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STUDIES ON A SOLUBLE DIPEPTIDASE FROM PIG INTESTINAL MUCOSA

I. PURIFICATION AND SPECIFICITY

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SUMMARY

Purification of a soluble dipeptidase (EC 3.4.3.2) from pig small intestine is described. 10–20 mg of the purified enzyme were obtained in each preparation (30 m small intestine). It was tested to be at least 99% pure on polyacrylamide electrophoresis and homogeneous in crossed immunoelectrophoresis. The specific activity of the enzyme, estimated against glycyl-L-leucine, varied between 1400–1700 units of activity per mg protein in different batches.

The enzyme has a wide specificity against dipeptides, although it has no prolidase activity. Among the dipeptides tested, L-alanyl-L-alanine, L-alanyl-L-serine and glycyl-L-leucine were hydrolyzed most rapidly.

The enzyme constitutes a true dipeptidase, as it has no tripeptidase or arylamidase activities. It is strictly stereospecific and requires free end-groups of the dipeptides. No activity of the enzyme was found against dipeptides containing β-amino acids.

INTRODUCTION

Much of the dipeptide hydrolysis is performed by soluble enzymes, and among these enzymes a particular interest has been paid to those related to the intestinal mucosa[1]. The knowledge about these enzymes has, however, greatly been hampered because most of the studies have been carried out with rather impure material, although early attempts of purifying them were made[2]. The aim of the present investigation was, therefore, to isolate and purify a water-soluble dipeptidase from pig intestinal mucosa and determine its specificity. Since the activity against glycyl-L-leucine has been proposed to represent a distinct enzyme (EC 3.4.3.2) (ref. 3) and the intestinal mucosa contains much of hydrolyzing activity against this dipeptide, glycyl-L-leucine was selected as substrate for assaying the dipeptidase activity during the purification.

Our report describes the purification of the enzyme and includes specificity studies, which show that it constitutes a true dipeptidase. A general outline of the purification procedure has previously been reported[4]. Very recently an intestinal dipeptidase from monkey has also been reported purified[5].

MATERIALS AND METHODS

Chemicals

DEAE-cellulose, DE 52, was purchased from Whatman Co., Maidstone, England. Sephadex G-100 and DEAE-Sephadex A-50 were obtained from Pharmacia, Uppsala, Sweden. Hydroxylapatite (Biogel HTP) was purchased from Bio-Rad Labs., Richmond, U.S.A., and agarose was obtained from l'Industrie Biologique Française, Gennevillier, France. Acrylamide and *N*,*N*′-methylenebisacrylamide were delivered by BDH Chemicals Ltd, Poole, England. Dialysis tubes were manufactured by Visking Co., Chicago, U.S.A. To remove inactivating material they were treated before use with 0.1 M EDTA (disodium salt) at 50 °C for a minimum of 12 h and subsequently washed thoroughly with water. All other chemicals used were of analytical grade and distilled and de-ionized water was used throughout.

Substrates

The substrates used in the specificity analysis were analyzed for purity by thin-layer chromatography. Suppliers of the substrates are given in Tables II–IV.

Source of enzyme

Small intestines of adult pigs were used as source of the enzyme. They were cut open immediately after slaughter and squeezed before use to remove intestinal contents. Pieces of 3-m length were taken from each intestine 2 m distal of the pylorus. They were opened longitudinally, cut into about 10-cm pieces, and put into precooled water (1 l water/3-m intestine, 10 °C) for extraction. After about 1 h of extraction with stirring in a cold room, the extract was filtered through a crude nylon net (mesh size 1.5 mm) and then centrifuged (15 000 × *g*, 60 min, 4 °C). The supernatant was collected and subsequently lyophilized. The resulting red-yellow powder (about 3 g/m intestine) was stored in closed bottles at —20 °C until used. No considerable loss of activity was observed under these conditions.

