

Enzymatic Properties of Pig Intestinal Proline Dipeptidase

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The basic enzymatic properties of pig intestinal proline dipeptidase (aminoacyl-L-proline hydrolase, EC 3.4.13.9) were studied. The enzyme was inhibited both by substrate and product. pH-optima and K_m of the reactions with L-alanyl-L-proline (6.8 and 0.29 mM) and glycyl-L-proline (7.4 and 0.13 mM) as also K_i of the proline inhibition (0.21 mM at pH 7.4 and 0.49 mM at pH 6.8) were determined. The values were used for the calculation of theoretical progression curves, which were in good accordance with the experimental ones. 2-Mercaptoethanol, used as a stabilizer of the enzyme preparation showed inhibitory properties. The prolidase activity was not affected by DFP whereas thiol-reagents inhibited the enzyme. Bivalent metal ions showed no influence on the activity. Inhibition by 1,10-phenanthroline, partially reversible by Zn^{2+} , suggested this metal to be involved in the catalysis.

Proline dipeptidase (also known as prolidase, aminoacyl-L-proline hydrolase, EC 3.4.13.9) is a dipeptide hydrolase which almost exclusively cleaves dipeptides of the aminoacyl-L-proline or aminoacyl-L-hydroxyproline type. It was first recognized by Bergmann and Fruton¹ and since then several reports on the enzyme from various animal sources have appeared.²⁻⁵ The basal enzymatic characteristics of the pure enzyme, necessary for further enzyme kinetic work, have now been investigated on the pig intestinal prolidase, recently obtained as a homogeneous preparation.⁶

The present report settles the optimal assay conditions for the enzyme and investigates the possibility of product inhibition. A theoretical progression curve, based on the experimental data, is calculated and compared to the one obtained experimentally. In addition, the prolidase is characterized in respect to some common enzyme inhibitors and activators.

EXPERIMENTAL PROCEDURES

Enzyme preparation. The enzyme was prepared according to final purification Procedure II as described elsewhere.⁶ It was stored frozen at -20°C in small samples in 0.05 M Tris-HCl buffer (pH 7.5), made 4 mM in respect to 2-mercaptoethanol. The concentration of the enzyme was about 50 units of activity per ml. Under these conditions the enzyme was found to be stable for several months. 2-Mercaptoethanol was present to prevent aggregation of the enzyme. For each experiment one of the stored samples was used. After thawing it was generally diluted with 0.1 M potassium phosphate buffer (pH 6.8) at 4°C to give a concentration of about 0.5 units of activity per ml, a concentration suitable for adequate substrate hydrolysis during a 10 min time period using the ordinary substrate concentration. Although the inhibitory effect observed for 2-mercaptoethanol at higher concentrations became negligible by this dilution, samples used for experiments involving low substrate concentrations were dialyzed (dialysis tubes, Visking Co., Chicago, U.S.A.) over-night against the buffer to further dilute the 2-mercaptoethanol.

Chemicals. L-Alanyl-L-proline was purchased from Fluka AG Chem. Fabrik, Buchs, Switzerland, and glycyl-L-proline was obtained from Sigma Chem. Comp., St. Louis, U.S.A. They were found chromatographically pure (thin layer chromatography, Merck DC Alufolien Cellulose 0.1 mm; butanol-acetic acid-water 4:1:1 by vol., ninhydrin reagent). In all experiments involving the continuously recording procedure the substrate concentration was calculated on the basis of the determined molar extinction coefficient of the peptide bond. Both substrates were dried as described by Bergmann *et al.*¹⁰ until constant weight was obtained. Di-isopropylfluorophosphate (DFP) and *p*-hydroxymercuribenzoate (crystalline sodium salt, PHMB) were products of Sigma, St. Louis, U.S.A. 1,10-phenanthroline was obtained from Merck, Darmstadt, Germany. De-ionized and glass distilled water was used

throughout. All other reagents used were of analytical grade.

