CONSECUTIVE USE OF ω-AMINOALKYLAGAROSES. RESOLUTION AND PURIFICATION OF CLOSTRIPAIN AND COLLAGENASE FROM CLOSTRIDIUM HISTOLYTICUM

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Received January 27,1976

Summary: Clostripain and collagenase A were resolved and purified from the culture filtrate of Clostridium histolyticum by consecutive use of two columns from the homologous series of ω-aminoalkylagaroses. Clostripain was extracted by passing the culture filtrate through ω-aminobutylagarose and purified ~9-fold with 70% overall yield. Collagenase A was then extracted by passing the excluded mixture of proteins through ω-aminoheptylagarose and was purified about 7-fold with an overall yield of 90%. This procedure yields two useful proteolytic enzymes with narrow specificity and also illustrates the potential of homologous series of hydrocarbon-coated agaroses for the development of consecutive fractionators. Such fractionators would separate several proteins out of the same crude extract, thus leading to maximal utilization of expensive or scarcely-available tissues.

From the culture filtrate of Clostridium histolyticum a number of enzymes have been isolated, among them collagenase A (clostridiopeptidase A, EC 3.4.24.3) and clostripain (clostridiopeptidase B, EC 3.4.22.8).

Collagenase A (1) is a hydrolytic enzyme which cleaves collagen in its native conformation, attacking the domains of the substrate pervaded by the collagen helix. Like other collagenases, this enzyme is a useful tool for studying the structure of collagens, their biosynthesis, folding, assembly and immunogenicity. Moreover, it seems to be very useful in the identification and location of collagenous components of tissues and for the dispersion of cells held in a net of connective tissue prior to their isolation.

Clostripain (2) is a sulfhydryl protease which considerably restricts its action to the carboxyl peptide bond of arginyl residues. This unique specificity of the enzymes makes it very valuable in protein chemistry, especially

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Abbreviations used: BAEE, N-benzoyl-L-arginine-ethylester; DTE, dithioerythritol; PZ, p-phenylazo-benzyloxycarbonyl; Seph-C_n, represents Sepharose 4B activated with CNBr and reacted with an α-aminoalkane n-carbon-atoms long; Seph-C_n-NH_2, represents Sepharose 4B activated with CNBr and reacted with an α,ω-diaminoalkane n-carbon-atoms long; SDS, sodium dodecylsulfate.

for sequence determinations and for fragmentation of proteins in structure-function studies.

The usefulness of these enzymes lies mainly in their narrow specificity and thus depends on their being free of other proteolytic activities. This paper describes the consecutive use of ω-aminoalkylagaroses in the resolution and purification of these two enzymes, and further illustrates the efficiency of hydrophobic chromatography using homologous series of hydrocarbon-coated agaroses (3,4).

MATERIALS AND METHODS

Enzymes: a lyophilized collagenase preparation (specially produced by Boehringer,Mannheim) containing several extracellular proteins from Clostridium histolyticum served as a source for both collagenase and clostripain.

Assays: collagenase activity was measured by the cleavage of the hydrophilic peptide PZ-L-Pro-L-Leu-Gly-L-Pro-D-Arg into the colored lipophilic peptide PZ-L-Pro-L-Leu and the non-colored peptide Gly-L-Pro-D-Arg, following the method of Wünsch and Heidrich (5). One unit of enzyme activity is defined as the amount of enzyme that causes (under the assay conditions) the formation of 1 μmole of PZ-L-Pro-L-Leu per min at 37°. The amount of the blocked dipeptide is determined by its absorption at 320 nm (ε_m = 2.1 x 10^4 M^{-1} cm^{-1}). Clostripain activity was measured by the hydrolytic cleavage of BAEE into benzoyl-L-arginine and ethanol. The reaction mixture (1 ml, pH 7.8) contained Tris (80 μmoles), KCl (100 μmoles), DTE (3 μmoles), BAEE (1.1 μmoles) and limiting amounts of enzyme. The assay was carried out by following the change in absorbance at 253 nm as a function of time. Under the above mentioned conditions the change in absorbance with time is linear up to an absorbance of 0.3. For calculating the activity of the enzyme, a molar absorptivity difference of 1.15 x 10^3 M^{-1} cm^{-1} (at 253 nm) was used. One unit of enzyme is defined as the amount of enzyme which cleaves 1 μmole of BAEE per min at 20°.

