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AN ALKALINE METALLO-PROTEINASE IN THE HUMAN UTERINE CERVIX AND CHANGES IN ITS ACTIVITY BY CERVICAL RIPENING

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Human uterine cervix at term pregnancy was found to contain an alkaline metallo-proteinase by use of a synthetic substrate, 2,4-dinitrophenyl-L-Pro-L-Gln-Gly-L-Ile-L-Ala-Gly-L-Gln-D-Arg. The enzyme (with a molecular weight of $3.8 \cdot 10^4$) was most active around pH 9.2 toward casein and Nα-benzoyl-DL-Arg-*p*-nitroanilide. [^{14}C]-Gelatin and proteoglycan subunit were also substrates for the enzyme, but [^{14}C]collagen was not. In particular, the enzyme digested gelatin 70-times faster than the novel neutral proteinase in the cervix. Although EDTA was a potent inhibitor, 1,10-phenanthroline, human serum, diisopropylfluorophosphate and elastatinal had no effect on the enzyme. Alkaline proteinase in term pregnant cervices was significantly higher than in non-pregnant ones.

Introduction

Recently, the presence of a neutral serine proteinase in uterine tissues has been reported and its physiological roles have been discussed; e.g., as an activator of latent collagenase [1,2] and plasminogen [3,4], and as a degradation enzyme of uterine actomyosin [5]. However, little is known about alkaline proteinases in the uterus.

Masui et al. [6] have reported that the octa-peptide, DNP-L-Pro-L-Gln-Gly-L-Ile-L-Ala-Gly-L-Gln-D-Arg (DNP-peptide), is a useful synthetic substrate for vertebrate collagenase, but this peptide is distinct from the hepta-peptide substrate used for a metal-dependent endopeptidase in rat uterus [7], and is known to be more specific for vertebrate collagenase [6]. During the course of earlier studies of human uterine cervical collagenase, we have found an alkaline proteinase which degrades DNP-peptide. This enzyme is predominantly found in the cervix at term pregnancy and is inhibited by EDTA, as well as vertebrate collagenase. This proteinase has no activity toward native collagen, but hydrolyses gelatin more rapidly than the neutral proteinase found in the uterine cervix [8].

In the present paper, some properties of human cervical alkaline proteinase are described.

Materials and Methods

Tissue sources. Non-pregnant human uterine cervical tissues were obtained from patients for hysterectomy in fibromyoma, and cervices in pregnancy at term were obtained by biopsy from patients at cesarean section. The stromal tissues were rinsed twice with 100 vol. cold 0.9% NaCl, and stored at −20°C until use.

Tissue homogenization. The tissue homogenate was usually prepared at 4°C as follows: the thawed and minced tissue was homogenized in 10 vol. 50 mM Tris-HCl/0.25% Triton X-100/0.1 M $CaCl_2$ (pH 7.4).

Abbreviations: DNP-; 2,4-dinitrophenyl, Bz-Arg-NAN; Nα-benzoyl-DL-Arg-*p*-nitroanilide, Pz-; 4-phenylazobenzyloxycarbonyl-, Suc-[Ala]$_3$-NAN; succinyl-[Ala]$_3$-*p*-nitroanilide, DFP; diisopropylfluorophosphate, PMSF; phenylmethylsulfonyl fluoride, TLCK; Nα-*p*-tosyl-L-Lys chloromethyl ketone, TPCK; Nα-*p*-tosyl-L-Phe chloromethyl ketone.

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The homogenization was done twice for 30 s each, with cooling in ice for 30 s after the first homogenization, at full speed in a VirTis 45 homogenizer. The sample was then centrifuged at 20 000 × *g* for 20 min.

