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PURIFICATION AND SPECIFICITY OF PIG INTESTINAL PROLIDASE

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SUMMARY

Purification of prolidase (imidodipeptidase, EC 3.4.3.7) from pig small intestine is described. Two alternative purification procedures have been developed, one of which utilizes the capability of the enzyme to aggregate to high molecular weight complexes and allows purification of 20 mg of prolidase in each preparation. The purity of the prolidase was found to be more than 99% when tested on polyacrylamide electrophoresis, and it showed homogeneity in crossed immunoelectrophoresis. The enzyme has a specific activity of about 200 units of activity per mg protein when determined against L-alanyl-L-proline.

The specificity of the enzyme is in accordance with the original definition of an imidodipeptidase, as its activity is limited to dipeptides with the constitution aminoacyl-L-proline or aminoacyl-L-hydroxyproline, and it has no activity against tripeptides.

INTRODUCTION

Dipeptide-splitting enzymes have been known since the beginning of this century and our knowledge about them has increased in parallel with the development of peptide synthesis. The extensive work done by the group of Smith (for a review see ref. 1), also including studies with partially purified enzyme preparations, provided the primary knowledge of this group of enzymes. During the last few years great interest has been focused on the peptidases in the small intestine, especially in relation to their role in the intestinal digestion and absorption of proteins Although much information has accumulated, the interpretation of the results has been limited, since very little is known about the specificity of this group of enzymes[2].

In 1937 Bergmann and Fruton[3], using glycyl-L-proline as a substrate, recognized a peptidase in the pig intestinal mucosa, capable of splitting peptides having the general constitution aminoacyl-L-proline or aminoacyl-L-hydroxyproline. This enzyme, prolidase (imidodipeptidase, EC 3.4.3.7), has later been shown to be present in many other tissues and purification experiments on the enzyme have been reported[4–6].

Recently a few other enzymes with similar specificity have also been reported[7–9].

The present report describes the purification of prolidase from pig intestinal mucosa and includes specificity data of the enzyme. The details of two alternative purification procedures are given, one of which has been outlined generally before[10].

MATERIALS AND METHODS

Chemicals

DEAE-cellulose, DE 32, was obtained from Whatman, Maidstone, England. Sephadex G-200, Sephadex G-200 (superfine) and DEAE-Sephadex A-50 were purchased from Pharmacia, Uppsala, Sweden. Agarose was purchased from L'Industrie Biologique Française, Gennevillier, France. Acrylamide and *N,N'*-methylenebisacrylamide were obtained from BDH Chemicals, Poole, England, and bovine serum albumin was purchased from the Armour Pharmaceutical Co., Eastbourne, England. All other chemicals used were of analytical grade and distilled and de-ionized water was used throughout.

Substrates

L-Alanyl-L-proline, obtained from Flura, Buchs, Switzerland, was used as the main substrate for the prolidase activity. The other dipeptides and the tripeptides used for the specificity studies were obtained from various manufacturers (*cf.* Table II). All substrates were tested for purity by thin-layer chromatography, using *n*-butanol–acetic acid–water (4:1:1, by vol.) or water-saturated phenol as solvents.

Source of the enzyme

Fresh pig intestines were obtained from a local slaughter house. They were removed according to the ordinary slaughter process and squeezed to remove intestinal contents. The portion between 2 and 5 m from the pylorus was used, and 18 intestines were taken routinely for each preparation. They were immediately cut open, divided into pieces of about 10 cm in length and subsequently extracted in cold water (1 l per 3 m intestine) for 1 h with continuous stirring. The resulting muddy solution was cleared by filtration through a crude nylon net (mesh size 1.5 mm) followed by centrifugation (15 000 × *g*, 60 min, 4 °C). The supernatant was lyophilized and the resulting red-yellow powder was stored at −20 °C until used (intestinal powder). Under these conditions no loss of prolidase activity was observed for a period of more than one year.

Equipment

For all spectrophotometric analyses a spectrophotometer, Model PMQ II (Carl Zeiss, Oberkochen, Germany), was used. pH measurements were performed with a pH-meter 28 (Radiometer, Copenhagen, Denmark). In certain chromatographic procedures the transmission of the eluate was continuously measured using an Uvicord II, LKB 8300 A, provided with a 3 mm measuring cell (LKB produkter, Stockholm, Sweden). The salt gradients were followed by measuring the conductance of the eluate using a conductance meter, Tetramatic (Kemotron, Copenhagen, Denmark). An amino acid analyszer, Multichrom, Model 4255 (Beckman, Munich, Germany), was used to analyse the enzymatic activity against certain substrates. Ultrafiltration was

performed, using filters of Type PM-10 and UM-10, essentially as described by the manufacturer (Amicon, Lexington, U.S.A.). Dialysis tubes were obtained from Visking Co. Ltd., Chicago, U.S.A. Precoated cellulose thin-layer chromatography plates (20 cm × 20 cm, 0.1 mm layer thickness, without fluorescence indicator) were obtained from Merck, Darmstadt, Germany.

