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Purification and Properties of the Pyrrolidonecarboxylate Peptidase of *Streptococcus faecium*

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Abstract

Pyrrolidonecarboxylate peptidase (EC 3.4.11.8) from *Streptococcus faecium* was purified by fractionation with streptomycin sulphate and ammonium sulphate, by chromatography on Sephadex G200 and DEAE-cellulose, and by preparative electrophoresis on Sephadex G25. The purified enzyme on acrylamide gel showed a strong protein band which contained enzyme activity and a very faint band which had no activity.

The subunit molecular weight of the purified enzyme was estimated by acrylamide gel electrophoresis in sodium dodecyl sulphate to be 42 000±1 000. The enzyme showed optimum activity at pH 7·6 and was unstable in the absence of 2-mercaptoethanol. The sensitivity of the enzyme to alkylating agents (*N*-ethylmaleimide and iodoacetamide) suggested that free sulphydryl groups were essential for enzyme activity. The enzyme was rapidly inactivated above 45°C. The values of the Michaelis constants (K_m) obtained with various L-pyrrolidonecarboxylyl dipeptides were similar although there was a 10-fold range in the maximal rates of hydrolysis of these substrates. Inhibition studies showed that the substrate analogues 2-pyrrolidone and pyrrolidonecarboxylate are competitive inhibitors of the enzyme. The binding of substrates and inhibitors to the active site of the enzyme is discussed.

Introduction

Pyrrolidonecarboxylate peptidase (L-pyroglutamyl-peptide hydrolase; EC 3.4.11.8) has been observed in various bacteria (Doolittle and Armentrout 1968; Mulczyk and Szewczuk 1970; Sullivan and Jago 1970; Fellows and Mudge 1971; Kwiatkowska *et al.* 1974), plants and animal tissues (Armentrout 1969; Szewczuk and Kwiatkowska 1970). It is an enzyme which specifically hydrolyses the peptide bond joining L-pyrrolidonecarboxylyl residues to peptides and proteins (Doolittle and Armentrout 1968; Kwiatkowska *et al.* 1974). The enzyme is therefore useful in determining the amino acid sequence in polypeptide chains which are blocked by a pyrrolidonecarboxylate moiety at the $-NH_2$ terminus. A purified enzyme has been obtained recently from *Klebsiella cloacae* (Kwiatkowska *et al.* 1974).

The observation that *Streptococcus faecium* (*Streptococcus durans*) is a rich source of pyrrolidonecarboxylate peptidase (Sullivan 1972) led to the present investigation in which the enzyme from this organism was purified and its properties studied.

Materials and Methods

Bacteria

S. faecium was maintained by daily subculture in sterile milk [10% (w/v) skim milk powder containing 0·2% yeast extract] and grown in quantity in broth containing tryptone (Oxoid), 30 g/l; yeast extract (Difco), 10 g/l; lactose, 30 g/l; KH_2PO_4, 5·0 g/l; and Lab-Lemco powder (Oxoid),

2 g/l. The medium was filtered and autoclaved at 121°C for 10 min. The lactose was autoclaved separately before addition to the sterile medium.

The broth, in 51 batches, was inoculated from the skim milk cultures and incubated at 30°C for 17 h. The pH of the broth was maintained at 6·3 by the addition of 10 M NaOH controlled by a magnetic valve connected to a Radiometer Titrator, Model TTT11b. Cells were harvested from the growth medium by centrifugation at 24 000 *g* for 10 min at 4°C, washed twice in 0·5% NaCl containing 0·05 M 2-mercaptoethanol and finally recovered by centrifugation at 35 000 *g* for 20 min at 4°C.

Cell-free extracts were prepared by suspending the washed cells in 0·9% NaCl containing 0·05 M 2-mercaptoethanol (1 g wet weight of cells per 3 ml buffer) and extruding them through a French pressure cell (French and Milner 1955) at a pressure of $1{\cdot}54\ N\,m^{-2}$. Unbroken cells and cellular debris were removed by centrifugation at 35 000 *g* for 30 min at 4°C.

