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CHEMICAL CHARACTERIZATION OF THE HOMOGENEOUS COLLAGENASE FROM *CLOSTRIDIUM HISTOLYTICUM*

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Summary

Pure collagenase (clostridiopeptidase A, EC 3.4.24.3) having a molecular weight of 70 000 was obtained from the culture medium of *Clostridium histolyticym* by a combination of ultrafiltrations, molecular sieve, affinity and hydrophobic chromatography. The value of its specific activity is the highest of those described previously but 6-times lower than that of the collagenase from *Achromobacter iophagus* (EC 3.4.24.8). Its amino acid composition differs from previous data, namely by the presence of cysteine, methionine, tryptophan and *O*-phosphoserine residues. In contrast to *Achromobacter* collagenase it does not dissociate in subunits during the deactivation by EDTA or LiCl/glycine buffer at pH 10.5. Existence of multiple forms of *Clostridium* collagenase previously described is discussed as being due to autolysis of a single molecular species or to a different degree of phosphorylation.

Introduction

The degradation of connective tissue during the infection by *Cl. histolyticum* as a proteolytic process was first described by Weinberg and Séguin at the Institut Pasteur more than 60 years ago [1]. Since that observation the purification and characterization of the *Clostridium* collagenase, responsible for the specific degradation of collagen, has been described in more than 130 papers which are summarized in several reviews [2—6]. From the same crude enzyme up to four different active collagenases were isolated [7,8]. Variations in ob-

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Abbreviation: SDS, sodium dodecyl sulphate

served molecular weight between 72 000 and 147 000 [7—14] can be attributed partly to the existence of different active enzyme forms and partly to the anomalous behaviour of collagenase in SDS-polyacrylamide gel electrophoresis [8]. A search was undertaken for the existence of subunits [15]. The data on the amino acid composition of *Clostridium* collagenase reflect different degrees of purity of the enzyme [12,13,16,17]. On the other hand, the enzyme of the highest specific activity [7] was not analyzed.

As part of a larger study of procaryote and eucaryote collagenases (for review see Ref. 16) we have elaborated several methods for the separation of the two major extracellular proteinases of *Clostridium*, collagenase and clostripain [18,21]. Recently, a large scale preparation allowed us to purify the *Clostridium* collagenase to homogeneity and to determine its chemical properties.

Materials and Methods

Enzyme production

All procedures were carried out at 4°C.

Step 1: Precipitation of culture filtrates. Filtrates (160 l) of the culture of *Cl. histolyticum* (strain G-54, Institut Pasteur) grown on medium VF were brought to 40% saturation with crystalline $(NH_4)_2SO_4$. After 24 h the precipitate was removed by centrifugation and the filtrate was saturated to 80% with crystalline $(NH_4)_2SO_4$ and the collected precipitate was dissolved in 50 mM Tris-HCl, pH 7.5.

Step 2: Ultrafiltration and gel filtration. The solution resulting from step 1 was fractionated by DC 2 hollow-fiber ultrafiltration (Amicon Corp., Lexington, MA). The fraction which passed through Diaflo membrane HP 100, but which was retained by membrane HP 10, was applied on a column of 8 l of Ultrogel AcA-44 (IBF, France) and stabilized in the same buffer. The fraction containing collagenase activity was concentrated by rapid ultrafiltration and lyophilized.

Steps 3 and 4: Affinity and hydrophobic chromatography. The chromatography of the crude lyophilized enzyme on a L-arginine-CH-Sepharose column was made essentially as described previously [18,19], after incubation in 50 mM Tris-HCl/50 mM $CaCl_2$ buffer, pH 7.4, made up to 2.5 mM in dithiothreitol 1 h prior to the application on the column. Collagenase emerged with the inactive volume. It was concentrated by ultrafiltration through a UM-10 membrane and washed on the membrane by 50 mM Tris-HCl buffer, pH 7.4 (step 3).

It was further purified by hydrophobic chromatography as described by Kula et al. [20] in the modification proposed by Gilles et al. [21]. The CC-Sepharose-C_7NH_2 column was stabilized in the same buffer as the sample. Elution of pure collagenase was obtained by 50 mM Tris-HCl/150 mM NaCl buffer, pH 7.4. The eluate was made up to 0.1 M in histidine [22], concentrated by ultrafiltration on a UM-10 membrane, washed three times by small volumes of chilled water and either kept frozen at —20°C or lyophilized (Step 4).