Analytical procedures

pH measurements were performed at room temperature using a pH-meter 28 (Radiometer, Copenhagen, Denmark) provided with a glass electrode. Spectrophotometric measurements were performed using a PMQ-II spectrophotometer (Zeiss, Oberkochen, Germany). The transmission at 280 nm of column chromatography effluents was continuously monitored with an LKB 8300 A Uvicord II, provided with a 3-mm measuring cell (LKB-Produkter AB, Stockholm, Sweden). The conductance of ion exchange column chromatography effluents was measured with a Tetramatic conductance meter (Kemotron, Copenhagen, Denmark) and transformed to NaCl concentration using a standard curve. Ultrafiltration was undertaken using Diaflo PM-10 filters essentially as described by the manufacturer (Amicon Corp., Lexington, U.S.A.). Polyacrylamide gel electrophoresis was run in 4 mm × 70 mm

vertical glass columns[6,7]. The gels were stained with Coomassie brilliant blue[8]. Crossed immunoelectrophoresis[9] was run on 1% agarose in 0.02 M sodium barbital buffer, pH 8.6. The identification of the precipitate corresponding to the dipeptidase in the crossed immunoelectrophoresis was performed as described by Krøll[10]. The precipitates of the immunoelectrophoresis were coloured as described by Axelsen[11]. Thin-layer chromatography was carried out on precoated cellulose TLC plates, 20 cm × 20 cm, 0.10 mm layer thickness (Merck, Darmstadt, Germany), using *n*-butanol–acetic acid–water (4:1:1, by vol.) or water-saturated phenol as solvents and ninhydrin reagent for staining. Peptides containing no free α-amino group were stained by the starch–iodide procedure[12].

Assays

The purification of the enzyme was followed by assaying the activity of the enzyme against glycyl-L-leucine according to the spectrophotometric procedure of Josefsson and Lindberg[13], using the following conditions in the incubation mixture: 11.0 mM dipeptide in 0.1 M sodium phosphate buffer, pH 7.8, at 25 °C. One unit of enzyme activity was defined as the activity hydrolyzing 1 μmole glycyl-L-leucine per min at 25 °C and pH 7.8.

The specificity studies (Tables II and IV) were performed at 25 °C using a substrate concentration of 8.5 mM (1.8 mM for L-alanyl-L-proline) in 0.1 M potassium phosphate buffer, pH 7.4. To assure a better precipitation of the proteins at the end of the incubation, ethanol was replaced by a 2.5% (w/v) metaphosphoric acid as precipitating agent. In those cases where the substrate was not suitable for the spectrophotometric assay[13], *i.e.* when the dipeptide included side chains with strong ultraviolet absorbance, the measurement of the hydrolysis was performed by using the 2:4:6-trinitrobenzenesulphonic acid (TNBS) reagent according to a modified procedure of that described by Binkley *et al.*[14]. The procedure was as follows: The hydrolysis in the incubation mixture was interrupted by 1 ml trichloroacetic acid (10%, w/v). 50 μl were withdrawn and given 1 ml of the TNBS reagent. After standing for 40 min at 25 °C to allow for development of colour, the TNBS reaction was stopped by adding 1 ml trichloroacetic acid (10%, w/v) to the reaction mixture. This procedure effectively stops further colour development because the reaction velocity is strongly retarded at pH below 6 (ref. 15). The absorbance of the solution was measured at 350 nm. A standard sample of fixed percentage of hydrolysis was included in every assay. The assay procedures used for analyzing the hydrolysis of aminoacyl-β-naphthyl amides and L-leucyl-*p*-nitroanilide (Table III) were those of Weber[16] and Nagel *et al.*[17], respectively.

Estimation of enzyme purification

The specific activity was expressed as units of enzyme activity per mg of protein, assuming an average absorbance for an 1% protein solution at 280 nm to be 10, using a path length of 1 cm.

Purification procedure

All operations were performed in the cold room (4 °C). In order to stabilize the activity of the enzyme all buffers contained 4 mM 2-mercaptoethanol and 12.5% (w/v) glycerol, unless otherwise stated.