Measurement of Enzyme Activity using L-*Pyrrolidonecarboxylyl-β-naphthylamide*

(i) N-*1-naphthyl-ethylenediamine* (*NNED*) *method*

The reaction mixture, total volume 1 ml, contained: 0·5 ml of assay buffer (0·1 M potassium phosphate buffer, pH 7·0, containing 0·01 M 2-mercaptoethanol and 0·01 M EDTA) and 0·4 ml of enzyme solution. After pre-incubation at 30°C for 5 min the enzyme reaction was started by the addition of 0·1 ml of 0·02 M L-pyrrolidonecarboxylyl-β-naphthylamide in methanol and the incubation was continued at 30°C for 1 h. The reaction was stopped by the addition of 1 ml of 25% trichloroacetic acid and the precipitated protein was removed by centrifugation. The β-naphthylamine liberated in 1 ml of the supernatant was determined by the method of Bratton and Marshall (1939) as modified by Goldbarg and Rutenberg (1958). As preliminary experiments had shown that the concentration of 2-mercaptoethanol in the incubation mixture greatly decreased the sensitivity of this method for estimating free β-naphthylamine, all standard curves were prepared using the same concentrations of 2-mercaptoethanol as were used in the reaction mixtures.

(ii) *Fast Blue B method*

The reaction mixture, total volume 1 ml, contained: 0·8 ml of 0·5 M tris buffer, pH 7·7, and 0·1 ml of enzyme solution. After pre-incubation at 30°C for 5 min, 0·1 ml of 0·02 M L-pyrrolidonecarboxylyl-β-naphthylamide in methanol was added to start the reaction. The mixture was further incubated at 30°C for 1 h. The absorbance of the red-coloured complex of β-naphthylamine and Fast Blue B salt (Gurr), formed by the method of Mulczyk and Szewczuk (1970), was measured at 500 nm.

(iii) *Direct method*

The reaction mixture, total volume 2 ml, usually contained 0·1 ml of enzyme solution, 0·1 ml of 0·02 M L-pyrrolidonecarboxylyl-β-naphthylamide and 1·8 ml of assay buffer, pH 7·0. The release of free β-naphthylamine at 30°C was followed according to the method of Lee *et al.* (1971) at 340 nm on a Zeiss spectrophotometer, model PMQII, attached to a Rikadenki recorder, model B-14.

Measurement of Enzyme Activity using Pyrrolidonecarboxylyl Peptides

The reaction mixture, total volume 1 ml, contained 0·1 ml of enzyme solution and 0·9 ml of dipeptide (0·9 μmol/ml) dissolved in the assay buffer, pH 7·0. Incubation was at 30°C. Aliquots of 0·1 ml were taken at appropriate time intervals and the enzymically released amino acid was estimated by the ninhydrin method of Matheson and Tattrie (1964). The amount of amino acid released from each substrate was calculated from a standard curve prepared with the respective amino acid.

Protein Estimation

Protein was estimated by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

Acrylamide Gel Electrophoresis

0·1 M tris, adjusted to pH 9·6 with boric acid, was used to prepare 5% acrylamide gels and as the electrode buffer. Samples of 50 μl, containing 15% sucrose and dye marker (3 μl bromphenol blue), were layered on the gels and run at 2 mA per tube until the marker dye reached 2–3 mm from the end of the tube. The gels were stained in 0·1% amido black in 7% acetic acid and destained by soaking in 7% acetic acid.

The enzyme was located on the gel by fluorescence, under ultraviolet light, of the β-naphthylamine released after incubation of an unstained gel in 4 ml of assay buffer containing L-pyrrolidonecarboxylyl-β-naphthylamide.