Protein concentrations were determined by the method of Lowry et al (6) after precipitation of the protein with 10% trichloroacetic acid to remove DTE. Crystalline ovalbumin was used as a standard. During chromatography, protein was monitored by following the absorbance at 280 nm.

Polyacrylamide gel electrophoresis on slabs was carried out with 7.5% gels in the presence of SDS (7). Samples were prepared by diluting an appropriate volume of the fraction to be tested with an equal volume of a 2-fold concentrated "sample buffer" and heating for 3 min at 100°.

Hydrocarbon-coated agaroses (Seph-C_n and Seph-C_n-NH_2, n = 0-12) were prepared by the procedures described elsewhere (8). Other chemicals used were commercial products of analytical grade.

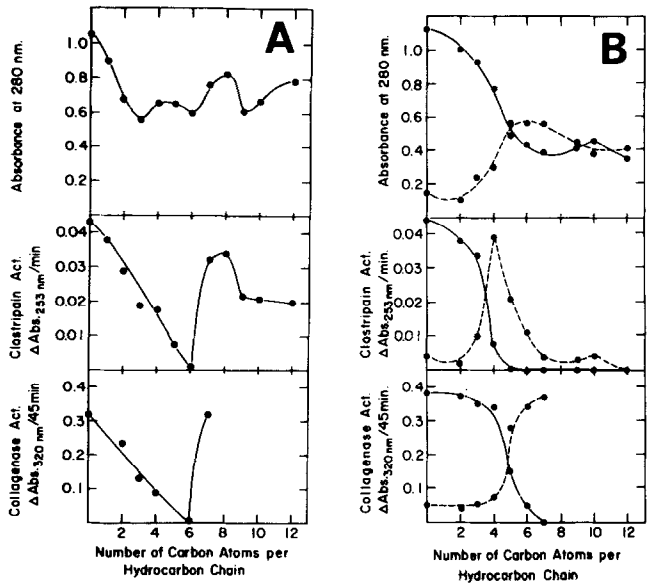

Fig. 1. Adsorption-elution profiles of collagenase and clostripain on homologous series of (A) alkylagarose columns (Seph-C_n) and (B) ω-amino-alkylagarose columns (Seph-C_n-NH_2). Exploratory kits of columns (n = 0-12) were equilibrated at 20° with a buffer composed of Tris (50 mM) and DTE (2 mM) adjusted to pH 7.5 with HCl. Aliquots (0.1 ml containing 1 mg protein) of the crude lyophilized collagenase preparation dissolved in the same buffer were applied on each of the columns (0.4 x 5 cm). Each column was then washed off with 2 ml of the same buffer and the absorbance of the effluent at 280 nm as well as its collagenase and clostripain activities were monitored (— ● —). In the Seph-C_n-NH_2 series (B), which discriminated between the two enzymes, each of the columns was subsequently washed with 2 ml of a buffer containing Tris (50 mM) DTE (2 mM) and NaCl (1 M) adjusted to pH 7.5 with HCl. Again the absorbance (at 280 nm) of each of the 2 ml fractions as well as its collagenase and clostripain activities were monitored (-- ● --) and plotted against the number of carbon atoms in each of the hydrocarbon chains attached to the agarose.