$(NH_4)_2SO_4$ fractionation. Lyophilized intestinal extract (67 g) was dissolved in precold glycerol-free 0.05 M sodium phosphate buffer, pH 7.5 (670 ml) and extracted for 1 h with continuous stirring. Insoluble material was centrifuged off (15 000 × *g*, 1 h) and the supernatant was filtered through a coarse glass filter to remove some floating lipid material. The resulting red, slightly opalescent solution (Supernatant, 650 ml) was fractionated with $(NH_4)_2SO_4$.

Solid $(NH_4)_2SO_4$ (220 mg/ml) was given to the supernatant under continuous stirring, and the solution was left for 30 min. The precipitate formed was centrifuged off (27 000 × *g*, 1 h) and the supernatant (590 ml) was collected. A further amount of solid $(NH_4)_2SO_4$ (345 mg/ml) was given under stirring to the supernatant and it was then left for another 30-min period. The precipitate formed was collected by centrifugation (27 000 × *g*, 1 h), washed twice with glycerol-free 0.05 M sodium phosphate buffer, pH 7.5, containing 350 mg $(NH_4)_2SO_4$/ml (75.0 ml). The final precipitate was dissolved in 100 ml 0.02 M Tris–HCl buffer, pH 7.0, and subsequently dialyzed against the same buffer (2 × 7 l) for 2 × 18 h. Small amounts of insoluble material formed during the dialysis were removed by centrifugation (48 000 × *g*, 2 h) and the yellow, slightly opalescent, supernatant was collected ($(NH_4)_2SO_4$ fraction, 135 ml).

Chromatography on DEAE-cellulose. The $(NH_4)_2SO_4$ fraction was applied to an 8.0 cm × 14.5 cm column of DEAE-cellulose, prepared in and equilibrated with 0.02 M Tris–HCl buffer, pH 7.0. After an initial wash with the same buffer (1575 ml) the column was eluted with a linear increasing NaCl-gradient (3600 ml) in the starting buffer, ending at 0.3 M NaCl. The elution was performed at a constant rate of 172 ml/h. Fractions (23 ml) were collected and assayed for enzyme activity (Fig. 1). Those containing more than 114 units of activity/ml were pooled (DEAE-cellulose fraction, 330 ml).

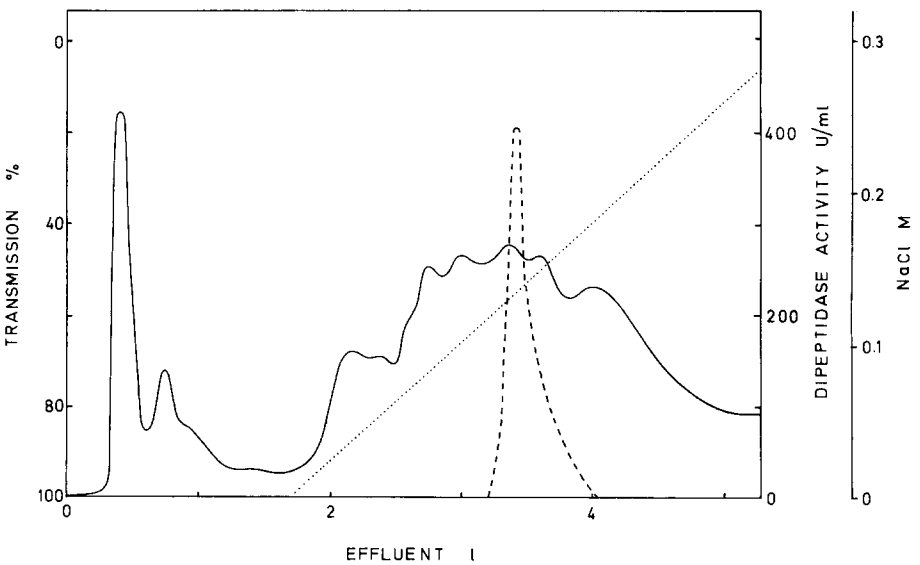

Fig. 1. Chromatography of the $(NH_4)_2SO_4$ fraction on DEAE-cellulose (see Purification procedure). — — —, transmission at 280 nm; ------, dipeptidase activity; · · · · · ·, NaCl concentration.

