Acta Chemica Scandinavica B 28 (1974) 711—716

Studies on a Soluble Dipeptidase from Pig Intestinal Mucosa. Enzymatic Properties

OVE NORÉN

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

The kinetics of a soluble dipeptidase (glycyl-L-leucine dipeptidase, EC 3.4.13.2) purified from pig intestinal mucosa were studied under stable assay conditions. The pH-optima for the reaction of L-alanyl-L-alanine and glycyl-L-leucine with the enzyme were settled to 8.4 and 8.0, respectively. In the reaction with these two substrates the enzyme followed Michaelis-Menten kinetics and the K_m-values found were 0.74 mM and 2.1 mM for L-alanyl-L-alanine and glycyl-L-leucine. Several L-amino acids inhibited the enzyme and a kinetic study on the L-leucine inhibition showed it to be competitive in nature. The soluble dipeptidase was also inhibited by SH-compounds, EDTA, and SH-reagents while glycerol and divalent metal ions only slightly influenced the activity of the enzyme. DFP had no inhibitory effect.

Only a few intestinal exopeptidases have hereto been purified.[1-4] We have in a preceding report described a preparative purification procedure of a soluble dipeptidase from pig intestinal mucosa (glycyl-L-leucine dipeptidase, EC 3.4.13.2) together with its specificity.[5] Earlier studied proteolytic enzymes of the gastrointestinal tract followed the Michaelis-Menten kinectics and showed a distinct dependence of common enzyme inhibitors.[6] The objective of this investigation was to put the purified soluble dipeptidase in relation to these well-characterized proteolytic enzymes by studying these parameters.

MATERIALS AND METHODS

Enzyme. The soluble dipeptidase was prepared as described earlier.[5] The enzyme had a specific activity of 1400 units of activity per mg protein. In order to avoid repeated freezing and thawing the enzyme was stored in small samples at −20 °C in 0.07 M sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl, 4 mM 2-mercaptoethanol, and 12.5 % (w/v) glycerol. The concentration of the enzyme was about 600 units of activity per ml. Under these conditions the enzyme was found to be stable for several months. One of the stored samples was used for each experiments.

Chemicals. Glycyl-L-leucine was purchased from Sigma Chem. Co., St. Louis, U.S.A., and L-alanyl-L-alanine was obtained from Cyclo Chem. Corp., Los Angeles, U.S.A. The purity of the two dipeptides was tested by thin layer chromatography (precoated cellulose plates, 0.1 mm, Merck, Darmstadt, Germany; butanol-acetic acid-water, 4:1:1 by vol.; ninhydrin-visualization). Amino acids were obtained from Mann Res. Labs., New York, U.S.A. DFP and sodium *p*-hydroxymercuribenzoate (PHMB) were purchased from Sigma Chem. Co., St. Louis, U.S.A. All other chemicals used were of analytical grade and de-ionized and glass destilled water was used throughout.

Assays. Dipeptidase activity was assayed according to one of the following procedures. *Fixed time procedure* followed in general the assay method of Josefsson and Lindberg [7] with the modifications of Sjöström.[8]

The dipeptides and amino acid solutions were prepared in 0.05 M Tris-HCl buffer solution at proper pH (pH-meter 28, Radiometer, Copenhagen, Denmark) in their suitable concentrations (12.3 mM and 8.5 mM for glycyl-L-leucine and L-alanyl-L-alanine, respectively, unless otherwise stated). The incubation mixtures, containing an enzyme concentration of about 0.2 units of activity per ml, were incubated at 25 °C for 10 min unless otherwise stated. In experiments where compounds with disturbing UV-absorbance were present the dipeptidase activity was assayed by using the 2,4,6-trinitrobenzenesulfonic acid (TNBS-) reagent as described earlier.[5] *Continuously recording procedure*, as described by Sjöström [8] was used for the determination of the kinetic coefficients of the enzyme. The molar extinction

coefficient of the peptide bond of the substrates was determined at each wavelength used.

Unit of dipeptidase activity. One unit of soluble dipeptidase activity is defined as the amount of enzyme hydrolyzing 1 μmol glycyl-L-leucine (12.3 mM) per min in 0.05 M Tris-HCl buffer (pH 8.0) at 25 °C.

