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PRODUCTION OF COLLAGENASE INHIBITOR BY MOUSE CALVARIA IN TISSUE CULTURE

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ABSTRACT

A collagenase inhibitor was identified in the culture medium of mouse calvaria after separation from mouse bone collagenase by Sephadex G-200 gel filtration equilibrated with 6M urea in Tris buffer. The simultaneous synthesis of both collagenase and inhibitor by mouse calvaria in tissue culture was consistent with the resuls of our previous study using a chick bone culture system and otehr recent studies.

Keywords: Collagenase, Collagenase inhibitor, Mouse calvaria, Cel filtration

INTRODUCTION

It has been controversial whether latent collagenase is a precusor zymogen which is then enzymatically converted into an active form[1,2] or bound complex of active enzyme and inhibitor.[3,4,5] In 1978, Sakamoto *et al.*[6] succeeded in isolating a latent collagenase from the tissue culture medium of embryonic chick bones. Furthermore, we have been able to isolate collagenase and collagenase inhibitor from the culture fluid of embryonic chick bones.[7] We also reported that embryonic chick bones produced latent collagenase and collagenase inhibitor in tissue culture, and proposed that the latent form of chick bone collagenase is a precursor zymogen and that the inhibitor is simultaneously synthesized and released by bone explants.[7] In this study, we report the production of a collagenase inhibitor by mouse calvaria which also produced collagenase in tissue culture.

MATERIALS AND METHODS

1. Tissue culture of bone

Calvaria of 5-day old Swiss Albino mice of Webster strain were removed aseptically and rinsed in Gay's balanced salt solution. Immediately thereafter, 10 calvaria were placed in a 75 cm^2 plastic culture flask (Falcon Plastics, Los Angeles, Cal.) containing 10 ml of tissue culture medium and cultured at 37°C in 95% O_2 and 5% CO_2. The tissue culture medium was composed of mammalian Tyrode solution containing amino acids, vitamins, L-glutamine, penicillin, and streptomycin as described by Shimizu *et al.*.[8]

For the time course study, cultures were fed daily and continued for 6 days. Culture systems

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with heparin (50 units/ml) and without heparin in the medium were employed. For the preparation of crude enzyme and inhibitor, cultures were fed every 2 days and continued for 6 or 7 days. The pooled culture media, which were frozen at −60°C and preserved were concentrated approximately 100-fold on PM-10 membrane (Amicon Corp., Lexington, Mass.).

2. Collagenase and collagenase inhibitor assay

Collagenase activity was assayed by measuring the release of ^{14}C-labelled peptides from reconstituted fibrils of radioactively labelled rat skin collagen as described by Sakamoto *et al.*.[9] Inhibitor activity was assayed by adding inhibitor preparations to a known amount of active chick bone or mouse bone collagenase as previously described.[7] Prior to assay, desalting was carried out by passing chromatographic fractions through PD-10 columns (Pharmacia Fine Chemicals, Piscataway, N.J.).

3. Activation of latent collagenase

Prior to collagenase activity assay, the samples of crude culture medium and chromatographic fractions were activated with p-APMA (p-aminophenylmelcuric acetate, Aldrich Chemiccal Co., Milwaukee, Wis.) for measurement of total (active plus latent) collagenase activity.

4. Column chromatography

The concentrated culture media were chromatographed in a cold room on a Sephadex G-200 gel filtration column (Pharmacia Fine Chemicals, Piscataway, N.J.) equilibrated with 50 mM Tris-HCl buffer, pH 7.6 containing 5 mM $CaCl_2$. 1 mM benzamine-HCl (Sigma Chemical Co., St. Louis, Mo.) and 1M NaCl or 6M urea. The samples of collected fractions were assayed for their collagenase and inhibitor activities.

RESULTS

The time course analysis of the crude culture media collected daily indicated that this culture system produced and released collagenase into the culture medium. The product of total collagenase in the system with heparin was sufficiently recognized. In this system, collagenase was synthesized daily and the maximum release of total collagenase was observed during the first and second days of the culture, and the product of collagenase decreased after the third day. On the sixth day, no release of collagenase was seen. No appreciable amount of collagenase was observed in the system without heparin (Fig. 1).

