LATENT COLLAGENASE FROM THE CULTURE MEDIUM OF EMBRYONIC CHICK BONES

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1. Introduction

Enzymatically inactive collagenases have been isolated from several tissues which may be activated by tissue culture medium or trypsin [1–5], suggesting synthesis by the cells of a precursor zymogen which is then enzymatically activated by scission of a portion of the larger precursor molecule. However, 3 M NaSCN was found [6,7], which dissociates a number of antigen–antibody complexes, was as effective as trypsin in converting the inactive form of the collagenase to an active form, suggesting that the inactive form is a non-covalently bound complex of active enzyme and inhibitor. Additional evidence for this latter proposal is provided [8–10]. We have recently been able to isolate a latent collagenase from the tissue culture medium of embryonic chick bones and have studied the nature of the latent enzyme.

2. Methods

2.1. *Tissue culture*

The cartilage-free portions of 14-day embryonic chick tibiae were cultured in Leighton-type roller tubes containing 50 units/ml heparin in a fashion similar to that for mouse bone [11]. Tubes containing 4 bones/2 ml medium were cultured at 37°C in 95% O_2, 5% CO_2, for 4 or 6 days without changing the medium. In some instances, the cultures were fed every second day and culture continued for 8 days.

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2.2. *Collagenase assay*

Collagenase activity was assayed by measuring the release of ^{14}C-labelled peptides from reconstituted fibrils of radioactively labelled rat skin collagen [11].

2.3. *Activation of latent collagenase*

Three methods were employed for the activation of latent collagenase:

2.3.1. Trypsin treatment [2]

Preliminary experiments were done to determine the concentrations of trypsin and the duration of incubation required for maximal activation of latent enzyme. Generally, culture media or chromatographic fractions were incubated with trypsin (Trypsin-TPCK, Sigma) at a concentration of 1–4 μg/ml for 5 min at ambient temperature (22°C). After incubation, soybean trypsin inhibitor (15X excess, Sigma) was added and incubated for 10 min prior to the collagenase assay.

2.3.2. NaSCN treatment [6]

Samples were dialyzed against 200-fold vol. 3 M NaSCN in 50 mM Tris–HCl buffer, pH 7.6, containing 200 mM NaCl and 5 mM $CaCl_2$ for 16 h at 4°C, then dialyzed against large volumes of the same buffer without NaSCN.

2.3.3. NaI treatment

Concentrated samples were activated by mixing with equal vol. 6 M NaI in Tris buffer. The mixture was then immediately diluted 10-fold with the same Tris buffer without NaI and concentrated with a Diaflo PM-10 membrane (Amicon Corp., Lexington, MA) to the original sample volume and charged on a column of Sephadex G-200 (Pharmacia Fine Chemi-

cals, Piscataway, NJ). In some instances, the mixture of latent enzyme sample and NaI was directly charged on the columns of Sephadex G-50 superfine [8] or Sephadex G-200. Alternatively, samples were dialyzed against 3 M NaI in Tris buffer for 16 h at 4°C and then dialyzed thoroughly against a large volume of the same buffer without NaI. In other experiments, samples were mixed with NaI and made to 3 M and then thoroughly dialyzed against Tris buffer to remove NaI completely.

2.4. *Sephadex G-200 gel filtration for molecular weight estimations*

Latent collagenase and activated collagenase samples were chromatographed on a calibrated column (2.5 × 90 cm) of Sephadex G-200. The column was run in 50 mM Tris–HCl buffer, pH 7.6, containing 200 mM NaCl and 5 mM $CaCl_2$. Molecular weights were estimated as in [12] using standard proteins of known molecular weights.

2.5. *Other methods*

Heparin concentration of the column effluent was affirmed as in [13] and the results expressed as A_{530}.