Chromatography on DEAE-Sephadex. The DEAE-cellulose fraction was dialyzed against 0.05 M Tris–HCl buffer, pH 7.0, containing 0.04 M NaCl (3000 ml) for 18 h and then applied to a 2.5 cm × 30.5 cm column of DEAE-Sephadex A-50. The column was prepared in 0.2 M Tris–HCl buffer, free of 2-mercaptoethanol and glycerol, and then equilibrated with 0.05 M Tris–HCl buffer, pH 7.0, containing 0.04 M NaCl. After an initial wash with the buffer (150 ml) the column was eluted with a linear increasing NaCl gradient (2000 ml) in the same buffer, ending at 0.3 M NaCl. The elution was performed at a constant rate of 25 ml/h and the effluent was collected in fractions (12.5 ml). The fractions were assayed for enzyme activity (Fig. 2) and those containing more than 157 units of activity/ml were combined (DEAE-Sephadex fraction, 124 ml).

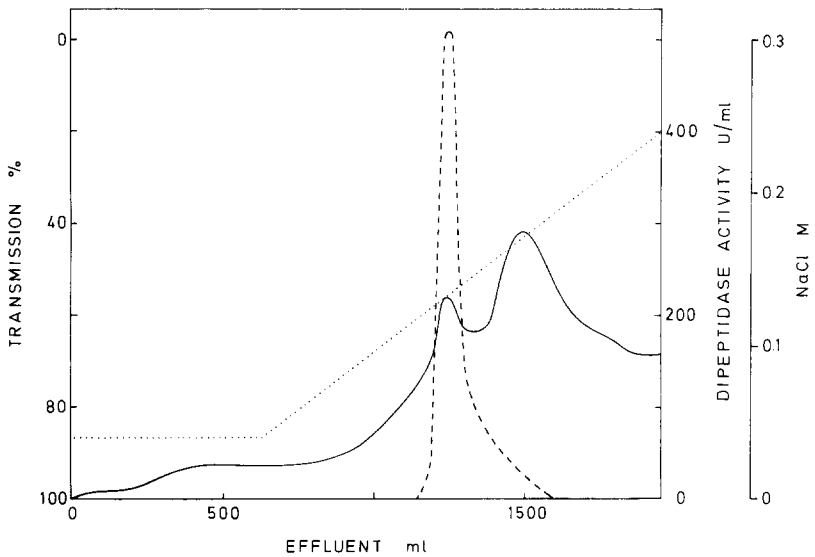

Fig. 2. Chromatography of the DEAE-cellulose fraction on DEAE-Sephadex (see Purification procedure). — — —, transmission at 280 nm; ------, dipeptidase activity; · · · · · ·, NaCl concentration.

Chromatography on hydroxylapatite. The DEAE-Sephadex fraction was applied to a 2.5 cm × 24.7 cm column of hydroxylapatite, prepared in and equilibrated with 0.025 M sodium phosphate buffer, pH 7.0, made 0.2 M with respect to NaCl. After an initial wash with the same buffer (450 ml) the column was eluted with 0.07 M sodium phosphate buffer, pH 7.0, containing 0.2 M NaCl. The elution was kept constant at a flow rate of 25 ml/h and the effluent was collected in fractions (6 ml). The fractions were analyzed for enzyme activity (Fig. 3) and those containing more than 38 units of activity/ml were pooled (hydroxylapatite fraction, 75.0 ml).

Chromatography on Sephadex G-100. The hydroxylapatite fraction was concentrated by means of ultrafiltration. A small precipitate formed during the ultrafiltration was centrifuged off (27 000 × *g*, 30 min) and the clear supernatant (3.2 ml) was applied underneath the eluant to a 2.5 cm × 93.5 cm column of Sephadex G-100. The column, prepared in and equilibrated with glycerol-free 0.07 M sodium phosphate buffer, pH 7.0, made 0.2 M with respect to NaCl, was eluted under constant pressure

