Partial Purification and Characterization of a Novel Neutral Proteinase from Human Uterine Cervix

Akira ITO, Hiroko IHARA and Yo MORI

Department of Biochemistry, Tokyo College of Pharmacy, Horinouchi, Hachioji, Tokyo 192-03, Japan

(Received 12 March 1979)

1. Human uterine cervical stroma was found to contain a Ca^{2+} -independent neutral proteinase against casein and *N*-benzoyl-DL-arginine *p*-nitroanilide (Bz-DL-Arg-Nan). This enzyme was tightly bound to an insoluble material (20000g pellet) and was solubilized by high concentrations of NaCl or KCl. High concentrations of them in the reaction system, however, inhibited reversibly the activity of this enzyme. 2. The neutral proteinase was partially purified by extraction with NaCl, gel filtration on Sephadex G-200 and affinity chromatography on casein–Sephadex. 3. The optimal pH of this partially purified enzyme was 7.4–8.0 against casein and Bz-DL-Arg-Nan. The molecular weight of the enzyme was found to be about 1.4×10^5 by gel filtration on Sephadex G-200. 4. The enzyme was significantly inhibited by di-isopropyl phosphorofluoridate (0.1 mM). High concentration of phenylmethanesulphonyl fluoride (5 mM), 7-amino-1-chloro-3-L-tosylamidoheptan-2-one (0.5 mM), antipain (10 μM) or leupeptin (10 μM) was also found to be inhibitory, but chymostatin (40 $\mu\text{g}/\text{ml}$), soya-bean trypsin inhibitor (2.5 mg/ml), human plasma (10%, v/v), *p*-chloromercuribenzoate (1 mM), EDTA (10 mM) and 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one (1 mM) had no effect on the enzyme. 5. The neutral proteinase hydrolysed casein, Bz-DL-Arg-Nan and heat-denatured collagen, but was inactive towards native collagen and several synthetic substrates, such as 4-phenylazobenzoyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg, 3-carboxypropionyl-Ala-Ala-Ala *p*-nitroanilide and 2,4-dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg, and also proteoglycan. The enzyme did not act as a plasminogen activator. 6. These properties suggested that a neutral proteinase in the human uterine cervix was different from enzymes previously reported.

Several enzymes, such as collagenase, acid proteinases and neutral proteinases, are generally known as enzymes that degrade connective tissues, and acid and neutral proteinases are particularly associated with the destruction of proteoglycans (Morrison *et al.*, 1973; Sapolsky *et al.*, 1976; Roughley & Barrett, 1977; Dingle *et al.*, 1977). Human uterine cervical stroma consists mainly of an extracellular matrix composed largely of collagen, structural glycoprotein and proteoglycan. It is well known that marked changes in these connective-

tissue components were caused in the cervical stroma during dilatation in pregnancy at term (Danforth *et al.*, 1974; Karube *et al.*, 1975; Kleissl *et al.*, 1978; Ito *et al.*, 1979a). However, little attention has been paid to the metabolism of connective tissue in the cervical stroma, with special reference to ripening. We have reported the isolation and partial characterization of the cervical enzymes that degrade the connective tissue, such as Pz-peptidase (Ito *et al.*, 1977), cathepsin D (Ito *et al.*, 1979b) and collagenase (K. Kitamura, A. Ito & Y. Mori, unpublished work), but no information on neutral proteinases has been obtained. During the course of the earlier studies, a neutral proteinase tightly bound to an insoluble fibrous material in the stroma was detected. This enzyme was characterized as a novel neutral proteinase different from the enzymes so far known.

In the present paper, the extraction, partial

* Abbreviations used: Bz-DL-Arg-Nan, *N*-benzoyl-DL-arginine *p*-nitroanilide; Dip-F, di-isopropyl phosphorofluoridate; Dnp-, 2,4-dinitrophenyl-; Pz-, 4-phenylazobenzoyloxycarbonyl-; Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one ('TLCK'); Tos-Phe-CH₂Cl, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK'); Suc-[Ala]₃-Nan, 3-carboxypropionyl-Ala-Ala-Ala *p*-nitroanilide; SDS, sodium dodecyl sulphate.

characterization and physiological activities of this neutral proteinase are described.

Experimental

Enzyme source

Cervical tissues from women of child-bearing age were obtained from patients scheduled for hysterectomy of fibromyoma not involving the cervix. Cervical epithelium and stroma were completely separated, and the stroma was repeatedly rinsed with cold 0.9% NaCl and then kept at -20°C until use.