Determination of Subunit Molecular Weight

This was estimated by electrophoresis on sodium dodecyl sulphate (SDS) acrylamide gels according to the method of Weber and Osborn (1969). The standard proteins used were crystalline bovine serum albumin (Mann Research Laboratories); glutamate dehydrogenase (C. F. Boehringer & Soehne); 5× crystallized ovalbumin (Pentex); crystalline glyceraldehyde-phosphate dehydrogenase (Sigma); and 3× crystallized β-lactoglobulin (Koch–Light). The subunit molecular weights of the above proteins were taken from Weber and Osborn (1969).

Processing of Kinetic Results

The data from kinetic experiments were fitted directly to the appropriate rate equation using a computer program which gives a least squares estimation of non-linear parameters by the method of Marquardt (1963). Further details, together with the rate equations, are reported elsewhere (Simpson and Davidson 1976).

Results

Purification of the Enzyme

All steps in the purification procedure were carried out at 4°C in the presence of 0·05 M 2-mercaptoethanol. The criterion for purity was the number of bands obtained after electrophoresis on acrylamide gels. The individual steps are detailed below.

(i) *Treatment with streptomycin sulphate*

A 10% (w/v) solution of streptomycin sulphate was added dropwise to the cell-free extract with constant stirring until no further precipitation occurred. After 4 h the precipitate was removed by centrifugation at 35 000 *g* for 20 min and discarded. The supernatant solution was dialysed overnight against 0·9% NaCl containing 0·05 M 2-mercaptoethanol.

(ii) *Fractionation with ammonium sulphate*

The dialysed solution was brought to 55% saturation with solid ammonium sulphate (35·1 g/100 ml). After 2 h the precipitate was removed by centrifugation (35 000 *g* for 20 min) and discarded, and the supernatant solution was brought to 75% saturation with solid ammonium sulphate (14·1 g/100 ml). The second precipitate, which contained the bulk of the enzyme activity, was recovered by centrifugation (35 000 *g* for 20 min).

(iii) *Gel filtration*

The 55–75% ammonium sulphate fraction was dissolved in a minimum volume of 0·9% NaCl containing 0·05 M 2-mercaptoethanol and applied to a column of Sephadex G200 (1·5 by 150 cm) pre-equilibrated with the same solution. The protein was eluted with this solution at a flow rate of 0·2 ml/min and 4-ml fractions were collected. The elution profile and the pooled fractions are shown in Fig. 1*a*.

(iv) *Anion exchange chromatography*

Fraction *A* from the Sephadex G200 column (Fig. 1*a*) was concentrated in an Amicon Diaflo apparatus with a UM10 filter, dialysed against 5 mM sodium phosphate buffer, pH 7·0, containing 0·05 M 2-mercaptoethanol and then applied to a column of DEAE-cellulose (2·5 by 62 cm) pre-equilibrated in this buffer. The protein was eluted first with 1200 ml of a linear NaCl gradient (0–0·6 M), and later with 1 M NaCl,

in the same buffer. The flow rate was 1·6 ml/min and 10-ml fractions were collected. The elution profile is shown in Fig. 1*b*.

(v) *Preparative electrophoresis on Sephadex G25*

Fraction *B* (Fig. 1*b*) from the DEAE-cellulose column was concentrated in the Diaflo apparatus, as above, and dialysed overnight against tris–glycine buffer (0·05 M tris, 0·38 M glycine, 0·05 M 2-mercaptoethanol), pH 8·3. The sample was subjected to electrophoresis for 7 h at 900 V and 20 mA on a column of Sephadex G25 (2·5 by 43 cm) as described by Whitehead *et al.* (1971). On completion, the column was eluted with tris–glycine buffer, pH 8·3, and the elution profile is shown in Fig. 1*c*.

