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INACTIVATION OF SUBSTANCE P BY GRANULATION TISSUE-DERIVED GELATINASE

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Abstract—An active gelatinase has been purified from the conditioned medium of granulation tissue culture formed by carrageenin injection in rats. The purified gelatinase gave a single band corresponding to a M_r of 57 kDa on both sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and SDS-gelatin PAGE. The granulation tissue-derived gelatinase selectively cleaved the Gln^6-Phe^7 bond of substance P (SP) with a K_m of 0.17 mM and a V_{max} of 0.027 nmol SP^{7-11}/min/μg protein, resulting in the generation of biologically inactive fragments, SP^{1-6} and SP^{7-11}. Our data suggest that the gelatinase produced by granulation tissue participates in the inactivation of SP in the inflammatory site.

The neuropeptide substance P (SP†) is released upon stimulation of the sensory nerves, and causes neurogenic inflammation [1]; it also stimulates inflammatory cells including mast cells, macrophages, neutrophils and lymphocytes [2]. It has been demonstrated that neutral endopeptidase which is a membrane-bound enzyme [3–5], kininase II (angiotensin I-converting enzyme) [6–9], cathepsin G [10], chymase [11] and stromelysin [12] are able to degrade and thus inactivate SP. We demonstrated in previous papers [13, 14] that an active gelatinase is present in exudate and in the collagen fiber fraction of granulation tissue induced by carrageenin in rats. In the present study, we observed that an active gelatinase produced by granulation tissue can selectively cleave the Gln^6-Phe^7 bond of SP, which resulted in the generation of biologically inactive fragments. Our results show that the granulation tissue-derived gelatinase is a SP-degrading enzyme.

MATERIALS AND METHODS

Granulation tissue culture. Inflammation was induced by subcutaneous injection of 4 mL of a 2% (w/v) carrageenin solution into preformed air-pouches on the backs of male Wistar rats weighing 160–190 g [15]. The granulation tissue formed in a capsular shape with 1–2 mm thickness was collected on day 7 after carrageenin injection, and washed extensively with Dulbecco's modified Eagle's medium (DMEM). The washed tissue was cut into small pieces (about 1 × 1 cm) and cultured for 6 days in DMEM (4 mL/piece) supplemented with 0.1% (w/v) bovine serum albumin, 25 mM HEPES, penicillin (0.1 mg/mL) and streptomycin (0.1 mg/mL) under 95% O_2/5% CO_2. The culture medium was changed every day. The conditioned media collected on days 3–6 were centrifuged at 1500 *g* for 20 min at 4°, and frozen at −30° until used for purification of gelatinase.

Purification of gelatinase. The frozen media (total 15 L) collected on days 3–6 of granulation tissue culture were thawed and pooled. Gelatinase in the medium was precipitated with $(NH_4)_2SO_4$ at 80% (w/v) saturation. The precipitate was dissolved in 0.05 M Tris–HCl buffer (pH 8.0), and dialyzed against the same buffer. The gelatinase was purified essentially by the same procedures described previously [14]; the dialyzed sample was chromatographed sequentially on heparin-Sepharose, DEAE-Sephacel and gelatin-Cellulofine. One unit of gelatinase activity is defined as the degradation of 1 μg fluorescein isothiocyanate (FITC)-labeled gelatin (substrate)/min. To confirm the homogeneity of the purified gelatinase sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and SDS-gelatin PAGE were performed on a slab gel as described by Laemmli [16] and Heussen and Dowdle [17], respectively.

Degradation of SP by granulation tissue gelatinase. SP (50 μg; Peptide Institute Inc., Osaka, Japan) was incubated at 37° with the purified granulation-tissue gelatinase (1.24 μg; 155 U/mg protein) in 0.2 mL of 0.05 M Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl, 5 mM $CaCl_2$, 1 mM 4-aminophenylmercuric acetate (APMA), 0.02% (w/v) NaN_3 and 0.01% (w/v) Brij35. After incubation for 2, 6 or 24 hr, the reaction was stopped by freezing of the reaction mixtures at −80°. The samples were thawed and loaded onto a C-18 reverse-phase column (0.46 × 25 cm; Tosoh Co., Tokyo, Japan). The degradation products of SP were eluted from the column with a linear concentration gradient of acetonitrile from 0% (v/v) to 80% (v/v) in 0.05% (v/v) trifluoroacetic acid at a flow rate of 0.8 mL/min.

