Substrate Specificity of a Highly Active Dipeptidase Purified from Monkey Small Intestine

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Mammalian small-intestinal mucosa is known to contain several di- and tri-peptidases (Smith & Bergman, 1944; Josefsson & Lindberg, 1965; Heizer & Laster, 1969; Peters, 1970). A series of dipeptidases have been reported on the basis of observations with crude extracts (Dolly *et al.*, 1971). Dipeptidases from hog kidney (Campbell *et al.*, 1966) and ascites-tumour cells (Hayman & Patterson, 1971) have been purified and their properties studied. Recently the purification of a pig intestinal dipeptidase (Noren *et al.*, 1971) has been reported, though the properties were not given. The present paper reports the substrate specificity of a highly active dipeptidase purified from monkey small-intestinal extracts, Gly-L-Leu being used as the test substrate during the purification.

With the crude extracts, Gly-L-Leu gave higher rates than many other dipeptides tested as substrate. The bulk of the enzyme activity (over 95%) was present in the soluble supernatant fraction (100000g) of the homogenate. The enzyme was assayed by a modification of the spectrophotometric method of Josefsson & Lindberg (1965). The standard reaction mixture (0.05 ml) contained the substrate (0.6 μ mol), sodium phosphate buffer, pH 7.7 (10 μ mol), ZnSO₄ (5 nmol) and the enzyme (about 0.03 unit). After incubation at 37°C for 5 min, the reaction was stopped by the addition of 1.95 ml of spectrophotometric-grade ethanol. The precipitate was removed by centrifugation, and the decrease in absorbance of the supernatant fluid at 210 nm was measured in a spectrophotometer (Carl Zeiss model PMQII) and compared with that of a control tube containing standard reaction mixture without the enzyme. One unit of enzyme activity was defined as the amount required to catalyse the hydrolysis of 1 μ mol of Gly-L-Leu/min at 37°C. Protein was determined by the method of Lowry *et al.* (1951). Polyacrylamide-gel electrophoresis was carried out by the method of Davis (1964) with a Buchler instrument, with 0.125M-sodium borate buffer, pH 8.1.

All operations during the purification of the enzyme were carried out at 0–4°C; dithiothreitol (0.1 mM) and ZnSO₄ (0.1 mM) were present throughout the fractionation procedure. A 20% (w/v) homogenate of the monkey small-intestinal mucosa in 0.1M-NaCl was centrifuged at 10000g for 30 min. The supernatant fluid (fraction 1) was subjected to fractionation with (NH₄)₂SO₄ and the fraction that

was precipitated at 55–75% saturation was collected. The precipitate was dissolved in 0.04M-sodium phosphate buffer, pH 6.5, dialysed against the same buffer for 6 h (fraction 2) and then subjected to fractional adsorption on alumina C_γ gel (prepared as described by Willstätter & Kraut, 1923) in six steps by the general procedure of Ochoa *et al.* (1948) with a total gel/protein ratio of 1:1 (w/w). Each of the gel fractions was eluted individually with 0.2M-potassium phosphate buffer, pH 8. The buffer eluates with the highest enzyme specific activity were pooled, dialysed against 0.04M-sodium phosphate buffer, pH 6.5, for 6 h (fraction 3) and subjected to a second fractional adsorption (six steps) on calcium phosphate gel (prepared as described by Keilin & Hartree, 1938) with a total gel/protein ratio of 2:1 (w/w). The buffer eluates with the highest specific activity were pooled, freeze-dried and then dialysed against 0.05M-potassium phosphate buffer, pH 6.5, for 6 h (fraction 4). Fraction 4 was applied to a DEAE-Sephadex column (1.4 cm × 16 cm; bed volume 25 ml) equilibrated with 0.05M-potassium phosphate buffer, pH 6.5, and washed with 5 bed volumes of the buffer. The enzyme was then eluted with 0.1M-potassium phosphate buffer, pH 6.5, and the fractions with high specific activity were pooled, concentrated by freeze-drying and dialysed against 0.02M-sodium phosphate buffer, pH 6.8, for 6 h (fraction 5). Fraction 5 was then applied to a column (1 cm × 12.5 cm; bed volume 10 ml) of cellulose-hydroxyapatite (1:2, w/w) (hydroxyapatite was prepared as described by Tiselius *et al.*, 1956) equilibrated with 0.02M-sodium phosphate buffer, pH 6.8. The column was eluted with increasing stepwise concentrations of sodium phosphate buffer, pH 6.8. The enzyme was eluted as a single peak at 0.15M-phosphate concentration. The final preparation gave a specific activity of 4106 μ mol of Gly-L-Leu hydrolysed/min per mg of protein. A summary of the purification is given in Table 1. In the purification procedure employed fractions 2, 3, 4 and 5 showed some variation in specific activity, but the final preparation (fraction 6), however, showed a remarkable constancy in this respect. The purified enzyme was extremely unstable even in the presence of dithiothreitol, and was stabilized to only a limited extent in the presence of 50%-saturated (NH₄)₂SO₄ or 12.5% (v/v) glycerol.

The purified enzyme gave a pH optimum of 7.75 for Gly-L-Leu and for two other closely related

Table 1. *Purification of Gly-L-Leu hydrolase from monkey small-intestinal mucosal extracts*

Experimental details are given in the text.