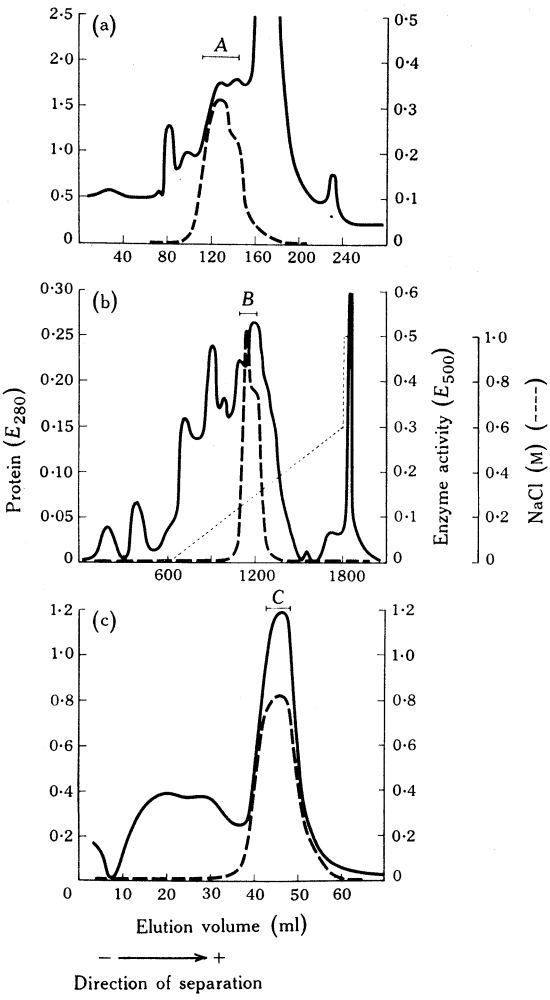

Fig. 1. Elution profiles of protein and pyrrolidonecarboxylate peptidase activity from (*a*) a Sephadex G200 column, void volume 80 ml; (*b*) a DEAE-cellulose column; and (*c*) a Sephadex G25 column after electrophoresis. —— Protein. --- Pyrrolidonecarboxylate peptidase activity. Assay of pyrrolidonecarboxylate peptidase activity was carried out using the Fast Blue B method (described in Methods) and protein by measurement of absorbance at 280 nm.

To ascertain the purity of the enzyme, electrophoresis on acrylamide gels was carried out. As shown in Fig. 2, two protein bands were evident in fraction *C* (Fig. 1*c*). The major band contained pyrrolidonecarboxylate peptidase activity whilst the minor band was without enzyme activity.

A summary of the purification steps with measures of their relative efficiency is presented in Table 1.

Table 1. Summary of the purification procedure for pyrrolidonecarboxylate peptidase
Pyrrolidonecarboxylate peptidase activity was assayed by the NNED method and protein was estimated as described in Methods. The loss in activity between steps 4–5 and 5–6 was due to the instability of the purified enzyme

Step	Fraction	Total activity (A_{570} h^{-1})	Total protein (mg)	Specific activity [A_{570} h^{-1} (mg protein)$^{-1}$]	Purification factor
1	Cell-free extract	879	3450	0·26	1·0
2	Streptomycin sulphate	781	2650	0·30	1·2
3	Ammonium sulphate	470	1050	0·45	1·7
4	Sephadex G200	563	212	2·66	10·2
5	Before DEAE-cellulose	234	192	1·22	4·7
	After DEAE-cellulose	215	10·7	20·1	77·3
6	Before Sephadex G25 electrophoresis	23	6·5	3·57	13·7
	After Sephadex G25 electrophoresis	14	2·21	6·50	25·0

