FIVE SEPHAROSE-BOUND LIGANDS FOR THE CHROMATOGRAPHIC PURIFICATION OF *CLOSTRIDIUM* COLLAGENASE AND CLOSTRIPAIN

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1. Introduction

Two of the extracellular proteolytic enzymes produced by the anaerobic microorganism *Clostridium histolyticum* have been studied in particular because of their restricted specificity: collagenase (clostridiopeptidase A, EC 3.4.24.3) which cleaves native collagen preferentially at the amino group of glycine residues and clostripain (clostridiopeptidase B, EC 3.4.22.8) which cleaves proteins exclusively at the carboxyl of arginine residues.

Several methods have been proposed for their separation and purification: the highest specific esterolytic activity values as yet obtained for clostripain were 1.1. μkat.mg^{-1} [1] and 2.72 μkat.mg^{-1} (after activation) [2]; the highest value of specific activity of purified collagenase on a synthetic substrate [3] was 0.27 μkat.mg^{-1} [4]. In both cases these values can still not be considered as upper limits. In this paper we describe several chromatographic separations using Sepharose-bound ligands: compounds which act as competitive inhibitors of clostripain, such as arginine [5], polyarginine [1], benzamidine [6] and soybean trypsin inhibitor [7], the SH-binding mercurial *p*-aminophenylmercuric acetate [8] and acridine dye Rivanol which is known to inhibit collagenase [9].

2. Materials and methods

2.1. *Materials*

Batches of crude collagenase from *Clostridium histolyticum* of different degrees of purity were products of Institut Pasteur Production. Sepharose 4B and CH–Sepharose 4B were purchased from Pharmacia (Uppsala). *p*-Aminophenylmercuric acetate–Sepharose was prepared from activated Sepharose 4B [10] and *p*-aminophenylmercuric acetate as described by Sluyterman and Wijdenes [8]. L-Arginine–Sepharose and Rivanol–Sepharose were prepared in a similar way using L-arginine monochlorhydrate (Fluka) and Rivanol (3,9-diamino-7-ethoxyacridine lactate, Vorm, Hoechst), respectively. Poly-L-arginine–Sepharose was prepared from Sepharose 4B and poly-L-arginine sulfate of mol. wt approx. 17 000 (type II B, Sigma). Mercuric and arginine derivatives linked to Sepharose by a six carbon spacer-group were obtained by coupling the ligand to CH–Sepharose 4B by the usual procedure using 1-ethyl-3(3-dimethylaminopropyl) carbodiimide. The amount of coupled L-arginine, as determined by amino acid analysis, was 10 μmol/ml swollen gel; the mercuric derivative retained 0.25 μmol thiol reagent/ml gel. The same coupling procedure was used to couple CH–Sepharose 4B with soybean trypsin inhibitor (STI, Sigma). STI (0.4 μmol) was bound/ml swollen gel. 4-Aminobenzamidine–succinyl–aminododecyl (–SAD–) cellulose was a gift of Merck (Darmstadt). It contained 100 μmol 4-amino-benzamidine/ml swollen gel.

2.2. *Enzyme purification*

All procedures were carried out at 4°C. Chromatographic separations were performed in closed-bed columns with minimum dead volume at constant flow-rate (Minipuls II, Gilson), with stepwise or gradient elutions (11 300 Ultrograde, LKB) and automatic recording.(LUV-Monitor, LDC). Conductivity values

Abbreviations: BAE, α-*N*-benzoylarginine ethyl ester; DTT, dithiothreitol; STI, soybean trypsin inhibitor

Table 1
Composition of buffer used for affinity chromatography (mM concentrations of all components are given)

Buffer	Tris	$CaCl_2$	DTT	NaCl	pH	Conductivity (mS)
A	5	5	–	1000	6.7	75
B	10	–	–	60	7.4	5.5
C	10	10	–	–	7.4	2.7
D	10	10	–	300	7.4	28
E	50	50	–	–	7.4	11.5
F	50	50	2.5	–	7.4	11.5
G	500	50	2.5	–	7.4	31.5

of buffers were checked on a CDM3 conductivity Meter (Radiometer, Copenhagen) equipped with a CDC 314 cell. The composition of buffers is summarized in table 1. Optical density of the fractions was evaluated at 280 nm; for collagenase and clostripain activity 100 ml and 10 μl were withdrawn, respectively. The use of coupled columns as described under sections 3.4. and 3.6. eliminates the intermediary treatment of enzyme solutions between two successive chromatographies.