Reagents used. The following reagents were commercially obtained; Sephadex G-75 and 150, and Blue Dextran 2000 from Pharmacia, Uppsala, Sweden; DNP-peptide, Suc-[Ala]$_3$-NAN, chymostatin, elastatinal and leupeptin from Protein Research Foundation, Osaka, Japan; Hammarsten casein from E. Merck, Tokyo, Japan; Bz-Arg-NAN, Pz-L-Pro-L-Leu-Gly-L-Pro-D-Arg (Pz-peptide) and Pz-L-Pro-L-Leu from Fluka, Buchs, Switzerland; egg albumin (grade VII), α-chymotrypsinogen A (bovine pancreas, type II), cytochrome *c* (horse heart, type IV), plasminogen (pig blood), soybean trypsin inhibitor, TLCK, TPCK, DFP and PMSF from Sigma, St. Louis, U.S.A.; urokinase (human urine) from Green-Cross, Co. Osaka, Japan; DEAE-cellulose (DE-52) from Whatman, Maidstone, U.K. Other reagents used were of analytical-reagent grade.

Enzyme assay methods. DNP-peptide hydrolysing activity was assayed by the method of Masui et al. [6]. An appropriate amount of the enzyme in 0.1 ml was mixed with an equal vol. of 0.5 mM DNP-peptide in 50 mM Tris-HCl/5 mM $CaCl_2$/0.15 M NaCl/0.02% bovine serum albumin (pH 7.4), and the mixture was incubated at 37°C for 18 h. The reaction was stopped by the addition of 0.5 ml 1 M HCl. The DNP-peptide fragment released was extracted by vigorous shaking with 1 ml ethyl acetate and determined by the absorbance of the organic layer at 365 nm. One unit of the activity is expressed as the amount that catalyses the liberation of 1 nmol DNP-peptide fragment/h under these conditions.

Hydrolysing activity for Bz-Arg-NAN was assayed as described previously [8] with slight modifications. The enzyme (0.25 ml) was incubated with 1.25 mM Bz-Arg-NAN (1.0 ml) in 50 mM glycine/NaOH (pH 9.2) for 18 h at 37°C, and after the addition of 30% (v/v) acetic acid (0.5 ml) to the reaction mixture, the absorbance at 410 nm was measured.

The caseinolytic activity was determined by using Hammarsten casein. 0.25 ml enzyme were added to 1.0 ml 2% (w/v) casein solution in 50 mM glycine/NaOH (pH 9.2) and incubated at 37°C. After incubation for 18 h, 1.25 ml 5% (w/v) trichloroacetic acid was added to terminate the reaction, and the mixture was filtered through Toyo filter paper No. 2. The released peptides in the filtrate were measured by using the Folin-Ciocalteu reagent (at 660 nm [9]).

Degrading activity toward proteoglycan subunit was assayed by using proteoglycan subunit-acrylamide gel beads [10]. 4 mg of dry proteoglycan subunit-beads containing 1 mg proteoglycan subunit were incubated at 37°C with the enzyme in a total vol. of 1.0 ml 50 mM glycine /NaOH (pH 9.2). After incubation for 18 h, the incubation mixture was chilled in ice-water and 0.5 ml chilled water was added to stop the reaction. The mixture was immediately filtered through Toyo filter paper No. 2, and hexuronic acid in the filtrate was determined by the method of Bitter and Muir [11].

Pz-peptide cleaving activity was assayed as described in a previous report [12]. [^{14}C]Glycine-labeled collagen from guinea pig skin was prepared and used for the collagenase assay by the method of Terato et al. [13]. Gelatinolytic activity was also assayed by using heat-denatured [^{14}C]collagen as described by Harris and Krane [14].

Action of alkaline proteinase on DNP-peptide and Pz-peptide. The cleaved DNP-peptide fragment containing DNP-group was extracted with ethyl acetate, dried off, and hydrolyzed in 6 M HCl at 110°C for 18 h under nitrogen gas and then dried again. Amino acid analysis was carried out with a Hitachi model 835 amino acid analyzer. Pz-peptide fragment extracted with ethyl acetate was also dried off, chromatographed on silica gel plates using a solvent system of *n*-butanol/acetic acid/water (4 : 1 : 1, v/v) and then compared with a standard of Pz-L-Pro-L-Leu.