3. Results

3.1. *Demonstration of latent collagenase in the culture medium*

No active collagenase activity was detected in the culture media of 14-day embryonic chick bones. However, if the culture medium was treated with either trypsin or NaSCN, considerable collagenase activity was demonstrated (table 1). When heparin was omitted from the medium, neither active nor latent collagenase could be detected. Addition of heparin to the medium after culture did not convert a latent enzyme to an active form, indicating that heparin affected the synthesis and/or release of latent collagenase from the bone explants into the culture medium during the incubation period, similar to results reported for mouse bone collagenase [11]. It is not clear whether in the absence of heparin the cells fail to synthesize or extrude a latent enzyme into the tissue culture media, or whether an inactive form of the enzyme is synthesized but not excreted, or excreted and not activated by either trypsin or NaSCN. Exposure of trypsin-activated culture medium with NaSCN increased the activity from 2.35–3.10 units/ml, but the treatment of NaSCN-activated culture medium with trypsin decreased the activity from 3.26–2.86 units/ml, suggesting that trypsin partially degraded the NaSCN-activated collagenase. No additive effect was observed when NaSCN and trypsin were added in that order.

When cultures were fed every second day and cultured for 8 days, 1.7, 1.63, 0.45 and 0 units/ml latent collagenase was recovered in the media at the 2nd, 4th, 6th and 8th days, respectively. When cultured for longer periods without the addition of fresh tissue culture media, less latent collagenase activity was also detected: 2.23 and 2.45 units/ml for 4 and 6 day incubations, respectively. No significant amount of active collagenase activity was detected whether the culture was fed every 2 days or continued without the addition of fresh media.

Table 1
Collagenase activity in the culture medium of embryonic chick bones (units/ml)[a]

	Untreated	Trypsin-treated	SCN^--treated	Trypsin-treated then SCN^--treated	SCN^--treated then trypsin-treated
Heparin absent	0	0	0.06	n.d.	n.d.
Heparin present[b]	0	2.35	3.26	3.10	2.86
Heparin added postculture	0	0	0	n.d.	n.d.

[a] One collagenase unit is defined as the amount of enzyme activity necessary to digest 1 μg collagen in 1 min under the above assay conditions
[b] 50 units/ml

3.2. *Activation of latent collagenase by trypsin*

Figure 1 shows the relationship between the amount of trypsin and the time of incubation for optimal activation of the latent collagenase obtained from two lots of culture media. Although the activation of latent enzyme in the culture media by trypsin varied to some extent, incubation with 1–4 μg/ml trypsin for 5 min was generally used for the activation of latent collagenase. For the activation of concentrated latent collagenase samples, a proportionately higher concentration of trypsin was used for 5 min.

3.3. *Gel-filtration studies of latent and activated chick bone collagenases*

Figure 2 shows the elution profiles of latent chick bone collagenase from a column of Sephadex G-200 after reaction with trypsin and with NaSCN. There is an apparent difference in the molecular weights of the latent chick bone collagenase before (fig.2A) and after (fig.2B) the ammonium sulfate fractionation used to partially purify the enzyme. A similar change was previously demonstrated for mouse bone collagenase [14]. The difference is due to the formation of a high molecular weight complex of collagenase with heparin. Heparin in the culture medium was virtually eliminated by ammonium sulfate fractionation. Figure 2A includes the distribution of heparin

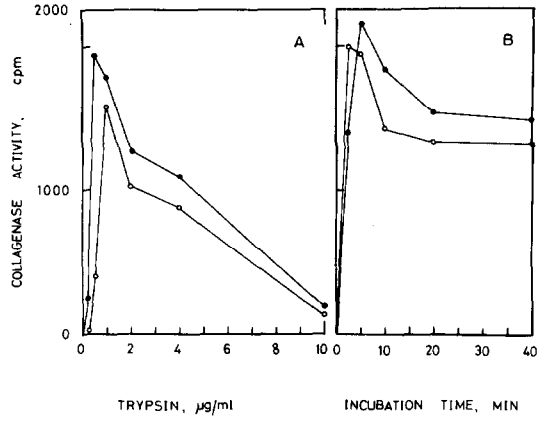

Fig.1. Relationship between the amount of trypsin in culture media of embryonic chick bones and incubation time for optimal activation of latent collagenase in two experiments (–●–; –○–). (A) Activity versus trypsin concentration; (B) activity versus incubation time.

