BBA 63432

Pyrrolidonecarboxylyl peptidase from rat liver

The enzyme pyrrolidonecarboxylyl peptidase was originally sought as a convenient and specific means of removing pyrrolidonecarboxylyl residues blocking the amino-terminal of peptides and proteins[1]. The pyrrolidonecarboxylyl residue is the naturally occurring amino terminus in a variety of proteins and also arises through cyclization of terminal glutaminyl groups in peptide fragments. The presence of a pyrrolidonecarboxylyl residue is a hinderance to sequencing due to the lack of a free terminal amino group. Until recently, there has been no specific way to chemically remove such groups[2]. Pyrrolidonecarboxylyl peptidase has been isolated and purified from a strain of *Pseudomonas fluorescens*[3,4]. This enzyme specifically removes pyrrolidonecarboxylyl residues from large proteins as well as small peptides. A similar activity has been observed in *B. subtilis*[3] and has also been substantially purified[5]. This paper reports the partial purification of pyrrolidonecarboxylyl peptidase from rat liver and compares it with the bacterial enzyme.

Rat liver homogenates fractionated by procedures similar to those employed with bacterial sonicates exhibited activity in the corresponding fractions. Activity was assayed, as previously described[3], with dipeptide L-Pyr–L-Ala as substrate. All manipulations were carried out at 0° where possible, otherwise, at 4°. Rats were decapitated and the liver was removed into 0.05 M phosphate buffer (pH 7.3) containing 0.25 M sucrose, 0.001 M EDTA and 0.01 M β-mercaptoethanol. The livers were minced and homogenized, followed by centrifugation for 30 min at 34 000 $\times g$. The supernatant was diluted (using the above buffer without sucrose, Buffer A) to an $A_{280\ nm}$ of about 50 and fractionated with $(NH_4)_2SO_4$. 92% of the activity was in the 20–50% cut, 8% in the 50–70% cut, and no activity in the 0–20% or 70–100% fractions. The 20–50% precipitate, representing 47% of the protein, was stored as a pellet at −20°.

When homogenate was layered over 30% sucrose (w/v in 0.01 M Tris, pH 7.3, 0.015 M $MgCl_2$, 0.02 M β-mercaptoethanol) after a clearing spin (4300 $\times g$, 10 min) and centrifuged at 100 000 $\times g$ for 1 h, activity was quantitatively recovered from the supernatant; none was found in the ribosomal fraction.

The sizes of rat liver and bacterial enzymes were compared by Sephadex G-200 chromatography. Equal volumes of the bacterial enzyme (42% $(NH_4)_2SO_4$ cut) and rat liver enzyme (20–50% cut) were applied to separate but identically prepared columns (40 cm × 2.5 cm) and run simultaneously. The fraction of the included volume occupied by the rat liver enzyme relative to the total included volume (V_P/V_i) was 0.656 (average of three determinations, ± 0.005) and for the bacterial enzyme, 0.453 (average of three determinations, ± 0.010). While both enzymes are well within the included volume, under these conditions pyrrolidonecarboxylyl peptidase from the rat liver behaved as if it had a significantly smaller molecular radius than the bacterial enzyme[6].

For purposes of purification of rat liver enzyme, the active fractions of the included volume were brought to 73% saturation with solid $(NH_4)_2SO_4$, centrifuged 30 min at 34 000 $\times g$ and the precipitates were stored at −20° (activity was maintained for at least 10 days). This procedure resulted in a 30-fold increase in specific activity.

Abbreviation: Pyr–, pyrrolidone carboxylyl–.

