PURIFICATION OF PROCOLLAGENASE AND COLLAGENASE BY AFFINITY CHROMATOGRAPHY ON SEPHAROSE–COLLAGEN

Chantal GILLET, Yves EECKHOUT and Gilbert VAES

Laboratoire de Chimie Physiologique, Université de Louvain and International Institute of Cellular and Molecular Pathology, UCL 7539, Avenue Hippocrate, 75, 1200 Bruxelles, Belgium

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1. Introduction

Specific animal collagenases (EC 3.4.24.3) have been purified by several methods including affinity chromatography on collagen coupled to agarose [1–3] or entrapped in polyacrylamide beads [4]. The occurrence of latent forms of collagenase – or procollagenases – is now well documented [5] but their molecular structure – zymogens or enzyme-inhibitor complexes – and their role in physiology or pathology remain to be elucidated. The present report demonstrates that the procollagenase which is released by mouse bone explants in culture [6] exhibits a striking affinity for native salt-soluble collagen coupled to agarose and that it can be extensively purified (to a specific activity of about 3500 units/mg protein) by affinity chromatography. This efficient, rapid and simple method should have broad applicability in the purification of other mammalian (including human) procollagenases. A preliminary abstracted account of this work has already been presented [7].

2. Materials and methods

Tissue culture of tibiae and calvaria from 5-day-old mice, subsequent treatment of the culture fluids, preparation of salt-soluble ^{14}C-labelled native collagen, activation of procollagenase by trypsin and collagenase assays on [^{14}C]collagen in solution at 25°C (maximum incubation time: 30 min) were as reported [6]. One unit of procollagenase corresponds to the amount of latent enzyme that gives rise, after its full-activation by trypsin, to one unit of collagenase, i.e., to an activity which degrades 1 μg of native soluble collagen per minute at 25°C (this corresponds approximately to the degradation of 1 μg gelified collagen/minute at 37°C).

Collagen was coupled to agarose (Sepharose 4 B, Pharmacia) following the procedure of Bauer et al. [1] with slight modifications. Twentyfive millilitres of packed CNBr-activated Sepharose 4 B were washed with 300 ml ice-cold 0.1 M $NaHCO_3$, pH 9 and promptly added to 25 ml of a solution of ^{14}C-labelled salt-soluble collagen (1 mg/ml in 0.2 M NaCl, 0.1 M $NaHCO_3$, pH 9). The mixture was gently stirred for 16–20 h at 4°C, packed in a refrigerated Pharmacia K 16/20 column (1.6 × 20 cm) and washed with 125 ml of 0.2 M lysine, pH 9 and 125 ml of CCA buffer (50 mM sodium cacodylate–HCl, 5 mM $CaCl_2$ and 0.2 mg/ml NaN_3, pH 7.0). The amount of collagen bound to Sepharose was estimated by the radioactivity measured in the washing fluid and in the Sepharose–collagen itself. In all cases the efficiency of linkage was at least 80% (0.8 mg collagen/ml packed gel).

Affinity chromatography (flow rate: 22 ml/h) was carried out at 4°C on 18 ml of Sepharose–collagen with continuous registration of light transmission at 280 nm. Protein concentrations were determined [8] using bovine serum albumin as a standard. All reagents used were of analytical grade.

3. Results

In preliminary experiments trypsin-activated culture fluids were applied to a column containing collagen–Sepharose which had been equilibrated with 0.05 M

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Tris–HCl, 5 mM $CaCl_2$, pH 7.5 and elution was accomplished by the addition of 1.0 M NaCl to the eluant buffer [1]. All the collagenase activity was retained on the column at low ionic strength but its recovery after elution by 1.0 M NaCl was only 10–20%. Better recoveries (70% ± 17%; mean ± SD of 5 experiments) were obtained when the Tris–HCl buffer was replaced by CCA buffer which is less sensitive to temperature and ionic strength.

As illustrated in fig.1, the procollagenase present in untreated culture fluids also exhibited an affinity for collagen–Sepharose and considerable purification was achieved by using for its elution a continuous gradient of NaCl concentration; good recoveries (74% ± 14%; mean ± S.D. of 12 experiments) were obtained. The specific activities of the most purified fractions were about 3500 units of procollagenase/mg protein. Although these fractions appeared as single bands on polyacrylamide gel electrophoresis, they still contained latent neutral caseinolytic protease activity [9]. Other