The presence of a collagenase inhibitor in the cluture medium was suspected because the addition of culture medium aliquots to active collagenase in the assay resulted in the inhibition of the enzyme. However, the quantification of collagenase inhibitor activity in the culture medium sample was difficult due to the concomitant activity of collagenase therein. Therefore, the chromatographic separation of collagenase and collagenase inhibitor was tried using the culture medium samples which contained heparin. The concentrated culture medium sample was gel-filtered with a column of Sephadex G-200 gel equilibrated with Tris buffer containing 1M NaCl. Table 1 shows that weak collagenase inhibitor activity was diffusively detected in the several fractions of higher molecular weight preceding the fractions of collagenase. The result was similar to that of the chick bone collagenase/inhibitor study.[7] When heparin-containing medium smaples were gel-filtered in 6M urea, the inhibitor activity was not separated from the enzyme (result not shown). Since the spent culture medium to which heparin had not been added was found in our preliminary studies to have a comparable amount of collagenase inhibitor activity with far less enzyme activity (Fig. 1), the concentrated culture medium (without heparin) was then gel-filtered in 6M urea. Fig. 2 shows

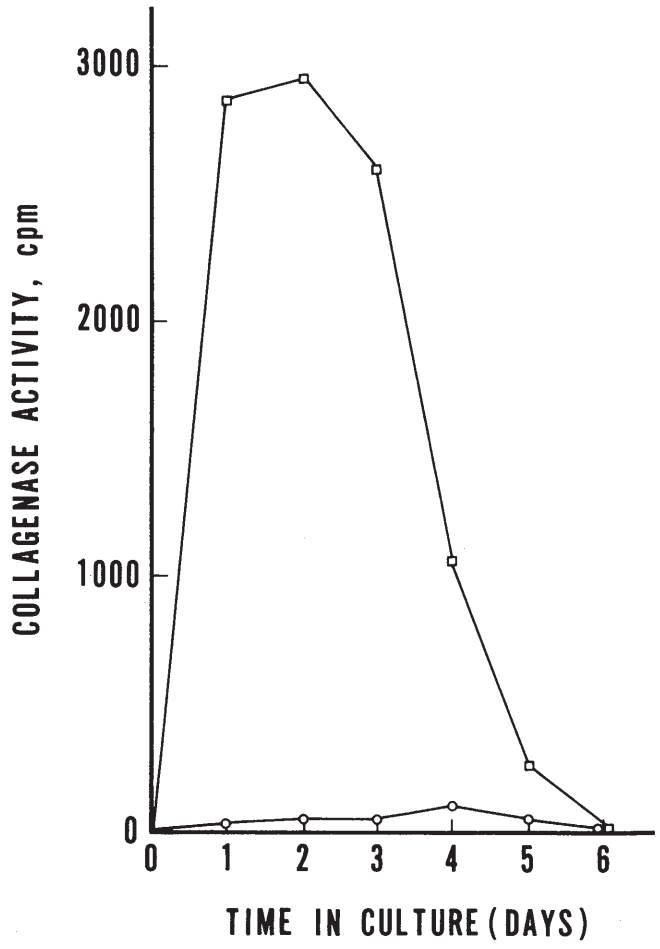

Fig. 1 The time course analysis on the release of total collagenase activity (latent plus active) from mouse calvarial explants. (□——□): production of collagenase in the presence of heparin (50 units/ml). (○——○): Production of collagenase in the absence of heparin. In both cases, the crude culture medium aliquots (25 μl) were assayed for their collagenase activities after activation with p-APMA. The incubation periods for the assay were approx. 20 h at 37°C in both cases.

that both collagenase and collagenase inhibitor were demonstrated and the two components could be well separated by the gel filtration in 6M urea.

Since the presence of a collagenase inhibitor in the cluture medium was confirmed by the above experiment, the time course synthesis and release of collagenase and collagenase inhibitor were then studied using the culture system without heparin. Fig. 3 shows that the collagenase was synthesized daily and the maximum release of total collagenase was observed on the third day of the culture, and on eighth day collagenase was scarcely found. The collagenase inhibitor was observed only on the first and second days of the cluture.

Table 1. Inhibition of mouse bone collagenase by the fractions of concentrated culture medium (with heparin) from Sephadex G-200 gel filtration in 1M NaCl.

Assay tube No.	Tris buffer μl	Enzyme μl	Sample fraction (desalted) Fr. # — μl	Activity cpm	Activity %	Inhibition %
1	250	0	0	340	—	—
2	225	25	0	1938	100	0
3	25	25	# 32 — 200	1557	76	24
4	25	25	# 34 — 200	1144	50	50
5	25	25	# 36 — 200	1671	83	17
6	25	25	# 38 — 200	1223	55	45
7	25	25	# 40 — 200	1643	82	18
8	25	25	# 42 — 200	1779	90	10
9	25	25	# 44 — 200	1475	71	29
10	25	25	# 46 — 200	1822	93	7
11	25	25	# 48 — 200	1522	74	26
12	25	25	# 52 — 200	2500	135	—
13	25	25	# 54 — 200	3064	170	—
14	25	25	# 56 — 200	3134	175	—

The chromatographic fractions from Sephadex G-200 gel filtration in Tris buffer containing 1M NaCl were desalted and used as presumptive inhibitor samples. Aliquots of mouse bone collagenase prepared by Sephadex G-200 gel filtration in 6M urea were used as the standard enzyme in this expeiment. The assay mixtures were incubated at 37°C for 2h.