RESULTS AND DISCUSSION

Separation of clostripain from collagenase was first attempted by bio-specific affinity chromatography (9). For that purpose we tried to take advantage of the finding that alkylguanidines are potent competitive inhibitors of clostripain (Inagami and Cole, quoted in reference 2 p. 712). It was hoped that columns such as ω-guanidobutylagarose or ω-guanidohexylagarose would retain clostripain and maybe separate it from collagenase. However, these columns failed to separate the two enzymes under a variety of experimental conditions tested. On the other hand, a control column of hexylagarose (Seph-C_6) sufficed to retain both clostripain and collagenase. Therefore we

considered the possibility of resolving the two enzymes by hydrophobic chromatography, using homologous series of hydrocarbon-coated agaroses (3,4). Since such series provide column materials with increasing hydrophobic character, it was assumed that the lipophilic interactions which apparently had caused the binding of the enzymes to hexylagarose might be adjusted so as to achieve optimal resolution with another member in the alkylagarose or ω-aminoalkylagarose series.

Using the exploratory kits of columns described previously (8) it could easily be shown that clostripain and collagenase have a similar adsorption profile on the Seph-C_n series (Fig. 1A) and therefore this series seems unsuitable for their separation. However, in the Seph-C_n-NH_2 series they exhibit different elution profiles: while clostripain was essentially retained already on Seph-C_4-NH_2, collagenase was excluded by this column, as indicated by the fact that about 90% of the enzyme was found in the excluded fraction we collected (Fig. 1B). Under our experimental conditions, a higher member in the series, Seph-C_7-NH_2 was required to completely retain collagenase (Fig. 1B). Moreover, the binding of the enzymes to Seph-C_4-NH_2 and Seph-C_7-NH_2 was easily reversible as they could be detached from the columns in 90-100% yield by including 1M NaCl in the eluent (Fig. 1B).

On the basis of this exploratory experiment, it was attempted to resolve and purify the two enzymes by passage of the crude preparation first on Seph-C_4-NH_2 to extract clostripain and then by applying the excluded proteins from this column on Seph-C_7-NH_2 in order to purify collagenase. The results depicted in Fig. 2 show that this consecutive usage of two members of the Seph-C_n-NH_2 series brings about resolution and purification of both enzymes.

A sample of the crude lyophilized collagenase preparation (10.7 mg) was applied on a Seph-C_4-NH_2 column (10 ml) under the experimental conditions described in the legend to Fig. 2A. This column retained about 80% of the clostripain activity in the mixture, which could be eluted by applying a NaCl gradient on the column. The specific activity of clostripain in the crude preparation was 18.6 units/mg. and it increased to 163 units/mg representing a $\sim$9 fold purification in one step, with an overall yield of 70% (based on activity measurements). It should be noted that the clostripain thus obtained was free of collagenase activity, but the excluded proteins which contained the collagenase activity still contained some ($\sim$20%) clostripain-type activity. This excluded protein mixture was then subjected to chromatography on Seph-C_7-NH_2 which extracted collagenase (Fig. 2B)**. The specific activity

**Only a negligible percentage of the clostripain-type activity co-elutes with the collagenase peak in the Seph-C_7-NH_2 column. The rest of the clostripain-type activity is retained more tightly on the column, but it can be removed by washing with 1M NaCl, so that the column can be re-used.