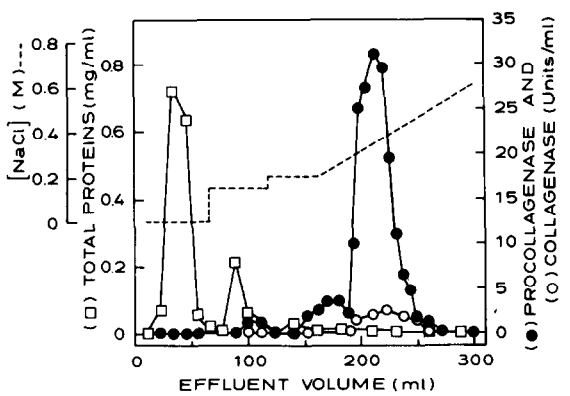

Fig.1. Affinity chromatography of procollagenase on collagen coupled to Sepharose. Crude concentrated culture fluid containing 1596 units of procollagenase (86 units/mg protein) was applied to a collagen-Sepharose column as described in Materials and methods. Elution was accomplished by increasing NaCl concentration in the eluant CCA buffer as indicated on the figure. The activity was assayed in the effluent fractions either directly (to evaluate the 'free' collagenase that could result from autoactivation of latent procollagenase during the fractionation procedure) or after activation by trypsin (to evaluate the 'total' collagenase activity of the fractions). The 'procollagenase' presented on the graph is the difference between total and free collagenase; its specific activity in the fraction corresponding to 230 ml effluent volume was 3870 units/mg protein and its recovery was of 74%.

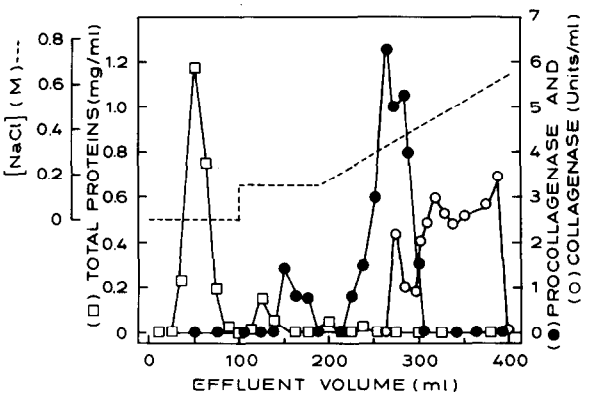

Fig.2. Affinity chromatography of a mixture of collagenase and procollagenase. A mixture containing 800 units of trypsin-activated collagenase and 800 units of procollagenase was chromatographed as described in Materials and methods and under fig.1. The specific activity of procollagenase in the fraction corresponding to 272 ml effluent volume was 1193 units/mg protein.

purification procedures such as chromatography on Sephadex G-150 or on various ion exchangers did not provide collagenase preparations that were free of neutral protease.

In order to compare the affinity of procollagenase to that of active collagenase for collagen-Sepharose, a mixture of both untreated (procollagenase) and trypsin-activated (collagenase) culture fluids was applied on a column containing collagen–Sepharose and eluted in a manner similar to that used in fig.1. The chromatogram on fig.2 shows that active collagenase was desorbed at higher NaCl concentrations and had a broader elution profile than procollagenase, indicating that, as expected, collagenase has a stronger affinity for its substrate than procollagenase. Partial separation of latent and active collagenase can thus be achieved with this method.

4. Discussion

The present paper reports the first evidence for a specific affinity of procollagenase for collagen and the use of this property for the purification of mouse bone procollagenase. The lacking affinity of tadpole procollagenase for collagen fibrils [10] might be due either to a species specificity or to different

experimental conditions (e.g., higher NaCl concentration or other type of collagen used).

The affinity of mouse bone procollagenase for collagen points to the existence of substrate-binding sites which differ from the catalytic site. If, as is suggested (but still not demonstrated) by other data [5,6,11], procollagenase is a physiological precursor of collagenase, its affinity for collagen might have important implications in the regulation of collagen degradation. It could indeed be stored extracellularly in its latent state on collagen fibers. These would thus contain the enzymatic equipment required for their own degradation (in a form that is not inhibited by the main extra-cellular inhibitor of collagenase, α_2-macroglobulin [11]) which might then be triggered by catalytic amounts of a procollagenase activator [5]. Detection of collagen-bound collagenase after its activation by trypsin [12] or by its immunoreactive properties [3] supports this hypothesis.

Our purification of procollagenase by affinity chromatography is, in terms of specific activity, at least 400-times higher than the only other reported purification of (tadpole) procollagenase [10] and it is of the same magnitude as the most purified collagenases [2,4,13]. Nevertheless, our purest preparations contain a latent neutral caseinolytic protease, which could still however be a contaminant [10].

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