RESULTS AND DISCUSSION

Stability of the enzyme. Stability studies were performed, using glycyl-L-leucine as substrate and the fixed time procedure, to assure stable assay conditions for the soluble dipeptidase. Solutions of varying enzyme concentrations (2 – 20 units of activity per ml) were prepared in two different buffers (0.05 M Tris-HCl, pH 8.0, and 0.1 M sodium phosphate, pH 7.8) and stored at 0 °C. Samples were withdrawn at once after the preparation of the solutions and then for each 30 min interval during a 2 h period and assayed for the dipeptidase activity. No decrease of enzyme activity was observed. Likewise, an enzyme solution (0.5 units of activity per ml) in 0.05 M Tris-HCl buffer (pH 8.0) stored at 25 °C and assayed for its activity at 6 min interval during a 36 min period showed no loss of activity. The stability of the soluble dipeptidase was also studied at different pH-values (0.05 M Tris-HCl buffer, pH 7.0, 8.0, and 9.0). At each pH a series of incubation mixtures with varying enzyme concentrations (0.82 – 0.13 units of activity per ml) were prepared and assayed for their activity, using incubation times for the separate mixtures inversely proportional to their enzyme concentrations (5 – 30 min). At each pH the various solutions showed the same percentage of glycyl-L-leucine hydrolysis, indicating no loss of activity during the time of incubation.

pH-Optima of the enzyme. The influence of pH on the reaction rate was studied in the pH range 6.9 – 8.9 (0.05 M Tris-HCl buffer) with glycyl-L-leucine and L-alanyl-L-alanine as substrate and using the fixed time procedure. pH-Measurements of the different reaction mixtures at the start and at the end of the incubation period made in parallel experiments showed a change of less than 0.03 pH-units. The pH-activity curves for the enzyme are presented in Fig. 1. The pH-optimum, 8.0, for the dipeptidase reaction with glycyl-L-leucine is in good accordance with the values earlier reported for pig intestinal

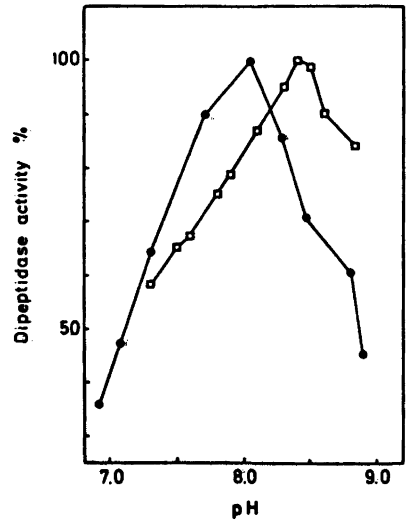

Fig. 1. pH-Activity curves for the soluble dipeptidase. Substrate: □ L-Alanyl-L-alanine; ● glycyl-L-leucine.

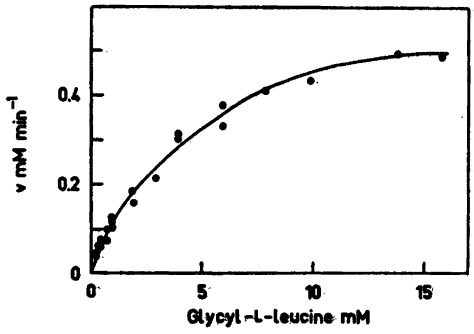

Fig. 2. Dipeptidase activity as a function of glycyl-L-leucine concentration.

mucosa homogenate[7] and for the purified monkey intestinal dipeptidase.[8] The pH-optimum for L-alanyl-L-alanine was found to be 8.4.

Kinetics of the enzyme. Experiments using the fixed time procedure were performed to determine the type of kinetic behaviour of the enzyme. The rate of hydrolysis of glycyl-L-leucine was measured in 0.05 M Tris-HCl buffer (pH 8.0) at different substrate concentrations (0.25 mM to 16 mM). The reactions were not allowed to proceed to more than 15 % of hydrolysis. The velocities calculated from these experiments were plotted *versus* the corresponding

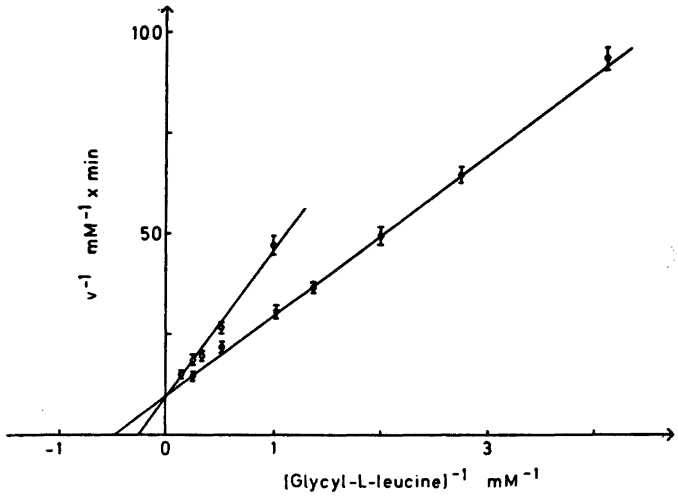

Fig. 3. Experimental data for the determination of the kinetic coefficients for the soluble dipeptidase without (●) and with (○) L-leucine (4 mM) using glycyl-L-leucine as substrate. ⊥ indicates 2 S. D. of the velocity measurements.