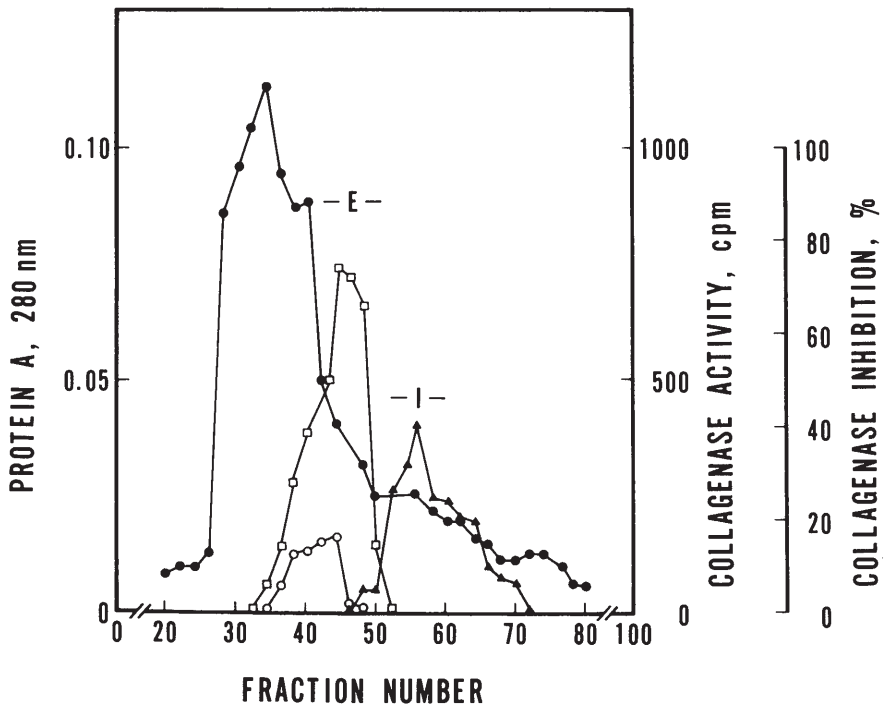

Fig. 2 Elution profile of mouse bone collagenase inhibitor and collagenase from a column (1.6 x 90 cm) of Sephadex G-200 gel, equilibrated with 6M urea in Tris buffer. The culture medium to which heparin had not been added was pooled, concentrated approx. 110-fold, and a sample of 2.5 ml was applied to the column. Fractions of 2.3 ml were collected at 7.5 ml/h. Desalted fractions (200 μl aliquots) were assayed for their abilities to inhibit the mouse bone collagenase (0.2 units) which had been isolated by Sephdex G-200 gel fitration in 1M NaCl. The results are expressed as percentage inhibition of the stated amount of enzyme. Desalted fractions (100 μl aliquots) were also assayed for collagenase activities after activation with p-APMA (total collagenase) or without activation (active collagenase). Incubation was carried out at 37°C for 2 h and 5 h, respectively.
(●——●): distribution of the protein measured in the absorbance at 280 nm.
(▲——▲): distribution of collagenase inhibitor activity. (□——□): distribution of total collagenase. (○——○): distribution of active collagenase,