The amounts of SP and its degradation products

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† Abbreviations: SP, substance P; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; APMA, 4-aminophenylmercuric acetate; and RP-HPLC, reverse-phase high performance liquid chromatography.

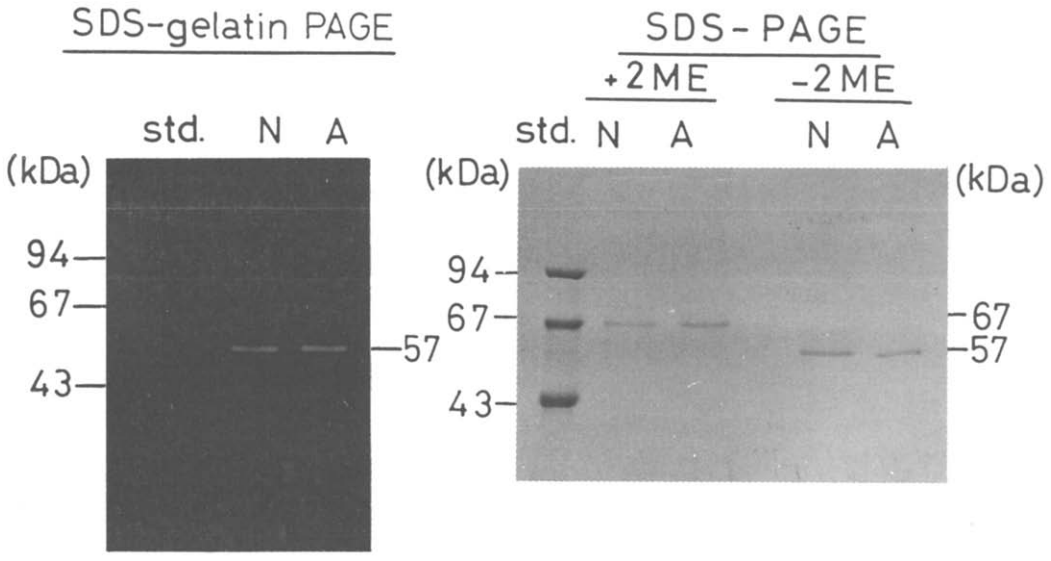

Fig. 1. SDS–PAGE and SDS-gelatin PAGE of the gelatinase purified from the conditioned medium of granulation tissue culture. The purified gelatinase was heated at 80° for 20 min in the presence (+2ME) or absence (−2ME) of 5% (v/v) 2-mercaptoethanol, and applied to 4% (w/v) stacking/7.5% (w/v) running gels for SDS–PAGE. The gelatinase was activated (A) or not activated (N) with 0.8 mM APMA before 2ME treatment. In the case of SDS-gelatin PAGE, the gelatinase was directly applied to the gels without 2-mercaptoethanol treatment. M_r standards (std.) are indicated to the left.

in the reaction mixtures were determined based on the areas of their elution peaks on RP-HPLC monitored at 220 nm using the linear calibration curve of SP.

Amino acid analysis. The degradation products isolated by RP-HPLC were hydrolyzed *in vacuo* in 6 M HCl containing 0.1% (v/v) phenol and 10 mM dithiothreitol for 24 hr at 110°. Amino acid composition was determined by RP-HPLC of phenylthiocarbamoyl derivatives [18].