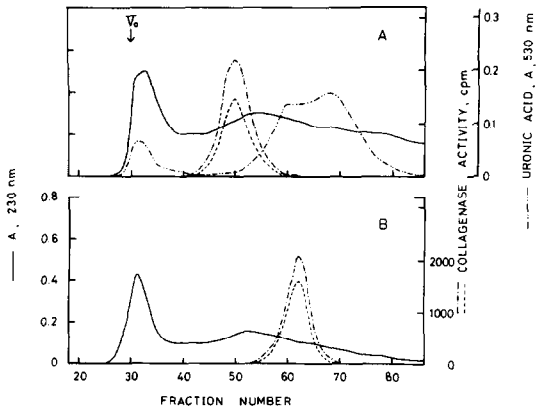

Fig.2. Elution profiles of latent chick bone collagenase from a column of Sephadex G-200. (A) 4 ml concentrated (20-fold) culture medium was applied to a column (2.5 × 90 cm) of Sephadex G-200 equilibrated with 50 mM Tris–HCl buffer, pH 7.6, containing 200 mM NaCl and 5 mM $CaCl_2$. Fractions, 5 ml, were collected at 15 ml/h. Aliquots of each fraction were activated by treatment with trypsin (- - - -), with NaSCN (–·–·–) and then assayed for collagenase activity. The distribution of heparin is indicated by A_{530} (–···–···–) for uronic acid. (B) 4 ml sample of latent collagenase obtained by ammonium sulfate fractionation from 200 ml pooled culture medium was gel filtered and analyzed as in (A). No uronic acid was detected in this experiment.

in the effluent fraction as determined by the amount of uronic acid.

Figure 3 shows the elution profiles of activated chick bone collagenase from a column of Sephadex G-200. The latent collagenase was precipitated out and the heparin removed by ammonium sulfate (0–60% saturation), and activated by trypsin (fig.3A), NaSCN (fig.3B), or NaI (fig.3C). The elution position of the active collagenase is similar regardless of how the latent enzyme was converted, suggesting that there is only one form of the latent collagenase in the culture medium.

3.4. *Apparent molecular weights of latent and active chick bone collagenases*

Latent chick bone collagenase was found to have an approx. mol. wt 54 000; the activated enzyme an approx. mol. wt 43 000 (fig.4). Latent chick bone collagenase was approx. mol. wt 130 000 when the enzyme existed as a complex with heparin (fig.2A).

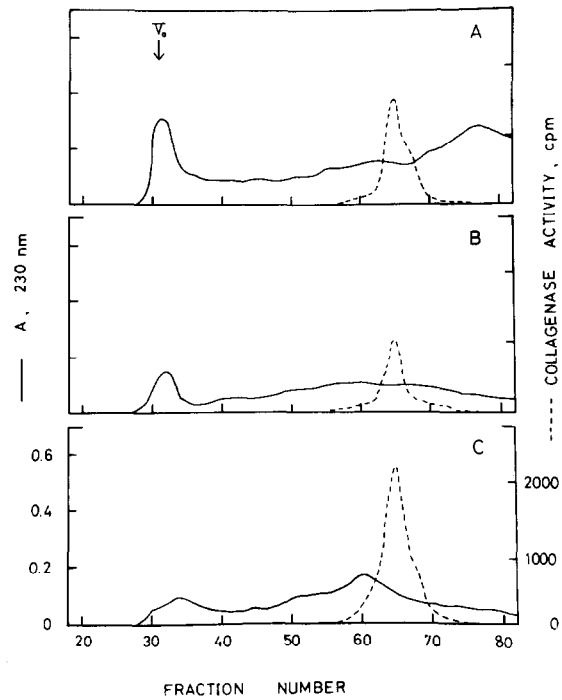

Fig.3. Elution profiles of activated chick bone collagenases from a column of Sephadex G-200. Aliquots of latent collagenase prepared by ammonium sulfate fractionation were activated by reaction with trypsin (A), NaSCN (B) and with NaI (C), and the activated collagenase gel-filtered and assayed for enzyme activity.

