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Dipeptidase Activity in Rat Carrageenin Granuloma

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Dipeptidase activity was determined in rat carrageenin granuloma as a chronic inflammation model. The specific activity of dipeptidase in the granulation tissue was about 230 and 40 times higher than those in the exudate and in the serum, respectively. Most part of this activity was distributed in the soluble fraction. The dipeptidase partially purified was most active in the pH range 8.1 to 9.3 and preferentially hydrolyzed dipeptides, such as Gly-L-Leu, L-Val-L-Leu, L-Leu-L-Ala, L-Val-L-Phe and L-Leu-L-Leu. This enzyme, however, did not split dipeptides containing C- or N-terminal L-proline, tripeptides, L-leucineamide, L-leucine-*p*-nitroanilide and hippuryl-L-Phe. After injection of carrageenin, the dipeptidase activity in the granulation tissue increased gradually and reached a maximum level on the 15th day.

Keywords——dipeptidase; aminopeptidase; carboxypeptidase; carrageenin granuloma; inflammation model

Dipeptidase (EC 3.4.13.-) which perferentially hydrolyzes dipeptides, digestion products from proteins by proteinases, was found in rat liver,[2–5] intestine,[6–8] guinea pig intestine,[9–11] mouse ascites tumor,[12] human kidney.[13] In granulation tissues, however, the presence of dipeptidase activity has not been confirmed. In this study, the distribution and partial characterization of dipeptidase in the rat carrageenin granuloma as an experimental model of chronic inflammation are described.

Materials and Methods

Materials——Chemicals and substrates were commercially purchased: DE-52 cellulose from Whatman, England; L-amino acid oxidase from *Crotalus adamanteus* (Type I), horseradish peroxidase (Type I), L-leucineamide, L-leucine-*p*-nitroanilide, hippuryl-L-Phe, dipeptides and tripeptides from Sigma, USA. Other reagents used were of analytical reagent grade.

Induction of Carrageenin Granuloma——The carrageenin granuloma pouch was induced as described previously[14] by using male rats of Donryu strain. The granulation tissue and the exudate formed were removed immediately after sacrifice and stored frozen at −20° until use. This granulation tissue was homogenized in 50 mM Tris-HCl buffer (pH 8.1) in a VirTis 45 homogenizer at a full speed for 1 min.

Enzyme Assay——Dipeptidase activity was estimated by the one step method of Sugiura *et al.*[13] with a minor modification; a commercial L-amino acid oxidase was used instead of an enzyme from *Agkistrodon caliginousus*. A mixture containing 0.1 ml of dipeptidase solution, 1 ml of 6 mM L-Leu-L-Leu in 50 mM

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Tris-HCl buffer (pH 8.1), 20 μl of L-amino acid oxidase solution (0.3 unit) and 2 ml of the color reagent was incubated at 37° for 20 min. The reaction was terminated by the addition of 1 ml of 0.1 N acetic acid and the absorption of the mixture at 550 nm was measured. The color reagent was composed of 8 mg of *p*-aminoantipyrine, 20 μl of N,N′-dimethylaniline and 300 units of the peroxidase in a total volume of 100 ml of 50 mM Tris-HCl buffer (pH 8.1). One unit of the enzyme activity is defined as 1 μmol of substrate hydrolyzed per min under the above conditions. Carboxypeptidase was assayed by the method of Bergmeyer *et al.*[15] using hippuryl-L-Phe, and amino peptidase was also determined according to the method of Appel *et al.*[16] by using L-leucineamide and L-leucine-*p*-nitroanilide.

Protein Determination——Protein was determined by the method of Lowry *et al.*[17] by using bovine serum albumine as a standard.

Results

Distribution of Dipeptidase Activity in Rat Carrageenin Granuloma

Carrageenin granuloma is composed of the exudate and capsule of the granulation tissue. Table I shows the distribution of dipeptidase activity in the tissues investigated by using

Table I. Distribution of Dipeptidase Activity in Rat Tissues

Tissue		Dipeptidase activity (mU/mg protein)
Granulation tissue	I*	287
	II*	357
Exudate	I*	1.3
	II**	1.5
Serum	I*	7.0
	II**	7.3

The granulation tissue was homogenized in buffered 0.25 M sucrose and centrifuged at 12000×*g* for 20 min, then the supernatant was used as an enzyme preparation. The dipeptidase activity was determined by the method described in the text. The substrate was L-Leu-L-Leu. I* and II**; on the 4th and 8th day after carrageenin injection, respectively.

Table II. Intracellular Distribution of Dipeptidase in Rat Carrageenin Granuloma

Granulation tissue	Dipeptidase activity	
	mU/mg protein	mU/ml supernatant
I: 4th day after carrageenin injection		
700×*g* supernatant	248	1.46
5000×*g* supernatant	287	1.37
100000×*g* supernatant	327	1.42
II: 8th day after carrageenin injection		
700×*g* supernatant	430	2.11
5000×*g* supernatant	459	2.06
100000×*g* supernatant	587	2.07

The granulation tissue was homogenized in buffered 0.25 M sucrose and 5% (w/v) of the homogenate was finally prepared. The homogenate was centrifuged at 700×*g* for 15 min, then at 5000×*g* for 15 min and finally 100000×*g* for 60 min and the residual activity in each supernatant was determined by using L-Leu-L-Leu as a substrate.