Determination of vascular permeability in vivo. Vascular permeability activity of samples was investigated in the skins of male Wistar rats (body wt 200–250 g) according to the method of Udaka *et al.* [19]. Briefly, Evans blue solution (2.5%, w/v; 1 mL/kg body wt) was injected intravenously via the tail veins of rats. SP^{1-11}, SP^{1-6} and SP^{7-11} were dissolved in Tyrode solution at a concentration of 10^{-5} M, and injected intradermally in volumes of 50 μL into shaved dorsal skins immediately after the dye injection. The rats were killed 30 min later, their dorsal skins were removed, and the injection sites (20-mm diameter) were punched out. Evans blue in the punched skin was extracted by incubation with 4 mL of formamide at 60° overnight, and the amount of Evans blue was calculated from the absorbance at 595 nm of the extract. Vascular permeability activity is expressed as the amounts (μg) of the dye per site after subtraction of the value of the skin injected with Tyrode's solution alone.

RESULTS

A gelatinase was purified from the conditioned medium of granulation tissue culture. The purified gelatinase gave a single band corresponding to a M_r of 57 kDa on both SDS–PAGE and SDS-gelatin PAGE (zymography) under nonreducing conditions, while the gelatinase showed a M_r of 67 kDa on SDS–PAGE under reducing conditions (Fig. 1). The APMA treatment did not cause the reduction of the M_r of the purified gelatinase (Fig. 1) and its gelatinase activity was detected without activation, indicating that the gelatinase is an active gelatinase. The purified gelatinase extensively degraded gelatin, but showed no proteolytic activity toward type I collagen and α-casein, a substrate of stromelysin (data not shown), suggesting that the purified gelatinase does not contain other proteinases including collagenase and stromelysin.

When SP was incubated with the purified gelatinase, the degradation products of SP increased with time; two major peaks and one minor peak of SP-derived fragments were found on RP-HPLC of the reaction products (Fig. 2). Amino acid analyses of the peaks (R-1–R-4 in Fig. 2) isolated by RP-HPLC revealed that R-1 is the native SP^{1-11}, and R-2, -3 and -4 correspond to SP^{1-6}, SP^{7-11} and SP^{1-9}, respectively (Table 1). The generation of each peptide in the course of incubation time is shown in Fig. 3. A rapid increase in the amounts of SP^{1-6} and SP^{7-11} was proportional to the rapid disappearance of SP^{1-11}, whereas the amount of SP^{1-9} retained a very low level throughout the experimental periods (Figs. 2 and 3). The results suggest that the Gln^6-Phe^7 bond of SP^{1-11} is selectively cleaved by the granulation tissue-derived gelatinase, whereas the cleavage of the Gly^9-Leu^{10} bond by the gelatinase is negligible.

Kinetic properties of the gelatinase-catalyzed

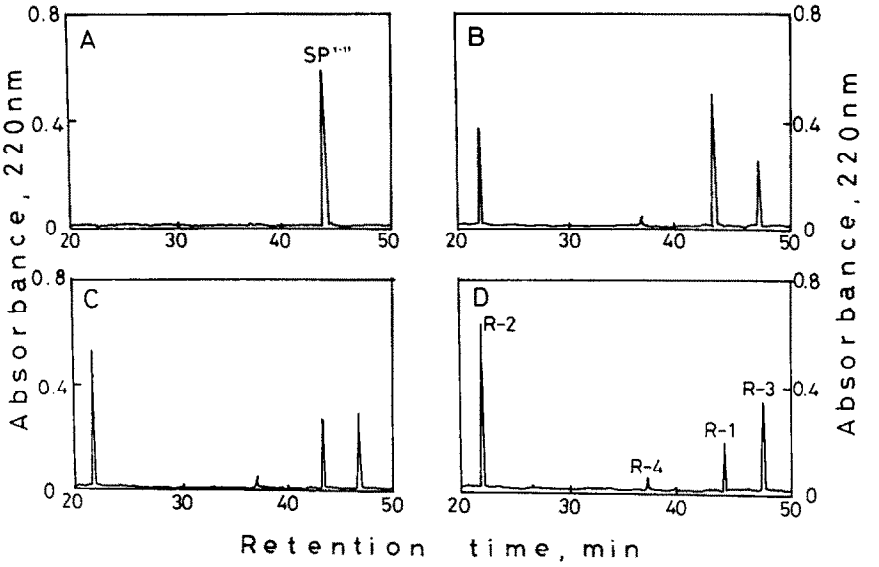

Fig. 2. Hydrolysis of SP by the granulation tissue-derived gelatinase. SP (50 μg) was incubated with the purified gelatinase (1.24 μg) at 37° for 0 hr (A; no incubation), 2 hr (B), 6 hr (C) and 24 hr (D), and then loaded onto a C-18 reverse-phase column (RP-HPLC).