Analytical electrophoretic procedures

Polyacrylamide gel electrophoresis was run according to Ornstein[11] and Davis[12]. The crossed immunoelectrophoresis[13] was run on 1% agarose in 0.02 M sodium 5,5-diethylbarbiturate buffer (pH 8.6) as described by Clarke and Freeman[14]. For the preparation of antibody-containing gels, 100 μl of the prepared immunoglobulin solution were mixed into each ml of the agarose solution, and the plates were made to contain 10 μl immunoglobulin solution per cm^2. After completed electrophoresis the proteins were visualized by using Coomassie Brilliant Blue[15].

Assays

During the purification procedures the prolidase activity was determined according to the method of Josefsson and Lindberg[16] (1.8 mM L-alanyl-L-proline in 0.1 M potassium phosphate buffer (pH 6.8, 25 °C). One unit of prolidase activity was defined as the activity hydrolyzing 1 μmole L-alanyl-L-proline per min at 25 °C and pH 6.8.

In the specificity studies the various constitutions of the substrates selected necessitated the use of different assay methods (*cf.* Table II). However, the incubations followed some general outlines. The dipeptides containing a C-terminal L-proline or L-hydroxyproline residue and the tripeptides were incubated in a concentration of 1.8 mM, while all other substrates were incubated in a concentration of 8.5 mM. The pH was kept at 7.4 (0.1 M potassium phosphate buffer) and the temperature at 25 °C in all the incubations. The incubation time was limited to a maximum of 60 min to prevent enzyme denaturation. The enzyme concentration was varied to obtain the optimum precision of the activity estimate without having too high a hydrolysis percentage. When using the spectrophotometric method[16] or the trinitrobenzenesulphonic acid (TNBS) reagent method[17], the procedures given in a parallel report[25] were followed. In the cases where the amino acid analysis procedure was used, a fixed volume of the supernatant obtained after the centrifugation step, using 0.2 M sodium citrate buffer (pH 2.2) as precipitating agent, was taken off and applied to the amino acid analyzer. Standard samples, corresponding to fixed percentages of hydrolysis, were included in every assay.

Estimation of purification

As an indication of enzyme purification, the ratio of units of prolidase activity to mg protein was used, assuming an average absorbance of 1 (280 nm, 1 cm pathlength) for a protein concentration of 1 mg/ml.

Purification procedures

All operations were carried out in the cold room (4 °C) and all buffers contained 2-mercaptoethanol (4 mM) and disodium EDTA (1 mM), unless otherwise stated. The purification procedure is given for the preparation of the enzyme from 100 g of intestinal powder.

Extraction. Intestinal powder was dissolved in 1 l 0.05 M KH_2PO_4 solution (intestinal powder solution) and the pH was subsequently adjusted to 5.2 with 0.5 M HCl. After standing for 2 days the insoluble and precipitated material was cleared from the solution by centrifugation (15 000 × *g*, 60 min). The supernatant was adjusted to pH 7.5 by 0.5 M NaOH and then filtered through a glass filter funnel under suction to remove some floating insoluble material (supernatant).

$(NH_4)_2SO_4$ fractionation. 190.1 g solid $(NH_4)_2SO_4$ per l was added to the supernatant under continuous stirring and then left for 1 h. The precipitate formed was centrifuged off (15 000 × *g*, 60 min) and discarded, and supernatant again added with 91.4 g solid $(NH_4)_2SO_4$ per l under stirring. After standing another hour and after centrifugation (15 000 × *g*, 60 min) a yellow white sediment was collected. This was washed twice with 500 ml 0.05 M potassium phosphate buffer (pH 7.5) to which 300 g solid $(NH_4)_2SO_4$ per l had been added. The final sediment was easily dissolved in 50 ml 0.025 M Tris–HCl buffer (pH 7.5), giving a clear yellow solution. After being dialyzed against the same buffer for at least 8 h with each of two portions (10 l), the solution was centrifuged (27 000 × *g*, 60 min). A small precipitate formed was discarded and the clear supernatant was collected ($(NH_4)_2SO_4$ fraction).

Chromatography on DEAE-cellulose. The $(NH_4)_2SO_4$ fraction was applied to a 400-ml column of DEAE-cellulose, prepared and equilibrated in 0.025 M Tris–HCl buffer (pH 7.5). The column was eluted over-night with the starting buffer (flow rate 7 $ml \cdot cm^{-2} \cdot h^{-1}$), followed by a linear increasing KCl gradient in the same buffer, ending at 0.3 M KCl (volume equivalent to 5 times bed volume). All processes were performed by pumping, and the effluent was continuously monitored for transmission at 280 nm. Fractions (10 ml) were collected and assayed for prolidase activity. Fractions containing more than 10% of the activity of the most active fraction were pooled (DEAE-cellulose fraction).

