# MULTIPLE STEPS IN THE ACTIVATION OF THE INACTIVE PRECURSOR OF BONE COLLAGENASE BY TRYPSIN

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## 1. Introduction

Neutral collagenase is released as an inactive precursor or proenzyme [1–3], a "pro"-collagenase, by mouse bone explants in culture. The proenzyme is activated by a limited proteolysis under the action of trypsin, chymotrypsin or purified lysosomes [2, 3]. Recent studies, here reported, indicate that trypsin has a double action: i) it activates a latent precursor of an enzyme which is able to activate the pro-collagenase, and ii) it renders the pro-collagenase susceptible to the action of that activator.

## 2. Experimental

Tissue culture of tibiae from 5-day-old mice, subsequent treatment of the culture media and collagenase assays [4] on radioactive collagen in solution (incubation time: 30 min) were as reported [3]. The results of the assays are expressed in units/ml of culture fluid, one unit referring to the decomposition of 1 μg of collagen/min.

The activation of the Tris-buffered (pH 7.5) culture media was done by preincubation at 25° with trypsin for various lengths of time before the addition of soya-bean trypsin inhibitor (40 μg/ml). *"Non-activated" media* are culture fluids that have been added to a preformed mixture of trypsin and soya-bean inhibitor so as to achieve final conc. of 2 μg of trypsin/ml and 40 μg of inhibitor/ml. *"Subactivated" media* have been in a limited contact with trypsin, insufficient to elicit any significant collagenase activity from its precursor. They were treated for either 1 or 4 min with 2 μg of trypsin/ml prior to the addition of the inhibitor, to provide, respectively, "subactivated (1 min)" or "subactivated (4 min)" media. *"Activated" media* were treated with 5 μg of trypsin/ml for 10 min at 25° before the addition of the inhibitor; the pro-collagenase was maximally activated by that treatment. In some experiments, DFP (diisopropylfluorophosphate, 2 mM) was substituted to the soya-bean inhibitor.

## 3. Results and discussion

Activation curves of pro-collagenase obtained by preincubating culture fluids for increasing time with various concentrations of trypsin are shown in fig. 1. The use of low doses of trypsin and of short preincubation times allows one to distinguish two steps in the activation, a latency period and a subsequent rapid activation; the latency increases with decreasing concentrations of trypsin. The same amount of active collagenase is reached by treatment with high or low doses of trypsin.

Culture fluids in which different levels of activation by trypsin had first been achieved, were subsequently incubated at 25° prior to the collagenase assays (fig. 2). The pro-collagenase present in "non-activated" medium (curve 1) remains latent over several hours of incubation at 25° whereas collagenase remains fully active over that period in "activated" medium (curve 2). Dilution of "activated" medium by half with "non-activated" medium merely reduces by half its collagenase activity (curve 3). "Activated" culture fluid is thus unable to elicit free collagenase from the pro-collagenase present in "non-activated" medium.



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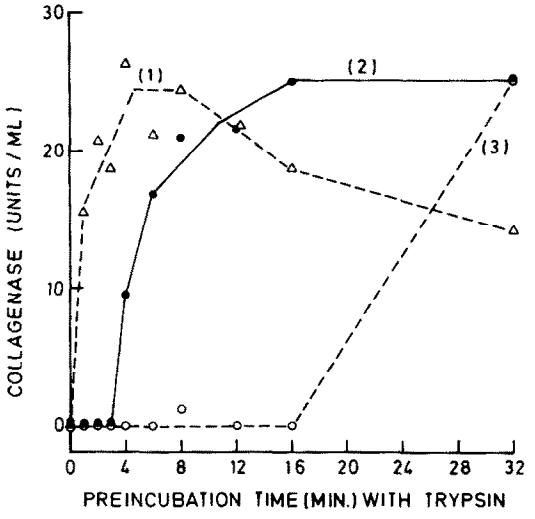


Fig. 1. Activation of bone pro-collagenase by various concentrations of trypsin. The culture media were preincubated for the time indicated with either 8 (curve 1, △), 2 (curve, 2, •) or 0.5 (curve 3, ○) μg of trypsin/ml before the addition of soya-bean trypsin inhibitor.