Analytical methods

SDS-polyacrylamide gel electrophoresis was performed in slab gels (5—15%)

as described by Ames [23] with the staining procedure of Weber and Osborn [24]. Molecular weight estimation by gel filtration was effected on a Sephadex G-150 column.

Amino acid analyses were performed in seven independent assays on a Multichrome B (Beckman) apparatus coupled with the microprocessor (LTT, France ICAP 10) using a single column procedure [25] and hydrolysed for 15, 48 and 72 h in 6 N HCl in vacuo at 110°C according to the method of Spackman et al. [26]. The tryptophan content was determined from two independent assays after hydrolysis with 4 M methanesulfonic acid at 105°C for 20 h according to the method of Liu and Chang [27], using a short column. Methionine and half-cystine were estimated after performic acid oxidation according to the method of Hirs [28] from two analyses.

High voltage electrophoresis was effected on Whatman No. 1 paper for 45 min at 3500 V in a Gilson Medical Electronic Apparatus, type D, in a mixture of diluted acetic acid and formic acid (2.5 and 15%, respectively).

Protein concentrations were determined colorimetrically by the method of Lowry et al. [29].

Enzyme assays and inhibition of collagenase by EDTA

The digestion of the native collagen (calf skin, type III, Sigma) was evaluated from the degradation of its helical structure according to the method of Keil et al. [30] on a recording polarimeter (Fica, France). The assay is based on the disappearance of the negative Cotton effect in the collagen solutions at 215 nm.

As synthetic peptide substrate, 4-phenylazobenzoyl-oxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine dihydrate (Pz-Pro-Leu-Gly-Pro-D-Arg) (Fluka) was used according to the method of Wünsch and Heidrich [31]. Numerical data have been recalculated on the basis of 1 nkat = 90 units according to [31]. Clostripain activity was determined spectrophotometrically [32].

For the inhibition and dissociation assays, 0.05 M Tris-HCl/0.15 M NaCl buffer, pH 7.4 was used made up to 10^{-3} M and 10^{-1} in EDTA at 4°C. The peptide substrate was adjusted to the same concentrations of EDTA. The incubation of the substrate with EDTA without enzyme gave zero control values.

Results

Purification of Clostridium collagenase (Table I).

The crude material which precipitates from the culture medium between 40 and 80% $(NH_4)_2SO_4$ saturation is liberated from molecules with a molecular weight greater than 100 000 and from the salts and small molecules by hollow-fiber ultrafiltrations. The following chromatography on a large column of gel removes the bulk of other proteinases and in particular, clostripain. The last traces of clostripain are removed by the affinity chromatography on Sepharose-bound arginine. The specific activity of the resulting collagenase, although electrophoretically quasi-homogeneous, is improved by hydrophobic chromatography [18,20] on CC-Sepharose-C_7NH_2 (Fig. 1). Further purification increases neither homogeneity nor specific activity.

The greatest losses are at the first steps where the whole mixture of protein-

TABLE I

PURIFICATION OF COLLAGENASE FROM THE CULTURE MEDIUM OF *CLOSTRIDIUM HISTOLYTICUM*

The specific activity on synthetic peptide is expressed in nkat/mg of Pz-Pro-Leu-Gly-Pro-D-Arg and the specific activity on the native collagen is expressed in mg of collagen/min per mg (ORD measurement). The yield is calculated on the synthetic substrate. n.d., not determined.

Purification steps	Protein (mg)	Spec. act.		Yield (%)
		Synth. peptide	Native collagen	
1. $(NH_4)_2SO_4$ (40—80%)	133 000	2.2	n.d.	100
2. HP100-HP10-AcA-44	432	91.7	n.d.	23.5
3. Sepharose-Arginine	292	121	2.0	12.9
4. CC-Sepharose C_7NH_2-UM 10	105	332 *	5.7 *	11.9

* In an independent preparation on a smaller scale the values were 343.3 and 5.8, respectively.

ases is present. The yields of the following steps are satisfactory if they are performed rapidly and if the last concentration by ultrafiltration is made in the presence of histidine [22]. The heterogeneity of the crude enzyme and of a crude commercial collagenase sample (Institut Pasteur), is demonstrated by electrophoresis (Fig. 2a, g). The same analysis shows the homogeneity of the purified enzyme (Fig. 2c, d, e).