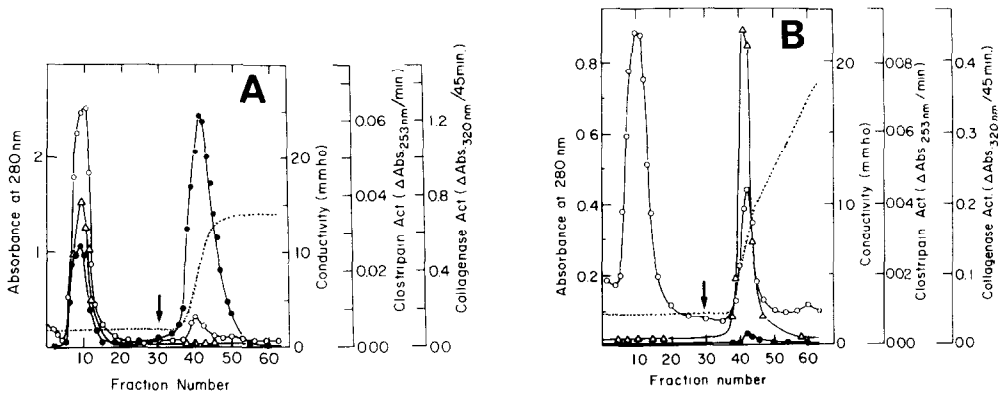

Fig. 2. Purification of clostripain and collagenase by consecutive use of ω-aminoalkylagaroses.
(A) Resolution of the enzymes on Seph-C_4-NH_2 and purification of clostripain. A sample (10.7 mg) of the crude lyophilized collagenase preparation was dissolved in a buffer composed of Tris (50 mM) and DTE (2 mM) adjusted to pH 7.5 with HCl. This sample was applied on a column (1.5 x 6.0 cm) which was equilibrated at 20° with the same buffer. Unadsorbed protein was washed off and then (arrow) a NaCl gradient (up to 1 M, in the same buffer) was applied. Fractions of 1.3 ml were collected and their absorbance at 280 nm (— O —), conductivity (.....) as well as their collagenase (—Δ—) and clostripain (— ● —) activities were monitored. The flow rate of the column (which was run at 20°) was 85 ml/h. The fractions were kept in the cold (0-4°).
(B) Purification of collagenase on Seph-C_7-NH_2.
Fractions 7-10 of the Seph-C_4-NH_2 column (see (A)) were combined and ~80% of the volume (4.1 ml) were applied on a Seph-C_7-NH_2 column (1.5 x 6.0 cm) which was washed and eluted as described in part (A) of this legend.
(— O —), Absorbance at 280 nm; (.....), conductivity; (—Δ—), collagenase activity; (— ● —), clostripain activity.

of collagenase increased from 1.06 units/mg to 2.98 units/mg in the pooled excluded fractions of the Seph-C_4-NH_2 column and to 7.56 units/mg after passage through the Seph-C_7-NH_2 column. This represents a ~7 fold overall purification for collagenase with an overall yield of 90% (by activity). The fact that the collagenase purified by this process is essentially free of clostripain-type activity (Fig. 2B) is of great importance (see reference 1 p. 663) particularly in the preparation and study of cells and tissue cultures (1,10,11).

The efficient resolution and purifications achieved by consecutive usage of ω-aminoalkylagaroses are also illustrated by subjecting the purified enzymes to acrylamide gel electrophoresis in the presence of SDS (Fig. 3). In both cases the enzymes migrated in one major protein band. It should be emphasized, however, that by loading the gel with larger samples it can be shown that both enzyme preparations still contain minor protein impurities.