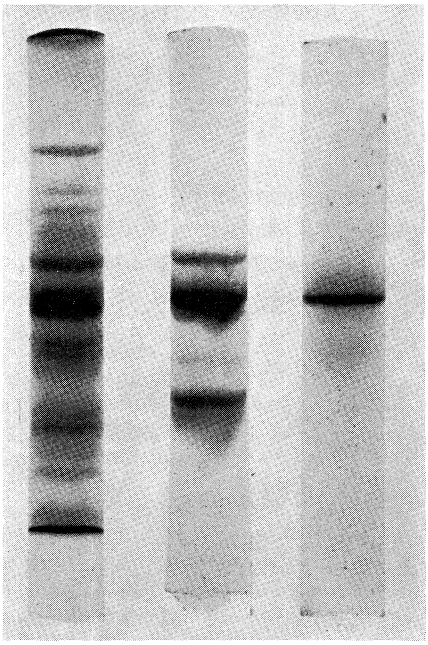

Fig. 2. Electrophoretic patterns on acrylamide gels. *1*, Cell-free extract. *2*, Fraction *A* (see Fig. 1) after gel filtration on Sephadex G200. *3*, Fraction *C* (see Fig. 1) after electrophoresis on Sephadex G25.

Properties of the Enzyme

Owing to the instability of the purified enzyme, all studies, except for the minimum molecular weight determinations, were carried out with fraction *A* obtained after gel filtration on Sephadex G200.

(i) *Subunit molecular weight*

Two separate determinations gave an average subunit molecular weight of 42 000±1 000.

(ii) *pH optimum*

The activity of the enzyme was measured in citrate, tris–maleate and glycine buffers, prepared as described by Gomori (1955), over the pH range 4·0–10·0 using L-pyrrolidonecarboxylyl-β-naphthylamide as the substrate. As shown in Fig. 3, a sharp peak of activity was observed at pH 7·6.

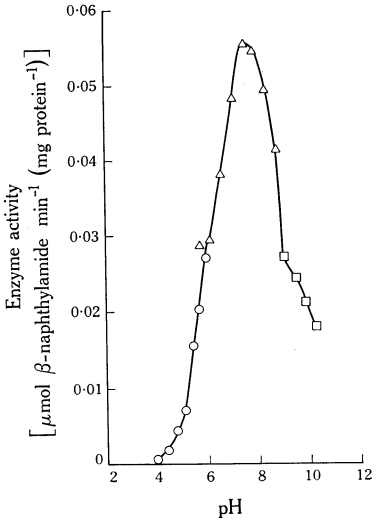

Fig. 3. Effect of pH on pyrrolidonecarboxylate peptidase activity. The reaction mixture, total volume 1 ml, contained: 0·1 ml (0·5 mg protein) of enzyme, 0·1 ml of 0·2 M L-pyrrolidonecarboxylyl-β-naphthylamide, and 0·8 ml of buffer containing 0·01 M 2-mercaptoethanol and 0·01 M EDTA. Incubation was at 30°C for 30 min and enzyme activity was determined by the NNED method described in Methods.
○ 0·05 M Sodium citrate buffer.
△ 0·05 M Tris–maleate buffer.
□ 0·05 M Sodium glycinate buffer.

(iii) *Stability*

The enzyme activity was stable for a period of at least 4 weeks when stored in 0·05 M 2-mercaptoethanol at 4°C. At lower concentrations of 2-mercaptoethanol the enzyme lost activity as shown in Fig. 4*a*. However, on increasing the temperature of storage to 30°C and above, the enzyme rapidly lost activity in 0·05 M 2-mercaptoethanol (Fig. 4*b*).

