Isolation and Purification of a Prolidase from Pig Intestinal Mucosa

HANS SJÖSTRÖM, OVE NORÉN and LARS JOSEFSSON

Department of Biochemistry C, University of Copenhagen, DK-2200 Copenhagen N, Denmark

Dipeptides of the general constitution X-L-proline, where X is an amino acid, contain a special type of peptide linkage, since proline is bound *via* its imino group. In 1937 Bergmann and Fruton [1] using glycyl-L-proline as a substrate, recognized an enzyme in the pig intestinal mucosa, which was capable of splitting this peptide bond. The enzyme, prolidase (E.C.3.4.3.7), has later been shown to be present in many other tissues.[2-4] Attempts to purify it have been reported, employing pig intestinal mucosa,[5] horse erythrocytes,[6] and pig kidney,[7] as sources of the enzyme. Of these only the last one gave a considerable purification. More recently, Dehm and Nordwig [8] have reported purification of a particle bound enzyme from pig kidney which splits glycyl-L-proline bonds. Purification of a similar enzyme from *E. coli* has been described.[9]

The present report describes purification of a prolidase from pig intestinal mucosa. Prolidase activity was determined spectrophotometrically using L-alanyl-L-proline (Sigma, Lot No. 47 B-1850) as substrate.[10,11] The assays were performed at optimal pH and 25°C. No metal ions were added. One unit (U) of prolidase activity is defined as the activity hydrolyzing 1 μmol/min of L-alanyl-L-proline at 25°. As an indication of enzyme purification, the ratio of U of activity/ml to 280 nm absorbancy was chosen. All preparative steps during the purification were carried out in the cold room. The buffer solutions used in the chromatographic separations before the gel electrophoresis contained 0.001 M EDTA (disodium salt) and 0.004 M 2-mercaptoethanol.

Pieces (3 m) of pig small intestines were taken 2 m distal from pylorus immediately after slaughter. They were squeezed to remove intestinal content, cut open and divided into 5 cm pieces. Without further disintegration they were extracted for 1 h in precooled distilled water (1 l per 3 m intestine). The extract was centrifuged and the supernatant was subsequently lyophilized. The brown-yellow powder (3 g/m intestine) was suspended (100 g/l) in 0.05 M phosphate, preadjusted to pH 2.0 with 0.1 M HCl, giving a final pH of the solution close to 5.2. After standing for 2 days the solution was cleared from insoluble and precipitated material by centrifugation. The pH of the supernatant was adjusted to 7.5 with 0.1 M NaOH and a small precipitate formed was centrifuged off (Fraction 1, Table 1). The supernatant was fractionated with $(NH_4)_2SO_4$, and the fraction precipitating between 35 and 50 % $(NH_4)_2SO_4$ saturation was collected. The precipitate was dissolved in 0.005 M Tris–HCl (pH 7.5) and subsequently dialyzed against 200 vols. of the buffer. A small precipitate formed during dialysis was centrifuged off.

The clear supernatant (Fraction 2, Table 1) was applied to a 2.5 cm × 21 cm column of DEAE-cellulose (Whatman, DE 32), prepared and equilibrated in 0.005 M Tris-HCl (pH 7.5). The column was eluted at a constant flow rate of 10 ml/cm² × h with a 0.3 M linear increasing KCl gradient (10 × column volume). The prolidase activity was eluted as a single peak and the effluent containing more than 12 U/ml of prolidase activity was collected (Fraction 3, Table 1). The enzyme was concentrated 20 times by means of ultrafiltration (Amicon, Diaflo filter PM-10) and applied to a 5 cm × 92 cm column of Sephadex G-100 (Pharmacia). The column, prepared and equilibrated in 0.005 M Tris-HCl (pH 7.5), made 0.3 M in respect to KCl, was eluted with the buffer at a constant flow rate of 1.5 ml/cm² × h. The prolidase activity was again eluted as a single peak and the effluent containing more than 22 U/ml of prolidase activity was collected (Fraction 4, Table 1). The prolidase solution was diluted with an appropriate volume of 0.005 M Tris-HCl (pH 7.5) to obtain the same conductivity as that of 0.005 M Tris-HCl (pH 7.5), made 0.08 M in respect to KCl. The solution was then applied to a 2.5 cm × 20 cm column of DEAE-Sephadex A-50 (Pharmacia), prepared and equilibrated in the second buffer. The elution was performed with a linear increasing KCl gradient as above. The flow rate was kept at 11 ml/cm² × h and the prolidase activity was eluted in a single peak. The effluent containing more than 13 U/ml of prolidase activity was collected (Fraction 5, Table 1).