Enzyme assay. Prolidase activity was assayed according to the method of Josefsson and Lindberg¹¹ with the following modifications. The dipeptide and amino acid solutions were prepared in their suitable concentrations using 0.1 M KH_2PO_4 buffer solution adjusted to proper pH with 0.1 M KOH (pH-meter 28, Radiometer, Copenhagen, Denmark). To get a higher precision of the method, 100 μl of the enzyme solution were mixed with 750 μl of the buffered substrate (2 mM) solution. Two samples were used for each digestion time. They were incubated at 25°C for a suitable time period (5–60 min). Because of the small amounts of protein in the samples the ethanol precipitation procedure was omitted and instead the hydrolysis was interrupted by the addition of 750 μl 0.5 M H_3PO_4 . The samples were directly read in a Zeiss spectrophotometer PMQ II at 226 nm (α -alanyl-L-proline) and 224 nm (glycyl-L-proline). In certain experiments the sensitivity of the method was increased by mixing a greater volume of enzyme solution into a smaller volume of substrate solution. The total volume of the incubation solution was always the same as in ordinary assays, as was the final substrate concentration.

In experiments where the substrate concentration was varied, alternative wavelengths from 205 nm and upwards were used to compensate for the absorbance differences. To get an approximation of the initial velocity a continuously recording procedure was chosen. The spectrophotometer was provided with a logarithm recorder (Servogor R. E. 514.9) and a thermostated cuvetteholder for cuvettes with 1 cm light path. The enzyme solution (20 μl) was mixed with pre-temperated, buffered substrate solution (1000 μl), and subsequently transferred to the cuvette. The mixing and transfer process took about 15 s. The enzyme concentration was chosen to allow approximation of the initial velocity and the reading wavelength was chosen to give a starting absorbance between 0.7 and 0.8. The spectrophotometer was set to zero before each experiment using a mixture of the enzyme solution and a buffered solution of the corresponding amino acids at appropriate concentrations.

Unit of prolidase activity. One unit of prolidase activity was defined as the amount of enzyme hydrolyzing 1 μmol α -alanyl-L-proline (1.8 mM) per min at 25°C and pH 6.8.

RESULTS

Factors affecting stable assay conditions

Stability as a function of pH. The buffer prepared for these studies was made from a

stock solution containing equimolar concentrations of H_3PO_4 , citric acid, and H_2BO_3 . After adjusting the solution to appropriate pH with 0.1 M KOH, water was added to give a final concentration of 0.1 M for each of the constituents. Samples of the enzyme stock solution were diluted ten times with the described buffer solution, preadjusted to pH-values varying from 2.9 to 10.3. The samples, containing 3 units of activity per ml, were stored for 30 min at 25°C and subsequently diluted another ten times with 0.1 M potassium phosphate buffer (pH 7.0) to accomplish a nearly neutral solution before the samples were assayed for their activity. All pH-values were controlled during the experiments in parallel experiments, where the enzyme stock solution was substituted with 0.05 M Tris-HCl buffer (pH 7.5). As shown in Fig. 1 there is no difference in enzyme stability in the pH-range between 5.5 and 8.5.

Stability as a function of time, temperature, and enzyme concentration. The stability of the prolidase at 0°C (melting ice) was studied in 0.1 M potassium phosphate buffer (pH 6.8), using an enzyme concentration of 0.5 units of activity per ml. Assays (1.8 mM α -alanyl-L-proline) were performed at suitable time intervals during a time period of 3 h from the time of preparing the diluted enzyme solutions. The results showed a small decrease of the activity during the first hour and then a more stable activity. Thus practically constant assay values were obtained during a 2 h period if the enzyme solution first was left for 1 h at 0°C.

The stability of the prolidase after being preincubated as above was also studied at 25°C at the enzyme concentrations 0.03, 0.06,

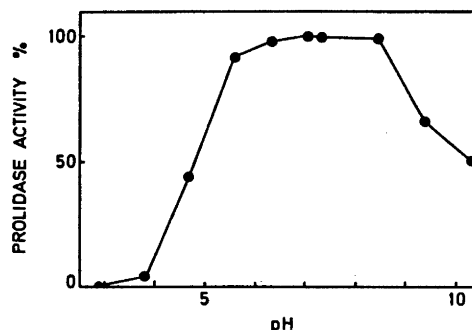


Fig. 1. Stability of prolidase as a function of pH. Substrate: α -alanyl-L-proline.

and 0.15 units of activity per ml. The same experimental procedure as above was used and the time period was 1 h. A continuously proceeding slow loss of activity was observed in all incubations, although the loss was most pronounced during the first 20 min period.