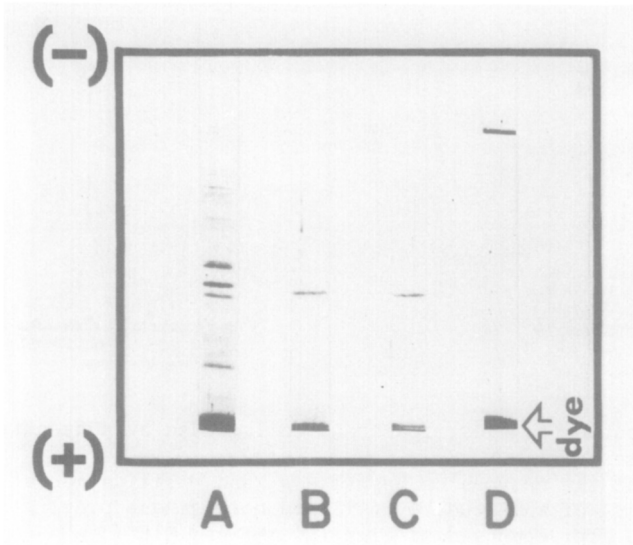

Fig. 3. SDS slab-gel electrophoresis of fractions obtained from the Seph-C_4-NH_2 and Seph-C_7-NH_2 columns (Fig. 2).
(A) Crude collagenase preparation (20 µg protein) which was applied on the Seph-C_4-NH_2 column; (B) and (C) represent aliquots (2 µg protein) of fractions 41 and 45 (Fig. 2A); (D) depicts an aliquot (2 µg protein) of fraction 41 (Fig. 2B). The band at the bottom of the gels (arrow) is the marker dye (bromophenol blue (7)) used for following the course of the electrophoresis. This band is of course present even before protein staining.

Hydrophobic interactions seem to play an important role in the resolution of the enzymes by the Seph-C_n-NH_2 series: On the one hand, retention can be achieved also by columns of the Seph-C_n series (e.g. n = 6, Fig. 1A) indicating that the amino groups at the tips of the hydrocarbon chains are not indispensible for retention. On the other hand, under identical experimental conditions there is a gradation in the binding of each of the enzymes to the various columns in the homologous series. This gradation is different for the two enzymes in the Seph-C_n-NH_2 series, which makes it possible to resolve them and purify each one.

In the case of collagenase, which cleaves the Y-Gly bond in Y-Gly-Pro-X sequences (where Y could be any of 17 different amino acids and X is most frequently Ala or Hyp (1)), there is no reason to assume that the interaction with the ω-aminoheptyl chains occurs at the active site. However, in the case of clostripain it could be argued that the ω-aminoalkyl chains bind the protein through its active site, since alkylamines were reported to be competitive inhibitors of the enzyme (2). Although this possibility cannot be entirely excluded, it does not seem very likely that a biospecific inter-

action involving exclusively the active site is responsible for the retention of the enzyme by Seph-C_4-NH_2, since (a) alkylamines are very poor competitive inhibitors of the enzyme (cf. Table V in ref. 2); (b) ethylamine and butylamine are equally efficient in inhibiting the clostripain-catalyzed hydrolysis of BAEE (K_I for ethylamine = K_I for butylamine = 27.0 mM (2)), yet Seph-C_2-NH_2 excludes the enzyme, while under the same conditions Seph-C_4-NH_2 retains it (Fig. 1B); (c) in spite of the fact that alkylguanidines are potent competitive inhibitors of clostripain (2) it was found that hexylagarose is not less efficient than ω-guanidohexylagarose in retaining clostripain (see above); (d) there are now quite a few enzymes that were shown to bind to alkylararoses, though they bear no special resemblance to the substrates or the known effectors of the enzymes (e.g. see references cited in (12) and (13))

The above procedure for the purification of clostripain and collagenase is rapid, achieves considerable purification factors with high yields and does not involve the use of columns with prohibitively expensive biospecific ligands. Furthermore, the above results illustrate the potential of homologous series of hydrocarbon-coated agaroses for consecutive extraction of several proteins out of the same tissue extract. We have previously shown, for example, that Seph-C_1 will retain glycogen synthetase I from a crude muscle extract and that upon subsequent passage of the excluded protein mixture through Seph-C_4, glycogen phosphorylase b can be extracted (3,4,8). Similarly, we have shown that consecutive extraction of proteins can be achieved by using columns of two different homologous series (3,4). These observations, together with the results presented here, form the basis for the development of consecutive fractionators for the purification of proteins by passage of a crude extract through Seph-C_1, Seph-C_2, ..., Seph-C_n. Each member of the homologous series will retain one or a few proteins out of the mixture. Some of the proteins may be purified by being excluded from all the members in the series (14). Consecutive extraction will thus lead to maximal utilization of expensive or scarcely-available tissues (12,15).

ACKNOWLEDGEMENT

This work was supported by a grant within the Biotechnology Program of the Bundesministerium für Forschung und Technologie der Bundesrepublik Deutschland and the Israeli National Council for Research and Development.

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