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L-Leu-L-Leu as a substrate. The highest dipeptidase activity was found in the granulation tissue and the specific activities in the granulation tissues were about 230 and 40 times higher than those in the exudate and in the serum, respectively. Therefore, the granulation tissue was used as an enzyme source for the subsequent investigations. The subcellular distribution of dipeptidase activity was examined. The homogenate of granulation tissue in 0.25 M sucrose was centrifuged in Hitachi ultracentrifugator 65P. As shown in Table II, similar results were observed in two granulation tissues examined; the residual activity per ml of each supernatant of fraction was about the same value. Therefore, the effect by treatment with Triton X-100 or with sonication on the enzyme distribution in the homogenate of granulation tissue was examined. The homogenate (5%, w/v) was treated with final 1% (w/v) of Triton X-100 for 30 min or with sonication at 20 KHz for 15 min. Neither of them affects the distribution of the dipeptidase. On the other hand, N-acetyl-β-glucosaminidase, a lysosomal enzyme, was easily solubilized. These results suggest most part of dipeptidase activity was originally present in the cytosol fraction.

Partial Purification of Dipeptidase

The granulation tissue on the 8th day after carrageenin injection was homogenized in 50 mM Tris-HCl buffer (pH 7.2) containing 20% (w/v) glycerol, and finally 30% (w/v) homogenate was prepared. The supernatant obtained by the centrifugation at 12000×g for 15 min was fractionated with $(NH_4)_2SO_4$ (30—80% saturation). The precipitate collected was dialyzed against the same buffer and adsorbed on a DE-52 cellulose column. Dipeptidase activity was eluted with a linear gradient system of NaCl. Figure. 1 shows that the fractions with dipeptidase activity were eluted between 0.06 to 0.13 M NaCl, and these fractions were collected together. The increase in the specific activity of the dipeptidase (about 3-times) and the total recovery of its activity (13%) were very low because its lability; after storing the effluent or the homogenate at 4° or −20° for 24 hr, the residual activity was only several percent of the original activity. Throughout the purification procedures, addition of glycerol (20%, w/v) to buffer used stabilized slightly the enzyme, but $MnCl_2$ (1 mM) had no effect on it. After gel electrophoresis of the preparation in pH 9.4 gel,[18] the gel was cut into 1 mm pieces. The detection of L-Leu-L-Leu dipeptidase activity in these pieces and the staining of protein in the gel indicated that the activity originated from one of the several protein bands. Therefore, the preparation was used for subsequent investigations.

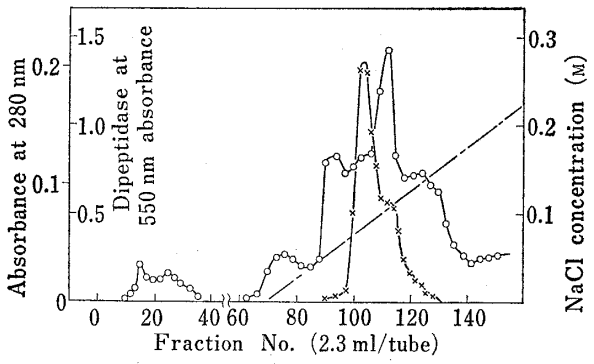

Fig. 1. Elution Pattern of Dipeptidase from DE-52 Cellulose

The enzyme preparation (18 mg) fractionated with $(NH_4)_2SO_4$ was adsorbed on a column (2×20 cm) of DE-52 cellulose equilibrated with 50 mM Tris-HCl buffer (pH 7.2) containing 20% (w/v) glycerol, and eluted with a linear gradient system of NaCl (0—0.4 M) in the same buffer. The enzyme activity in the effluent was determined by using L-Leu-L-Leu as a substrate. ○—○: absorbance at 280 nm, ×—×: dipeptidase activity, ———: NaCl concentration.

Optimum pH for Dipeptidase

Effect of pH on the reaction rate of dipeptidase was investigated by using L-Leu-L-Leu and Gly-L-Leu. The enzyme was most active in the pH range 8.1 to 9.3 towards both substrates, and at pH 7.0 and 10.0, about 90% of the maximum activity was observed.

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Substrate Specificity of Dipeptidase

Table III shows substrate specificity of dipeptidase. The enzyme hydrolyzed preferentially dipeptides, such as Gly-L-Leu, L-Val-L-Leu, L-Leu-L-Ala, L-Val-L-Phe and L-Leu-L-Leu, whereas it acted neither on dipeptides as such L-Leu-L-Pro, L-Pro-L-Leu, L-Phe-L-Pro, L-Pro-L-Phe and L-Phe-Gly nor on two tripeptides such as L-Leu-L-Leu-L-Leu and L-Leu-Gly-Gly. L-Leucineamide and L-leucine-*p*-nitroanilide as a substrate of aminopeptidase and hippuryl-L-Phe as a substrate of carboxypeptidase were not hydrolyzed by this enzyme. These results suggested that dipeptides were hydrolyzed by the action of original dipeptidase.