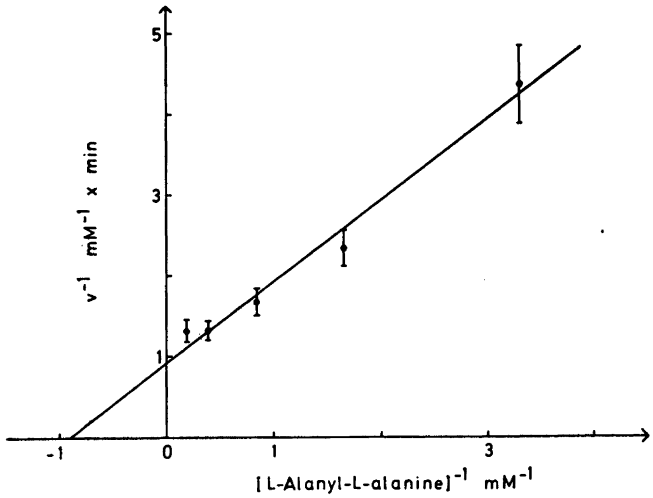

Fig. 4. Experimental data for the determination of the kinetic coefficients for the soluble dipeptidase using L-alanyl-L-alanine as substrate. A vertical bar indicates 2 S.D. of the velocity measurements.

substrate concentrations (Fig. 2). They were found to be located along a hyperbola and even at low substrate concentrations no sigmoid shape was observed. At high substrate concentrations the curve approached asymptotically a maximum value of velocity and substrate inhibition was not observed within the concentration range studied.

The determination of K_m- and V-values for the soluble dipeptidase were performed using the continuously recording procedure. To get a rapid and objective method for the calculation of the kinetic coefficients, with their standard errors, the statistical estimation method of Wilkinson [9] was used. The calculation procedure was translated into Univac-Algol and the ex-

perimental data were processed in a computer (Univac 1106, Recku, Copenhagen, Denmark). The experiments were performed in 0.05 M Tris-HCl buffer at pH 8.0 with glycyl-L-leucine and at pH 8.4 with L-alanyl-L-alanine, varying the peptide concentrations between 0.24 – 3.87 mM and 0.30 – 4.84 mM, respectively. The results obtained are presented in Fig. 3 and 4. Six different velocity measurements were made at each substrate concentration in the experiments with glycyl-L-leucine and the calculations resulted in a K_m of 2.1 (S.E. 0.13) mM and a V of 0.11 (S.E. 0.0034) mM per min. In the experiments with L-alanyl-L-alanine five separate velocity measurements were made at each substrate concentration and the calculations gave a K_m of 0.74 (S. E. 0.10) mM and a V of 0.94 (S. E. 0.039) mM per min.

Previous studies on soluble intestinal dipeptidase activities [10,11] have shown kinetic in accordance with the theory of Michaelis and Menten. This finding is also valid for studies performed on membrane bound intestinal peptidase activities using L-phenylalanyl-glycine and glycyl-L-phenylalanine as substrates [11] and for the renal dipeptidase.[12] K_m values have also been reported for the purified soluble monkey intestinal dipeptidase [8] without details on its kinetic behaviour. In the present study it has been found that for the two substrates studied the Michaelis-Menten kinetics may be applied. The found K_m values are of the same magnitude as those reported for the dipeptides with the enzymes mentioned above.

Using these kinetic data the molecular activity can be calculated to 2.4×10^5 min^{-1} and 6.4×10^5 min^{-1} for glycyl-L-leucine and L-alanyl-L-alanine, respectively, by using a molar extinction coefficient of 14.6×10^4 M^{-1} cm^{-1} for the soluble dipeptidase.[13]

Inhibitors of the enzyme

Amino acids. In the study of possible product inhibition of the dipeptidase reaction with glycyl-L-leucine it was observed, that L-leucine inhibited the reaction markedly, while glycine was only slightly inhibitory. Keeping the wide specificity of the soluble dipeptidase in mind [5] these observations prompted a more general study on the inhibitory effect of the amino acids.