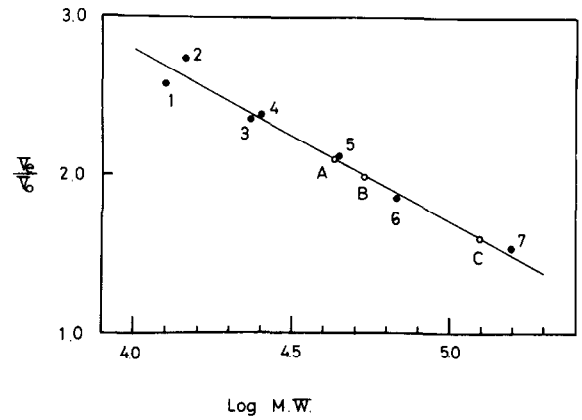

Fig.4. Whitaker plot of latent and active collagenases and of standard protein for determination of the approximate molecular weights of the enzymes. (A) Activated collagenase; (B) latent collagenase; (C) latent collagenase complex with heparin; (1) cytochrome C; (2) ribonuclease; (3) trypsin-DFP; (4) γ-chymotrypsinogen A; (5) egg albumin; (6) bovine serum albumin; (7) aldolase.

3.5. *Reinactivation of NaI activated collagenase after the removal of NaI*

When the sample containing latent collagenase and NaI was charged on a column of Sephadex G-50 superfine [8], we were frequently unable to recover enzyme activity. On the other hand, when the mixture was charged on a column of Sephadex G-200, activated collagenase activity was readily recovered with a yield of 60–70%. These observations led us to suspect that an intact inhibitor might be present which was binding to the activated enzyme after the removal of NaI. Table 2 presents the results of experiments in which collagenase activity could be elicited with trypsin,

Table 2
Reversible activation of latent collagenase by NaI

Activation procedure		Activity (units/ml)	% Activity recovery
A.	Trypsin	2.91	100[a]
B.	3 M NaSCN	3.06	105
C.	Dialysis versus 3 M NaI	0.61	21
D.	Mixed with NaI to 3 M, then:		
	(a) Gel filtered with Sephadex G-200	1.89	65
	(b) Dialyzed versus Tris buffer	0	0
	(c) Dialyzed versus Tris buffer, then treated with trypsin	2.47	85

[a]Yield by trypsin method was set as 100% activity recovery

with NaSCN, with NaI and with a combination of NaI and trypsin.

Latent collagenase was activated to a lesser extent (21% of that with trypsin) by dialysis against 3 M NaI in a similar way to that by 3 M NaSCN. When the sample containing latent collagenase and NaI was gel-filtered on a column of Sephadex G-200, the recovery of enzyme activity in the effluent fractions was 65% of the starting activity determined by activation of the latent enzyme with trypsin. However, no activity was detectable when the mixture was dialyzed against a buffer in order to remove NaI. However, when the dialyzed sample was subsequently treated with trypsin, collagenase activity was reactivated with recovery of approx. 85% starting enzyme activity.

4. Discussion

The collagenase released into the culture medium of 14-day embryonic chick bones is virtually all in the form of a latent enzyme. There is no significant conversion of the latent form to the active form when cultured for 8 days. It is not clear to what extent this is due to an excess of inhibitors and/or to the relative absence of activating factor(s) or both.

Heparin was found to be essential for the synthesis and/or release of latent chick bone collagenase into the culture medium from the explants. These results are similar to those reported for mouse bone collagenase [15]. Heparin was not effective, however, in stimulating production of collagenase in cultures of mouse skin and human gingiva. Although latent collagenase has been isolated [8] from the culture medium of embryonic chick skin to which no heparin was added, it was shown [16] that heparin not only stimulates the release of collagenolytic activity from sarcoma and carcinoma cells, but also maintains their viability throughout the culture period. It is therefore not clear how heparin affects the synthesis and/or release of tissue collagenases in culture. Although heparin has made it possible to demonstrate and obtain latent collagenase in the culture medium of embryonic chick bones, it has hampered the subsequent characterization of the enzyme, since it forms high molecular weight complexes with the enzyme (fig.2A). When this preparation of latent collagenase is activated by treatment with trypsin and gel-filtered, the activated enzyme is approx. mol. wt 80 000–90 000, as compared with mol. wt 54 000 for latent collagenase and 43 000 for activated collagenase after heparin is removed.