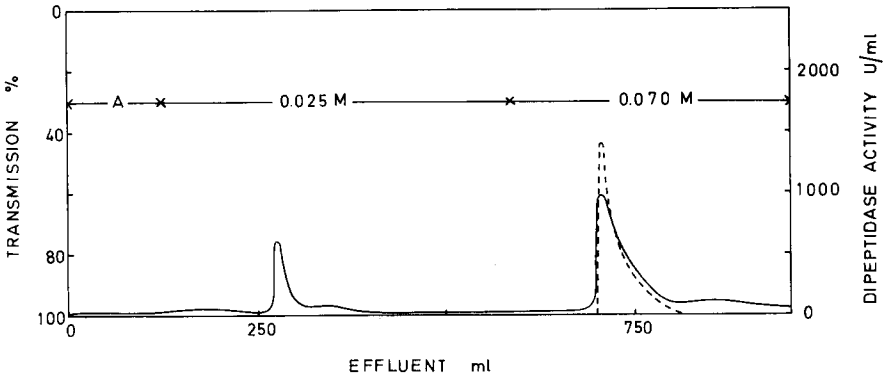

Fig. 3. Chromatography of the DEAE-Sephadex fraction on hydroxylapatite (see Purification procedure). A, applied volume; — — —, transmission at 280 nm; ------, dipeptidase activity.

at a flow rate of 12 ml/h with the same buffer. Fractions (3.1 ml) were collected and assayed for absorbance at 280 nm and for enzyme activity (Fig. 4). The glycyl-L-leucine hydrolase activity was obtained in two separate peaks, of which the one with the lowest molecular size was responsible for most of the activity and corresponded to the main protein peak. Fractions containing more than 275 units of activity/ml were combined (purified dipeptidase, 21.7 ml). This enzyme solution, constituting the purified dipeptidase, was made 12.5% with respect to glycerol and then stored frozen (—20 °C) until used.

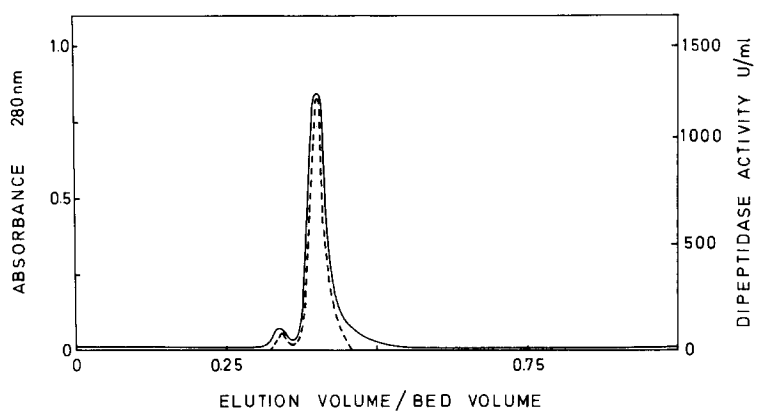

Fig. 4. Chromatography of the hydroxylapatite fraction on Sephadex G-100 (see Purification procedure). ———, absorbance; ------, dipeptidase activity.

Preparation of intestinal extract for specificity studies

Lyophilized intestinal extract (0.5 g) was dissolved in 0.1 M potassium phosphate buffer, pH 7.4 (5 ml), containing 12.5% (w/v) glycerol, and dialyzed over-night against the same buffer (1000 ml) to remove disturbing low molecular weight material. Insoluble material was centrifuged off (27 000 × *g*, 30 min) and the supernatant was used directly in the assays (Extract solution). A new extract solution was prepared for every day of experiment.

Preparation of rabbit immunoglobulins to the DEAE-Sephadex fraction

A DEAE-Sephadex fraction (see Purification procedure) was concentrated by ultrafiltration to 11.4 mg protein/ml. 50 μl of this solutuon together with 50 μl of Freund's incomplete adjuvant were injected intracutaneously into white rabbits. A booster injection was given every fourteenth day during a six week period. After this period the interval between each injection was extended to 45 days. Ten days after injection the rabbits were bled for about 40 ml, the first bleeding following the fourth injection. The serum was collected and used for the preparation of immunoglobulins[11]. 20 ml serum resulted in 6.8 ml purified immunoglobulin solution.