Aqueous solutions of active enzymes in eluates were protected against autolysis by addition of reversible inhibitors (0.1 M histidine for collagenase [11], 0.1 M arginine for clostripain). Concentration of eluate fractions was made by ultrafiltration (Amicon UM 10) prior to desalting by dialysis or by Sephadex G-25 chromatography.

2.3. *Enzyme assays and characterization*

Specific activities of different batches of crude collagenase used in the particular experiments described below are included in table 2.

Clostripain activity was determined spectrophotometrically [1]: the initial rate of hydrolysis of BAE was recorded with a Zeiss POM-2 spectrophotometer at 25°C. Molar absorptivity difference of 1150 M^{-1} cm^{-1} at 253 nm was used for the substrate hydrolysis. Substrate solution (3 ml) contained 2.5×10^{-4} M BAE (Fluka), 2.5 mM DTT[+] and 50 mM Tris–HCl buffer (pH 7.4) made 50 mM in $CaCl_2$. Specific activity was expressed in μkat.mg^{-1} protein. A unit of activity as used by [1] corresponds to 16.67 nkat.

Collagenase activity was measured colorimetrically using 4-phenylazobenzoyl–oxycarbonyl–L-prolyl–L-leucyl–glycyl–L-prolyl–D-arginine dihydrate (Fluka) according to Wünsch and Heidrich [3]. Numerical data have been recalculated on the basis of 1 nkat = 90 units according to [3].

Protein concentrations were determined colorimetrically by the method of Lowry et al. [12].

Descending gel-filtration for the estimation of molecular weights was performed according to Andrews [13] through an AcA44 Ultrogel (LKB) column (176 X 1.2) in buffer E using serum albumin, ovalbumin, chymotrypsinogen and ribonuclease as standard markers. Dodecylsulfate–gel electrophoresis was carried out on slabs according to Ames [14] at pH 8.5, using a 5–15% gel gradient and the same standards as above. The protein bands were stained with Coomassie Blue R 250. Homogeneity at pH 8.5 was checked by polyacrylamide gel electrophoresis as described by Uriel [15].

3. Results

3.1. *L-Arginine–CH–Sepharose, poly(L-arginine)–Sepharose and 4-aminobenzamidine–SAD–cellulose*

The procedure described below is essentially identical for the derivatives of arginine with or without the spacer group as well as for the derivative of poly-(L-arginine).

Crude *Clostridium* collagenase (30 mg) was activated 1 h at 4°C in 2 ml buffer F (table 1) before its application to a column of arginine–CH–Sepharose stabilized in the same buffer. Buffer F eluted two peaks of collagenase activity, the subsequent gradient between

Table 2
Comparison of affinity chromatographies for the purification of clostripain and *Clostridium* collagenase

Heading in results	Purification step	Clostripain				Collagenase			
		Specific activity[a]			% Activity recovered	Specific activity[b]			% Activity recovered
		Crude	Purified	Purification		Crude	Purified	Purification	
3.1.	Arg–Sepharose	0.038	2.08	55 ×	85[c]	1.52	23.9[e]	16 ×[e]	90
3.1.	Benzamidine–Cellulose	0.078	1.07	14 ×	84[c]	0.83			
3.2.	Mercuri–Sepharose	0.078	1.1	14 ×	88.6[c]	0.83			
3.3.	STI–Sepharose	0.025	0.064	1.5 ×	19	1.16	13.9	12 ×	39
3.4.	Mercuri–Sepharose - → Poly-(Arg)–Sepharose	0.025	1.83	73 ×	79	0.21	–	–	n.d.
3.5.	Rivanol - → Sephadex - → Arg–Sepharose → re-Arg–Sepharose	0.045	4.94	109 ×	16.4[d]	0.30	34	117 ×	n.d.
3.6.	DE-32-Cellulose - → STI–Sepharose	0.058	–	–	n.d.	75.5	655	8.7 ×	50