Table 1. Influence of amino acids on the activity of the soluble dipeptidase.

Amino acid (L-forms) added (10 mM)	Dipeptidase activity (%)	Assay method[a]
–	100	A
Ala	95	A
Arg	80	A
Asp	95	A
Glu	99	A
Gly	96	A
His	37	B
Ile	59	A
Leu	55	A
Lys	94	A
Met	75	A
Phe	97	B
Pro	99	A
Ser	80	A
Thr	91	A
Trp	58	B
Val	73	A

[a] A. Spectrophotometric method.[7] B. TNBS-method.[5]

Each amino acid (Table 1) was mixed separately with glycyl-L-leucine and incubated with a solution of the dipeptidase. The activity was measured using the fixed time procedure. The results (Table 1) showed that L-histidine inhibited the reaction most strongly but also L-isoleucine, L-leucine, and L-tryptophan showed a marked inhibitory effect. L-Arginine, L-methionine, L-serine, and L-valine moderately slowed down the reaction rate, while the other amino acids tested had no or only a slight inhibitory effect on the dipeptidase reaction. This variation in the inhibitory effect of the amino acids, is, however, not parallel to our knowledge about the specificity of the dipeptidase,[5] and requires therefore a more detailed study to elucidate the inhibitory mechanism of the amino acids.

The type of amino acid inhibition was studied using L-leucine as inhibitor. L-Leucine (4 mM) was added separately to a series of glycyl-L-leucine solutions (0.99 – 5.94 mM). The reaction velocity of a dipeptidase solution was then assayed with the various substrate concentrations using the continuously recording procedure (0.05 M Tris-HCl buffer, pH 8.0). Five different velocity measurements were made at each substrate concentration. The experimental data

are presented in the double reciprocal form in Fig. 3. The apparent K_m and V were calculated to be 3.8 (S.E. 0.35) mM and 0.11 (S. E. 0.0054) mM per min, respectively, using the statistical estimation method described above. The K_m-values obtained for glycyl-L-leucine in the absence and presence of L-leucine were compared using the t-test [14] and were found to be significantly different (degrees of freedom 63, P = 99.9. As the V-values obtained showed no difference L-leucine can be regarded as a competitive inhibitor. From the determined kinetic coefficients K_i for L-leucine was calculated to be 5.0 mM.

Glycyl-D-leucine. Glycyl-D-leucine, not hydrolyzed by the enzyme,[5] was mixed with the L-form of the dipeptide in equal concentrations (12.3 mM) and incubated with an enzyme solution, using the fixed time procedure (0.1 M sodium phosphate buffer, pH 7.8). No change of the rate of hydrolysis was observed in this mixture when compared with a parallel incubation mixture containing the same amount of enzyme but glycyl-L-leucine only. Thus glycyl-D-leucine does not interfere with the substrate binding site on the enzyme.

SH-compounds. To ascertain stability of the dipeptidase during its purification, all solutions had to contain 2-mercaptoethanol (4 mM),[5] although this compound strongly inhibited the dipeptidase activity (Table 2). Other SH-compounds, when present in the same concentration, also inhibited the dipeptidase reaction with glycyl-L-leucine (Table 2). As a consequence of its presence in the enzyme stock solutions, the influence of 2-mercaptoethanol on the dipeptidase reaction was further investigated in concentrations valid for the assay mixtures. An enzyme stock solution was passed through a column of Sephadex G-25 Fine (0.07 M sodium phosphate buffer, pH 7.0, added 0.2 M NaCl) to remove the 2-mercaptoethanol and glycerol present. This enzyme solution was assayed according to the fixed time procedure against two series of glycyl-L-leucine solutions (0.25 mM and 12.3 mM), each series added 2-mercaptoethanol in concentration varying from 0 to 12 μM. No influence on the dipeptidase reaction was observed in the series with 12.3 mM substrate concentration, while a 2-mercaptoethanol concentration above 6 μM showed a slight inhibition of the activity in the series with 0.25 mM substrate concentration. To avoid inhibition in the kinetic experiments (see above), precautions were therefore taken not to allow the 2-mercaptoethanol concentration in the incubation mixtures to rise above 6 μM.

EDTA. The influence of EDTA (1 mM) on the dipeptidase reaction was measured using the fixed time procedure with glycyl-L-leucine as a substrate. EDTA was added to the substrate-buffer solution and no preincubation of the dipeptidase was undertaken. The inhibition observed (Table 3), suggests that the enzyme is dependent of a metal ion for its proper function.