The enzyme solution was concentrated by ultrafiltration and further purified by polyacrylamide gel electrophoresis.[12] The electro-

Table 1. Purification of a prolidase from pig intestinal mucosa.

Fraction	Vol. (ml)	A_{280}	Prolidase activity (U/ml)	Recovery (%)	Purification factor
1. Supernatant pH 7.5	810	63.8	20.3	100	1
2. Dialyzed $(NH_4)_2SO_4$ precipitate	105	48.3	63.0	40	4.1
3. Pooled active fractions of DEAE-cellulose effluent	220	2.1	20.2	27	30
4. Pooled active fractions of Sephadex G-100 effluent	90	1.0	41.4	23	127
5. Pooled active fractions of DEAE-Sephadex effluent	119	0.27	18.0	13	207
6. Pooled active fractions of Sephadex G-100 effluent	68.5	0.08	13.2	5.5	501

Figures are given for 85 g lyophilized intestinal extract.

phoresis was run in 6 parallel tubes of 10 mm inner diameter. 250 µl of the solution (corresponding to about 1 mg protein) were applied to each tube. 10 mA was applied to each gel. After electrophoresis the gels were sliced into discs of about 2 mm thickness and extracted each with 1 ml 0.05 M Tris-HCl (pH 7.5). The separate extracts were analyzed for 280 nm absorbancy and for prolidase activity. A

Fig. 1. Analytical polyacrylamide gel electrophoresis of pig intestinal prolidase. Parallel extracts of the most active discs from a preparative polyacrylamide gel electrophoresis experiment were pooled and 50 µl of the solution were applied. Current 2.2 mA. The gel was stained with Coomassie blue.

complete correspondence was found between the activity and the major absorbancy peak. The parallel extracts from the six separate gels were pooled and a sample from each solution was subsequently analyzed for homogeneity by analytical polyacrylamide gel electrophoresis (Fig. 1).

The homogeneous enzyme solutions were pooled, concentrated by ultrafiltration and applied to a column (see Fig. 2) of Sephadex G-100, equilibrated and eluted with 0.05 M Tris-HCl (pH 7.5). The prolidase activity was obtained in two partly separated peaks showing correspondence with the 280 nm absorbancy (Fig. 2). The active fractions were pooled and could be stored without loss of

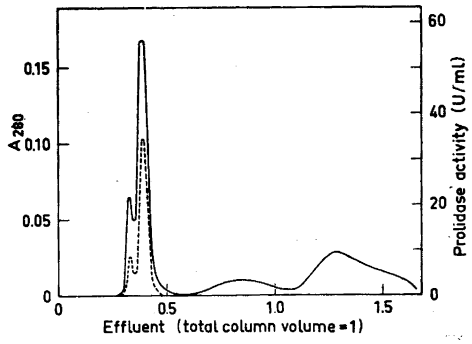

Fig. 2. Chromatography of pig intestinal prolidase on Sephadex G-100. 1.1 ml concentrated enzyme solution after polyacrylamide gel electrophoresis was applied to a 2.5 cm × 91 cm column of Sephadex G-100, equilibrated and eluted with 0.05 M Tris-HCl (pH 7.5). Flow rate 4.5 ml/cm² × h. Prolidase activity (– – –); A_{280} (———).

activity when kept frozen at −20°C (Fraction 6, Table 1).