TABLE I

PURIFICATION OF PYRROLIDONECARBOYYLYL PEPTIDASE FROM RAT LIVER

Fraction	*Total protein ($A_{280\ nm}$ units)*	*Specific activity*[4]	*Total units of enzyme activity*[4]
34 000 × *g* supernatant of homogenate	3182	2.1	390
20–50% $(NH_4)_2SO_4$	1508	4.2	358
Sephadex G-200 enzyme	118	64.9	286

The Sephadex G-200 enzyme was sufficiently pure for the use in the experiments described in the remainder of this paper. There are no radical differences in the purification behavior of the rat liver and the bacterial enzyme, as the procedures outlined (Table I) are very similar to those employed in the preliminary fractionation of bacterial sonicates[3,4].

The two enzymes exhibit further similarity in that they both appear to contain sulfhydryl groups[3]. The rat liver activity is extremely sensitive to inactivation in the absence of a reducing agent. It is, in fact, more labile than the bacterial enzyme: 1-h dialysis (0°) against Buffer A without β-mercaptoethanol is sufficient to completely inactive the rat liver enzyme (when 0.01 M β-mercaptoethanol is present, activity is unaffected).

The rat liver activity is unstable when stored in dilute buffer solution at 0°. In the case of the bacterial enzyme, similar instability was prevented by addition of 2-pyrrolidone, a reversible, noncompetitive inhibitor, to the buffer solution[4]. The rat liver enzyme also is both stabilized as well as reversibly inhibited by 2-pyrrolidone (81% inhibition of activity with 0.1 M 2-pyrrolidone at K_m levels of Pyr–Ala). The effects of 2-pyrrolidone on the stability of the rat liver and bacterial enzymes are compared in Fig. 1.

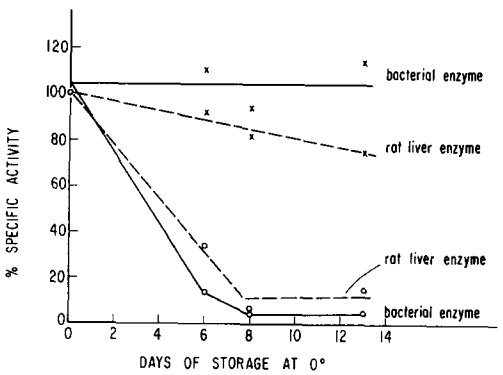

Fig. 1. Stabilizing effect of 2-pyrrolidone on solutions of rat liver and bacterial enzymes. Aliquots of rat liver and bacterial Sephadex G-200 enzymes, prepared simultaneously, were stored at 0° in Buffer A with (×) and without (○) 0.1 M 2-pyrrolidone. At intervals, samples were removed, dialyzed briefly against Buffer A (whether or not they contained 2-pyrrolidone) and assayed.

TABLE II

RELATIVE RATES OF HYDROLYSIS OF REPRESENTATIVE DIPEPTIDES BY PYRROLIDONECARBOXYLYL PEPTIDASE

Bacterial data from ULIANA AND DOOLITTLE[7]. K_m value for Pyr–Ala (rat liver enzyme) established by reciprocal plot. The same dipeptide solutions (stored frozen) and similar assay conditions were used to establish rates with the rat liver enzyme as were previously employed with the bacterial enzyme. The substrates were in excess and the rates obtained represented maximum initial velocities.

Substrate	*Relative rate of hydrolysis (%) by*	
	Rat liver enzyme	*Bacterial enzyme*
L-Pyr–L-Ala	100 ($K_m = 1.5 \cdot 10^{-3}$)	100 ($K_m = 2.0 \cdot 10^{-3}$)[4]
L-Pyr–L-Ile	35	50
L-Pyr–L-Phe	29	14

The relative rates of hydrolysis of nine pyrrolidonecarboxylyl dipeptides by the bacterial enzyme have been established[7]. These rates form three distinct groups represented by the order Pyr–Ala > Pyr–Ile > Pyr–Phe, each rate being about twice as great as the one below it. The rat liver enzyme hydrolyzes these three representative dipeptides with the same order of rates: Pyr–Ala > Pyr–Ile > Pyr–Phe, although