SH-reagents. The influence of Hg^{2+} and PHMB on the dipeptidase reaction was studied. The reagents were added to the substrate-buffer solution and the dipeptidase activity was measured using the fixed time procedure with glycyl-L-leucine as substrate. In the concentrations used both reagents had strong inhibitory effect on the

Table 2. Influence of SH-compounds on the activity of the soluble dipeptidase. Assay conditions: Fixed time procedure (0.1 M sodium phosphate buffer, pH 7.8).

SH-compound added (4 mM)	Dipeptidase activity (%)
–	100
2-Mercaptoethanol	10
L-Cysteine	10
Thioglycolic acid	40
Cysteamine	30

Table 3. Influence of miscellaneous compounds on the activity of the soluble dipeptidase.

Compound added	Concentration mM	Dipeptidase activity (%)	Assay method[a]
–	–	100	A
EDTA	1.0	40	A
DFP	0.15	100	A
PCMB	0.1	44	B
Hg^{2+}	0.001	55	B
Co^{2+}	0.005	83	A
Mn^{2+}	0.005	84	A
Mg^{2+}	0.005	95	A
Zn^{2+}	0.005	90	A
Glycerol	50	95	A

[a] A. Spectrophotometric method.[7] B. TNBS-method.[5]

dipeptidase reaction (Table 3), suggesting that thiol group(s) of the soluble dipeptidase are necessary for its function.

Glycerol. During the purification procedure glycerol was used to achieve stable conditions for the dipeptidase.[5] Its influence on the dipeptidase reaction was therefore studied using the fixed time procedure with glycyl-L-leucine as substrate and with glycerol present in the substrate-buffer solution. In the concentration used only a slight inhibitory effect was recognized (Table 3).

DFP. The inhibitory effect of DFP was studied by preincubating a dipeptidase solution (0.8 units of activity per ml) for 20 min at 25 °C in an 0.1 M sodium phosphate buffer (pH 7.7), made 0.15 mM in respect to DFP. Parallel samples, pre-incubated as above, but without DFP added, served as a control. The dipeptidase activity was measured against glycyl-L-leucine using the fixed time procedure. No effect on the dipeptidase activity was observed under these conditions (Table 3). Chymotrypsin, included in the study and assayed against casein, showed a total inactivation under the same experimental conditions.[15]

Divalent metal ions. Co^{2+}, Mn^{2+}, Mg^{2+}, and Zn^{2+} were added directly to the glycyl-L-leucine solution. The activity of the soluble dipeptidase measured using the fixed time procedure, was only slightly influenced (Table 3), which is in accordance with observations earlier reported on the glycyl-L-leucine dipeptidase activity in crude extracts of pig intestinal mucosa.[7]

Acknowledgement. For valuable discussions during the preparation of this work the author is grateful to Professor Lars Josefsson. Thanks are also directed to Mrs. D. Anthonsen who has performed skilful technical assistance. The investigation was aided by grants from the Danish Natural Science Research Council (Project No. 511).

REFERENCES

1. Sjöström, H., Norén, O. and Josefsson, L. *Acta Chem. Scand. 25* (1971) 1911.
2. Norén, O., Sjöström, H. and Josefsson, L. *Acta Chem. Scand. 25* (1971) 1913.
3. Das, M. and Radhakrischnan, A. N. *Biochem. J. 128* (1972) 463.
4. Maroux, S., Louvard, D. and Baratti, J. *Biochim. Biophys. Acta 321* (1973) 282.
5. Norén, O., Sjöström, H. and Josefsson, L. *Biochim. Biophys. Acta 327* (1973) 446.
6. Boyer, P. D. *Enzymes 3* (1971) 1.
7. Josefsson, L. and Lindberg, T. *Biochim. Biophys. Acta 105* (1965) 149.
8. Sjöström, H. *Acta Chem. Scand. B 28* (1974) 802.
9. Wilkinson, G. N. *Biochem. J. 80* (1961) 324.
10. Heizer, W. D. and Laster, L. *Biochim. Biophys. Acta 185* (1969) 409.
11. Heizer, W. D., Kerley, R. L. and Isselbacher, K. J. *Biochim. Biophys. Acta 264* (1972) 450.
12. René, A. M. and Campbell, B. J. *J. Biol. Chem. 244* (1969) 1445.
13. Norén, O. and Sjöström, H. *Acta Chem. Scand. B 28* (1974) 787.
14. Cleland, W. W. *Advan. Enzymol. 29* (1967) 1.
15. Jansen, E. F., Fellows Nutting, M.-D., Jang, R. and Balls, A. K. *J. Biol. Chem. 179* (1949) 189.

Received March 2, 1974.