Fraction no.	Step	Volume (ml)	Protein (mg/ml)	Total enzyme units	Specific activity (units/mg of protein)
1	Crude extract	800	10	176000	22
2	(NH ₄) ₂ SO ₄ (55–75% satn.)	246	10	128000	52
3	Alumina C _γ gel	241	3.66	105000	118
4	Calcium phosphate gel	94	3.4	50760	160
5	DEAE-Sephadex	8.6	3.47	47470	1300
6	Hydroxyapatite	8	0.5	16430	4106

substrates, namely Gly-L-Val and Gly-L-Ile. The K_m value for Gly-L-Leu was 10.6mM, which was lower than those determined for Gly-L-Val (20mM) and Gly-L-Ile (21mM). The enzyme was completely inhibited by EDTA (5mM) and by *p*-hydroxy-mercuribenzoate (0.1mM). Although dithiothreitol protected the enzyme during purification, at higher concentrations (above 1mM) it caused inhibition, which was complete at 10mM. The final enzyme preparation was activated by about 35% by the addition of Zn²⁺ (0.1mM), but not by the other bivalent metal ions Mn²⁺, Mg²⁺ and Co²⁺, which indeed inhibited the enzyme slightly.

The substrate specificity of the enzyme was studied by using a large excess of enzyme and a long incubation period, and the results are given in Table 2. It is noteworthy that the enzyme is free from Gly-Gly, Gly-L-Pro and L-Pro-Gly hydrolase activities, which were present in the crude extract. Partial hydrolysis of Gly-L-Trp and L-Trp-L-Leu was observed (not shown in Table 2), probably because of their very low solubility in water. It would appear that both the -NH₂ and the -CO₂H groups of Gly-L-Leu are essential for activity, since Z-Gly-L-Leu and Gly-L-Leu-NH₂ were not hydrolysed. Also, the enzyme appears to be a true dipeptidase, since extension of the peptide chain of Gly-L-Leu even by a single residue, with glycine, the smallest of the amino acids, resulted in inactive substrates. By disc electrophoresis the purified enzyme gave a major protein band corresponding to enzyme activity and traces of three other protein bands. The substrate specificity obtained with the enzyme band isolated by disc electrophoresis was found to be the same as that with the purified enzyme (fraction 6), although the hydrolysis was not complete because of the low amounts of the enzyme used.

With the standard assay procedure, i.e. with 12mM substrate concentration, the relative rates of hydrolysis, with that of Gly-L-Leu taken as 100%, are: Gly-L-Val (76%), Gly-L-Ile (70%), L-Ala-L-Leu

Table 2. *Substrate specificity of purified monkey small-intestinal Gly-L-Leu dipeptidase*

Standard incubation mixtures were employed, except for a large excess of the enzyme (3.6 units) and for the longer incubation period (30min). After incubation 0.02ml portions were applied (5μl spot size) to a Whatman no. 1 paper and the chromatograms developed with butan-1-ol-acetic acid-water (4:1:1, by vol.) as solvent. The peptides and the constituent amino acids, detected by ninhydrin, gave excellent and unambiguous separation in each case. Abbreviation: Z, *N*-benzyloxycarbonyl.

Complete hydrolysis	No detectable hydrolysis
Gly-L-Leu	Gly-Gly
Gly-L-Val	Gly-D-Leu
Gly-L-Ile	Gly-L-Pro
Gly-L-Phe	L-Pro-Gly
L-Ala-L-Leu	Gly-L-His
L-Leu-L-Leu	Z-Gly-L-Leu
L-Phe-L-Leu	Gly-L-Leu-NH ₂
L-Leu-Gly	Gly-Gly-L-Leu
	Gly-L-Leu-Gly

(25%) and L-Leu-Gly (18%). High rates of hydrolysis were observed with Gly-L-Leu and with peptides in which leucine was replaced by the related branched-chain amino acids valine and isoleucine, but the rates decreased sharply when the glycine residue in Gly-L-Leu was replaced by L-alanine or L-leucine. The poor solubility of L-Leu-L-Leu and the interference of Gly-L-Phe and L-Phe-L-Leu with the spectrophotometric assay procedure prevented a study of these susceptible peptides. It should be mentioned that these assays were carried out at lower-than-optimal concentrations because of the limitations in the available assay procedures, and

require verification when better assay procedures become available.

A comparison of the substrate specificity of the intestinal dipeptidase with those of two other purified dipeptidases shows that the intestinal dipeptidase is more similar to the soluble ascites-tumour dipeptidase (Hayman & Patterson, 1971) than to the kidney particulate dipeptidase (Campbell *et al.*, 1966), especially in the observed high specific activity with Gly-L-Leu and lack of activity with Gly-D-Leu. Gly-Gly was not hydrolysed at all by the purified intestinal enzyme, although it was the best substrate for the kidney enzyme and was hydrolysed to a small extent by the purified tumour enzyme. The crystalline kidney enzyme also hydrolysed Gly-D-Leu.

Further studies on the properties of the dipeptidase were hampered by the extreme lability of the purified enzyme and by the limitations of the spectrophotometric assay procedure. The specific activity of the purified intestinal dipeptidase (4106 units/mg of protein) is higher than that of most purified or crystalline soluble enzymes. An estimate of 107000 was obtained for the molecular weight of the enzyme by employing the procedure of Andrews (1965) with a column of Sephadex G-200 calibrated with marker proteins. On the basis of this result the molecular activity was in the range 10^5 – 10^6 mol of Gly-L-Leu hydrolysed/min per mol of enzyme. Gly-L-Leu dipeptidase, as indicated above, is present mainly in the soluble fraction of intestinal mucosa, unlike the disaccharidases, which are present in the brush border (Eichholz & Crane, 1965). The mechanisms of absorption and transport of dipeptides and disaccharides are likely to be different in view of this

obvious difference in the location of the key hydrolases.

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