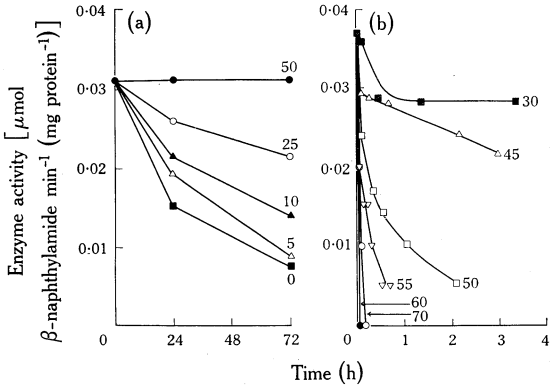

Fig. 4. Effect of (*a*) 2-mercaptoethanol and (*b*) temperature on the stability of pyrrolidonecarboxylate peptidase.
(*a*) 0·3-ml aliquots of the enzyme were dialysed at 4°C against 200 ml of 0·9% NaCl containing 0–0·5 M 2-mercaptoethanol for 24 and 72 h, after which enzyme activity was estimated by the direct method (described in Methods). 2-Mercaptoethanol concentrations (mM) are shown beside the graphs. (*b*) The enzyme was heated at the temperature (°C) indicated beside each graph. At the appropriate time intervals 0·1-ml aliquots of the enzyme solution were assayed for activity by the direct method.

(iv) *Affinity for substrates*

The enzyme was examined for its ability to hydrolyse L-pyrrolidonecarboxylyl dipeptides which contained either L-alanine, L-glutamate, L-valine or L-proline as the other amino acid residue. L-Pyrrolidonecarboxylyl-L-proline was not hydrolysed

under the conditions used, whilst the other three dipeptides had simple linear double reciprocal plots (Fig. 5). The rate of reaction was highest when L-pyrrolidonecarboxylyl-L-alanine was the substrate, although the K_m for this substrate was not significantly different from that obtained for L-pyrrolidonecarboxylyl-L-valine, L-pyrrolidonecarboxylyl-L-glutamate and L-pyrrolidonecarboxylyl-β-naphthylamide (Table 2).

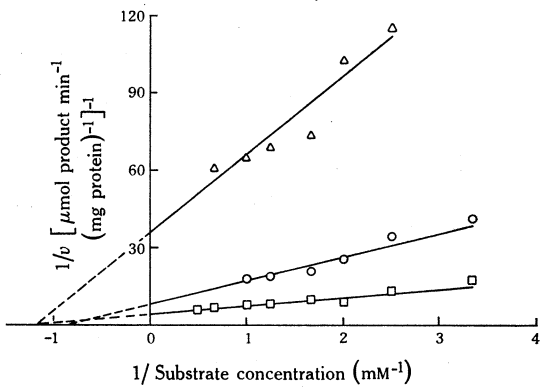

Fig. 5. Lineweaver–Burke plots of the activity of pyrrolidonecarboxylate peptidase on L-pyrrolidonecarboxylyl dipeptides. The reaction mixture, total volume 1 ml, contained: 0·1 ml (0·25 mg protein) of enzyme, 0–0·2 ml of 10 mM dipeptide, and assay buffer, pH 7·0, to 1 ml. Incubation was at 30°C and 0·1-ml aliquots of the reaction mixture were assayed by the ninhydrin method (described in Methods). □ L-Pyrrolidonecarboxylyl-L-alanine. ○ L-Pyrrolidonecarboxylyl-L-glutamate. △ L-Pyrrolidonecarboxylyl-L-valine.

Table 2. Kinetic constants for various substrates and inhibitors of pyrrolidonecarboxylate peptidase
Data were fitted to the rate equation as described in Methods. Standard errors have been calculated at the 95% confidence level

Substrate	Inhibitor	K_m (mM)	K_{is}	V
L-Pyrrolidonecarboxylyl-L-alanine	—	0·83±0·18	—	0·26±0·03
L-Pyrrolidonecarboxylyl-L-glutamate	—	1·19±0·23	—	0·13±0·02
L-Pyrrolidonecarboxylyl-L-valine	—	0·85±0·24	—	0·028±0·003
L-Pyrrolidonecarboxylyl-β-naphthylamide	—	0·86±0·08	—	0·06±0·01
L-Pyrrolidonecarboxylyl-β-naphthylamide	2-Pyrrolidone	—	1·7±0·6	—
L-Pyrrolidonecarboxylyl-β-naphthylamide	L-Pyrrolidonecarboxylate	—	1·0±0·3	—