Table 1. Amino acid compositions of SP and its degradation peptides isolated by RP-HPLC*

Amino acid	R-1	R-2	R-3	R-4
Gln	2.1 (2)	1.7 (2)	—†	2.2 (2)
Gly	1.0 (1)	—	1.0 (1)	1.3 (1)
Arg	1.0 (1)	1.0 (1)	—	1.0 (1)
Pro	2.1 (2)	1.7 (2)	—	1.7 (2)
Met	1.4 (1)	—	0.9 (1)	—
Leu	1.1 (1)	—	1.0 (1)	—
Phe	1.7 (2)	—	2.0 (2)	2.1 (2)
Lys	0.8 (1)	0.9 (1)	—	1.1 (1)
Positions	SP^{1-11}	SP^{1-6}	SP^{7-11}	SP^{1-9}

* SP was incubated with the granulation tissue-derived gelatinase, and the peptides (R-1–R-4) in the reaction mixture were isolated by RP-HPLC as described in Fig. 2. The theoretical value of each amino acid is shown in parentheses.

† Not detectable.

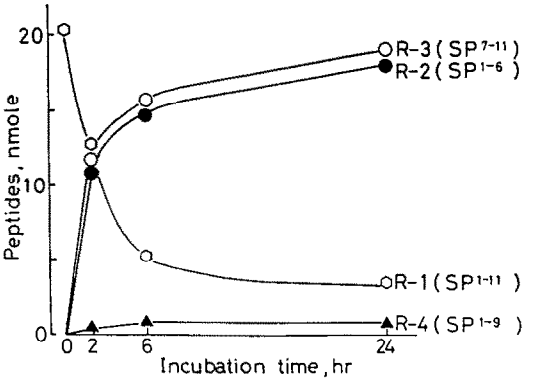

Fig. 3. Time courses of the changes in the amounts of SP and its degradation products. The areas under the peptide peaks (R-1–R-4) shown in Fig. 2 were converted to nanomoles of the peptides using the calibration curve of SP.

hydrolysis of SP were studied as a function of substrate concentrations. From the Lineweaver–Burk analysis (data not shown) of the generation of SP^{7-11} from SP^{1-11} (10–200 μM) by the gelatinase (3.3 μg), K_m, V_{max} and k_{cat} values were calculated to be 0.17 mM, 0.027 nmol SP^{7-11}/min/μg protein and 4.85/min, respectively.

To clarify the contamination of SP-degrading enzymes in the purified granulation tissue-derived gelatinase, the effects of thiorphan and captopril on the degradation of SP by the gelatinase were studied. Cleavage of the Gln^6-Phe^7 bond of SP by the gelatinase was not inhibited by either 0.03 mM thiorphan or 0.03 mM captopril, which are specific inhibitors of neutral endopeptidase and kininase II, respectively (data not shown). The results suggest that the purified gelatinase does not contain neutral endopeptidase and kininase II (angiotensin I-converting enzyme).

The major degradation products, SP^{1-6} and SP^{7-11}, had no biological activity; no increase in the vascular permeability was observed when SP^{1-6} and SP^{7-11} at 10^{-5} M were injected intradermally into the rat skins, though native SP (10^{-5} M) caused a significant increase in the vascular permeability (Fig. 4).