Assay for neutral proteinase

The activity of neutral proteinase was assayed by the method of Erlanger *et al.* (1961) by using Bz-DL-Arg-Nan as a substrate. An appropriate amount of the enzyme (0.25 ml) was mixed with 1.0 ml of 1.25 mM-substrate in 50 mM-Tris/HCl, pH 7.4, and the mixture was incubated for 30 min at 37°C , and then 0.5 ml of 30% (v/v) acetic acid was added to terminate the reaction. The amount of liberated *p*-nitroaniline was calculated from the A_{410} of the mixture. One unit of the enzyme is expressed as the amount that catalyses the liberation of $1\text{ }\mu\text{mol}$ of *p*-nitroaniline/min under these conditions. Caseinolytic activity was also analysed by the method of Anson (1938). To 2 ml of 2% (w/v) casein solution in 50 mM-Tris/HCl, pH 7.4, 0.5 ml of the enzyme was added and incubated at 37°C for 30 min. After the incubation, 2.5 ml of 5% (w/v) trichloroacetic acid was added to terminate the reaction, and the mixture was filtered through Toyo filter paper no. 2. The digested peptides in the filtrate were measured by using the Folin-Ciocalteu reagent (at 660 nm; Folin & Ciocalteu, 1927).

Assay for plasminogen activator

Plasminogen activation was assayed by the method of Katz *et al.* (1976) by using azo-casein in place of protamine as a substrate (Fritz *et al.*, 1974). A mixture of 0.18 unit (A_{275} of HClO_4 -soluble product from casein) of plasminogen and an appropriate amount of neutral proteinase in a total volume of 2.0 ml of 25 mM-Tris/HCl, pH 7.4, was first incubated at 37°C . After 60 min, 0.5 ml of the mixture was added to 1.0 ml of 2% (w/v) azo-casein in 25 mM-Tris/HCl, pH 7.4, and incubated for 60 min at 37°C . The enzyme reaction was stopped by the addition of 1.5 ml of 5% (w/v) trichloroacetic acid, and the mixture was filtered. The liberated peptides in the filtrate were monitored by photometric measurement of A_{365} and by using the Folin-Ciocalteu reagent (A_{660}). Controls were similarly assayed by using 240 units of urokinase (human urine) or 75 units (*N*-benzoyl-L-arginine ethyl ester

units) of trypsin in place of the cervical neutral proteinase.

SDS/polyacrylamide-disc-gel electrophoresis

This was performed by the method of Scott & Veis (1976) and Laemmli (1970), by using a 7.5% acrylamide gel in 50 mM-sodium phosphate/0.1% SDS, pH 7.2, or a 5% gel in 25 mM-Tris/192 mM-glycine/0.1% SDS, pH 8.3, respectively. Gels were stained with Coomassie Brilliant Blue R.

Preparation of casein-Sephrose

Casein (38 mg) was coupled to 3 g of CNBr-activated Sepharose 4B by the method of Axén & Ernback (1971); more than 90% of casein was constantly bound.

Extraction of proteoglycan from bovine nasal cartilage

Proteoglycan was isolated by the dissociative extraction method of Sajdera & Hascall (1969). Defatted bovine nasal cartilage was extracted for 48 h in 60 vol. of 4 M-guanidinium chloride in 50 mM-sodium acetate, pH 5.8, with stirring. The non-extracted cartilage residue was removed by centrifugation at 30000 g for 60 min, and the proteoglycan was precipitated with 4 vol. of ethanol containing 2% (w/v) potassium acetate. The precipitate was redissolved in deionized water and reprecipitated in the same way. The precipitate dissolved in the water was dialysed against water for 48 h and then freeze-dried. By these procedures, approx. 50 mg of proteoglycan was obtained from about 500 mg of defatted bovine nasal cartilage.

Reagents used

The following reagents were commercially obtained: Hammarsten casein from E. Merck, Tokyo, Japan; Bz-DL-Arg-Nan and Pz-Pro-Leu-Gly-Pro-D-Arg (Pz-peptide) from Fluka, Buchs, Switzerland; Sephadex G-200, Blue Dextran 2000, Sepharose 4B and CNBr-activated Sepharose 4B from Pharmacia, Uppsala, Sweden; soya-bean trypsin inhibitor and azo-casein from Miles, Stoke Poges, Bucks., U.K.; bovine serum albumin ($4\times$ crystallized) and egg albumin ($5\times$ crystallized) from ICN Pharmaceuticals, Cleveland, OH, U.S.A.; antipain, chymostatin, leupeptin, pepstatin, Suc-[Ala]₃-Nan and Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg (Dnp-peptide) from Protein Research Foundation, Osaka, Japan; trypsin (bovine pancreas, type XII, 7500 *N*-benzoyl-L-arginine ethyl ester units/mg), γ -globulin (human Cohn fraction II), plasminogen (pig blood, 1.1 A_{275} units/mg), Tos-Lys-CH₂Cl, Tos-Phe-CH₂Cl, Dip-F and phenylmethanesulphonyl fluoride from Sigma, St. Louis, MO, U.S.A.; urokinase (human urine) from Midori-juji Co., Osaka, Japan. Other reagents used were of analytical-reagent grade.