[a] In μkat.mg^{-1}
[b] In nkat.mg^{-1}
[c] Activity recovery under the peak in % total activity recovered. Direct comparison between the initial and final values were not calculated because the activation process continues during the chromatography
[d] Crude collagenase 10 g, containing non-activated clostripain yielded 15 mg rechromatographied active clostripain
[e] Second peak in fig.1a
n.d. Not determined

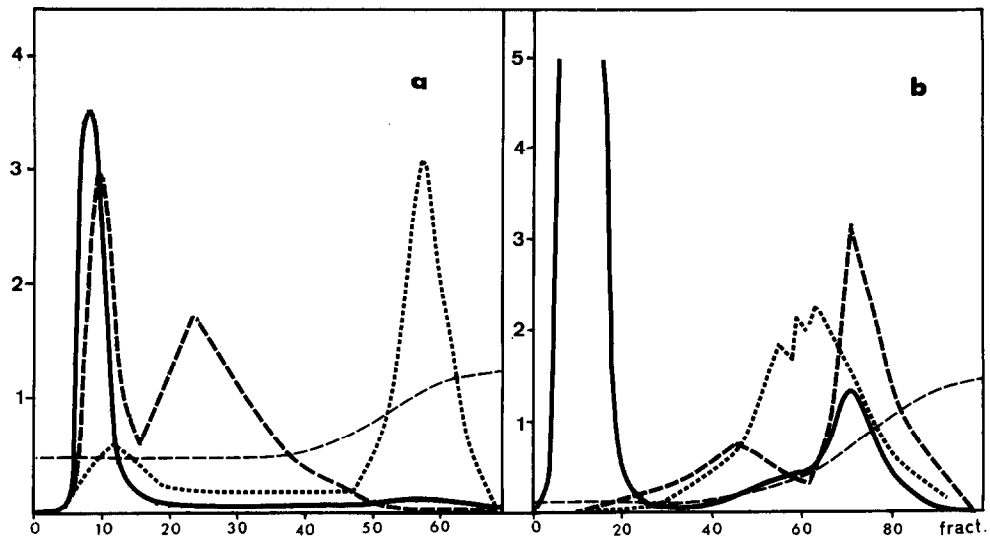

Fig.1. Chromatography of crude collagenase on Arg–CH–Sepharose (1a) and on STI–Sepharose (1b). Column size: 10.5 × 1.2 cm. Fractions: 2 ml/5 min (1a), 3 ml/15 min (1b). Curves are designated as follows: (———) Protein, optical density at 280 nm (× 2 for 1b). (– – – –) Collagenase activity against hexapeptide in nkat.ml^{-1} (× 5 for 1a, × 2 for 1b). (- - - - - - - - -) Clostripain activity against BAE in nkat.ml^{-1} (× 25 for 1a, × 100 for 1b). (– – – – –) Conductivity in mS (× 24 for 1a, × 20 for 1b).

buffers F and G eluted clostripain (fig.1a and table 2, section 3.1.). The chromatography on 4-aminobenzamidine–SAD–cellulose was performed under identical conditions. The result of the separation is in table 2, section 3.1. The L-arginine–Sepharose column can be reused after washing with buffer F without perceptible loss of activity. On the contrary, poly-(L-arginine)–Sepharose is much less stable. During eight successive runs the retaining capacity for clostripain fell from 80–25%.

3.2. p-*Aminophenylmercuric acetate–Sepharose*

The stabilization of the column was made by successive washing with buffer E, buffer F and again buffer E. Crude collagenase (30 mg) dissolved in 3 ml buffer E was adsorbed on the top layer of the column and left one hour for activation. Elution with buffer E was then continued until the elution of the inactive material absorbing at 280 nm was completed. Clostripain was then desorbed with the buffer F (table 2, section 3.2.).

3.3. *STI-Sepharose*

Crude *Clostridium* collagenase (250 mg) was dissolved in 2 ml buffer C and applied to a column of STI–Sepharose stabilized in the same buffer. A programmed concave gradient was made from buffers C and D. The result of the separation is shown in fig.1b and table 2, 3.3.

