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# ISOLATION AND CHARACTERIZATION OF MOUSE BONE COLLAGENASE INHIBITOR

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## ABSTRACT

A specific collagenase inhibitor was isolated by Sephadex G-200 gel filtration in 6M urea from the culture medium of mouse bone, which simultaneously produced collagenase. The mouse bone collagenase inhibitor apparently blocked collagenases prepared from mouse bone, chick bone, and rabbit cornea. As the result of gel electrophoresis, the molecular weight of the inhibitor was estimated to be approx. 40,000 daltons. This inhibitor appeared to be similar to collagenase inhibitors isolated from other tissues of various species.

Keywords: Collagenase, Collagenase inhibitor, Gel filtration, SDS gel electrophoresis

## INTRODUCTION

Recently, Welgus *et al.*[1] suceeded in purifying a specific collagenase inhibitor of vertebrate collagenase produced by human skin fibrobrast. Vater *et al.*[2] also purified the inhibitor isolated from human tendon and demonstrated the irreversible inhibition of collagenase by the inhibitor. The molecular weights of these inhibitors were estimated to be 31,000 and 25,000 daltons, respectively.

We demonstrated the presence of a collagenase inhibitor in the culture medium of mouse bone.[3] We report here our further study for isolation and characterization of the inhibitor.

## MATERIALS AND METHODS

### 1. *Preparation of enzyme and inhibitor*

Calvaria of 5-day old Swiss Albino mice of Webster strain were cultured. Plastic culture flasks (Falcon Plastics, Los Angeles, Cal.) containing 10 calvaria/10 ml medium were incubated at 37° C in 95% $O_2$ and 5% $CO_2$ for 6 or 7 days consecutively. The culture medium was composed of mammalian Tyrode solution containing amino acids, vitamins, L-glutamin, penicillin and streptomycin as described by Shimizu *et al.*[4] No heparin was added to the medium. The media were changed at 2 days, 4 days of the culture up to 6 or 7 days. The pooled culture media which were frozen at −60° C were concentrated approx. 100-fold on PM-10 membrane (Amicon Corp. Lexington, Mass.) before being applied to the column.

Partially purified rabbit corneal collagenase was kindly provided by Dr. B. Johnson-Wint, Developmental Biology Laboratory at the Massachusetts General Hospital, Boston, Massachusetts, U.S.A..

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*2. Collagenase and collagenase inhibitor assay*

Collagenase activity was assayed as previously described.[5] Prior to assay, the samples of chromatographic fractions were activated with p-APMA (p-aminophenylmercuric acetate, Aldric Chemical Co., Milwaukee, Wisc.) for activation of latent collagenase.

Inhibitor activity was assayed by adding inhibitor preparations to a known amount of active chick bone collagenase as previously described.[6] Prior to assay, desalting was carried out by passing the sample fractions through PD-10 columns (Pharmacia Fine Chemicals, Piscataway, N.J.).

*3. Column Chromatography*

The concentrated culture media were chromatographed in a cold room on a column (1.6× 90 cm) of Sephadex G-200 (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.) as described.[3] The chromatographic fractions corresponding to the collagenase and the collagenase inhibitor were collected, and the samples were assayed for their collagenase activities and abilities to inhibit the active collagenase.

*4. Dialysis and lyophilization of the inhibitor*

The collected fractions were concentrated to approx. 5 ml on PM-10 membrane and were frozen at −60°C. The large amount of concentrates corresponding to the inhibitor were put together and were further concentrated to approx. 10 ml on PM-10 membrane. Then the concentrated sample was dialyzed against deionized water and thereafter was lyophilized.

*5. Gel electrophoresis*

The method used was that of Fairbanks *et al.*[7] with 5.6% polyacrylamide gels. This system employs a Tris/acetate/EDTA buffer at pH 7.4 containing 1% sodium dodecyl sulfate (SDS).

As standard protein samples, the kit of SDS gel electrophoresis standards (Bio-Rad laboratories, Richmond, Cal.) which consists of a series of natural proteins with molecular weights ranging from 14,400 (lysozyme) to 92,500 daltons (phosphorylase B) was used. The standard samples as well as the lyophilized inhibitor were dissolved in the sample solution as described.[7]

## RESULTS

Fig. 1 shows the elution profile of mouse bone collagenase and collagenase inhibitor from a column of Sephadex G-200 gel, equilibrated with 6M urea in Tris buffer. As indicated, the elution was monitored by the absorbance at 280 nm. The major protein peak was obtained at the elution position corresponding to fraction number 28 to 34 where neither appreciable amount of enzyme nor inhibitor was detected in the eluate.

Active and latent collagenase emerged in the identical elution position corresponding to fraction number 36 to 48.