Chromatography on Sephadex G-200. The DEAE-cellulose fraction was concentrated by means of ultrafiltration to a volume of about 7 ml. A slight precipitate formed during the ultrafiltration was centrifuged off (27 000 × *g*, 30 min). The clear yellow-green supernatant was passed through a 1700-ml column of Sephadex G-200, prepared and equilibrated in 0.025 M Tris–HCl buffer (pH 7.5), added with KCl (0.3 M). The elution was performed with a pump (flow rate 1.5 $ml \cdot cm^{-2} \cdot h^{-1}$), using the same buffer, and the transmission at 280 nm of the effluent was continuously monitored. Fractions (10 ml) were collected and assayed for prolidase activity. Those containing more than 30% of the activity of the most active fraction were pooled (Sephadex G-200 fraction).

Final purification Procedure I

Polyacrylamide gel electrophoresis. The Sephadex G-200 fraction was dialyzed over-night against 200 vol. of an 0.05 M Tris–HCl buffer (pH 7.5) and subsequently concentrated by ultrafiltration to a volume, where the absorbance at 280 nm of the solution became close to ten. 100 µl of the solution were applied to each of polyacrylamide gels prepared in electrophoresis tubes with an inner diameter of 10 mm and with a separating gel length of 50 mm. The tubes were run according to Ornstein[11] and Davis[12], applying a current of 10 mA per gel. The electrophoresis was interrupted when the salt frontier, which could be easily followed, had reached a distance of 5 mm from the bottom of the tubes. The gels were removed from the tubes and then separately scanned for absorbance at 280 nm by placing them on a specially designed

sleigh, adapted to the spectrophotometer. The area with the highest absorbance was marked out on the gels. They were then separately placed in a half cylindrical form and subsequently frozen. The marked area of the gels was sliced into discs of about 1 mm thickness, using a slicer consisting of a movable sleigh with the gel on it and a guilliotine made from a razor-blade. The best slices were obtained when the gel was just about to thaw. The slices were extracted separately in 0.05 M Tris–HCl buffer (pH 7.5) with continuous shaking over-night. The extracted solutions were assayed for prolidase activity, which showed that most of the activity was localized into three of the slices from each gel. After being tested for homogeneity by analytical polyacrylamide electrophoresis, the extracts were pooled (polyacrylamide electrophoresis fraction).

Chromatography on Sephadex G-200 (superfine). The polyacrylamide electrophoresis fraction was concentrated by ultrafiltration and then passed through a column of Sephadex G-200 (superfine), as described in the legend to Fig. 3. Fractions were collected and analyzed for absorbance at 280 nm and for prolidase activity. The active fractions were pooled (prolidase Preparation I).

Final purification Procedure II

This procedure was developed after it was found that the prolidase could be reversibly transformed into aggregates of higher molecular weights by removing 2-mercaptoethanol and EDTA from the solution and then concentrating it.

Chromatography of aggregated enzyme. The Sephadex G-200 fraction was passed through a column of Sephadex G-25 (bed volume 5 times the applied volume), prepared and equilibrated in 0.05 M Tris–HCl buffer (pH 7.5), added with KCl (0.3 M) but not containing 2-mercaptoethanol or EDTA. The transmission at 280 nm of the effluent was continuously monitored and the peak beginning at the void volume was collected as one fraction. It was subsequently concentrated by ultrafiltration until the protein concentration reached about 20%, and was then left for a few days to allow the aggregation to proceed. The solution was next diluted by 2 vol. of the above-mentioned buffer to permit application of the sample to a column of Sephadex G-200 (superfine, bed volume 50–100 times the applied volume), prepared and equilibrated in the same buffer. The column was eluted using a Mariotte flask with a flow rate of 1 $ml \cdot cm^{-2} \cdot h^{-1}$. Fractions (0.5–1% of the bed volume) were collected and analyzed for absorbance at 280 nm and for prolidase activity. The active fractions, corresponding to the aggregated forms and starting at void volume, were combined (aggregated fraction).

Chromatography of de-aggregated enzyme. The aggregated fraction was made 0.1 M with respect to 2-mercaptoethanol and left at room temperature for 8 h to allow complete reversion of the aggregation to take place. To re-establish the ordinary buffer conditions the solution was dialyzed against an 0.05 M Tris–HCl buffer (pH 7.5), containing the usual concentration of EDTA, until the concentration of 2-mercaptoethanol in the solution reached a level of 4 mM (de-aggregated fraction).

The de-aggregated fraction was concentrated to a volume of 1–2 ml by ultrafiltration and then passed through a column of Sephadex G-200 (superfine) as described in the legend to Fig. 5. Fractions were collected and assayed for absorbance at 280 nm and for prolidase activity. The active fractions were combined (prolidase Preparation II).