Results and Discussion

Effect of neutral salts on neutral proteinase in the human uterine cervix

Table 1 shows the effect of the concentration of NaCl on the extraction of neutral proteinase. The NaCl concentration in 10mM-sodium phosphate, pH 6.0, containing 5mM-EDTA was found to be of critical importance in the extraction procedure. Although the neutral proteinase was hardly solubilized by the buffer alone, most of the enzyme was effectively extracted by the addition of 0.5M- or 1.0M-NaCl to the buffer. The specific activity of the proteinase in the 0.5M-NaCl extract was found to be higher than that in the 1.0M-NaCl extract. Therefore, for subsequent experiments, we extracted the neutral proteinase with 0.5M-NaCl in the buffer. Similar results were observed with KCl extraction (results not shown), whereas an insignificant amount of the enzyme was solubilized by buffered 1% (w/v) Triton X-100, 1% (w/v) SDS, 0.5% (w/v) cholic acid or 0.1% (w/v) Brij 35. The finding that neutral proteinase bound to an insoluble fibrous material is active towards casein and Bz-DL-Arg-Nan indicated that the enzyme is not a latent form. These observations suggested that effects of neutral salts on the solubilization of the enzyme depend on the dissociation of the interaction between the enzyme and the insoluble material. Notides *et al.* (1973) reported the presence of neutral proteinase in the myometrium of the human uterus and the solubilization of about 30–50% of its total activity with 40mM-Tris/5mM-EDTA/50mM-KCl, pH 7.4. We have also confirmed that more than 25% of the total activity in the myometrium was usually extracted with 10mM-sodium phosphate/5mM-EDTA, pH 6.0, and that this indicated the difference in the solubility of the enzyme from that of the cervical one. A similar effect of neutral salts on the extraction was des-

cribed for the enzymes from human spleen (Starkey & Barrett, 1976), rabbit skin (Lazarus & Barrett, 1974) and rat uterine myometrium (Katz *et al.*, 1976; Afting *et al.*, 1979). It was also reported that the activities of neutral proteinase of the first two tissues were stimulated by neutral salts, such as NaCl and KCl, and also by some detergents. However, hydrolysing activities towards casein and Bz-DL-Arg-Nan in the human uterine cervix were reversibly inactivated by the addition of NaCl to the reaction system, as shown in Fig. 1. Two hydrolysing activities were completely inhibited by 1.0M-NaCl, and similar inhibitory effects were also observed with KCl (results not shown).

Partial purification of neutral proteinase in the human uterine cervix

The thawed cervical stroma (about 5g) was minced with scissors and mixed well with 50ml of 10mM-sodium phosphate, pH 6.0, containing 5mM-EDTA. The mixture was homogenized twice for 30s each, with cooling on ice for 30s after the first homogenization, at full speed in a VirTis 45 homogenizer. After centrifugation at 20000g for 20min, acid proteinase, Pz-peptidase and trypsin inhibitor were mostly found in the supernatant. Neutral proteinase activity, however, resided in the pellet. The residual pellet was suspended in the above buffer containing 0.5M-NaCl and rehomogenized as described above. After centrifugation at 20000g for 20min, extracted neutral proteinase in the supernatant was condensed with poly(ethylene glycol) 6000 and dialysed against the same buffer overnight. By the dialysis, a considerable amount of protein without activity was precipitated and the enzyme's specific activity increased significantly. After removal of the precipitate by centrifugation as

Table 1. *Effect of the concentration of NaCl on the extraction of neutral proteinase*

Portions of cervical stroma (0.5g) were homogenized in 5.0ml of 10mM-sodium phosphate/5mM-EDTA, pH 6.0, containing various amounts of NaCl. The homogenization was done twice for 30s each, with cooling in ice for 30s after the first homogenization, at full speed in a VirTis 45 homogenizer. The supernatant was centrifuged at 20000g for 20min and assayed for activity of neutral proteinase. Each value is the average of duplicate assays.