(v) *Inhibitors*

As shown in Table 3, 10 mM 2-pyrrolidone decreased the rate of hydrolysis of L-pyrrolidonecarboxylyl-L-alanine by approximately 50% although further increases in the inhibitor concentration did not decrease the rate of hydrolysis proportionately. By using a range of inhibitor and substrate concentrations, the inhibition by 2-pyrrolidone was examined to determine which of the models for competitive, non-competitive, or uncompetitive inhibition (Cleland 1970) described it best. The results indicated that the inhibition was competitive (Fig. 6 and Table 2). L-Pyrrolidonecarboxylate was also found to be a competitive inhibitor of approximately the same effectiveness as 2-pyrrolidone (Table 2).

The addition of L-alanine, L-glutamate, L-valine, L-proline and L-hydroxyproline at 5 mM concentration did not inhibit the enzyme when L-pyrrolidonecarboxylyl-β-naphthylamide was the substrate used.

Table 3. Effect of 2-pyrrolidone on pyrrolidonecarboxylate peptidase activity

The reaction mixture, total volume 1 ml, contained: 0·1 ml of 10 mM L-pyrrolidonecarboxylyl-L-alanine, 0·1 ml (0·25 mg protein) of enzyme, 0·01 ml of 1 M 2-pyrrolidone and assay buffer, pH 7·0, to 1 ml. Incubation was at 30°C and 0·1-ml aliquots of the reaction mixture were assayed by the ninhydrin method (described in Methods)

2-Pyrrolidone concn (mM)	0	10	30	50	100
Enzyme activity (arbitrary units)	100	48	31	23	18

Preincubation of the enzyme with either iodoacetamide or *N*-ethylmaleimide (final concentration 2·5 mM) for 1 min prior to assay with L-pyrrolidonecarboxylyl-β-naphthylamide, resulted in a complete loss of enzyme activity.

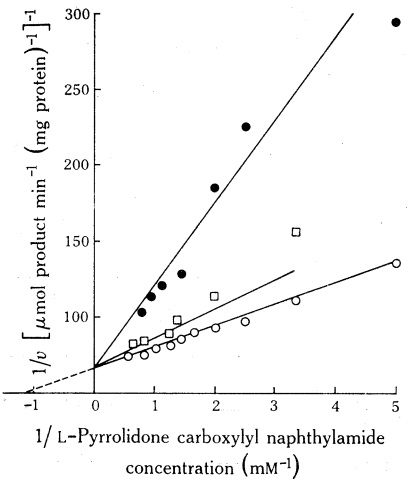

Fig. 6. Lineweaver–Burke plots showing the effect of varying 2-pyrrolidone concentration on pyrrolidonecarboxylate peptidase activity. The incubation mixture, total volume 1 ml, contained: 0·1 ml (0·25 mg protein) of enzyme, 0–0·2 ml of 0·02 M L-pyrrolidonecarboxylyl-β-naphthylamide, 0·1 ml of inhibitor, and assay buffer, pH 7·0, to 1 ml. The rate of reaction, which was carried out in a thermostatted cuvette at 30°C, was determined by the direct method (described in Methods). ○ No inhibitor. □ 0·5 mM 2-Pyrrolidone. ● 5 mM 2-Pyrrolidone. The lines are the lines of best fit of the data to the rate equation for competition inhibition: $v = V_s/[K_m(1+1/K_{is})+s]$.