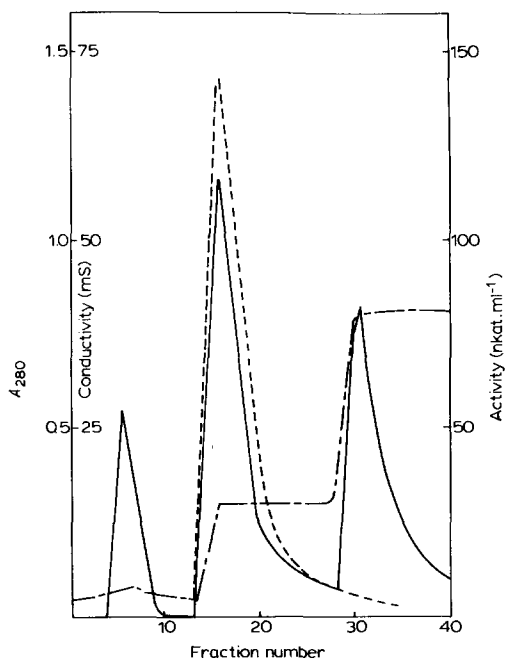

Fig. 1. Purification of collagenase by hydrophobic chromatography. Column size: 1.2 X 10 cm. Fractions: 3 ml/6 min; protein (———) absorbance at 280 nm; collagenase activity against Pz-Pro-Leu-Gly-Pro-D-Arg (- - - - -); conductivity (· — · — ·).

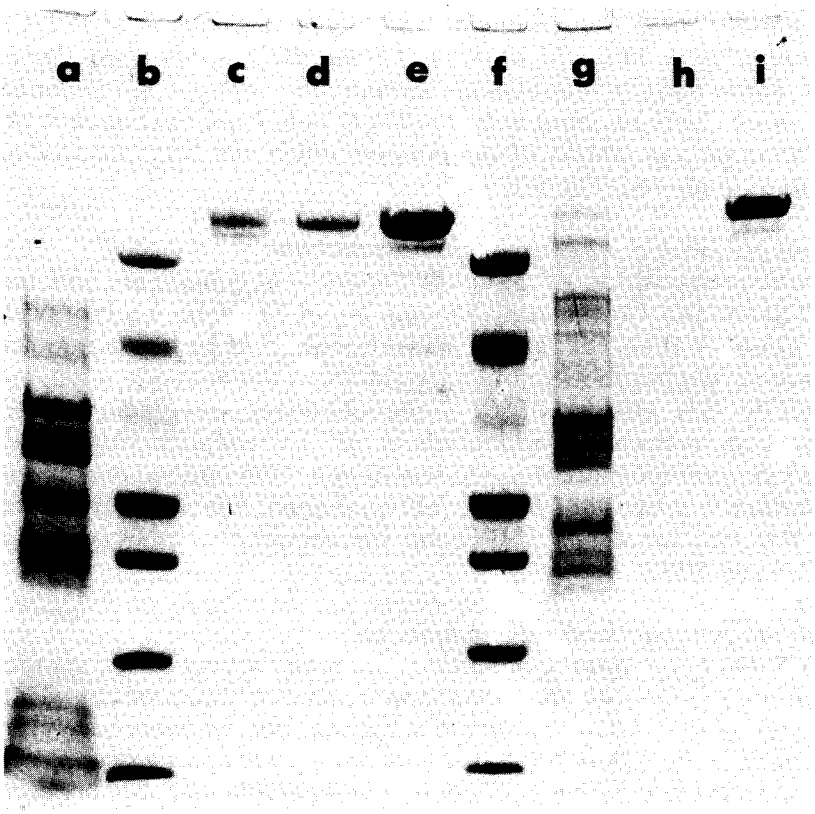

Fig. 2. SDS-polyacrylamide slab gel electrophoresis (5—15% gradient). (a) Crude collagenase (step 1); (b,f) proteins standard (phosphorylase, 94 000; bovine serum albumin, 68 000; aldolase, 40 000; carbonic anhydrase, 29 000; trypsinogen, 24 000; parvalbumin, 13 000); (c) after affinity chromatography (step 3); (d) (1X) and (e) (10X) after hydrophobic chromatography (step 4); (g) commercial colagenase; (h) incubation 16 h 4°C in glycine-LiCl, pH 10.5; (i) in 0.1 M EDTA.