NaCl concn. (M)	Extracted neutral proteinase (munits/100mg wet wt. of tissue)
0	2.3
0.1	18.4
0.3	27.6
0.5	52.7
1.0	55.9

Fig. 1. *Inhibitory effect of NaCl on the reaction rate of neutral proteinase*

The indicated concentration of NaCl was present in the enzyme assay system, and other assay conditions are described in the text. Substrate: O, Bz-DL-Arg-Nan; Δ, casein.

above, the enzyme preparation (5 ml) was applied to a Sephadex G-200 column (3.2 cm $\times$ 65 cm) equilibrated previously and eluted with 10 mM-sodium phosphate, pH 6.0, containing 5 mM-EDTA and 0.5 M-NaCl. Fig. 2 shows the elution pattern. Neutral proteinase activity was eluted at fractions distinct from the void-volume ones. The active fractions (nos. 52–69, 86 ml) were collected together, concentrated to about 8 ml with poly(ethylene glycol) 6000, and dialysed against 10 mM-sodium phosphate/5 mM-EDTA, pH 6.0, for 12 h, and then centrifuged at 20000g for 20 min to remove insolubilized material. The supernatant was applied to a column (1.4 cm $\times$ 8 cm) of casein–Sepharose equilibrated previously with the same buffer, and eluted stepwise with NaCl in the buffer as shown in Fig. 3. Although the neutral proteinase was eluted by two concentrations of NaCl, a considerable amount of the enzyme was present in the 0.5 M-NaCl fraction, and its specific activity in that fraction was about 14 times that in the 0.1 M-NaCl fraction. By these procedures, the neutral proteinase in the 0.5 M-NaCl fraction was purified about 70-fold over the crude extract. The proteinase activity eluted by 0.1 M-NaCl when rechromatographed was eluted by both 0.1 M-

Fig. 2. Gel chromatography of neutral proteinase extracted with NaCl on Sephadex G-200

Enzyme (8 ml) was applied to a column (3.2 cm $\times$ 65 cm) of Sephadex G-200 equilibrated previously with 10 mM-sodium phosphate/5 mM-EDTA/0.5 M-NaCl, pH 6.0, and eluted with the same buffer at a flow rate of 36 ml/h. O, A_{280} ; Δ , neutral proteinase.

and 0.5 M-NaCl (as in the first chromatographic separation, Fig. 3). The result indicates that the enzyme in the effluents is not due to an over-application of the enzyme. No further study was performed with this enzyme. The enzyme chromatographed on casein–Sepharose was not purified further because of its lability, by analogy with the proteinase in the rat and pig uterus (Afting *et al.*, 1979). During the course of purification, the enzyme in the cervix was mostly inactivated by the use of 25 mM-Tris/HCl, pH 7.5; thus the purification procedures were carried out by using 10 mM-sodium phosphate, pH 6.0, at 4°C, to prevent autolysis and digestion of casein coupled to Sepharose. The neutral proteinase was adsorbed on DEAE-cellulose and concanavalin A–Sepharose at pH 6.0, but no activity was recovered, suggesting its strong binding to affinity adsorbents. A typical purification procedure of this enzyme is summarized in Table 2. The neutral proteinase thus purified was used for subsequent experiments.

Fig. 3. Affinity chromatography of partially purified neutral proteinase on casein–Sepharose

Condensed neutral proteinase (8.7 ml) was adsorbed on a column (1.4 cm $\times$ 8 cm) of casein–Sepharose equilibrated previously with 10 mM-sodium phosphate/5 mM-EDTA, pH 6.0, at a flow rate of 9 ml/h, and eluted stepwise with NaCl (0, 0.1 and 0.5 M) in the same buffer at the same flow rate. O, A_{280} ; Δ , neutral proteinase.

Table 2. Purification of neutral proteinase from human uterine cervix

Data for a typical purification are shown. The values given are those of a single preparation from 5 g of the tissue. Protein was measured by A_{280} , and $A_{280}^{1\%}$ is calculated as 10.0.

Source	Protein (mg)	Total activity (munits)	Specific activity (munits/mg)	Yield (%)
Homogenate (0.5 M-NaCl extract)	223.7	720	3.2	100
Diffusate	96.6	650	6.8	90.3
Sephadex G-200	15.9	358	22.5	49.7
Diffusate	8.3	250	30.0	34.7
Affinity chromatography	0.34	71	210.0	9.9

Effect of pH on the reaction rate

As shown in Fig. 4, for the hydrolysis of Bz-DL-Arg-Nan the neutral proteinase showed its maximum activity in the pH range 7.4–8.0, and at pH 6.0 and 9.4 approx. 50% of the maximum activity was detected, but the enzyme was virtually inactive below pH 5.0. A similar activity curve was also observed with casein as a substrate (results not shown). These results indicate that the enzyme is a typical neutral proteinase.