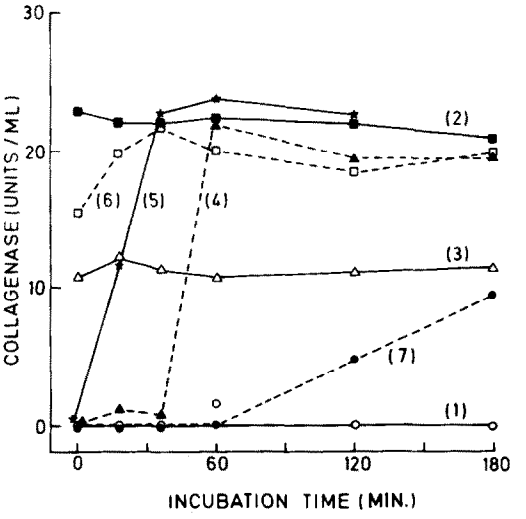


Fig. 2. Activation of bone pro-collagenase subsequent to the action of trypsin. Collagenase was assayed, either directly or after incubation at 25° for various lengths of time, in the following media: "non activated" medium (curve 1, ○); "activated" medium (curve 2, ■); "activated" medium diluted by half with "non-activated" medium (curve 3, △); "subactivated (1 min)" medium (curve 4, ▲); "subactivated (4 min)" medium (curve 5, ★); "subactivated (1 min)" medium diluted by half with "activated" medium (curve 6, □); "subactivated (1 min)" medium diluted by half with "non-activated" medium (curve 7, •). Curves similar to curves 2, 4 or 6 were observed when DFP was substituted to the soya-bean inhibitor to block the trypsin action in either "activated" or "subactivated (1 min)" media.

However, "subactivated (1 min)" medium activates itself spontaneously after a latency period of 30–60 min (curve 4). By increasing the time of action of trypsin, as done in "subactivated (4 min)" medium (curve 5), the latency period is considerably shortened but the rate of activation of pro-collagenase is not significantly changed and the same amount of active collagenase is obtained. Thus a limited action of trypsin on the culture fluids, that was insufficient by itself to elicit any free collagenase activity from the pro-collagenase, has nevertheless triggered an endogenous mechanism leading to the activation of pro-collagenase after a latency period of variable length. This latency decreases when trypsin has been allowed to act for a longer time on the medium before being blocked by the soya-bean inhibitor.

The "subactivated (1 min)" medium was also diluted by half with either the fully "activated" medium (curve 6) or the "non-activated" medium (curve 7). "Activated" medium activates rapidly the pro-collagenase present in the "subactivated (1 min)" medium whereas, on the contrary, "non-activated" medium does not activate it (and even retards its spontaneous activation). Thus when the pro-collagenase has been in contact with trypsin but is still in the latency phase, as is the case in "subactivated" medium, its incubation with a fully "activated" medium causes its direct activation without latency. This indicates that "activated" medium contains an "activator" of pro-collagenase which has been elicited by trypsin from an inactive precursor and which is able to activate the pro-collagenase present in "subactivated" medium. The kinetics of its action is shown in fig. 3. With increasing concentrations of activator ("activated" medium) or with longer times of incubation, increasing amounts of collagenase are elicited from the pro-collagenase of the "subactivated (1 min)" medium. The low rate of reaction at low concentrations of activator could be interpreted as indicating that low concentrations of activator are inhibited by some component of either the "subactivated (1 min)" or the "non-activated" medium.

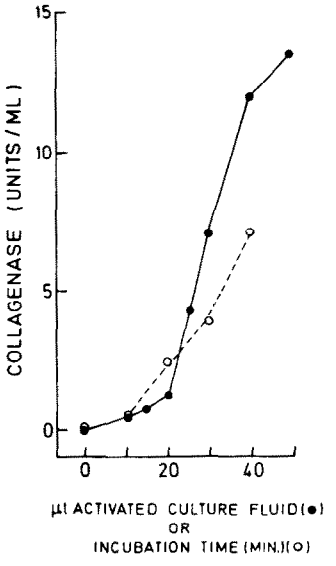


Fig. 3. Kinetics of the activation of "subactivated" medium by the endogenous activator. "Subactivated (1 min)" medium (400 µl) or, for the blanks, "non-activated" medium, was incubated at 25° with "activated" medium before the collagenase assays. The curves show the amounts of collagenase that have been elicted from the "subactivated" medium by its incubation either for 10 min with 50 µl of a mixture of "activated" and "non-activated" medium containing increasing amounts of "activated" medium (●) or for increasing time with a similar mixture containing 10 µl of "activated" medium (○).

The molecular weight of that "activator", estimated from its elution behaviour upon fractionation of the culture fluids on columns of Sephadex G-200, is close to 100,000 thus similar to that of collagenase itself. Its activity is completely lost by heating for 15 min at 100° but it is only partially decreased by heating for 10 min at 60°, conditions that inactivate completely the collagenase. Diisopropylfluorophosphate (0.33 or 3.33 mM) has no effect on either activity. Addition of 5% (v/v) human serum inhibits completely the activator but only partially (−80 to −85%) the collagenase. Casein inhibits the activator (−50 to −80%) at concentrations (1 to 4 mg/ml) that have little effect on the activity of collagenase. It appears thus likely that the activator is an enzyme, possibly a proteolytic enzyme that could be identical with a neutral protease, active on casein, which has been found in a latent, trypsin-activatable state in the culture media (G. Vaes, unpublished work).

These data may tentatively be interpreted as indicating that trypsin causes the activation of the latent precursor of collagenase by a combination of two actions. Indeed i) activation occurs only on the pro-collagenase present in culture fluids that have been in contact with trypsin and ii) it occurs then under the action of an activator enzyme that has been progessively elcited by trypsin from an inactive precursor, either a proenzyme (a "proactivator") or a dissociable enzyme–inhibitor complex. The molecular nature of the two transformations induced by trypsin is presently under study.

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