Preparation of immunoglobulin solution

The antigen used was withdrawn from the purification procedure after the first gel filtration step. It was ultrafiltrated to a protein concentration of 17.2 mg/ml, corresponding to 550 units of prolidase activity per ml. 50 μl of the antigen were suspended in 50 μl of Freunds incomplete adjuvant and then injected intracutaneously into rabbits. The injection was repeated each 14 days for the first 4 times and then each 45 days. The rabbits were bled (40 ml from an ear vein) 10 days after an injection, the first bleeding following the fourth injection, and the immunoglobulin was prepared[18] (6–9 ml purified immunoglobulin solution per bleeding).

Enzyme solutions for specificity studies

Mucosal extract. Intestinal powder was dissolved (10%, w/v) in 0.1 M potassium phosphate buffer (pH 7.4, 4 °C), and a solution was freshly prepared for each day of the experiment. Before being used, it was dialyzed against the buffer solution (4 °C) over-night to remove low molecular weight ninhydrin-positive material and then centrifuged (27 000 × *g*, 30 min, 4 °C) to remove insoluble or precipitated material.

Prolidase Preparation I and II. Purified enzyme solution was dialyzed against 0.05 M Tris–HCl buffer (pH 7.4, 4 °C) over-night, and then frozen in portions suitable for each assay. Stored under these conditions the enzyme activity was stable for several months, when tested against L-alanyl-L-proline.

RESULTS AND DISCUSSION

Purification

Figures from a purification experiment are given in Table I. The addition of EDTA and 2-mercaptoethanol to the buffer solutions assured stable conditions for the prolidase during the purification procedure, without any evidence that the two compounds caused an irreversible inhibition of the enzyme activity.

By adding 2-mercaptoethanol to the enzyme solution partial aggregation of the prolidase molecules was prevented and the enzyme activity was eluted in a narrow well-defined zone in the DEAE-cellulose and Sephadex G-200 chromatographies (Figs 1 and 2).

The polyacrylamide gel electrophoresis procedure is well-suited for preparation of the pure enzyme in small quantities, large enough for many kinds of studies, *e.g.* studies on its immunological and enzymatic properties. The impurities originating from the polyacrylamide gel were removed by the following Sephadex G-200 chromatography (Fig. 3). The gel electrophoresis step limits, however, the capacity of this purification procedure to the number of gels run. By using 6 gels (corresponding to 1 run in the electrophoresis apparatus) it yielded about 0.3 mg of the pure enzyme. The all-over recovery was 5–8% and the prolidase had a specific activity of about 180 units of activity per mg protein. As the intestinal powder solution contained 200–300 units of prolidase activity per g powder, the purification factor was 600–900-fold.

The purification procedure utilizing the capability of the enzyme to aggregate to high molecular weight complexes was accompanied by some unavoidable loss of activity because the aggregation reaction did not proceed to completion under our experimental conditions. To assure a pure enzyme in the next step of purification, part of the aggregated fraction, *i.e.* that with the lowest molecular weight, was discarded

TABLE I

PURIFICATION OF PROLIDASE FROM PIG INTESTINAL MUCOSA

Figures are given for 100 g of intestinal powder.

Fraction	*Total activity (units)*	*Spec. act. (units/mg protein)*	*Recovery (%)*	*Purification factor*
Intestinal powder solution	28 200	0.305	100	1.0
Supernatant	20 600	0.266	73	0.87
$(NH_4)_2SO_4$ fraction	18 800	2.48	67	8.1
DEAE-cellulose fraction	15 900	26.2	56	86
Sephadex G-200 fraction	11 900	54.5	42	180
Purification Procedure I*				
polyacrylamide electrophoresis fraction	5 540		20	
prolidase Preparation I	2 130	186	7.6	610
Purification Procedure II*				
aggregated fraction	3 090	72.5	11	240
prolidase Preparation II	2 460	223	8.7	730

* The figures, related to 100 g of intestinal powder, are based on an experiment where 2.4% of the Sephadex G-200 fraction were purified according to Procedure I and the rest according to Procedure II.

together with the non-aggregated fraction (Fig. 4), causing a further loss of activity in this purification step.

The de-aggregation reaction was not followed by any loss of prolidase activity and the following chromatography on Sephadex G-200 resulted in a pure enzyme preparation (Fig. 5).