Effect of various reagents on the neutral proteinase

As summarized in Table 3, the neutral proteinase from the human uterine cervix was not inactivated by soya-bean trypsin inhibitor, human plasma and serum, whereas trypsin was completely inactivated by them (results not shown). The enzyme was also insensitive to chymostatin, HgCl₂, *p*-chloromercuribenzoate, pepstatin, Tos-Phe-CH₂Cl, EDTA, 1,10-phenanthroline and CaCl₂. Dip-F was found to be a potent inhibitor of the enzyme, and high concentrations of phenylmethanesulphonyl fluoride, Tos-Lys-CH₂Cl, leupeptin and antipain also inhibited the enzyme significantly. These results indicate that the neutral proteinase in the human uterine cervix appears to be a serine proteinase, as it is sensitive to Dip-F and phenylmethanesulphonyl fluoride. It is very surprising that human plasma and serum were not inhibitors of the enzyme, and the specific inhibitor was also not detectable in the cervix (results not shown). Therefore we have an interest in the regulation system of the proteinase activity in the cervix. In the human uterine myometrium, a Ca²⁺-independent and Dip-F-sensitive neutral proteinase (Notides *et al.*, 1973) and trypsin-like enzymes (Rybák *et al.*, 1974) are present, but no other infor-

mation is yet known. With uterus from ovariectomized rats (Levitz *et al.*, 1974; Katz *et al.*, 1976) and mice (Katz *et al.*, 1977), it was reported that oestradiol or oestradiol plus progesterone induced a trypsin-like proteinase in the nucleus and 12000g pellet which was not inhibited by soya-bean trypsin inhibitor. On the other hand, Woessner (1977, 1979) and Afting *et al.* (1979) reported the presence of a trypsin-inhibitor-sensitive serine proteinase in the insoluble fraction of normal rat uterus; this enzyme was also inhibited significantly by a tissue proteinase inhibitor. There seem to be two or three kinds of neutral proteinase in rat uterus. Therefore the data from inhibition experiments do not indicate clearly the difference between the human cer-

Table 3. Effect of reagents on the activity of neutral proteinase from the human uterine cervix

The mixture of neutral proteinase and a reagent in 25 mm-Tris/HCl, pH 7.5, was kept at 0°C for 30 min, and then hydrolysing activity against casein was determined as described in the text.

Reagent	Concn. (mm)	Relative activity (%)
Control	—	100
Dip-F	0.1	13
	0.5	5
Phenylmethanesulphonyl fluoride	2.5	64
	5.0	20
<i>N</i> -Bromosuccinimide	1.0	0
Tos-Lys-CH ₂ Cl	0.1	80
	0.5	36
	1.0	32
Leupeptin	0.001	80
	0.01	60
	0.1	24
Antipain	0.001	88
	0.01	48
	0.1	28
EDTA	10	105
1,10-Phenanthroline	1.0	95
CaCl ₂	1.0	109
CuSO ₄	1.0	100
CoCl ₂	1.0	73
HgCl ₂	1.0	95
<i>p</i> -Chloromercuribenzoate	1.0	95
Monoiodoacetic acid	1.0	107
Dithiothreitol	1.0	109
Tos-Phe-CH ₂ Cl	0.5	98
	1.0	100
Pepstatin	1.0	96
Chymostatin	40 µg/ml	94
Soya-bean trypsin inhibitor	1.0 mg/ml	100
	2.5 mg/ml	100
Human plasma	2% (v/v)	95
	10% (v/v)	98
Human serum	2% (v/v)	97
	10% (v/v)	100

Fig. 4. Effect of pH on the hydrolysis of Bz-DL-Arg-Nan by neutral proteinase

The neutral proteinase was assayed as described in the text. Buffers used: pH 3–7.5, 50 mm-phosphate/citrate; pH 7–9, 50 mm-Tris/HCl; pH 9–10, 50 mm-Na₂CO₃/NaHCO₃.

vical proteinase and the various enzymes. The particular properties of the human enzyme are described in the following sections.