Action of alkaline proteinase on plasminogen and latent collagenase. Plasminogen activation was assayed by the method of Katz et al. [3]. Alkaline proteinase and plasminogen were preincubated at 37°C for 1 or 12 h and then the increase in proteolytic activity was determined using casein as substrate. The activation was compared with that of human urokinase.

The latent collagenase was prepared from the insoluble fraction of human uterine cervix by the method of Weeks et al. [15] and treated with alkaline proteinase at 37°C for 6 h, and then the increase in DNP-peptide cleavage was assayed for evaluation of latent collagenase activation. In this case, activation

of latent collagenase was compared with that of 1 mM 4-aminophenylmercuric acetate [16].

Results and Discussion

Extraction of alkaline proteinase from the human uterine cervix

When the human uterine cervix at term pregnancy was homogenized in 50 mM Tris-HCl/0.1 M $CaCl_2$/0.25% Triton X-100 (pH 7.4) and then the supernatant at 20000 × *g* was chromatographed on Sephadex G-150, three DNP-peptide hydrolysing activities were separated and designated as Peaks I, II and III, respectively, as shown in Fig. 1.

Peak III eluted just before the total volume of the column was confirmed to coincide with both Bz-Arg-NAN and casein hydrolysing activity peaks, and also had a hydrolysis action toward proteoglycan and Pz-peptide (data not shown). Peak II (fraction Nos. 46–56) was found to correspond with the peak of [^{14}C]collagen hydrolysing activity. Recently, we have reported that Peak I, at the void volume, and Peak II corresponded to α_2-macroglobulin-collagenase complex and active collagenase, respectively [17].

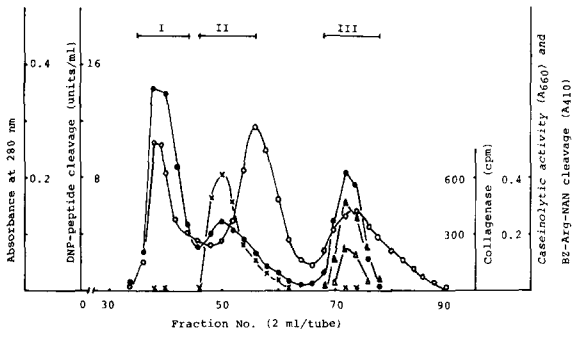

Fig. 1. Elution profile of DNP-peptide hydrolysing activity on Sephadex G-150. The crude enzyme preparation (1.5 ml) of pregnant cervix was chromatographed on a column (1.7 × 60 cm) of Sephadex G-150 previously equilibrated with 50 mM Tris-HCl/10 mM $CaCl_2$/0.15 M NaCl (pH 7.4). Elution was carried out by the same buffer at a flow rate of 8 ml/h. The activity of each fraction was assayed using Bz-Arg-NAN (pH 9.2), casein (pH 9.2) and DNP-peptide (pH 7.4). The peak (Frc. Nos. 66–80) of absorbance at 280 nm corresponded mainly to Triton X-100. •———•, DNP-peptide cleavage; X———X, collagenase; ▲———▲, caseinolytic activity; △———△, Bz-Arg-NAN cleavage; ○———○, absorbance at 280 nm.

In order to examine the extractability of Peak III, the thawed cervix at term pregnancy was homogenized in the above buffer without Triton X-100 and centrifuged as described under Materials and Methods. However, Peak III was not sought in the supernatant by gel filtration on Sephadex G-150. When the resided pellet here was rehomogenized in the same buffer containing 0.25% Triton X-100, Peak III was found to be extracted effectively by the procedure.