Sometimes rechromatography steps on Sephadex G-200 were necessary to ob-

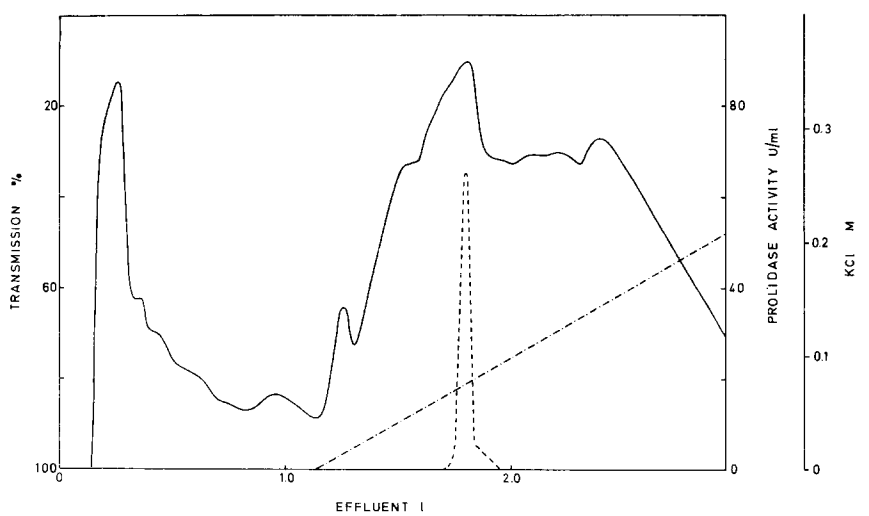

Fig. 1. Chromatography of an $(NH_4)_2SO_4$ fraction on DEAE-cellulose. 125 ml of a solution, corresponding to 100 g of intestinal powder, were applied. Column size 3.4 cm × 43.0 cm. ------, prolidase activity; ———, $T_{280\ nm}$; –·–·–·– KCl concentration.

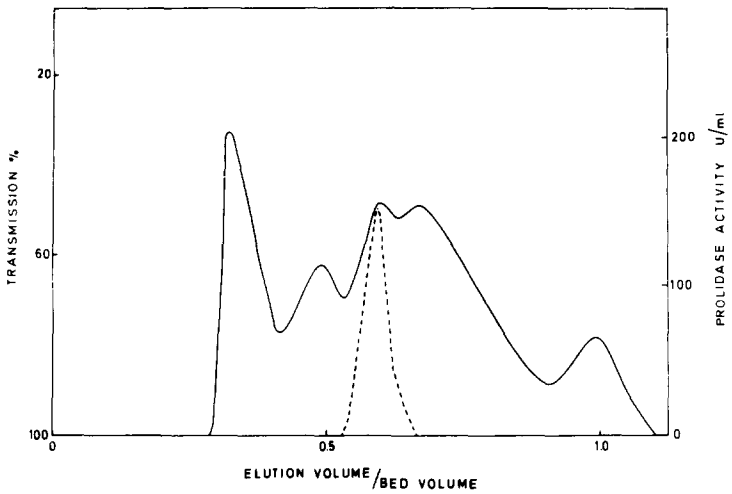

Fig. 2. Chromatography of a DEAE-cellulose fraction on Sephadex G-200. Thirteen ml of a solution, corresponding to 200 g of Intestinal powder, were applied. Column size 5.0 cm × 86.6 cm. ------, prolidase activity; ———, $T_{280\ nm}$.

tain a pure prolidase preparation. In the experiment given in Table I, a rechromatography was performed to obtain the aggregated fraction.

The last purification alternative allowed a much higher capacity than the former, *i.e.* preparation of as much as 20 mg prolidase in one experiment when performed on 200 g intestinal powder. The specific activity was about 200 units of activity per mg protein and the all-over recovery was about the same as in the other purification procedure, *i.e.* 5–10%, although the aggregation step included a rather great loss of activity. The purification factor obtained was also similar in the two alternative

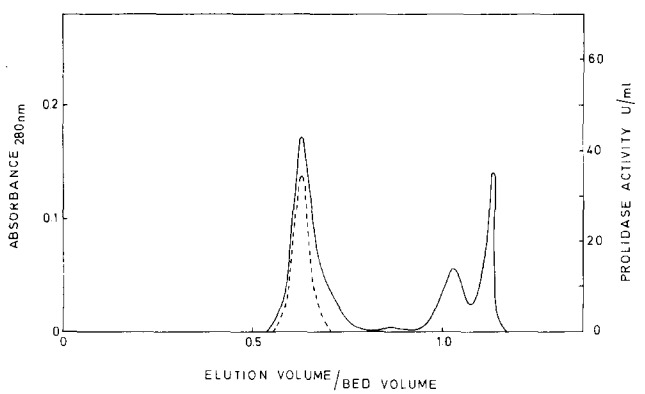

Fig. 3. Chromatography of a polyacrylamide electrophoresis fraction on Sephadex G-200 (superfine). 0.37 ml of a solution, corresponding to 2.4 g of intestinal powder, was applied. Column size 0.9 cm × 55.0 cm. The column was equilibrated and eluted with an 0.05 M Tris–HCl buffer (pH 7.5), containing 2-mercaptoethnaol and EDTA in the usual concentrations, at a constant flow rate of 1.7 ml·cm^{-2}·h^{-1}. Fraction volume 0.37 ml. ------, prolidase activity; ———, $A_{280\ nm}$.