This slow loss of the enzyme activity was, however, without influence in the assay procedure, as it was found that progression curves (1.8 mM L-alanyl-L-proline) when followed to 80 % hydrolysis at the three different enzyme concentrations showed identical profiles, *i.e.* the enzyme concentrations of 0.03 units of activity per ml resulted in the same hydrolysis figure, when incubated for 100 min as did the one containing 0.15 units of activity per ml, when incubated for 20 min. This finding thus suggests a substrate stabilisation of prolidase.

Factors affecting optimal assay conditions

Reaction velocity as a function of pH. The influence of pH on the reaction velocity was investigated in the pH-range from 6.0 to 7.9 using 0.1 M potassium phosphate buffer. Measurements of the pH at the start and at the end of the incubation period, made in parallel experiments, showed a change of less than 0.04 pH-units. The results, shown in Fig. 2, gave a velocity maximum for the enzyme reaction at pH 6.8 when L-alanyl-L-proline was the substrate. A similar experiment displayed a pH-optimum of 7.4 for the enzyme reaction when glycyl-L-proline was the substrate.

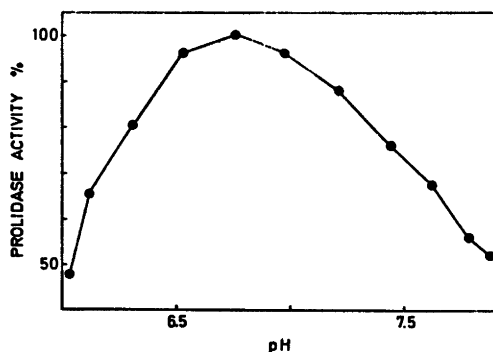


Fig. 2. Prolidase activity as a function of pH. Substrate: L-alanyl-L-proline.

Reaction velocity as a function of substrate concentration. The dependence of reaction velocity on substrate concentration was studied at five different substrate concentrations using L-alanyl-L-proline and glycyl-L-proline as substrates. Each experiment consisted of a double analysis at each substrate concentration and was run at the pH-optima proper to each of the reactions. The enzyme solution, dialyzed against 4000 vol. of 0.05 M Tris-HCl buffer (pH 7.5) over-night and subsequently frozen in portions suitable for each experiment, was appropriately diluted and kept at 0 °C for 1 h before the start of each experiment. Five experiments of this type were run for each substrate. A reference assay made at the beginning and end of each experiment showed neither an alteration of the activity during the test period nor a difference between the five separate experiments. Therefore the data could be statistically analyzed as one experiment with ten measurements at each substrate concentration. Assuming a classical Michaelis-Menten kinetics, the method of Wilkinson¹² could be used. The calculation including 4 of the 5 concentrations was performed on a Univac 1106 computer and resulted in a K_m of 0.29 (S.E. 0.025) mM and a V of 0.077 (S.E. 0.0033) mM/min for the reaction with L-alanyl-L-proline and a K_m of 0.13 (S.E. 0.015) mM and a V of 0.062 (S.E. 0.0011) mM/min for the reaction with glycyl-L-proline. Fig. 3a – b gives the data plotted as suggested by Lineweaver and Burk.

Product inhibition

Most of the naturally occurring amino acids were separately added to the buffered L-alanyl-L-proline solution in a concentration of 2 mM to investigate their possible role as inhibitors for the prolidase reaction. L-Tyrosine, L-phenylalanine and L-tryptophan were excluded because they disturbed the assay method. Of the various amino acids L-proline in consistency with earlier findings³ was found to be a rather strong inhibitor, whereas the other amino acids besides cysteine, caused no or only slight inhibition. The inhibition induced by cysteine is probably explained by its thiol-group (*cf.* influence on the activity with 2-mercaptoethanol).