Next, we investigated whether or not the above hydrolysing activities in Peak III originated from a single enzyme. After the dialysis of Peak III against 25 mM Tris-HCl/10 mM $CaCl_2$ (pH 7.4) overnight, the dialysate had a specific activity of 240 DNP-peptide units/mg protein and corresponded to 140 units/g wet tissue. The dialysate was chromatographed on DE-52 cellulose. As shown in Fig. 2, three hydrolysing activities toward DNP-peptide, proteoglycan and Bz-Arg-NAN were found to adsorb to the DE-52 cellulose column and eluted simultaneously with a less than 0.1 M NaCl difference. Peak III concentrated by Amicon DM 5 membrane was rechromatographed on Sephadex G-75. As shown in Fig. 3, both DNP-peptide and Bz-Arg-NAN hydrolysing activities were simultaneously eluted in the same fractions. Their molecular weights were identical at $3.8 \cdot 10^4$, and were significantly less than those of $1.4 \cdot 10^5$ for neutral proteinase [8] and $6.7 \cdot 10^4$ for Pz-

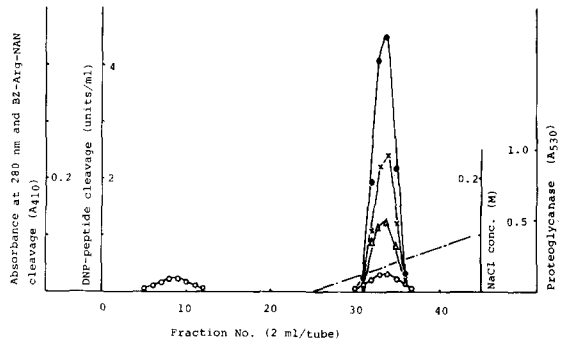

Fig. 2. Ion-exchange chromatography of enzyme preparation from Sephadex G-150 on cellulose DE-52. The dialysate (13 ml) was applied to a column (1.3 × 10 cm) of cellulose DE-52 previously equilibrated with 25 mM Tris-HCl/10 mM $CaCl_2$ (pH 7.4) at a flow rate of 8 ml/h. The elution was carried out by a linear gradient of 0–0.3 M NaCl in 120 ml of the same buffer. ○———○, absorbance at 280 nm; •———•, DNP-peptide cleavage; X———X, proteoglycanase; △———△, Bz-Arg-NAN cleavage; · – · – ·, NaCl concentration.

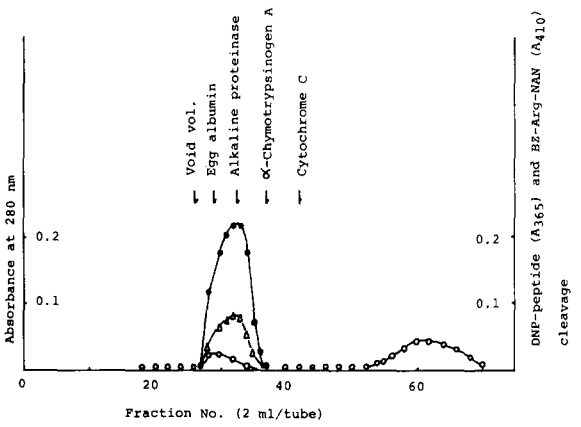

Fig. 3. Rechromatography of enzyme preparation from Sephadex G-150 on Sephadex G-75. Condensed enzyme pool was applied to a column (1.7 X 70 cm) of Sephadex G-75 as prepared in Fig. 1. The molecular weights of egg albumin, α-chymotrypsinogen A and cytochrome *c* were taken as $4.5 \cdot 10^4$, $2.5 \cdot 10^4$ and $1.3 \cdot 10^4$, respectively. ○———○, absorbance at 280 nm; ●———●, DNP–peptide cleavage; △———△, Bz-Arg-NAN cleavage.