RESULTS AND DISCUSSION

Purification

The results of a typical purification experiment are presented in Table I. In a series of experiments the purification procedure has yielded about 10–20 mg

TABLE I

PURIFICATION OF A DIPEPTIDASE FROM PIG INTESTINAL MUCOSA

Figures are given for 67 g lyophilized intestinal extract. Dipeptidase activity is measured against glycyl-L-leucine.

Fraction	*Total activity (units)*	*Specific activity (units/mg protein)*
Supernatant	276 000	4.01
$(NH_4)_2SO_4$ fraction	143 000	23.4
DEAE-cellulose fraction	84 200	197
DEAE-Sephadex fraction	66 800	900
Hydroxylapatite fraction	35 500	1320
Purified dipeptidase	17 900	1380

of the purified dipeptidase per 100 g of lyophilized intestinal extract. This represents about 5–10% of the original activity present in the starting material. The specific activity of the purified enzyme preparations has varied between 1400–1700 units of activity per mg protein.

Purity

The various fractions pooled from the main peak of activity in the Sephadex G-100 chromatography showed constant specific activity when assayed separately. A further test of the homogeneity of the preparation was obtained by polyacrylamide electrophoresis and by crossed immunoelectrophoresis.

The result obtained in the polyacrylamide electrophoresis (Fig. 5) showed that the purified dipeptidase besides a main component, corresponding to the dipeptidase activity, also gave rise to two minor bands. However, when re-electrophoresis was performed on the main component, after it had been eluted from the gels obtained in preparative electrophoresis experiments[18], the pattern obtained was identical to the one obtained in the first electrophoresis. This result showed that the two minor bands originate from the purified dipeptidase during the experimental procedures of the polyacrylamide electrophoresis. The sensitivity of the Coomassie brilliant blue

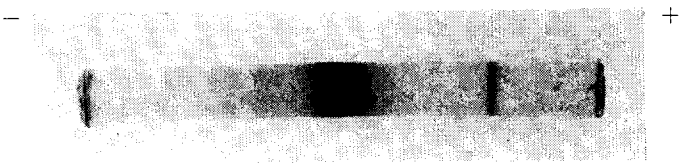

Fig. 5. Polyacrylamide electrophoresis pattern of a purified dipeptidase. 8 μg enzyme was electrophorized at 2.5 mA for 1.5 h.

reaction with protein, allowing identification of amounts down to 70 ng of protein, was used to estimate the purity of the enzyme preparation in the polyacrylamide electrophoresis experiments, and showed a purity of at least 99%.

Crossed immunoelectrophoresis was performed on samples of the supernatant and of the purified dipeptidase in the same experiment. Approximately equal amounts of dipeptidase activity were applied. The results showed, besides polyvalency of the immunoglobulin solution, which of the various precipitates from the multicomponent pattern of the supernatant that was related to the purified dipeptidase (Fig. 6A). A separate run with the purified enzyme showed only one component, giving further evidence for the homogeneity of the dipeptidase preparation (Fig. 6B).