The purification has usually been run with about 80 g of lyophilized mucosa extracts as starting material in each preparation. It has regularly resulted in an enzyme preparation having about 500 times higher specific activity than that of the acid extract. The overall recovery in the purification procedure has been about 5 %, equivalent to about 100 μg purified enzyme protein per gram of lyophilized mucosa extract.

This investigation was supported by grants from the *Swedish Medical Research Council* (Project No. 13X-17), and *Albert Påhlssons stiftelse*.

1. Bergmann, M. and Fruton, J. S. *J. Biol. Chem.* **117** (1937) 189.
2. Smith, E. L. *Advan. Enzymol.* **12** (1951) 191.
3. Lewis, W. H. P. and Harris, H. *Nature* **215** (1967) 351.
4. Josefsson, L., Norén, O. and Sjöström, H. *Acta Physiol. Scand.* **72** (1968) 108.
5. Smith, E. L. and Bergmann, M. *J. Biol. Chem.* **153** (1944) 627.
6. Adams, E. and Smith, E. L. *J. Biol. Chem.* **198** (1952) 671.
7. Davis, N. C. and Smith, E. L. *J. Biol. Chem.* **224** (1957) 261.
8. Dehm, P. and Nordwig, A. *Eur. J. Biochem.* **17** (1970) 364.
9. Yaron, A. and Mlynar, D. *Biochem. Biophys. Res. Commun.* **32** (1968) 658.
10. Josefsson, L. and Lindberg, T. *Biochim. Biophys. Acta* **105** (1965) 149.
11. Josefsson, L. and Lindberg, T. *Acta Physiol. Scand.* **66** (1966) 410.
12. Davis, B. J. *Ann. N. Y. Acad. Sci.* **121** (1964) 404.

Received May 14, 1971.

Preparation of a Highly Purified Glycyl-L-leucine Dipeptidase from Pig Intestinal Mucosa

OVE NORÉN, HANS SJÖSTRÖM and LARS JOSEFSSON

Department of Biochemistry C, University of Copenhagen, DK-2200 Copenhagen N, Denmark

The final digestion of proteins in the intestine involves several separate dipeptide splitting enzymes. Our knowledge of these enzymes has so far been based on their behaviour in crude extracts or in partly purified preparations. A more reliable information about their enzymatic properties and molecular constitution requires, however, their purification. In the following we wish to report a method for purification of a glycyl-L-leucine dipeptidase (glycyl-L-leucine hydrolase, EC 3.4.3.2) from pig intestinal mucosa.

Glycyl-L-leucine dipeptidase activity was determined under optimal pH-conditions at 25°C according to the method of Josefsson and Lindberg,[1] using glycyl-L-leucine (Sigma, Lot. No. 127 B−1460) as substrate. No metal ions were added. One unit (U) of enzyme activity is defined as the activity hydrolyzing 1 μmol/min of glycyl-L-leucine at 25°C. The absorbance at 280 nm was used as a measure of protein concentration. All purification procedures were performed in the cold room. Besides at the $(NH_4)_2SO_4$ precipitations, all buffers contained 2-mercaptoethanol (4 mM) and glycerol (12.5 % w/v) in order to stabilize the enzyme activity.

Fresh pieces (3 m taken 2 m distal from pylorus) of small intestines of adult pigs were used as source of enzyme. After being squeezed to remove intestinal content they were cut open and divided into 5 cm pieces. Without further disintegration the pieces were subsequently extracted with stirring for 1 h in precooled distilled water (1 l per 3 m intestine). After centrifugation the supernatant was lyophilized. The lyophilized, brown-yellow powder (90 g/30 m intestine) was suspended in 0.05 M phosphate (pH 7.5). Insoluble material was removed by centrifugation. The opalescent supernatant (Fraction 1, Table 1) was fractionated with $(NH_4)_2SO_4$. The fraction, precipitating between 40 and 65 % saturation of