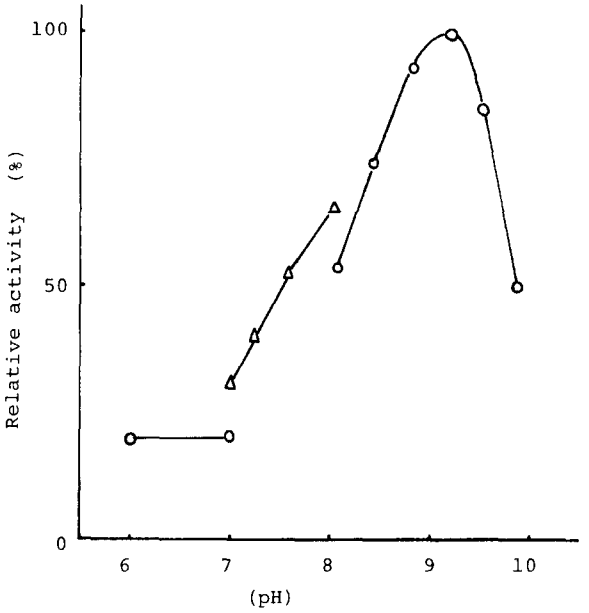

Fig. 4. Effect of pH on the activity of alkaline proteinase. The enzyme preparation from Sephadex G-150 and casein as a substrate were used. Buffers used: ●———●, McIlvain (pH 6.0–7.0); △———△, 50 mM Tris-HCl (pH 7.0–8.2); ○———○, 50 mM glycine/NaOH (pH 8.0–9.8).

peptidase [18] in the human uterine cervix.

As shown in Fig. 4, degrading activity toward casein in Peak III was most active around pH 9.2, and at pH 7.4 and 9.8 approx. 50% of the maximum activity was detected. A similar result was also observed with Bz-Arg-NAN as a substrate (data not shown).

These results suggest that the above hydrolysing activities in Peak III originate from a single enzyme. Peak III obtained from the different cervices was used for subsequent investigations.

Enzymatic properties of alkaline proteinase

Table I shows the effect of reagents on the activity of alkaline proteinase. The enzyme was significantly inhibited by EDTA as well as human cervical collagenase [17] and vertebrate collagenase, but not by chymostatin, elastatinal DFP, TLCK, TPCK, PMSF and soybean trypsin inhibitor, indicating that the enzyme was a metal-dependent proteinase. The proteinase was also not affected by 1,10-phenanthroline and human serum. We have observed that a neutral serine proteinase in the cervix was also not inhibited by human serum [8]. These indicate that proteinases in the human uterine cervix might be less sensitive toward serum inhibitors than other neutral proteinases so far known.

Quantitative amino acid analysis of DNP-peptide fragments produced by alkaline proteinase gave the following amino acid composition: proline (4.3 nmol); glutamic acid (8.7 nmol); glycine (8.8 nmol). This result indicated that the enzyme cleaved DNP-peptide at only the Gly-Ile bond, since DNP-proline was unstable under the usual conditions of hydrolysis [19]. Accordingly the mode of action of the proteinase on DNP-peptide is similar to that of collagenase from tadpole [6], human leukocytes [20] and human uterine cervix [17]. Recently, Kobayashi and Nagai [20] have reported that both elastase and gelatinase in human leukocytes can also hydrolyse DNP-peptide. Our results support recent reports indicating that great care must be exercised about using DNP-peptide as a substrate for impure collagenase preparation.

Alkaline proteinase also cleaved Pz-peptide at the Leu-Gly bond, a Pz-peptidase from the cervix [12], whereas its hydrolysing action was slower than that of the Pz-peptidase. Proteoglycan subunit in polyacrylamide gel beads was also a substrate, but native collagen and Suc-$[Ala]_3$-NAN a synthetic elastase substrate [21] were not. We have already reported

TABLE I

EFFECT OF REAGENTS ON ALKALINE PROTEINASE FROM HUMAN UTERINE CERVIX

The enzyme was treated with each reagent at 37°C for 30 min in 50 mM glycine/NaOH (pH 9.2), and then the remaining activity was assayed.