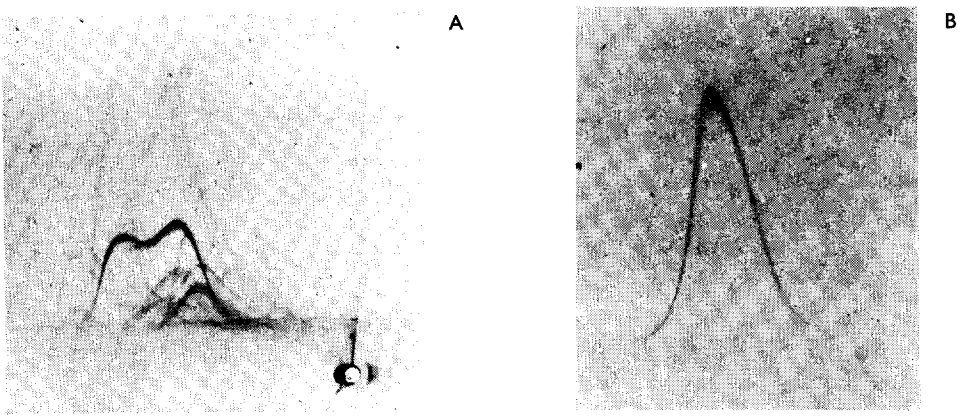

Fig. 6. Crossed immunoelectrophoresis patterns. A. To the left well 0.8 mg of a supernatant and to the right well 0.6 μg of a purified dipeptidase was applied. The first dimension was run for 75 min applying 8 V/cm and the second dimension was run for 17 h applying 2.5 V/cm. The concentration of immunoglobulin solution was 5 μl/cm^2. B. 2.5 μg of a purified dipeptidase was applied. The first dimension was run for 1 h applying 10 V/cm and the second dimension was run for 1 h applying 2.4 V/cm. The concentration of immunoglobulin solution was 10 μl/cm^2.

Specificity

The results of the specificity studies are given in Tables II–IV. The peptidase and arylamidase activities in the extract solution are also presented (Tables II and III).

Regarding the purified enzyme, it is evident from Table II, that it has a rather wide specificity against dipeptides. Of the various dipeptides studied the enzyme has the highest activity against L-alanyl-L-alanine, which is hydrolyzed at a rate about three times greater than glycyl-L-leucine. Only a few of the dipeptides are not hydrolyzed at all by the purified dipeptidase. Among these L-alanyl-L-proline has

TABLE II

RELATIVE RATES OF HYDROLYSIS FOR DIFFERENT SUBSTRATES OF THE INTESTINAL MUCOSA EXTRACT AND THE PURIFIED DIPEPTIDASE

The enzyme activities are expressed as relative rates of hydrolysis of the various substrates as a percentage of the hydrolysis rate of glycyl-L-leucine. Zero percent activity means that the activity found was less than 0.1 percent of that against glycyl-L-leucine.

Substrate (L-forms)	*Supplier*	*Assay method*	*Hydrolysis (%)*	
			Extract solution	*Dipeptidase*
Gly–Leu	1	A	100	100
Ala–Ala	2	A	280	300
Ala–Leu	1	A	39	53
Leu–Ala	1	A	27	35
Ala–Ser	3	A	190	170
Ser–Ala	2	A	60	52
Ala–Glu	1	A	59	56
Glu–Ala	1	A	11	7.3
Ala–Lys	1	A	20	19
Lys–Ala	1	A	3.7	0
Ala–Pro	3	A	5.4	0
Pro–Ala	1	A	3.7	2.0
Ala–Tyr	1	B	9.3	7.3
Tyr–Ala	1	B	27	14
Ala–Trp	1	B	77	37
Trp–Ala	1	B	6.2	0
Gly–Gln	2	A	38	18
Gln–Gly	2	A	30	28
Gly–Gly	4	B	3.1	0.25
Leu–Tyr	5	B	16	12
Ala–Ala–Ala	2	A	high activity	0
Gly–Leu–Tyr	2	B	high activity	0

1, Sigma Chem. Co., St. Louis, USA.
2, Cyclo Chem. Corp., Los Angeles, USA.
3, Fluka AG Chem. Fabrik, Buchs, Switzerland.
4, Mann Res. Labas., New York, USA.
5, Yeda Res. and Devel. Comp., Rehovoth, Israel.
A, Spectrophotometric method[13].
B, TNBS-method[14].

earlier been proposed to be hydrolyzed by a distinct enzyme, *i.e.* prolidase[19], now substantiated by its purification[23]. The findings that L-lysyl-L-alanine and L-tryptophanyl-L-alanine are not hydrolyzed by the purified dipeptidase may indicate the existence of additional dipeptidases in the intestine not yet described.

The purified enzyme must be classified as a true dipeptidase as it lacks activity against the tested tripeptides. It showed no activity against aminoacyl-β-naphthylamides and L-leucyl-p-nitroanilide (Table III), which indicates that there exists no relationship between the purified dipeptidase and the previously described arylamidases[20,21].