Optical properties and molecular weight

The specific absorption coefficient of the pure enzyme at 280 nm is $f = 1.250$. Circular dichroism measurements of the sample are described elsewhere [33].

According to the molecular sieve chromatography on a column of Sephadex G-150, standardized with pure proteins, the apparent molecular weight of pure *Clostridium* collagenase is 68 000—70 000. In the SDS-polyacrylamide gel electrophoresis, the enzyme migrates as a sharp band with a mobility corresponding to a molecular weight larger than 100 000 (Fig. 2).

Effect of EDTA on activity and molecular weight

When collagenase (5 μg/ml) was incubated for 10 min, 2 and 16 h in a solution made up to 10^{-3} M in EDTA, the enzyme lost 14, 22 and 35% of its activity, respectively. In solutions made up to 10^{-1} M in EDTA, the inhibition was 86, 93 and 100%, respectively.

In the search for supposed subunits, the pure enzyme was exposed to conditions under which the dimer of another microbial collagenase from *Achromobacter iophagus*, dissociates in subunits of 35 000 [22]; in one experiment, the

enzyme was incubated at 4°C for 16 h in 0.1 M glycine buffer/0.28 M LiCl, pH 10.5, in another series in 0.1 M EDTA for 2 and 16 h as described above. Electrophoresis shows that in the alkaline solvent, the enzyme disintegrated without traces of products susceptible to the Coomassie staining (Fig. 2h). On the contrary, the molecular weight of the enzyme which was fully inactivated by EDTA remained unaltered (Fig. 2i).

Amino acid composition and presence of O-phosphoserine

The amino acid composition of the pure *Clostridium* collagenase and the values reported previously for the same enzyme are in Table II. A minor peak preceding the peaks of the acidic amino acids prompted us to investigate the possible presence of *O*-phosphoamino acid residues in the enzyme. After hydrolysis for 4 h in 6 N HCl at 85°C, *O*-phosphoserine was demonstrated qualitatively by high voltage electrophoresis at pH 1.9 [34,35]. Automated amino acid analysis gave most satisfactory results when the optimal conditions from those previously proposed [36,37] were adopted; the hydrolysis was effected with 6 N HCl for 4 h at 110°C and the sample was analyzed on a column at pH 1.0 and 30°C.

Specific acitivity on synthetic and natural substrate

As shown in Table I, the specific activity of the pure enzyme on the synthetic pentapeptide Pz-Pro-Leu-Gly-Pro-D-Arg gave a value of 332 nkat/mg (19.9 μmol/min per mg) and on native collagen 5.7 mg/min per mg, respectively. In order to find whether the *Clostridium* and *Achromobacter* collagenases cleave the synthetic substrate and native collagen at a comparable rate, the collagen

TABLE II

AMINO ACID COMPOSITION OF THE COLLAGENASE FROM *CLOSTRIDIUM HISTOLYTICUM*

All values were recalculated in residues per mol on the basis of M_r 70 000. n.d., not determined.

Amino acid	This work	Ref.				
		12	16	13	17	38
Lys	54.4	35	65	61	61	51
His	11.6	29	10	9	9	11
Arg	20.7	22	18	22	17	17
Asp + Asn	92.0	43	97	95	102	96
Thr	34.2	34	44	32	36	42
Ser	43.9	70	44	16	16	40
Glu + Gln	58.3	49	56	51	54	60
Pro	23.1	60	16	33	24	19
Gly	47.2	153	53	57	57	57
Ala	26.5	117	38	43	41	36
Half-Cys	2.1	—	—	—	—	—
Val	38.5	33	37	39	39	32
Met	11.1	8	8	—	—	4
Ile	37.1	21	34	33	35	33
Leu	46.3	29	43	43	48	48
Tyr	38.5	15	43	30	29	49
Phe	22.7	16	26	30	29	29
Trp	5.3	—	—	—	—	n.d.
Ser-P	2.1	n.d.	n.d.	n.d.	n.d.	n.d.