Reagent	(mM)	Relative activity (%)		
		Bz-Arg-NAN	DNP-petide	Proteoglycan
Control	–	100	100	100
Na_2 · EDTA	10.0	0	0	0
	5.0	18	15	30
1,10-Phenanthroline	10.0	107	90	88
DFP	0.1	90		
PMSF	1.0	103		
TLCK	0.1	100		
TPCK	0.1	100		
Leupeptin	0.1	100		
Chymostatin	40 (μg/ml)	97		
Elastatinal	2 (μg/ml)	103		
Soybean trypsin inhibitor	1 (mg/ml)	94 [a]		
Human serum	5 (%, v/v)	108 [a]		

[a] Casein as substrate.

that a neutral proteinase in the cervix did not hydrolyse the DNP-peptide, Pz-peptide and proteoglycan subunit [8]. Less action of alkaline proteinase was observed on activation of plasminogen and the latent form collagenase of human uterine cervix.

Table II represents the substrate specificity of alkaline proteinase. When the caseinolytic activity of both alkaline and neutral proteinases was arranged, Bz-Arg-NAN was cleaved at a similar rate by both enzymes. Alkaline proteinase, however, digested gelatin about 70-times faster than the neutral enzyme indicating that the former hydrolyses gelatin more specifically and is clearly distinct from the latter enzyme.

TABLE II

SUBSTRATE SPECIFICITY OF ALKALINE PROTEINASE

Hydrolytic activities toward casein and Bz-Arg-NAN were assayed as described. Gelatin digestion was assayed as follows; [^{14}C]-glycine-labelled gelatin (600 μg, 8500 cpm) and enzyme (0.2 ml) were incubated at 37°C for 18 h in a total vol. of 0.6 ml containing 50 mM Tris-HCl/10 mM $CaCl_2$/0.15 M NaCl (pH 7.4) for neutral proteinase and 50 mM glycine/NaOH/10 mM $CaCl_2$/0.15 M NaCl (pH 9.2) for alkaline proteinase. After the incubation, 0.2 ml 60% (w/v) trichloroacetic acid was added to terminate the reaction, and then the mixture was centrifuged at 1 000 × g for 20 min and the radioactivity in the supernatant was counted in Bray's solution in a liquid scintillation counter.

Enzyme	Relative activity		
	Casein (μmol as tyrosine/ml [a] per h)	Bz-Arg-NAN (μmol/ml [a] per h)	Gelatin (μg/ml [a] per h)
Neutral proteinase	0.014	0.0091	1.6
Alkaline proteinase	0.014	0.0095	105.5

[a] ml enzyme solution.

Woessner [7] has reported the presence of a new metal-dependent endopeptidase in rat uterus of insoluble fraction which cleaved DNP-L-Pro-L-Leu-Gly-L-Ile-L-Ala-L-Arg amide. However, its enzymatic properties are obviously different from those of our enzyme; e.g., Triton X-100 did not have any effect on the peptidase extractability, whereas the heat-extraction effectively solubilized it from the insoluble fraction of rat uterus. Its molecular weight of $7 \cdot 10^4$ was also larger than that of alkaline proteinase.

More recently, Sellers and Woessner [22] have reported the extraction of a neutral metal-dependent proteinase from involuting rat uterus. This enzyme is present as a latent form in the insoluble fraction, solubilized and activated in part by the heat-extraction. The molecular weight of $1.2 \cdot 10^4$ for active proteinase was less than that of alkaline proteinase. Although its cleaving activity for DNP-peptide is not clear, these properties suggest that the enzyme is distinct from our alkaline proteinase.

Fig. 5 shows the effect of pregnancy on alkaline proteinase in the cervix. The enzyme activity extracted from pregnant cervices was significantly higher than that from non-pregnant ones, and the total activity is as high as active collagenase (left peaks). It is well known that the human uterine cervix at term pregnancy becomes softer and increasingly dilatable in structure. Therefore, it is possible that the above increase in proteinase is due to the different extractability of the enzyme between two groups. During the course of studies on the effect of dehydroepiandrosterone sulphate on rabbit cervical repining, however, alkaline proteinase extracted from term pregnant cervices was also significantly higher than that from non-pregnant tissues which were softer than non-pregnant human cervices [23]. In addition, alkaline proteinase was not detectable in such pregnant corpora (unpublished data). These suggest that the higher level of alkaline proteinase in the pregnant group might have originated in the actual increase of