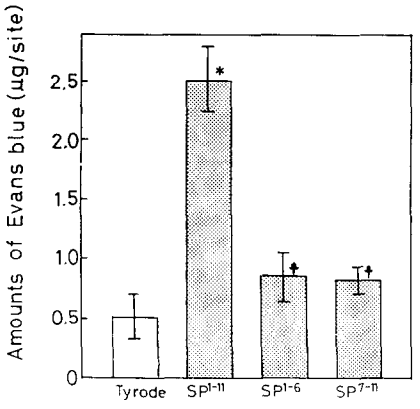

Fig. 4. Effects of SP and its degradation products on the vascular permeability in rat skins. Evans blue solution (2.5%, 1 mL/kg body wt) was injected intravenously, and native SP (SP^{1-11}), SP^{1-6} or SP^{7-11} at a concentration of 10^{-5} M were injected intradermally on the backs of rats. Experimental details are described under Materials and Methods. Data are shown as the means with bars representing SEM of twelve rats. Key: (*) statistically significant difference from control (Tyrode-injected group), $P < 0.01$; and (†) statistically significant difference from SP^{1-11}-injected group, $P < 0.01$.

DISCUSSION

A high level of gelatinase activity was found in the exudate during the course of granulation, and an active gelatinase with a M_r of 57 kDa on SDS-gelatin PAGE has been purified from the conditioned medium of granulation tissue culture (Fig. 1). In a previous paper [13] we demonstrated that granulation tissue-derived fibroblasts in culture mainly produce a 64-kDa gelatinase, which is converted to a 57-kDa species upon treatment with APMA, while rat macrophages and neutrophils mainly secrete a 96-kDa species. These findings strongly suggest that the active 57-kDa gelatinase purified from the conditioned medium of granulation tissue culture is synthesized by fibroblasts in the tissue induced by carrageenin in rats. In addition, it has been found that cytokines including tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) stimulated the production of active gelatinases including a 57-kDa species by the granulation tissue in culture [20]. Further studies are necessary to establish whether an active gelatinase produced by cytokine-stimulated fibroblasts plays an important role in the inactivation of inflammatory mediators such as SP in the inflammatory lesion.

The granulation tissue-derived gelatinase selectively cleaved the Gln^6-Phe^7 bond. This cleavage site of SP by the gelatinase is different from that by neutral endopeptidase, cathepsin G and chymase; the most susceptible bond of SP by neutral endopeptidase is the Gly^9-Leu^{10} bond [5], though the Gln^6-Phe^7 and Phe^7-Phe^8 bonds are also cleaved, while both cathepsin G and chymase hydrolyze SP primarily between the two aromatic residues Phe^7-Phe^8 [10, 11]. On the other hand, human fibroblast stromelysin exclusively cleaves the Gln^6-Phe^7 bond of SP [12], which is the same cleavage site as that by the gelatinase. The purified gelatinase did not contain stromelysin, because α-casein, a substrate of stromelysin, was not degraded by the gelatinase, and no proteinase band corresponding to M_r (45 and 28 kDa) of stromelysin [21] was found on SDS-gelatin PAGE (Fig. 1). These findings indicate that in the present experiments the cleavage of the Gln^6-Phe^7 bond of SP occurred by action of the gelatinase, and not by other SP-degrading proteinases including stromelysin.

Recently, it has been reported [22] that glucocorticoids suppress the SP-mediated plasma extravasation in rat tracheal mucosa by an increase in the activity of SP-degrading enzymes such as neutral endopeptidase. SP-degrading enzymes, therefore, may participate in the regulation of SP activity in inflammatory lesions. Our results suggest that the active gelatinase produced by cytokine-stimulated fibroblasts is capable of degrading/inactivating SP in the inflammatory site where IL-1β and TNF-α are secreted by activated inflammatory cells, including macrophages.