The extent and kind of inhibition with L-proline were further investigated by adding

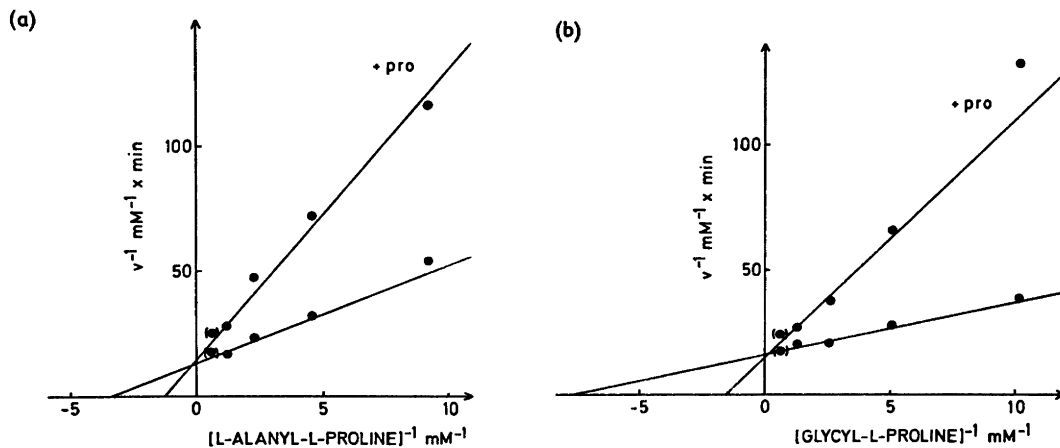


Fig. 3. Lineweaver-Burk plots of prolidase hydrolysis of (a) L-alanyl-L-proline and (b) glycyl-L-proline and the effect of L-proline (0.8 mM) on the reactions. ● Mean value of 10 measurements. Enzyme concentration during incubation (a) 0.03 units of activity per ml; (b) 0.1 units of activity per ml.

the amino acid (0.8 mM) to incubations with varying substrate concentrations. The experiment was run in the same way as described for the determination of the kinetic coefficients. In fact, the experiments described above and the inhibitor experiments were mixed with each other for making comparisons of the V -values more justified. Using the same statistical analysis procedure as above the apparent K_m was found to be 0.76 (S.E. 0.10) mM in the experiments with L-alanyl-L-proline and 0.62 (S.E. 0.039) mM in the experiments with glycyl-L-proline. The corresponding values for V were 0.069 (S.E. 0.0063) mM/min and 0.068 (S.E. 0.0038) mM/min, respectively. Fig. 3a–b gives the experimental data plotted as suggested by Lineweaver and Burk.

Other inhibitors

Influence of DFP on the activity. 150 μ l of an 0.2 mM solution of DFP in 0.1 M potassium phosphate buffer (pH 7.7) were added to 50 μ l of the enzyme solution, corresponding to a thousand-fold excess of the reagent. The samples in parallel with controls, in which the DFP solution was replaced by the buffer, were incubated at 25 °C for 20 min and then assayed (1.8 mM L-alanyl-L-proline) for their enzyme activity. No decrease of activity was observed in the DFP-containing samples when compared

to the controls. Using the same experimental conditions the DFP solution was found to cause a complete inactivation of chymotrypsin,¹⁸ when assayed against casein.

Influence of thiol-reagents on the activity. The essentiality of free SH-groups for the activity of prolidase was demonstrated in experiments with PHMB, Ag⁺, Cu²⁺, and Hg²⁺, all known to react with thiol-groups of proteins. The enzyme was incubated at 25 °C for 10 min with the compounds added separately to a buffered L-alanyl-L-proline solution (pH 6.8) at concentrations of 1, 10, and 100 μ M (Table 1). Preincubations of prolidase with a hundred-fold excess of PHMB (molar basis) for 16 h in 0.05 M Tris-HCl buffer (pH 7.5, 4 °C) resulted in a complete inactivation, which remained also after the excess of PHMB had been removed by dialysis.

