.
39 Koida, M. & Walter, R. (1976) *J. Biol. Chem.* 251, 7593–7599.
40 Walter, R. & Yoshimoto, T. (1978) *Biochemistry* 17, 4139–4144.
41 Yoshimoto, T., Fischl, M., Orlowski, R.C. & Walter, R. (1978) *J. Biol. Chem.* 253, 3708–3716.
42 Barret, A.J. (1977) in *Proteinases in Mammalian Cells & Tissues* (Barret, A.J., ed.) pp. 1–56, Elsevier North-Holland.
43 Kerr, M.A. & Kenny, A.J. (1974) *Biochem. J.* 137, 477–488.
44 Kerr, M.A. & Kenny, A.J. (1974) *Biochem. J.* 137, 489–495.
45 Vannier, C., Louvard, D., Maroux, S. & Desnuelle, P. (1976) *Biochim. Biophys. Acta* 455, 185–199.

Petr Kocna, II. Vědecké odd. gastroenterologické FVL UK, Karlovo náměstí 32, Praha 2, CS-121-11 Czechoslovakia.