The quotients between the hydrolysis rates of the substrates, which are split by the purified enzyme, are in most the cases very similar to the quotients obtained for the corresponding substrates with the extract solution, suggesting that the enzyme is responsible for all of these activities. On the other side the exceptions lend further support to the existence of other dipeptidases in the intestine.

TABLE III

ARYLAMIDASE ACTIVITY OF THE INTESTINAL MUCOSA EXTRACT AND THE PURIFIED DIPEPTIDASE

Substrate	*Supplier*	*Assay method*	*Hydrolysis ($\mu moles \cdot min^{-1} \cdot ml^{-1}$)*	
			Extract solution	*Dipeptidase*
L-Leu-β-naphthylamide	1	ref. 16	4.4	0
L-Ala-β-naphtylamide	2	ref. 16	6.1	0
L-Leu-*p*-nitroanilide	3	ref. 17	0.58	0

1, Fluka AG Chem. Fabrik, Buchs, Switzerland.
2, Sigma Chem. Co., St. Louis, USA.
3, Boehringer Mannheim GmbH., Mannheim, Germany.

The purified dipeptidase shows a complete stereospecificity, as judged from the results obtained with substrates containing D-forms of the amino acids (Table IV).

Experiments performed with compounds having no free *N*-terminal amino group or a substitution in the *C*-terminal carboxyl group showed that the enzyme requires free end-groups of the dipeptides to hydrolyze them effectively (Table IV). The very low but significant activity that was observed for the enzyme against *N*-methyl-glycyl-L-leucine may be a manifestation of the prolinase activity as this derivative has an *N*-terminal imino group like L-prolyl-L-alanine. This prolinase activity can be kept by the enzyme or represent trace amounts of a contaminating prolinase-like enzyme. A change in the distance between the free amino or carboxyl group and the peptide linkage in the dipeptide, *i.e.* β-alanyl-L-leucine and L-leucyl-β-alanine (Table IV), abolished the enzyme activity completely.

TABLE IV

SPECIFICITY STUDIES ON THE PURIFIED DIPEPTIDASE

The enzyme activities are expressed as relative rates of hydrolysis of the various compounds as a percentage of the hydrolysis rate of glycyl-L-leucine. Zero percent activity means that the activity found was less than 0.1 percent of that against glycyl-L-leucine.

Substrate	*Supplier*	*Assay method*	*Hydrolysis (%)*
Gly-L-Leu	1	A	100
D-Ala-L-Ala	2	A	0
L-Ala-D-Ala	2	A	0
Gly-D-Leu	1	A	0
D-Ala-D-Ala	2	A	0
N-Acetyl-L-Leu	2	A	0
N-Methyl-Gly-L-Leu	2	A	0.1
pGlu-L-Ala	3	B	0
pGlu-L-Val	3	B	0
Gly-L-Leu-NH_2	2	A	0
β-Ala-L-Leu	2	A	0
L-Leu-β-Ala	2	A	0

1, Sigma Chem. Co., St. Louis, USA.
2, Cyclo Chem. Corp., Los Angeles, USA.
3, Personal gift from Dr. T. Lindberg, Malmö, Sweden.
A, Spectrophotometric method[13].
B, TNBS-method[14].

Assays performed with a mixture of glycyl-L-leucine and L-alanyl-L-serine, present in equal concentrations (8.5 mM) in the incubation mixture, showed that these substrates were hydrolyzed at the same active site. The rate of hydrolysis measured was 75 nmoles/min, a figure close to half of the value obtained, when the corresponding figures (57 and 95 nmoles/min, respectively) from parallel assays, performed with the two substrates incubated separately with the same amount of enzyme, were added up. These results offered further support to the homogeneity of the purified dipeptidase[22].

A comparison between the specificity of the present dipeptidase and the recently described monkey enzyme[5] shows great similarities, although no activity of the monkey enzyme was reported for glycyl-glycine and L-prolyl-L-amino acids.

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