3.4. *Purification of clostripain on the combined columns of mercuri- and poly-L-arginine–Sepharose*

Column I packed with *p*-aminophenylmercuric acetate–Sepharose and column II packed with poly-(L-arginine)–Sepharose were connected as shown in fig.2 and washed for several hours with buffer E. Crude collagenase (100 mg) was dissolved in 2 ml buffer E. The columns were disconnected, the sample was adsorbed on the column I and eluted with buffer E into a fraction collector as shown in fig.2, Step 1. Most of the impurities and a small fraction of clostripain activity were desorbed. Columns I and II were then connected (Step 2) and eluted with buffer F in the same fraction collector. Clostripain was desorbed in this way from column I and withheld by column II; only inactive proteins were eluted. The columns were then disconnected and clostripain eluted directly from column II by buffer G (Step 3). The efficiency and yield are in table 2, section 3.4.

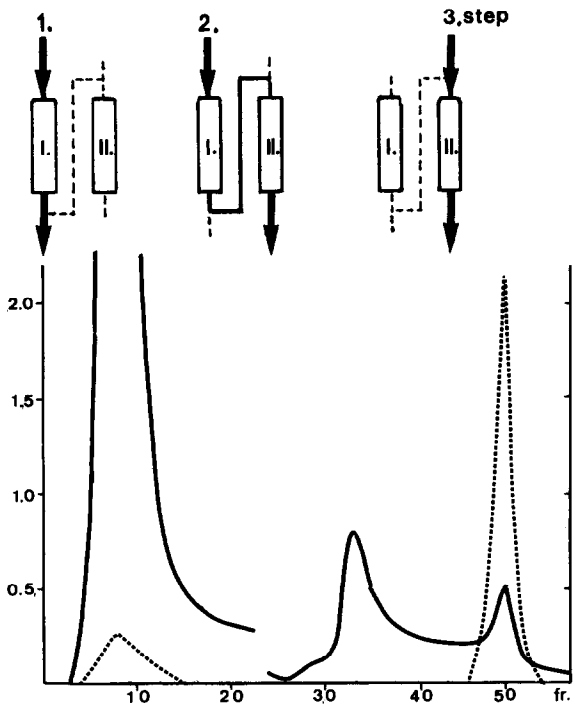

Fig.2. Combined columns of mercuri- and poly-(L-Arg)–Sepharose. Column sizes, 4 × 1 cm; fractions 2 ml/5 min. (————) Protein, optical density at 280 nm. (- - - - - - - - -) Clostripain activity against BAE in nkat.ml^{-1} · 200. Steps 1–3 are described in the text.

3.5. *Separation of collagenase and clostripain by successive application of Rivanol–Sepharose, Sephadex G-75 and arginine–Sepharose*

The binding of the enzymes to the Rivanol–Sepharose is dependent on salt concentration: at low ionic strength and pH 6.7, clostripain emerges with the dead volume (fig.3a), on the contrary, both clostripain and collagenase are retained from water solutions (fig.3b).

A column of Rivanol–Sepharose (32 × 4) was washed with distilled water and loaded with 210 ml solution containing 10 g crude collagenase pre-treated by dialysis (pH 6.7, conductivity 0.23 mS). Inactive components were washed from the column by water. Collagenase and clostripain were eluted with buffer A, concentrated by ultrafiltration through membrane PM 10 to volume 11 ml and applied on a column of Sephadex G-75 (Pharmacia, Uppsala, 190 × 2.8) washed with buffer E. Fractions containing collagenase were stabilized by addition of histidine hydrochloride, concentrated and lyophilized as described in Materials and methods. Fractions containing clostripain were applied to a column of L-arginine–CH–Sepharose (35 × 3.8) pretreated with buffer F. Clostripain was desorbed with buffer G, concentrated and washed with water by ultrafiltration using PM 10 membrane to volume