The inhibitor was found in the elution position, fraction number 50 to 60. It was observed that the inhibitor was almost completely separated from the enzyme. Those fractions corresponding to the inhibitor were collected, concentrated, and preserved in a freezer at −60°C. The pooled inhibitor sample was rechromatographed on a column (2.5 × 90 cm) of Sephadex G-200 equilibrated with 6M urea in Tris buffer. And, as shown in Fig. 2, a discrete peak of collagenase inhibition was obtained. No collagenase activity was detected in the preceding fractions (not shown in Fig. 2). The eluate corresponding to fraction number 57 to 66 was collected as the pure collagenase inhibitor.

In addition to mouse bone collagenase, the isolated mouse bone collagenase inhibitor also blocked collagenases isolated from the tissues of different species. Table I shows the

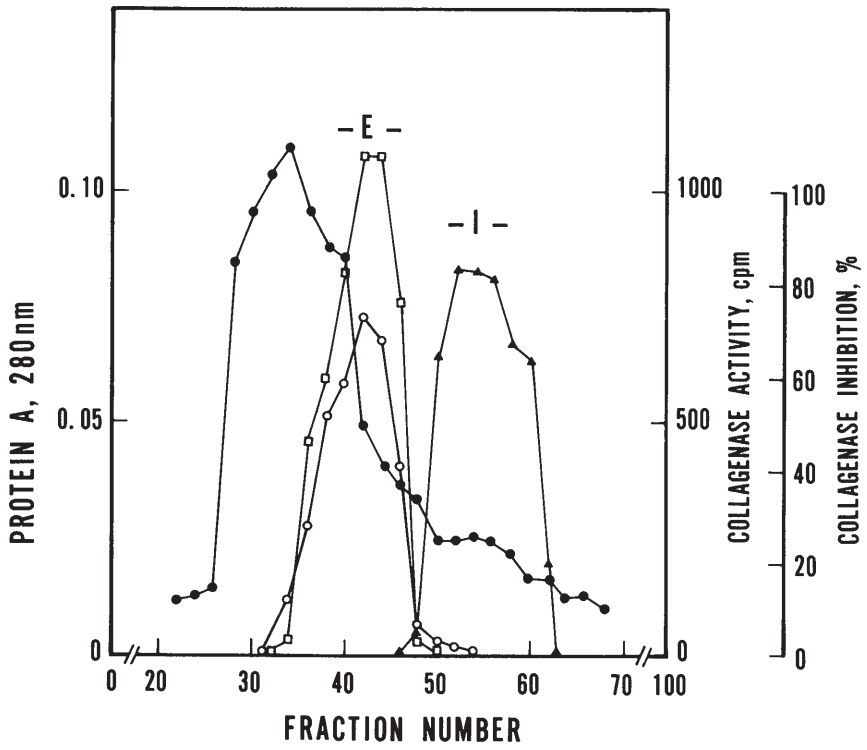


Fig. 1 Collagenase and collagenase inhibitor from mouse bone culture chromatographed by Sephadex G-200 gel column (1.6 × 90 cm), equilibrated with 6M urea in Tris buffer.
The pooled culture media (approx. 100-fold concentrated, 2.5 ml) was gel-filtered. The flow rate was 7.5 ml/h and fractions of 2.3 ml were collected. Desalted fractions (50 μl aliquots) were assayed for their collagenase activities after activation with p-APMA (total collagenase) or without activation (active collagenase). Desalted fractions (200 μl aliquots) were also assayed for their abilities to inhibit chick bone collagenase (25 μl aliquots). The results are expressed as percentage inhibition of the stated amount of enzyme.
The incubation periods for the assays were 21 h and 17 h at 37°C, respectively.
(●——●): Protein monitored by the absorbance at 280 nm. (□——□): total collagenase. (○——○): active collagenase. (▲——▲): collagenase inhibitor activity.