Table 1. Effect of thiol-reagents on the prolidase activity.

Concentration μ M	Activity % Ag ⁺	Cu ²⁺	Hg ²⁺	PHMB
0	100	100	100	100
1	0	100	100	50
10	0	60	10	40
100	0	30	0	20

Influence of 2-mercaptoethanol on the activity.

An enzyme solution, free from 2-mercaptoethanol by dialysis, was assayed according to the general procedure but using a series of buffered L-alanyl-L-proline solutions, containing varying concentrations (0–4 mM) of 2-mercaptoethanol. The results revealed a 50 % inhibition at a 2-mercaptoethanol concentration of 0.7 mM, while very low 2-mercaptoethanol concentrations (10 μ M) did not affect the prolidase activity.

Influence of certain metal ions on the activity.

CoCl₂, MgCl₂, MnCl₂ and ZnCl₂ from aqueous stock solutions (0.02 M) were separately added to buffered L-alanyl-L-proline and glycy-L-proline solutions at concentrations of 10 and 100 μ M. The activity of a prolidase solution was assayed according to the general assay procedure using the various substrate solutions. No obvious effect on the enzyme activity was observed with any of the metal ions when compared to an incubation with no metal added. The influence of the metal ions on the prolidase activity was also investigated after the enzyme was treated with 1,10-phenantroline. An enzyme solution (0.05 M Tris-HCl buffer pH 7.5) was stored during a fortnight at 4 °C with and without 1,10-phenantroline (1 mM) and then assayed against L-alanyl-L-proline. The results showed a 90 % decrease of the activity in the 1,10-phenantroline containing solution compared to only a 20 % decrease in the control solution. 1,10-phenantroline was then removed by dialysis and the enzyme solution was given the four metal ions separately at concentrations as above. Repeated assays of the prolidase activity then showed partial reactivation in the samples containing Zn²⁺, while no effect was observed in the other samples.

DISCUSSION

The omission of the centrifugation step in the original assay procedure²² increased the precision of the method as did the increase of the volumes, thereby making it more suitable for these studies. At a substrate concentration of 1.8 mM the coefficient of variation of the assay was found to be 4.5 % ($n=20$). The sensitivity of the method can be increased by decreasing the substrate concentration, as the

inadequacy caused by not measuring the reaction at maximal velocity can be compensated for by the use of the general formula given in this report. Systematic errors due to the hygroscopic nature of the substrates, *i.e.* varying water content, can be overcome by using the molar extinction coefficient of the peptide bond. The applicability of Lambert-Beers law was verified within the whole wavelength region used.

The pH-optima for the prolidase reaction with L-alanyl-L-proline and glycy-L-proline agree well with the values earlier reported for crude intestinal extracts of various species.^{5–7,14} Peters,¹⁵ however, reported a pH-optimum of 8.0 for the glycy-L-proline hydrolysis by a quinea-pig intestinal extract. This pH-optimum for the glycy-L-proline reaction was also given for the purified pig kidney prolidase.⁴

As separate experiments with substrate concentrations higher than 2 mM demonstrated substrate inhibition, the data for obtaining the kinetic coefficients were processed both with and without the values of 1.6 mM substrate concentration (Fig. 3). The kinetic coefficients obtained with a four point analysis fitted best in the progression curve (see below) thus making these values more probable in spite of the lower statistical precision.

Using the t-test according to Cleland,¹⁶ it was found that the two values of K_m and the two apparent K_m values obtained from the inhibition experiments with proline differed ($P=99.9\%$). No differences between the corresponding V values could be demonstrated (L-alanyl-L-proline, $P=90\%$, glycy-L-proline, $P=90\%$). These findings suggest L-proline as a competitive inhibitor and result in a K_i for the inhibition of 0.49 mM at pH 6.8 and 0.21 mM at pH 7.4.

Since variation of buffer (0.1 M Tris-HCl, pH 7.5, and 0.1 M potassium phosphate, pH 7.5) did not affect the prolidase activity and the enzyme activity was stable during the incubation period, the experimental progression curve could be completely explained by product inhibition and by the decrease of enzyme saturation with substrate.