Molecular weight of the human cervical proteinase

The molecular weight of partially purified neutral proteinase was determined by gel filtration on Sephadex G-200 by the method of Whitaker (1963). A column (1.8 cm $\times$ 67 cm) of Sephadex G-200 was previously equilibrated with 10 mM-sodium phosphate/0.5 M-NaCl, pH 6.0. Elution was carried out with the same buffer at a flow rate of 10 ml/h, and 2 ml fractions were collected. The void volume was estimated with Blue Dextran 2000, and the molecular weights of γ -globulin, bovine serum albumin and egg albumin were taken as 1.6×10^5 , 6.9×10^4 and 4.5×10^4 respectively. Their V_e/V_0 values were 1.23, 1.54 and 1.69 respectively and gave a linear relation to their log (molecular weight). Its V_e/V_0 value of 1.26 indicated that the molecular weight of human cervical neutral proteinase is approx. 1.4×10^5 . Rybák *et al.* (1974) separated two trypsin-like proteinases in the human uterine myometrium by Sephadex G-200 gel chromatography, but no information on their molecular weights was reported. We confirmed with human uterine cervix that only one peak of neutral proteinase activity was detected by gel chromatography, as shown in Fig. 2. The molecular weight of the cervical proteinase was higher than for those of human spleen (17000; Starkey & Barrett, 1976), polymorphonuclear leucocytes (12000, 14000 and 16000; Schmidt & Havemann, 1977) and rat bone-marrow mitochondria (31800; Aoki, 1978).

Substrate specificity of neutral proteinase

Fig. 5 shows the hydrolysing activities of partially purified proteinase towards casein and Bz-DL-Arg-Nan, and indicates that both substrates are degraded in proportion to the incubation period.

Fig. 5. Progress curves of hydrolysis of Bz-DL-Arg-Nan and casein by neutral proteinase

About 1 munit of neutral proteinase was added to the standard assay system as described in the text. Substrate: O, Bz-DL-Arg-Nan; Δ , casein.

Phenylmethanesulphonyl fluoride was also confirmed to inhibit similarly the hydrolysis of both substrates. These results suggest that the hydrolysing

Fig. 6. Hydrolysis activity of neutral proteinase towards native and heat-denatured rat tail-tendon collagens. Collagen (2 mg) with or without denaturation at 55°C for 30 min was incubated with 1.18 munits of neutral proteinase in a total volume of 1.0 ml of 25 mM-Tris/HCl, pH 7.5, and then 10 μ l of the mixture was subjected to SDS/polyacrylamide-disc-gel electrophoresis. Gels: (1), native collagen; (2), native collagen + neutral proteinase; (3) and (5), denatured collagen; (4) and (6), denatured collagen + neutral proteinase. Gels (1)–(4) are 5% acrylamide, and gels (5) and (6) are 7.5% acrylamide.

Fig. 7. Gel chromatography on Sepharose 4B of proteoglycan before and after a 20 h incubation with neutral proteinase and trypsin

A 10 mg portion of proteoglycan from bovine nasal cartilage and 1.97 munits of neutral proteinase or trypsin were incubated at 37°C for 20 h in a total volume of 1.0 ml of 20 mM-Tris/HCl/0.1 M-NaCl, pH 7.5, and then the mixture was centrifuged at 20000g for 30 min. The supernatant (0.6 ml) was applied to a column (1.5 cm $\times$ 45 cm) of Sepharose 4B equilibrated previously and eluted with the same buffer containing 0.2 M-NaCl. Flow rate was 6.5 ml/h, and uronic acid (A_{530}) in 0.5 ml of each fraction was determined by the method of Bitter & Muir (1962). O, Before incubation; Δ , after incubation with neutral proteinase; $\bullet$, after incubation with trypsin.

activities towards casein and Bz-DL-Arg-Nan are due to one enzyme. Fig. 6 shows the mode of action of neutral proteinase on native and heat-denatured collagens from rat tail tendon. The neutral proteinase was entirely inactive towards native collagen, whereas the enzyme easily hydrolysed heat-denatured collagen (gelatin) into small fragments, which migrated near the buffer front in 5% acrylamide gel and were found to be composed of many fragments smaller than α -chain in 7.5% acrylamide gel. The neutral proteinase is inactive towards Dnp-peptide as a synthetic collagenase substrate (Masui *et al.*, 1977), Pz-peptide as a collagen peptidase substrate (Morales & Woessner, 1977) and Suc-[Ala]₃-Nan as an elastase substrate (Bieth *et al.*, 1974). The enzyme did not hydrolyse proteoglycan of bovine nasal cartilage included in polyacrylamide-gel beads (results not shown; method of Nagase, 1977) or in solution, whereas trypsin as a control easily hydrolysed both substrates. Fig. 7 shows the results with proteoglycan in solution examined by gel filtration on Sepharose 4B. In spite of the addition of the same activity to the reaction system, the cervical neutral proteinase was found to be characteristically inactive towards proteoglycan.