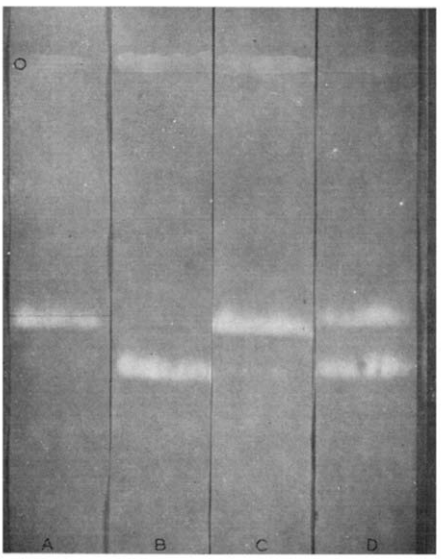

Fig. 2. Removal of pyrrolidonecarboxylyl residues from bovine fibrinopeptide B by rat liver and bacterial pyrrolidonecarboxylyl peptidase (as described by DOOLITTLE AND ARMENTROUT[3]). O, origin; A, rat liver enzyme and peptide, o-h digestion; B, rat liver enzyme and peptide, 3-h digestion; C, bacterial enzyme and peptide, o-h digestion; D, bacterial enzyme and peptide, 3-h digestion. 500 nmoles of bovine fibrinopeptide B (terminating in the sequence Pyr–Phe–) was divided into two equal portions (0.2 ml). To one aliquot was added rat liver Sephadex G-200 enzyme (1.0 unit) and to the other, bacterial Sephadex G-200 enzyme (2.3 units). At intervals 20-μl samples were removed and electrophoresed at 300 V, pH 2.0, 3 h. Strips were stained with arginine stain.

quantitative differences do exist. In contrast to the bacterial enzyme, the hydrolysis of Pyr–Ile and Pyr–Phe proceed at very similar rates (Table II).

The above results imply similarities between pyrrolidonecarboxylyl peptidase from the two sources which are confirmed in the most critical aspect, that of specificity. The rat liver enzyme is capable of specifically removing the amino-terminal Pyr residue from the bovine fibrinopeptide B. In Fig. 2, the results of digestion of this peptide with rat liver and bacterial Sephadex G-200 enzymes are compared. After 3 h of digestion with the rat liver enzyme, all detectable peptide has shifted to a single, more basic position. This band corresponds to the band produced by digestion with the bacterial enzyme, shown to be the peptide *minus* its pyrrolidonecarboxylyl terminus[3]. Even after 16-h digestion, no other band was detected. It is interesting to note that the rat liver enzyme removes the terminal pyrrolidonecarboxylyl residue from this long peptide much more rapidly than the bacterial enzyme despite the fact that less than half as many enzyme units (as determined by hydrolysis of Pyr–Ala) were present.

In summary, rat liver contains a pyrrolidonecarboxylyl peptidase which is generally similar to the bacterial enzyme with regard to purification, requirement for a reducing environment, stabilization and inhibition by 2-pyrrolidone, order of reaction rates on 3 dipeptides, and specificity. The rat liver enzyme very efficiently removes the pyrrolidonecarboxylyl residue from a natural peptide of 21 residues without detectable damage to the remainder of the molecule.

Although no definitive role has been established for this enzyme in either bacteria or rats, it might be expected that in soil bacteria, such as Pseudomonas and *B. subtilis*, it could serve a purely digestive purpose. Such a function for the rat liver activity would seem less likely. Generally speaking, the liver is not a particularly good source for digestive enzymes, (compared with the pancreas, for example). It is, on the other hand, an organ very much involved in active protein synthesis. In this regard, the role of the rat liver enzyme is of considerable interest, especially in light of recent work implying a possible role for pyrrolidonecarboxylic acid in the initiation of protein synthesis in mammalian cells, including rat liver[8].

This work was done in the laboratory of Dr. R. F. Doolittle, whom I thank for help.

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Received August 11th, 1969

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