REFERENCES

1. Jancso LN, Jancso-Gabor A and Szolcsanyi J, Direct evidence for neurogenic inflammation and its prevention by denervation and by pretreatment with capsaicin. *Br J Pharmacol Chemother* **31**: 138–151, 1967.
2. Payan DG, Neuropeptides and inflammation: The role of substance P. *Annu Rev Med* **40**: 341–352, 1989.
3. Kerr MA and Kenny AJ, The purification and specificity of a neutral endopeptidase from rabbit kidney brush border. *Biochem J* **137**: 477–488, 1974.
4. Malfroy B, Swerts JP, Guyon A, Roques BP and Schwartz J-C, High affinity enkephalin-degrading peptidase in brain is increased after morphine. *Nature* **276**: 523–526, 1978.
5. Matsas R, Fulcher IS, Kenny AJ and Turner AJ, Substance P and [Leu]enkephalin are hydrolyzed by an enzyme in pig caudate synaptic membranes that is identical with the endopeptidase of kidney microvilli. *Proc Natl Acad Sci USA* **80**: 3111–3115, 1983.
6. Erdös EG, Kininases. In: *Handbook of Experimental Pharmacology* (Ed. Erdös EG), Vol. XXV Supplement, pp. 427–487. Springer, Berlin, 1979.
7. Yokosawa H, Endo S, Ogura Y and Ishii S-I, A new feature of angiotensin-converting enzyme in the brain: Hydrolysis of substance P. *Biochem Biophys Res Commun* **116**: 735–742, 1983.
8. Skidgel RA, Engelbrecht S, Johnson RA and Erdos EG, Hydrolysis of substance P and neurotensin by converting enzyme and neutral endopeptidase. *Peptides* **5**: 769–776, 1984.
9. Cascieri MA, Bull HG, Mumford RA, Patchett AA, Thornberry NA and Liang T, Carboxy-terminal tripeptidyl hydrolysis of substance P activity *in vivo* by captopril and MK-422. *Mol Pharmacol* **25**: 287–293, 1984.
10. Skidgel RA, Jackman HL and Erdos EG, Metabolism of substance P and bradykinin by human neutrophils. *Biochem Pharmacol* **41**: 1335–1344, 1991.
11. Caughey GH, Leidig F, Viro NF and Nadel JA, Substance P and vasoactive intestinal peptide degradation by mast cell tryptase and chymase. *J Pharmacol Exp Ther* **244**: 133–137, 1988.
12. Harrison R, Teahan J and Stein R, A semicontinuous,

high-performance liquid chromatography-based assay for stromelysin. *Anal Biochem* **180**: 110–113, 1989.
13. Sakata K. Hoshino K and Nakagawa H, Purification and characterization of exudate gelatinases in the chronic-phase of carrageenin-induced inflammation in rats. *J Biochem (Tokyo)* **105**: 384–389, 1989.
14. Nakagawa H, Hirata M, Hoshino K, Sakata K and Hatakeyama S, Purification of an active gelatinase from collagen fiber fraction of granulation tissue in rats. *J Biochem (Tokyo)* **108**: 494–498, 1990.
15. Fukuhara M and Tsurufuji S, The effect of locally injected anti-inflammatory drugs on the carrageenin granuloma in rats. *Biochem Pharmacol* **18**: 475–484, 1969.
16. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
17. Heussen C and Dowdle EB, Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Anal Biochem* **102**: 196–202, 1980.
18. Heinrikson RL and Meredith SC, Amino acid analysis by reverse-phase high-performance liquid chromatography: Precolumn derivatization with phenylisothiocyanate. *Anal Biochem* **136**: 65–74, 1984.
19. Udaka K, Takeuch Y and Movat HZ, Simple method for quantitation of enhanced vascular permeability. *Proc Soc Exp Biol Med* **133**: 1384–1387, 1970.
20. Nakagawa H, Kitagawa H and Aikawa Y, Tumor necrosis factor stimulates galatinase and collagenase production by granulation tissue in culture. *Biochem Biophys Res Commun* **142**: 791–797, 1987.
21. Okada Y, Nagase H and Harris ED, Jr, A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. *J Biol Chem* **261**: 14245–14255, 1986.
22. Piedimonte G, McDonald DM and Nadel JA, Neutral endopeptidase and kininase II mediate glucocorticoid inhibition of neurogenic inflammation in the rat trachea. *J Clin Invest* **88**: 40–44, 1991.