The difference in molecular weight between latent and active enzyme is approx. 11 000. Although we have not yet been able to isolate this low molecular weight component derived from latent collagenase by activation, it seems reasonable to conclude that it represents the component responsible for the latency of the enzyme. Moreover, the conversion of the latent enzyme to the active form by NaI and NaSCN, makes it very likely that latent chick bone collagenase exists as a non-covalently bound complex of active enzyme and inhibitor(s).

It is interesting that latent collagenase activated by NaI becomes inactive when the NaI removed by dialysis, but can again be reactivated by the addition of trypsin for example. It seems most likely that this is owing to the reassociation of an inhibitor(s) with the active enzyme after the NaI is removed by dialysis.

Since the completion of this study the isolation of a latent collagenase in the culture medium of human fetal skin has been reported [17] where active collagenase activity was recovered after dialyzing the preparation free of NaI. Although this difference between latent human fetal skin collagenase and chick bone collagenase may be species dependent, further studies are needed in order to determine whether certain tissues contain relatively high molecular weight inhibitors as well as low molecular weight inhibitors, and the relative roles of each in regulating enzyme activity.

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References

[1] Harper, E., Bloch, K. J. and Gross, J. (1971) Biochemistry 10, 3035–3041.

[2] Vaes, G. (1972) Biochem. J. 126, 275–289.
[3] Bauer, A. E., Stricklin, G. P., Jeffrey, J. J. and Eisen, A. Z. (1975) Biochem. Biophys. Res. Commun. 64, 232–240.
[4] Birkedal-Hansen, H., Cobb, C. M., Taylor, R. E. and Fullmer, H. M. (1975) Scand. J. Dent. Res. 83, 302–305.
[5] Birkedal-Hansen, H., Cobb, C. M., Taylor, R. E. and Fullmer, H. M. (1976) Biochim. Biophys. Acta 426, 229–238.
[6] Abe, S. and Nagai, Y. (1972) J. Biochem. (Tokyo) 71, 919–922.
[7] Abe, S., Shinmei, M. and Nagai, Y. (1973) J. Biochem. (Tokyo) 73, 1007–1011.
[8] Shinkai, H., Kawamoto, T., Hori, H. and Nagai, Y. (1977) J. Biochem. (Tokyo) 81, 261–263.
[9] Sellers, A., Cartwright, E., Murphy, G. and Reynolds, J. J. (1977) Biochem. J. 163, 303–307.
[10] Sellers, A., Cartwright, E., Murphy, G. and Reynolds, J. J. (1977) Biochem. Soc. Trans. 5, 227–229.
[11] Sakamoto, S., Goldhaber, P. and Glimcher, M. J. (1972) Calcif. Tiss. Res. 10, 142–151.
[12] Whitaker, J. R. (1963) Anal. Chem. 35, 1950–1953.
[13] Dische, Z. (1947) J. Biol. Chem. 167, 189–198.
[14] Sakamoto, S., Sakamoto, M., Goldhaber, P. and Glimcher, M. J. (1975) Biochim. Biophys. Acta 385, 41–50.
[15] Sakamoto, S., Goldhaber, P. and Glimcher, M. J. (1973) Calcif. Tiss. Res. 12, 247–258.
[16] Kuettner, K. E., Soble, L., Croxen, R. L., Marczynska, B., Hiti, J. and Harper, E. (1977) Science 196, 653–654.
[17] Shinkai, H. and Nagai, Y. (1977) J. Biochem. (Tokyo) 81, 1261–1268.