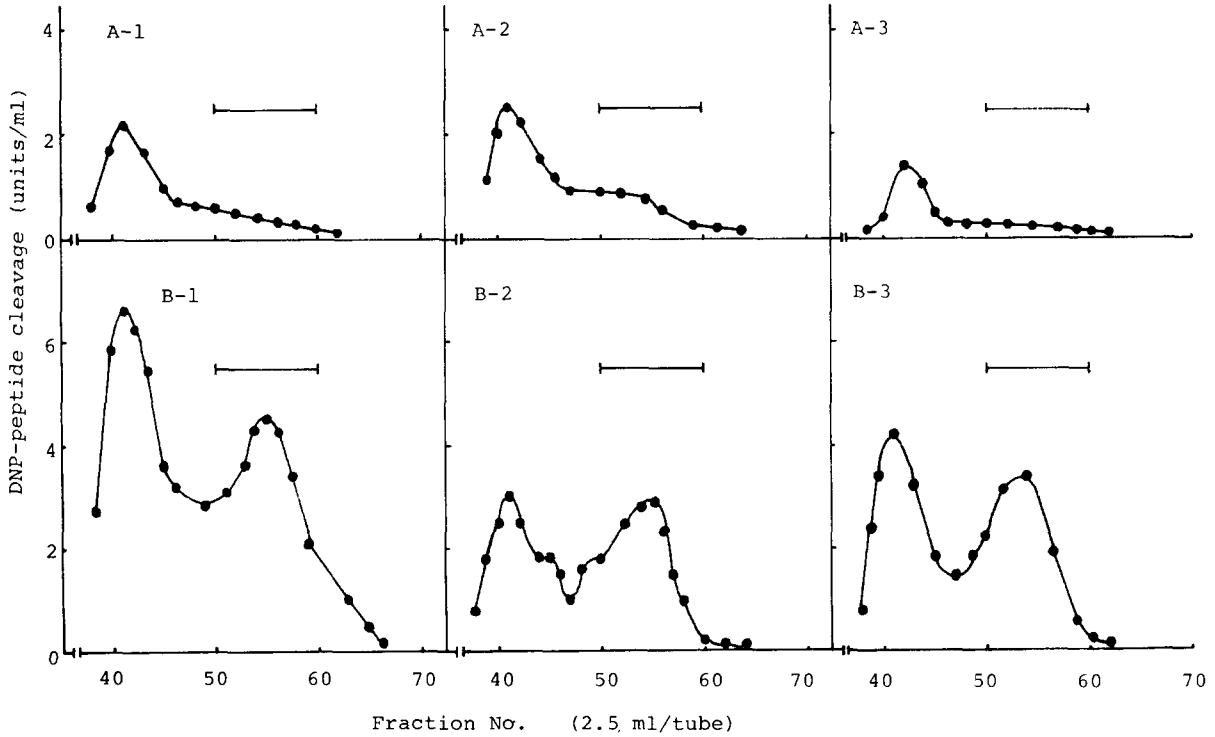

Fig. 5. Effect of pregnancy on alkaline proteinase activity in the human uterine cervix. The supernatant (1.5 ml) of the cervical homogenate was applied to a column (1.7 X 65 cm) of Sephadex G-150. Other conditions were the same in Fig. 1. Al-3 and Bl-3 are non-pregnant and term pregnant cervix, respectively. ——— shows the alkaline proteinase fractions. DNP-peptide hydrolysing activity in the left peak (Frcs, 38–45) is corresponding to active collagenase (Peak II in Fig. 1).

the enzyme activity, but not in the different extractability of the enzyme.

In this paper, we have described the presence of alkaline proteinase and its characterization in human uterine cervix, but its physiological roles are not clarified. However, the results of the present study indicate that the increase in alkaline proteinase is one of the important biochemical changes underlying cervical ripening at parturition.

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