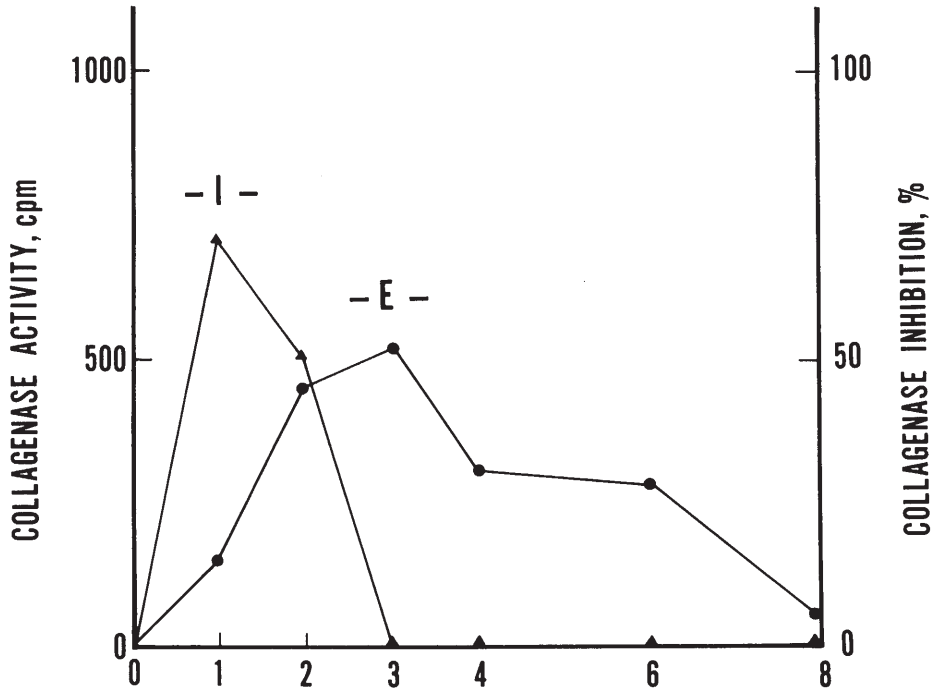

Fig. 3 The synthesis of collagenase inhibitor and collagenase in the tissue culture of mouse calvaria. The media collected on the definite days of culture were used. For the assay of collagenase inhibitor, the media were concentrated 7.5-fold, deslted, and then 100 μl aliquot samples were assayed for their abilities to inhibit the chick bone collagenase obtained from the Sephadex G-200 gel filtration column. The samples (100 μl aliquots) of the medium were also assayed for their collagenase activities after activation with p-APMA (25 μl). In both assays, Tris buffer was added to each assay tube to a total volume of 250 μl in all cases. The mixtures were incubated at 37°C for 17 h and 24 h, respectively.

DISCUSSION

Goldhaber[10] initially showed that the addition of small amount of a commercial heparin to the culture medium of mouse calvaria enhanced the bone resorption produced by suboptimal concentration of parathyroid extract (PTE). Moreover, Kaufman *et al.*[11] reported that the collagenolytic activity was increased by addition of PTE to the tissue culture medium, and still further enhanced when both PTE and heparin were added. Sakamoto *et al.*[9, 12] extended these studies and confirmed that heparin increased the amount of collagenase released into the culture medium from bone explants and also enhanced the activity of mouse bone collagenase. However, the mechanism involved herein remains unknown. In the present study, the time course analysis of the crude culture media revealed that the addtion of heparin markedly increased the activity of the collagenase (Fig. 1). The resluts are consistent with those of the studies noted above.

The gel filtration of the culture media which contained heparin in Tris buffer containing 1M NaCl failed to separate the collagenase inhibitor from the enzyme, although a substantial amount of total collagenase could be recovered (Table 1). In fact, the collagenase inhibitor

might exist in chromatographic fractions by gel filtration in 1M NaCl. However, it was speculated that the collagenase inhibitor could not be isolated due to possible interaction between the enzyme and inhibitor under this chromatographic condition.

We previously demonstrated that the yield of the chick bone collagenase inhibitor was approximately 1.4-fold higher by gel filtration in 6M urea than in 1M NaCl.[7] It was thus expected that gel filtration of the heparin-containing sample in 6M urea than in 1M NaCl.[7] It was thus expected that gel filtration of the heparin-containing sample in 6M urea would result in better separation of the inhibitor from the enzyme. However, this experiment also failed to separate the inhibitor from the enzyme. On the other hand, a similar gel filtration experiment using the medium sample which did not contain heparin discretely demonstrated both collagenase activity and collagenase inhibition as shown in Fig. 2. It is possible that the separation of the inhibitor from the enzyme could not be effected in the case of the heparin-containing samples partly because the samples contained larger amounts of enzyme than the chick bone culture medium samples,[7] but chiefly because the molecular weight of the mouse bone collagenase inhibitor[13] was larger than that of the chick bone collagenase inhibitor.[7]

The simultaneous synthesis of collagenase and collagenase inhibitor by the system of mouse calvaria culture is consistent with the results obtained from our previous study of the embryonic chick bone culture.[7] The result is also consistent with other recent studies.[14,15]

The control of the activity of bone collagenase may depend in part on the activity of the collagenase inhibitor which is also synthesized in bone tissue. The details of this control mechanism remain to be studied.

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