Table III. Substrate Specificity of partially Purified Dipeptidase from Rat Carrageenin Granuloma

Substrate	Dipeptidase activity (U/mg protein)
Gly-L-Leu	1.53
L-Val-L-Leu	1.58
L-Leu-L-Leu	0.89
L-Leu-L-Ala	1.07
L-Leu-Gly	0.56
L-Val-L-Phe	1.04
Gly-L-Phe	0.70
L-Phe-Gly	0.02
L-Leu-L-Try	0.28
L-Phe-L-Ala	0.17
L-Leu-L-Pro	0.22
L-Pro-L-Leu	0.09
L-Phe-L-Pro	0.15
L-Pro-L-Phe	0.21
L-Leu-L-Leu-L-Leu	0.05
L-Leu-Gly-Gly	0.05
L-Leu-*p*-nitroanilide	0.00
L-Leucineamide	0.00
Hippuryl-L-Phe	0.00

The substrate concentration was finally 1.6 mM and other conditions were the same as described in the text.

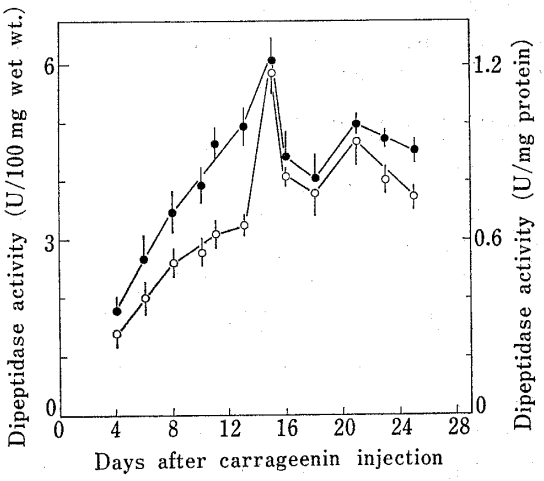

Fig. 2. Change in Dipeptidase Activity during Development of Carrageenin Granuloma

The granuloma homogenate prepared as described in the text was centrifuged at 20000×*g* for 20 min and then L-Leu-L-Leu dipeptidase activity in the supernatant was determined. All values are shown as mean of five animals±S.D. ●—●: dipeptidase activity of U/ 100 mg wet wt., ○—○: dipeptidase activity of U/mg protein.

Effects of Reagents and Metal Salts on Dipeptidase

Marked inhibition (*i.e.* greater than 90%) was observed when the partially purified enzyme was assayed in the presence of 1 mM N-bromosuccinimide, *p*-chloromercuribenzoate, cysteine, *o*-phenanthroline, EDTA, $HgCl_2$, $ZnSO_4$, $CuSO_4$ and $CoCl_2$.

Changes in Dipeptidase Activity during Development of Carrageenin Granuloma

Figure 2 shows daily change in dipeptidase activity of the granulation tissue. Both specific activities of units per mg protein and 100 mg wet wt. increased linearly from the 4th day to the 15th day after carrageenin injection, and their maximum activities on the 15th day were about 5 times higher than the activities on the 4th day, and then both activities decreased slowly.

Discussion

Subcellular distribution of dipeptidase has been examined in detail with the rat liver,[2–5] intestine[8] and guinea pig intestine[9,10] and described that the enzyme has been distributed mainly in lysosome[2,3,5] and soluble fraction.[4,8–10] In the rat carrageenin granuloma, about

230 times of dipeptidase activity of the exudate is found in the granulation tissue and most part of this activity is in soluble fraction of $100000 \times g$, but exact cellular source of the enzyme was not known. Optimum pH of dipeptidase from the granuloma was different from those of the lysosomal dipeptidases in the rat liver[2,3,5] and similar to that of the rat[8] and guinea pig intestinal enzymes[9,10] (pH 7.5—8.5). Inhibitory effects of EDTA and *o*-phenanthroline suggest that our dipeptidase is a metallo-enzyme as the enzyme of mouse ascites tumor.[12] Complete inactivation of dipeptidase by *p*-chloromercuribenzoate and $HgCl_2$ also suggests that the sulfhydryl group(s) in the enzyme is essential to keep its enzyme activity.

A physiological role of dipeptidase in the intestine has been suggested to stimulate the absorption of dipeptides,[6,19] but any role in the other tissues have not been discussed. Dipeptidase activity in the carrageenin granuloma increased gradually and its specific activity reached a maximum level on the 15th day after carrageenin injection, and then decreased slowly. Since Shubin and Thomas[20] have reported that non-collagenous proteins in the granulation tissue are substituted by collagen by approximately the 15th day, locally high distribution of dipeptidase in the granulation tissue and the change in the enzyme activity during development of carrageenin granuloma might be concerned with the obvious change in the tissue components. Further investigations are necessary to discuss the physiological role of this peptidase.

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