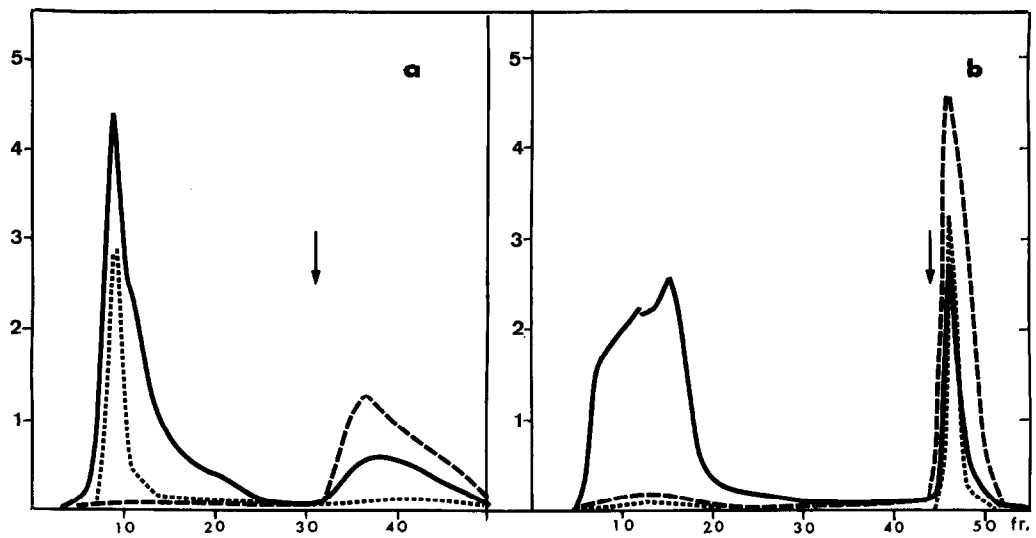

Fig.3. Chromatography of crude collagenase on Rivanol–Sepharose. Column size 14 × 1.2 cm; fractions, 2.5 ml/20 min. (————) Protein, optical density at 280 nm. (– – – – – –) Collagenase activity against hexapeptide. (- - - - - - - - -) Clostripain activity against BAE. (3a) Column run in Tris–$CaCl_2$ buffer 5 mmol, pH 6.7, arrow indicates the beginning of linear gradient elution with buffer A. (3b) Column run in water, arrow indicates elution with buffer A.

7 ml and conductivity 11 mS. The concentrate was rechromatographied on the same column by a linear-gradient elution. The whole four-step purification is summarized in table 2, section 3.5.

After lyophilization, clostripain gave a major single band on electrophoresis, at pH 8.5, but two protein bands (mol. wt 45 000 and 12 000) on dodecylsulfate electrophoresis. Gel-filtration on an AcA44 column (see Materials and methods) gave a single peak of activity, apparent mol. wt 43 000, as estimated from the elution volumes of standard proteins run on the same column.

3.6. *Purification of collagenase by pretreatment with DEAE-cellulose and chromatography on STI–Sepharose*

Column I (10 × 1.2) of DE-32 Cellulose (Whatman) and column II (8 × 1.8) of STI–Sepharose, both stabilized with buffer B, were connected and on column I was applied 30 mg of collagenase (spec. act. 75.5 nkat.mg^{-1}) in 8 ml buffer B. Under these conditions collagenase passes with the dead volume through column I and it is retained on the top of column II. A large part of the impurities remain on the top of column I. After the disconnection of the two columns, the impurities can be washed out of column I by 1 M NaCl; collagenase is desorbed from column II by buffer D (table 2, section 3.6.).

4. Discussion

As shown in table 2, four inhibitors of clostripain (arginine, poly-(arginine), arylmercurial and benzamidine) when bound to an insoluble matrix, retain clostripain selectively. The subsequent elution yields this enzyme in highly purified state: values of spec. act. 4.94 μkat (296 U) approach the theoretical values deduced from kinetic studies by Porter et al. [6].

Rivanol is an inhibitor of bacterial collagenases [9]. Independently it was proposed for selective precipitation of proteins [16]. Results in fig.3 show that immobilized Rivanol can serve for protein fractionation using chromatographic technique; its use could possibly be extended from this special case to other protein mixtures.

Soybean trypsin inhibitor was selected as another promising ligand because it inhibits clostripain in a competitive way [7]. The chromatography of the crude enzyme mixture showed, however, that collagenase is retained by this column even better than clostripain (fig.1b). At present, we have no explanation for this binding. When a purified collagenase sample 75.5 nkat.mg^{-1}) with low clostripain content (58 nkat.mg^{-1}) was passed through a two column system of combined ion-exchanger and STI–Sepharose (Exp. 3.6), the resulting collagenase had the highest specific activity ever observed (0.655 μkat.mg^{-1}). However, electrophoretically it was still not homogeneous. This indicates that the specific activity of pure *Clostridium* collagenase is even higher than this value and therefore that crude commercial collagenase samples containing clostripain are rather crude clostripain samples containing collagenase.

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