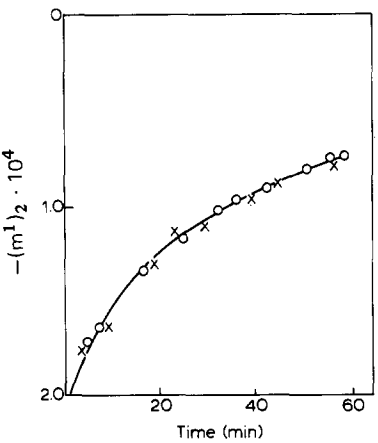

Fig. 3. Degradation of collagen by *Clostridium* (xx) and *Achromobacter* (**) collagenase at 30°C as followed by optical rotary dispersion at 215 nm [30]. Collagen (calf skin, type III, Sigma) was dissolved in 0.1 M Tris-HCl/5 mM $CaCl_2$/0.4 M NaCl buffer, pH 7.2; the concentration was 0.032%, cuvette volume 3 ml. 10 µl of enzyme solution contained 8.5 nkat (*Clostridium*) and 9.0 nkat (*Achromobacter*) of enzyme.

assay was effected in enzyme-substrate ratios which, in an independent assay, gave an identical catalytic activity on the synthetic peptide for the two enzymes (Fig. 3). Under these conditions, the cleavage rates of native collagen by the two enzymes follow the same pattern.

Discussion

We found difficulty in obtaining a pure *Clostridium* collagenase from a crude lyophilized commercial enzyme. Such samples had already undergone proteolytic alterations which created a complex mixture. It is known that *Achromobacter* collagenase yields, by limited autolysis, products which remain enzymatically active [22]; a similar process could occur in the crude *Clostridium* enzyme. On the contrary, if the isolation was effected rapidly from the culture medium, only one collagenase results. Its molecular weight of 68 000—70 000, obtained by molecular sieve chromatography, is close to the value obtained by Lwebuga-Mukasa et al. [8] for the different isolated multiple forms of the enzyme, 72 000 and 81 000, respectively. The value over 100 000, obtained by SDS-polyacrylamide gel electrophoresis is much less probable, firstly because molecules over 100 000 were eliminated by the HP-100 ultrafiltration during the preparation of the enzyme and secondly, because Lwebuga-Mukasa et al. [8] have already described the anomalous behaviour of collagenase in this system.

The molecular weight values are unusually high for a proteolytic enzyme. In the case of *Achromobacter* collagenase, the dissociation of the active dimer of M_r 70 000 was obtained either in alkaline pH or by inactivation by EDTA [22]. However, *Clostridium* collagenase under these conditions either remains unaltered, or disintegrates completely.

The amino acid composition of *Clostridium* collagenase (Table II) differs from the data previously published, particularly by the presence of cysteine,

methionine, tryptophan and *O*-phosphoserine.

The existence of two cysteine and two *O*-phosphoserine residues per molecule, together with spontaneous proteolysis yielding still active forms, could explain the difficulties in always isolating the same form of the enzyme. It is probable that different conditions of culture and isolation yielded enzyme forms of different degree of post-transcriptional phosphorylation, disulfide crosslinking and partial degradation.

The specific activity of pure *Clostridium* collagenase on the synthetic substrate Pz-Pro-Leu-Gly-Pro-D-Arg varies from one preparation to another between 325 and 340 nkat/mg (19.5—20.5 μmol/min) which is about 23% higher than fraction A-α (16.2 μmol/min per mg) published by Kono [7]. In this fraction Kono also found the highest activity on native collagen (5.1 as compared with our values of 5.7), whereas his other fractions (B-α and B-β) were much less active. This predominance of one highly active form is in accordance with our conclusion that *Clostridium* produces only one collagenase which in turn can yield less active forms.

As shown in Fig. 3, the ratio of the values obtained by the two assays on native collagen and synthetic substrate are similar for the collagenase *Clostridium* and *Achromobacter*. By both methods, the pure *Clostridium* enzyme has about 6-times lower specific activity than the *Achromobacter* enzyme.

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