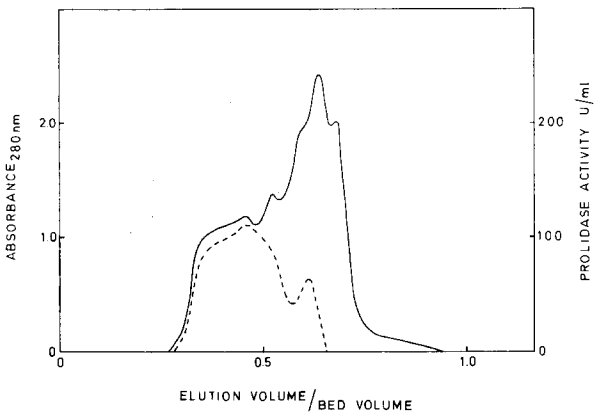

Fig. 4. Chromatography of a Sephadex G-200 fraction on Sephadex G-200 (superfine), after accomplished aggregation reaction. 7.5 ml of a solution, corresponding to 200 g of intestinal powder, were applied. Column size 2.5 cm × 87.8 cm. ------, prolidase activity; ———, $A_{280\ nm}$.

procedures, as demonstrated in Table I, which gives the results from an experiment where the final purification of the enzyme was performed in parallel by the two alternative procedures from the same Sephadex G-200 fraction.

Purity of the enzyme

The purity of the various prolidase preparations was analyzed by crossed immunoelectrophoresis and by disc electrophoresis. The immunoelectrophoresis was per-

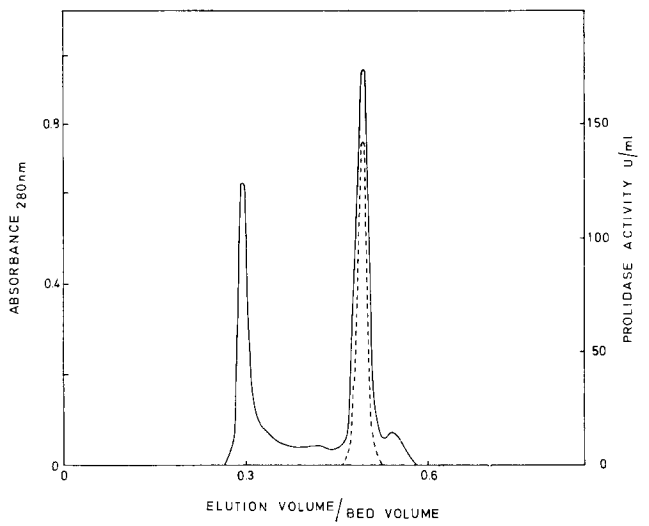

Fig. 5. Chromatography of a de-aggregated fraction on Sephadex G-200 (superfine). 0.72 ml of a solution, corresponding to 68 g of intestinal powder, was applied. The column (1.5 cm × 86.3 cm) was equilibrated and eluted with an 0.05 M Tris–HCl buffer (pH 7.5), containing 2-mercaptoethanol and EDTA in the usual concentrations. Flow rate 0.7 ml·cm^{-2}·h^{-1}. Fraction volume 0.5 ml. ------, prolidase activity; ———, $A_{280\ nm}$.

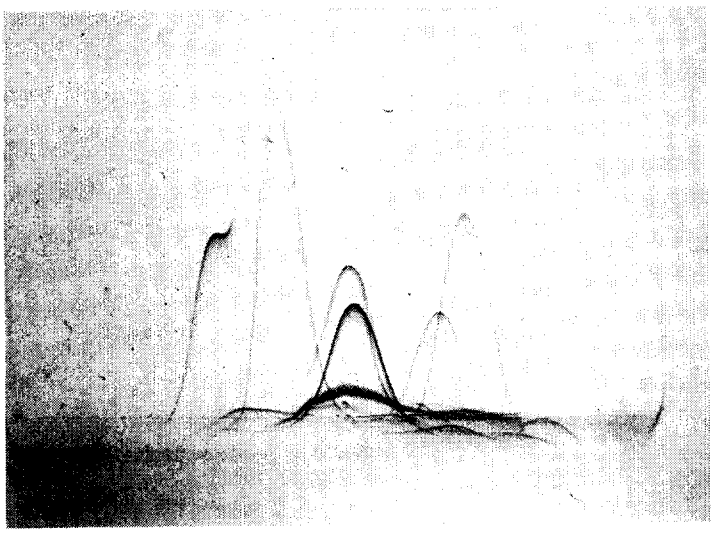

Fig. 6. Crossed immunoelectrophoresis of samples from Sephadex G-200 fraction and prolidase Preparation II. Two wells (diameter 2 mm) were punched. To the left one, 1 μl of a prolidase Preparation II (72 units of activity/ml) was applied, and to the right one, 1 μl of a Sephadex G-200 fraction (550 units of activity/ml), was applied. The electrophoresis was run at 10 V/cm for 1 h in the first dimension, and at 3 V/cm for 18 h in the second dimension.