Action of the cervical neutral proteinase on plasminogen

As shown in Table 4, in three experiments, the value in the column of neutral proteinase plus plasminogen was nearly equal to the sum of each value of neutral proteinase and plasminogen. The same results are also confirmed with the results by spectrophotometric determination of A_{365} . On the other hand, urokinase or trypsin as a control was found to act as the activator in the experiments. These results indicated that the cervical neutral proteinase did not activate plasminogen. In human uterus, Rybák *et al.* (1974) reported the presence of trypsin-like proteinase activity which is correlated with plasminogen activation. Troll *et al.* (1975) and Katz *et al.* (1976) described for ovariectomized rat uterus that oestradiol or oestradiol plus progesterone induced a trypsin-like but soya-bean-trypsin-inhibitor-insensitive proteinase which acted as a plasminogen activator. Although a similar

induction of proteinase activity was also reported with uterus of ovariectomized mice by Katz *et al.* (1977), different actions towards plasminogen were detectable in the activity; i.e. the activity bound to the uterus pellet fraction is a plasminogen activator, but the soluble activity is not. However, it was not clear yet whether the source of the bound and the soluble activity was identical or different. As described in the previous section, the identity or difference between human cervical proteinase and the above enzymes was not clarified by the data obtained with specific inhibitors, but the studies on activation of plasminogen seem to indicate a fundamental difference.

The enzymic properties of neutral proteinase in the human uterine cervix, with respect to the inactivation by NaCl and KCl, the molecular weight, sensitivity to inhibitors and inactivity towards plasminogen and proteoglycan, are rather different from those of neutral proteinases hitherto reported.

The physiological significance of the enzyme in the cervix, however, is not clear yet. Physiological roles of uterine proteinases so far known, other than as plasminogen activator, are reported as follows. The enzyme in the human uterine myometrium changes the size of rat oestrogen-receptor complex from 8S to 4.5S (Notides *et al.*, 1973). Rybák *et al.* (1974) also reported that trypsin-like enzymes obtained from human uterine wall caused contraction of rat uterus. Woessner (1977, 1979) has described that neutral proteinase bound to an insoluble material from rat uterus was able to activate the latent collagenase to an active form, and the activation was inhibited by soya-bean trypsin inhibitor. However, such activation was not confirmed with human uterine cervical proteinase (results not shown). Afting *et al.* (1979) has also reported that neutral proteinase in the rat and pig uterus degraded the uterine actomyosin, degradation of which was concerned with the involution of the uterus. We have confirmed the presence of Pz-peptidase (Ito *et al.*, 1977) and collagenase (K. Kitamura, A. Ito & Y. Mori, unpublished work) in the human uterine cervix and a significant increase in their activities during dilatation in pregnancy at term (Kitamura *et al.*, 1979). No activity of gelatinase, however, has been

Table 4. *Action of human cervical neutral proteinase on plasminogen*

Detailed experimental conditions are described in the text. Amounts of neutral proteinase used in Expts. I, II and III were 7.4, 11.3 and 16.1 munits, respectively, and in Expt. II plasminogen was treated with the enzyme for 120 min. Abbreviations: P, plasminogen; NP, neutral proteinase; U, urokinase; T, trypsin.

	Proteolytic activity (A_{660})						
	P	NP	U	T	P + NP	P + U	P + T
Expt. I	0.020	0.091	0.005	0.050	0.097	0.275	0.200
Expt. II	0.013	0.127	—	—	0.134	—	—
Expt. III	0.026	0.165	—	—	0.190	—	—

detected yet in the human uterine cervix. These observations suggest that the neutral proteinase might act as a gelatinase during collagen degradation in the human uterine cervix, but the cervical enzyme is different from the gelatin-specific neutral proteinase in human leucocytes, which is inhibited by EDTA and human serum (Sopata & Dancewicz, 1974; Kobayashi & Nagai, 1978). Further investigations are needed to explain the physiological significance of the enzyme in the cervix, i.e. during dilatation in pregnancy at term.

We express our gratitude to Dr. Shun Hirakawa, Department of Obstetrics and Gynecology, School of Medicine, Toho University, for supplying the enzyme source, and also to Dr. Hideaki Nagase, Department of Biochemistry, School of Medicine, Miami University, for his advice for proteinase assay by using proteoglycan-polyacrylamide-gel beads.