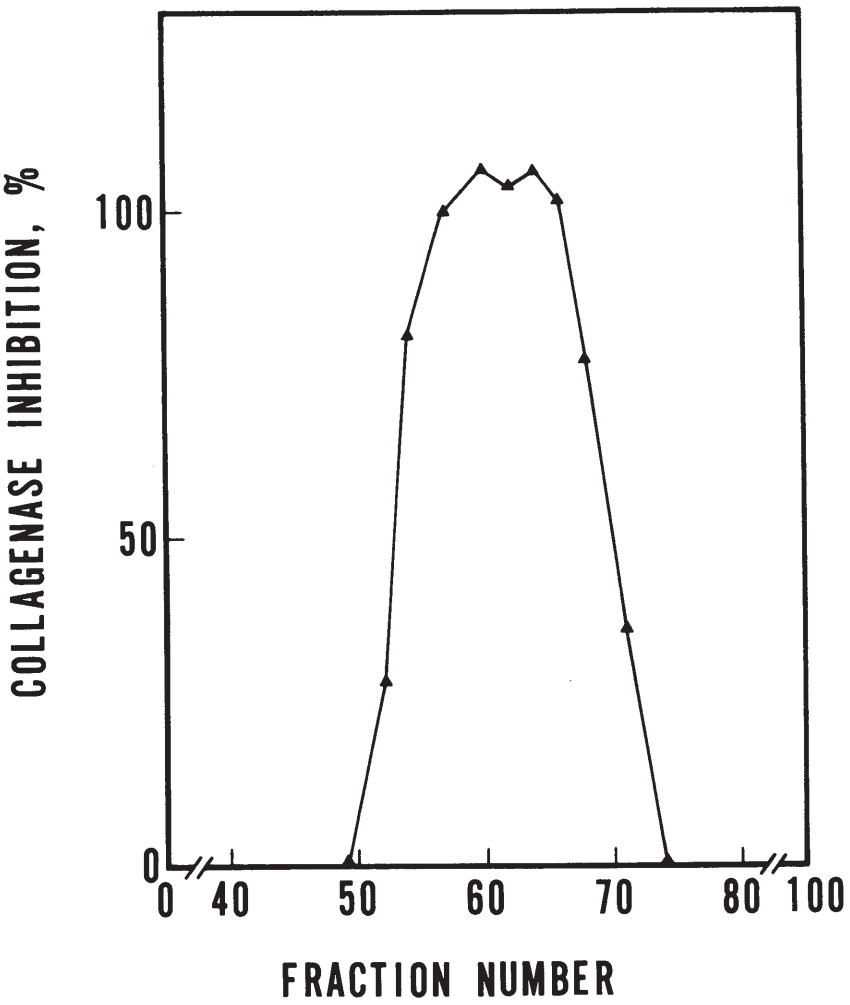


Fig. 2 Elution profile of mouse bone collagenase inhibitor from a column (2.5×90 cm) of Sephadex G-200 gel, equilibrated with 6M urea in Tris buffer (rechromatography).
The pooled inhibitor fractions (concentrated, approx. 5 ml) were gel-filtered. The flow rate was 15 ml/h and fractions of 4.6 ml were collected. Desalted fractions (50 $\mu$l aliquots) were assayed for their abilities to inhibit 25 $\mu$l aliquots of chick bone collagenase preparation. The results are expressed as percentage inhibition of the stated amount of enzyme. Incubation was carried out at 37°C for approx. 16 h.

Table 1. Inhibition of rabbit corneal collagenase by mouse bone collagenase inhibitor

| Rabbit Corneal Collagenase μl | Inhibitor Fraction μl | Activity cpm | Activity % | Inhibition % |
|---|---|---|---|---|
| 50 | 0 | 994 | 100 | 0 |
| 50 | 25 | 979 | 98 | 2 |
| 50 | 50 | 1009 | 102 | 0 |
| 50 | 100 | 673 | 49 | 51 |
| 50 | 200 | 526 | 25 | 75 |

Mouse bone collagenase inhibitor purified by 6M urea-Sephadex G-200 gel filtration (fraction #82, diluted 10-fold) and rabbit corneal collagenase (0.528 units/ml) were used. Tris buffer was added to each experimental tube to a total volume of 250 μl in all cases. The mixtures were incubated at 37°C for 18.5 h.

inhibition of rabbit corneal collagenase by the inhibitor. This inhibitor blocked chick and collagenase as well.

SDS gel electrophoresis of this inhibitor revealed one major band with an approx. molecular weight of 40,000 daltons, However, the band was not clear; therefore precise molecular weight of the inhibitor could not be determined.

Further purification and characterization of this inhibitor are in progress.

## DISCUSSION

We previously demonstrated that the inhibitor collected from culture medium of chick bone was a specific collagenase inhibitor and was different from $\alpha_2$-macroglobulin,[6] the principal collagenase inhibitor in serum,[1] but similar to other collagenase inhibitors isolated from human skin fibrobrast,[1] human tendon,[20] and smooth muscle cells.[3] Similarly, it was thought that the collagenase inhibitor derived from mouse bone culture was a specific one. The inhibitor was obtained from serum-free culture medium and its molecular weight was estimated to be approx. 40,000 daltons; therefore the inhibitor was different from $\alpha_2$-macroglobulin.[9] It was supposed that the difference in molecular weight between the chick bone[6] and mouse bone collagenase inhibitor was attributable to the species specificity. However, the mouse bone collagenase inhibitor blocked chick bone collagenase (Fig. 1, 2) as well as mouse bone collagenase[3] and rabbit corneal collagenase. Thus this inhibitor appears to have no species specificity on different collagenase preparations. The result was consistent with other recent studies.[1,2,6]

Purification and characterization of the mouse bone collagenase inhibitor was not sufficient enough to elucidate the role of this inhibitor in the latency of mouse bone collagenase and this subject remains to be studied.

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