Jennings and Niemann¹⁷ have described an integrated rate equation (1) for a product inhibited enzyme model. This equation, using a K_m of 0.29 mM and a K_i of 0.49 mM for the

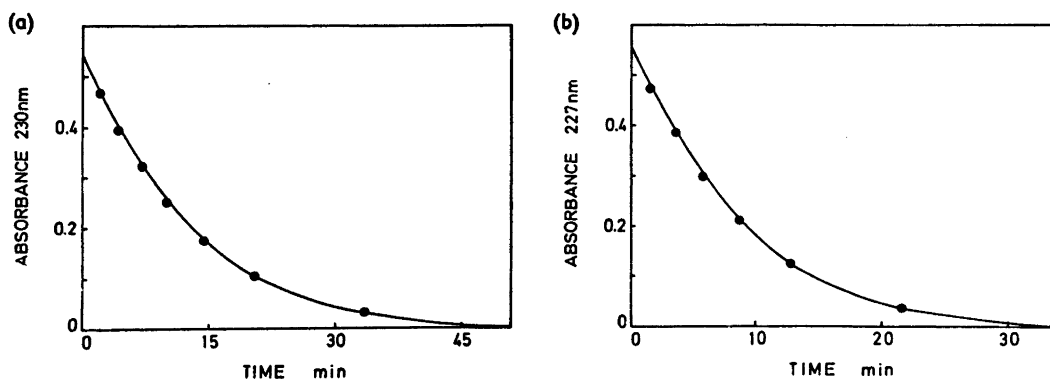


Fig. 4. Progression curves of prolidase with (a) 0.71 mM L-alanyl-L-proline and (b) 0.64 mM glycyL-L-proline as substrates. The theoretical values were calculated according to eqn. (1) at a constant interval of substrate concentration. The fitting of the equation to the enzymatic activity of the experiments were made as described in the text. — Experimental curve; ● calculated values.

prolidase reaction with L-alanyl-L-proline and a K_m of 0.13 mM and a K_i of 0.21 mM for the reaction with glycyL-L-proline is plotted in Fig. 4. In each of the curves, one point (substrate concentration, S_t ; time, t) around 50% hydrolysis was first taken from the experimental data to obtain $k_3 \times E$, and thereby fit the equation to the experimental curves.

$$k_3 E t =$$

$$K_m (1 + S_0/K_i) \ln S_0/S_t + (1 - K_m/K_i)(S_0 - S_t) \quad (1)$$

E enzyme concentration
 S_0 substrate concentration to time zero
 S_t substrate concentration to time t
 k_3 rate constant of the enzyme-substrate complex dissociation

This equation permits evaluation of the kinetic coefficients from a few progression curves instead of their estimation from many separate determinations of initial velocities.

The equation was also used to calculate the molecular activity (k_3) for the enzyme at 25 °C, using a molar extinction coefficient of $11.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for prolidase (280 nm)¹⁸. The molecular activity was found to be 3×10^4 and $8 \times 10^3 \text{ min}^{-1}$ for L-alanyl-L-proline and glycyL-L-proline, respectively. The value of the glycyL-L-proline hydrolysis is low as compared to that reported by Davis and Smith.⁴ The discrepancy is, however, partly explained by the higher experimental temperature used by them.

The experiments with DFP and thiol-reagents suggest prolidase as an enzyme de-

pendent on free SH-group(s) for its activity, which is in agreement with earlier observations on pig kidney prolidase.⁴

The inhibition observed in this study with the thiol-compounds, earlier also reported with glutathione,⁴ could be explained by the binding of the sulfur to an essential metal of the enzyme. This metal may be zinc, as suggested from the experiments including 1,10-phenanthroline. As the enzyme activity in our studies, in contrast to earlier findings with higher metal concentrations,^{2-4,6,7,18} was found to be unaffected with Co^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} and also stable in the presence of 1 mM EDTA,⁹ the metal is proposed to be strongly bound to the enzyme.

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