References

- Afing, E.-G., Becker, M. L. & Elce, J. S. (1979) *Biochem. J.* **177**, 99–106
- Anson, M. L. (1938) *J. Gen. Physiol.* **22**, 79–85
- Aoki, Y. (1978) *J. Biol. Chem.* **253**, 2026–2032
- Axén, R. & Ernback, S. (1971) *Eur. J. Biochem.* **18**, 351–360
- Bieth, J., Spiess, B. & Wermuth, C. G. (1974) *Biochem. Med.* **11**, 350–357
- Bitter, T. & Muir, M. (1962) *Anal. Biochem.* **4**, 330–334
- Danforth, D. N., Veis, A., Breen, M., Weinstein, H. G., Buckingham, J. C. & Monalo, P. (1974) *Am. J. Obstet. Gynecol.* **120**, 641–651
- Dingle, J. T., Blow, A. M. T., Barrett, A. J. & Martin, P. E. N. (1977) *Biochem. J.* **167**, 775–785
- Erlanger, B. F., Kokowsky, N. & Cohen, W. (1961) *Arch. Biochem. Biophys.* **95**, 271–278
- Folin, O. & Ciocalteu, V. (1927) *J. Biol. Chem.* **73**, 627–650
- Fritz, H., Trautschold, I. & Werle, E. (1974) in *Methods in Enzymatic Analysis* (Bergmeyer, H. U., ed.), vol. 2, pp. 1071–1074, Academic Press, New York
- Ito, A., Nagane, K., Mori, Y., Hirakawa, S. & Hayashi, M. (1977) *Clin. Chim. Acta* **78**, 267–270
- Ito, A., Kitamura, K., Mori, Y. & Hirakawa, S. (1979a) *Biochem. Med.* **21**, 262–270
- Ito, A., Mori, Y. & Hirakawa, S. (1979b) *Chem. Pharm. Bull.* **27**, 969–973
- Karube, H., Kanke, Y., Mori, Y., Hirakawa, S. & Hayashi, M. (1975) *Endocrinol. Jpn.* **22**, 445–448
- Katz, J., Troll, W., Levy, M., Flikins, K., Russo, J. & Levitz, M. (1976) *Arch. Biochem. Biophys.* **173**, 347–354
- Katz, J., Troll, W., Adler, S. W. & Levitz, M. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 3754–3757
- Kitamura, K., Ito, A., Hirakawa, S. & Mori, Y. (1979) *Biochem. Med.* in the press
- Kleissl, H. P., Rest, M. V. D., Naftolin, F., Glorieux, F. H. & Leon, A. D. (1978) *Am. J. Obstet. Gynecol.* **130**, 748–753
- Kobayashi, S. & Nagai, Y. (1978) *J. Biochem. (Tokyo)* **84**, 559–567
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lazarus, G. S. & Barrett, A. J. (1974) *Biochim. Biophys. Acta* **350**, 1–12
- Levitz, M., Katz, J., Krone, P., Prochoroff, N. N. & Troll, W. (1974) *Endocrinology* **94**, 633–640
- Masui, Y., Takemoto, T., Sakakibara, S., Hori, H. & Nagai, Y. (1977) *Biochem. Med.* **17**, 215–221
- Morales, T. & Woessner, J. F., Jr. (1977) *J. Biol. Chem.* **252**, 4855–4860
- Morrison, R. I. G., Barrett, A. J., Dingle, J. T. & Prior, D. (1973) *Biochim. Biophys. Acta* **302**, 411–419
- Nagase, H. (1977) Ph.D. Thesis, Miami University
- Notides, A. C., Hamilton, D. E. & Rudolph, J. H. (1973) *Endocrinology* **93**, 210–216
- Roughley, P. J. & Barrett, A. J. (1977) *Biochem. J.* **167**, 629–637
- Rybák, M., Rybáková, B. & Koutský, J. (1974) *Physiol. Bohemoslov.* **23**, 467–473
- Sajdera, S. W. & Hascall, V. C. (1969) *J. Biol. Chem.* **244**, 77–87
- Sapolsky, A. I., Kleiser, H., Howell, D. S. & Woessner, J. F., Jr. (1976) *J. Clin. Invest.* **58**, 1030–1041
- Schmidt, W. & Havemann, K. (1977) *Hoppe-Seyler's Z. Physiol. Chem.* **358**, s.555–564
- Scott, P. G. & Veis, A. (1976) *Connect. Tissue Res.* **4**, 107–116
- Sopata, I. & Dancewicz, A. M. (1974) *Biochim. Biophys. Acta* **370**, 510–523
- Starkey, P. M. & Barrett, A. J. (1976) *Biochem. J.* **155**, 255–263
- Troll, W., Rossman, T., Katz, J., Levitz, M. & Sugimura, T. (1975) in *Cold Spring Harbor Conferences on Cell Proliferation: Proteases and Biological Control* (Reich, E., Rifkin, D. B. & Shaw, E., eds.), vol. 2, pp. 977–987, Cold Spring Harbor Laboratories, Cold Spring Harbor
- Whitaker, J. R. (1963) *Anal. Chem.* **35**, 1950–1957
- Woessner, J. F., Jr. (1977) *Biochem. J.* **161**, 535–542
- Woessner, J. F., Jr. (1979) *Biochem. J.* **180**, 95–102