formed on samples of prolidase preparations purified according to the two alternative procedures. Typical patterns from the experiments are given in Figs 6–8. The polyvalency of the immunoglobulin solution used is demonstrated in Fig. 6, which also shows the purified prolidase identified as one of the large peaks running out of the plate[19]. Regarding the purity of the two different types of enzyme preparations, indications of small impurities in prolidase Preparation I (Fig. 7) were sometimes found,

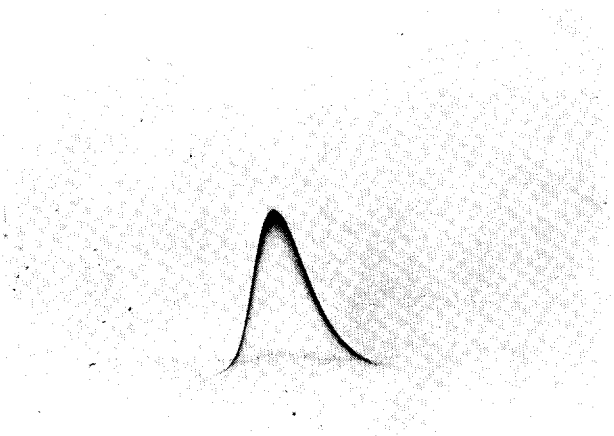

Fig. 7. Crossed immunoelectrophoresis of prolidase Preparation I. 10 μl of a prolidase Preparation I (15 units of activity/ml) were applied to a punched well (diameter 4 mm). The electrophoresis was run at 10 V/cm for 1 h in the first dimension, and at 3 V/cm for 21 h in the second dimension.

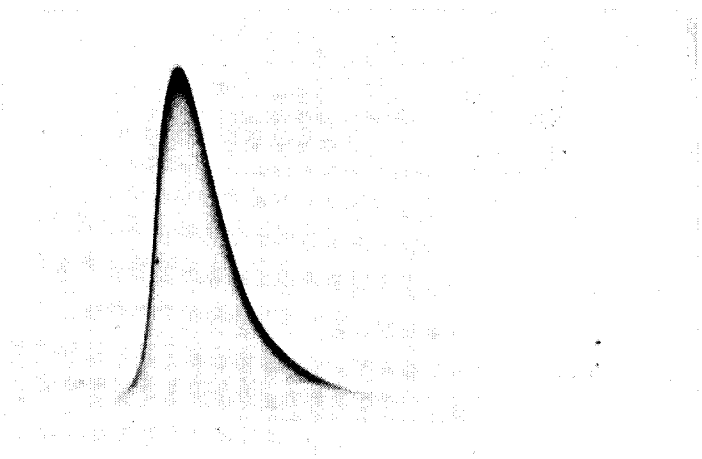

Fig. 8. Crossed immunoelectrophoresis of prolidase Preparation II. Six μl of a prolidase Preparation II (74 units of activity/ml) were applied to a punched well (diameter 4 mm). The electrophoresis was run at 10 V/cm for 1 h in the first dimension and at 3 V/cm for 21 h in the second dimension.

while prolidase Preparation II always was immunological pure, as demonstrated in Fig. 8.

The results obtained in the disc electrophoresis experiments confirmed the high grade of purity of the enzyme preparations (Fig. 9), although multiple aggregated forms of the enzyme regularly occurred on the polyacrylamide gels. Their intensity was strongly reduced by the treatment with 2-mercaptoethanol as described above, but it was difficult to remove them completely. One of them may be seen indistinctly in the figure. Taking advantage of the sensitivity of the Coomassie Brilliant Blue reaction, which allows the detection of small amounts of protein (20 ng)[20], disc electrophoresis was also utilized to quantitatively estimate the grade of purity of the prolidase preparations. Various amounts of the enzyme preparations were applied to two separate series of gels and to a third group of gels a standard concentration series of albumin was applied. The gels were run in parallel, and by determining the quantity of enzyme applied where impurities could be observed, it was possible to estimate the purity of the enzyme preparations to more than 99%. These results, together with the constant specific activity obtained in the various fractions within the prolidase peak in the final Sephadex G-200 chromatography, exclude the presence of significant impurities in the prolidase preparations.