Discussion

Armentrout and Doolittle (1969) attempted purification of the pyrrolidonecarboxylate peptidase from *Pseudomonas fluorescens* and obtained in their final purification step a preparation which showed two strong bands on acrylamide gel. Szewczuk and Mulczyk (1969) partially purified the pyrrolidonecarboxylyl peptidase from *Bacillus subtilis* and Szewczuk and Kwiatkowska (1970) partially purified the enzyme from pigeon liver. More recently Kwiatkowska *et al.* (1974) reported on the purification in low yield of the pyrrolidonecarboxylate peptidase from *Klebsiella cloacae*. A comparison of the polyacrylamide gel electrophoresis patterns of the purified enzymes from *K. cloacae* and *S. faecium* indicates that the final purity of the enzyme from the latter source is at least as good as that obtained from *K. cloacae*. The much higher enzyme levels in extracts of *S. faecium* is obviously an advantage in purifying the enzyme from this source.

Using crude extracts from pigeon and rabbit livers, Szewczuk and Kwiatkowska (1970) obtained a major and a minor peak of enzyme activity on Sephadex G75

corresponding to molecular weights of 33 000 and 80 000 respectively, whilst Kwiatkowska *et al.* (1974) found the apparent molecular weight of the *K. cloacae* enzyme to be 74 000. Assuming that the enzymes from the different species are of similar structure, the value of 42 000 for the subunit molecular weight of the *S. faecium* enzyme suggests that the native enzyme is a dimer.

Some of the properties of the *S. faecium* enzyme were similar to those of the enzymes obtained from other sources — for example, the stabilizing effect of 2-mercaptoethanol, the inhibition by sulphydryl reagents and the pH optimum of 7–9 (Armentrout and Doolittle 1969; Szewczuk and Mulczyk 1969; Szewczuk and Kwiatkowska 1970; Kwiatkowska *et al.* 1974). The first two observations confirm suggestions that at least one sulphydryl group in the enzyme is essential for activity (Doolittle and Armentrout 1969; Szewczuk and Mulczyk 1969).

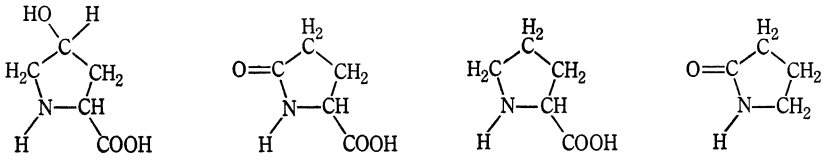

Fig. 7. L-Pyrrolidonecarboxylic acid and its structural analogues. (1) Hydroxyproline. (2) Pyrrolidonecarboxylic acid. (3) Proline. (4) 2-Pyrrolidone.

Substrate specificity studies with various L-pyrrolidonecarboxylyl dipeptides indicated that while there was no significant difference in their K_m values there was a marked difference in their relative maximal rates of hydrolysis by the enzyme. In decreasing rate order they were found to be: L-pyrrolidonecarboxylyl-L-alanine > L-pyrrolidonecarboxylyl-L-glutamate > L-pyrrolidonecarboxylyl-L-valine. The observation that L-pyrrolidonecarboxylyl-L-proline was not hydrolysed by the *S. faecium* enzyme is in contrast to the result found for the *K. cloacae* peptidase (Kwiatkowska *et al.* 1974).

The inhibition of the enzyme by both 2-pyrrolidone and pyrrolidonecarboxylate was found to be competitive, indicating that these compounds bind to the active site of the enzyme in place of the substrate. This result with 2-pyrrolidone is in contrast to the previous observation of non-competitive inhibition of the *Pseudomonas fluorescens* enzyme by this compound. On the basis of the inhibition being competitive, the stabilizing effect of 2-pyrrolidone (Armentrout and Doolittle 1969; Kwiatkowska *et al.* 1974) would probably be a consequence of its binding to the active site. L-Proline and L-hydroxyproline which have structures related to L-pyrrolidonecarboxylic acid (see Fig. 7) did not inhibit under the same conditions. As L-pyrrolidonecarboxylate and 2-pyrrolidone have a carbonyl group on position 2 whilst proline and hydroxyproline do not, it would appear that the carbonyl group is important for binding of compounds to the active site of the enzyme.

Acknowledgment

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