Specificity of the enzyme

In the investigation of the specificity of the purified enzyme a main interest was to settle if it really constituted a strictly defined prolidase and whether such a prolidase can be classified as a dipeptidase. The earlier work of Smith and co-workers[4–6] on the prolidase activity suggested that the enzyme had a rather limited specificity, hydrolyzing only dipeptides with the composition aminoacyl-L-proline or aminoacyl-L-hydroxyproline. Such a strict dipeptidase classification has, however, later been questioned[21] and prolidase preparations have also been used in sequence analysis of

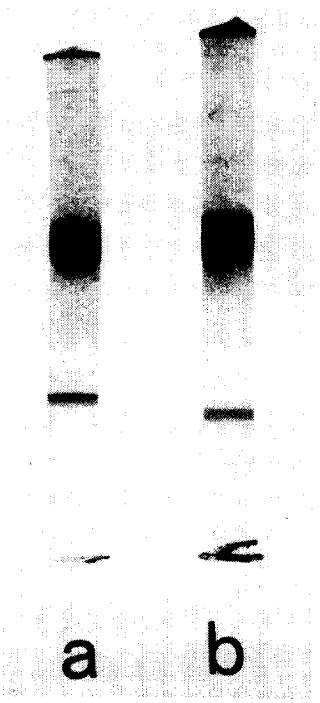

Fig. 9. Analytical polyacrylamide gel electrophoresis of prolidase Preparation I and II. 2.5 mA/gel were applied for 1.5 h. (a) 4 μg of a prolidase Preparation I in 50 μl were applied. (b) 9 μg of a prolidase Preparation II in 5 μl were applied.

polypeptides having their N-terminal amino acid residue localized adjacent to a proline residue[22–24].

The selection of dipeptides for the specificity studies, other than the classical prolidase substrates, was reduced to include representatives of dipeptides composed of the various types of amino acids, *i.e.* acidic, basic, aromatic *etc.*, mainly in combination with L-alanine. Glycylsarcosine was also included because of an earlier suggestion, that prolidase was capable of splitting this compound[6].

From the results (Table II) it is evident that the specificity of the purified enzyme is in good accordance with the strict definition of a prolidase. Furthermore, the constant relation between the figures for the various prolidase activities obtained with the different enzyme solutions, suggests that the purified enzyme is responsible for all the prolidase activity in the mucosal extract.

The tripeptides studied were hydrolyzed by the mucosal extract, but no tripeptidase activity was observed when they were incubated with the purified prolidase. This is in contrast to Hill and Schmidt[21], who found that their prolidase preparation split glycyl-L-prolyl-L-leucine, although the sensitivity of our assay procedure was somewhat higher (2–10 times) than theirs. Therefore, the prolidase seems to constitute a true dipeptidase.

Finally considering the activity of the enzyme against the other substrates tested, it is evident that, although the figures are low compared to those obtained with the mucosal extract, the enzyme exhibits a significant activity against many of them,

TABLE II

SUBSTRATE SPECIFICITY OF PIG INTESTINAL PROLIDASE IN RELATION TO MUCOSAL EXTRACT

Figures are given as a percentage of the activity against L-alanyl-L-proline. Zero activity in the table means an activity less than 0.1% of the activity against L-alanyl-L-proline. Exceptions are only obtained in the figures for the activity of the Mucosal extract against L-prolyl-L-proline and glycyl-sarcosine where the assay method did not permit estimations less than 5%.—

Substrate (L-forms)	*Manufacturer*	*Method*	*Activity*		
			Mucosal extract	*Prolidase Preparation I*	*Prolidase Preparation II*
Ala–Pro	4	A	100	100	100
Gly–Pro	1	A	30	30	30
Met–Pro	2	A	40	50	40
Glu–Pro	3	A	40	30	30
Ser–Pro	2	A	90	80	90
Gly–Hyp	2	A	0.3	0.5	0.4
Pro–Pro	2	A	0	0	0
Gly–Gly–Pro	2	C	+	—	—
Gly–Pro–Ala	2	C	+	—	—
Gly–Sarc	1	A	0	3	1
Gly–Gly	5	B	60	0	0
Ala–Leu	1	A	700	0	0
Leu–Ala	1	A	500	4	7
Ala–Ser	4	A	4000	0.2	0.2
Ser–Ala	2	A	1000	1	2
Ala–Glu	1	A	1000	0	0
Glu–Ala	1	A	200	2	3
Ala–Lys	1	A	400	0	0.5
Lys–Ala	1	A	70	6	7
Gly–Gln	2	A	700	0.1	0
Gln–Gly	2	A	600	0.1	0.2
Pro–Ala	1	A	70	0.3	0.4
Ala–Tyr	1	B	200	0	0
Tyr–Ala	1	B	500	6	0.4
Ala–Trp	1	B	1000	0	0
Trp–Ala	1	B	100	2	0.8

1, Sigma Chem. Comp., St. Louis, U.S.A.
2, Cyclo Chem. Corp., Los Angeles, U.S.A.
3, Yeda Res. and Devel. Comp., Rehovoth, Israel.
4, Fluka AG Chem. Fabrik, Buchs, Switzerland.
5, Mann Res. Labs., New York, U.S.A.
A, Spectrophotometric method[16].
B, TNBS method[17].
C, Amino acid analysis (+ means activity, — means no activity, see text).

indicating a more general capability of the enzyme to slowly hydrolyze dipeptides not classified as prolidase substrates. That these activities are not related to impurities in the enzyme preparations is suggested by the close similarities between the two different preparations.

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