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CHEMICAL CHARACTERIZATION AND STUDY OF THE AUTODIGESTION OF PURE COLLAGENASE FROM *ACHROMOBACTER IOPHAGUS*

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Summary

Only one collagenase (EC 3.4.24.3) is produced by the non-pathogenic *Achromobacter iophagus* strain. The chromatography of the crude enzyme on DE-32 cellulose followed by gel filtration on Sephadex G-100 in the presence of 1 M sodium chloride led to the isolation of a homogeneous enzyme. Its specific activity (1.642 μkat/mg) represents the highest value ever obtained for a bacterial collagenase. The amino acid composition of *A. iophagus* collagenase differs from that of *Clostridium histolyticum* mainly in the sulfur-containing amino acids. 1 mol of zinc was found for 1 mol of enzyme of molecular weight 104 000. The autodegradation of the *A. iophagus* collagenase results in the formation of at least three active fractions which can be separated by preparative polyacrylamide gel electrophoresis as well as rechromatography on DE-32 cellulose. They are active towards the synthetic substrate as well as towards the native collagen. The results of ORD have shown that the digestion of the last one occurs in the helical parts of the substrate.

Introduction

The collagenase (EC 3.4.24.3) from a non-pathogenic *Achromobacter iophagus* strain is an enzyme that splits the X-Gly bond in the sequence -Pro-X-Gly-Pro, both in the native collagen and in the synthetic substrates [1].

In our previous study we have already described the partial chromatographic purification of the collagenase from *A. iophagus* [2]. Although the specific activity of the enzyme was 1.587 μkat/mg (the highest activity obtained as yet for any collagenase), we found by polyacrylamide gel electrophoresis that the enzyme was heterogeneous and contained different active fractions.

A separation of the two electrophoretically different collagenases from *A. iophagus* was also described by Welton and Woods [3].

The present study was initiated to elucidate whether different isoenzymes are present even in the purified enzyme or whether the various enzymatically active fractions resulted from the degradation of the single collagenolytic enzyme produced by the bacterial cell. This report describes the isolation, characterization and stabilization of the pure enzyme and proves that the electrophoretically different fractions, all active towards native collagen and synthetic substrate, resulted from the autodigestion of the single original collagenase.

Methods

Purification of collagenase

Freshly prepared crude collagenase from *A. iophagus* of specific activity 0.17 μkat/mg was a gift from Institut Pasteur Production. Partial purification of the enzyme was accomplished by DE-32 cellulose chromatography as described earlier [2]. The final purification of collagenase was achieved by gel filtration on a Sephadex G-100 column (100 cm × 4 cm) which was equilibrated with 0.3 M Tris · HCl/0.01 M $CaCl_2$/1 M NaCl buffer (pH 7.0). For the desalting of collagenase samples a Sephadex G-100 column (100 cm × 2 cm) equilibrated with 0.01 M $CaCl_2$ (pH 7.0) was used.

Gel electrophoresis

Analytical disc electrophoreses were performed according to Jovin et al. [4] on a Buchler apparatus with 12-cm long tubes. Preparative disc electrophoresis was carried out according to the technique of Davis [5]. The upper gel was omitted. 50—100 μ of protein were applied in the analytical runs and 25 mg were applied in the preparative run. The fractions from two preparative runs which corresponded to each of the peaks were pooled together and concentrated by ultrafiltration to a volume of 1 ml.

Assay

Hydrolysis of the synthetic peptide. Collagenase activity was measured colorimetrically using 4-phenylazo-benzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine dihydrate (Pz-Pro-Leu-Gly-Pro-D-Arg, Fluka) according to Wünsch and Heidrich [6]. The buffer was brought to a concentration of 3 mM $CaCl_2$. Numerical data have been recalculated on the basis of 1 pkat = 0.09 units, according to Wünsch and Heidrich.

Hydrolysis of soluble collagen. The digestion of the native collagen (calf skin, Worthington) was evaluated by two methods. The measurement of the initial rate of increase of free α-amino groups was determined colorimetrically after reaction with trinitrobenzenesulphonic acid [7].

The disappearance of the helical structure of the native collagen during its digestion by collagenase and differently degraded fractions, was studied according to Keil et al. [1] on a SICA recording polarimeter. The assay is based on the disappearance of the negative Cotton effect in the collagen solutions at 215 nm.

Protein determination

The concentration of protein in the solution was determined by the method

of Lowry et al. [8]. Protein elution profiles of the chromatographic and preparative electrophoretic columns were monitored at 280 nm.

Amino acid and zinc analyses

Amino acid analyses were performed with a Beckman Unichrome amino acid analyser. Protein hydrolysis was carried out at 110° C under nitrogen, in sealed Pyrex tubes, for 24, 48 and 72 h. Values for the serine, threonine, valine, isoleucine and tyrosine content were obtained by extrapolation to zero time. The cysteine and methionine content was obtained after oxidation with performic acid [9]. Tryptophan was determined after hydrolysis by methanesulphonic acid, according to Liu and Chang [10].

The zinc content was determined colorimetrically according to Sandell [11].

N-Terminal determinations

The first two steps of N-terminal sequence of degraded forms of the enzyme were determined according to Edman [12].

Results

Isolation of pure collagenase

The results of the chromatography of two collagenase samples on DE-32 cellulose are shown in Fig. 1. Only one peak of the collagenase activity was eluted from DE-32 cellulose column when crude collagenase was directly applied to the column (Fig. 1a). Another sample of the same preparation of crude collagenase was incubated in 10 mM $CaCl_2$ at 4° C and pH 7.0 in the presence of 0.1 mM *p*-chloromercuribenzoate. The *p*-chloromercuribenzoate was added in order to inhibit non-collagenase proteolytic activity, as was described earlier [2]. After 10 days, the specific activity of the enzyme remained at the original value of 0.17 μkat/mg. Nevertheless the chromatography of a pre-incubated sample on DE-32 cellulose (Fig. 1b) resulted in a considerable diminution of the most acidic peak and in the formation of less acidic products that were collagenolytically active.

The *A. iophagus* collagenase (last peak, Fig.1a) is electrophoretically homogeneous after purification on DE-32 cellulose (Fig. 2a). Its specific activity is 1.587 μkat/mg. Nevertheless it still contains low molecular weight impurities which can be eliminated by gel filtration, as we have already described [2]. If this step was performed in a diluted buffer, a partial decomposition of the enzyme was observed (Fig. 2b), and its specific activity decreased to 1.043 μkat/mg. On the other hand, we found that collagenase purified by DE-32 cellulose was rather stable in a solution containing 1 M NaCl at pH 7.0. This stabilizing effect of 1 M NaCl was used in the second step of the purification. A solution of 20 mM $CaCl_2$ (pH 7.0) which we have used in gel filtration in previous work [2] was replaced by a buffer of the following composition: 0.3 M Tris · HCl/1 M NaCl/10 mM $CaCl_2$ (pH 7.0). The results shown in Fig. 2c indicate that this change permitted us to obtain an electrophoretically homogeneous enzyme. Its specific activity was increased to 1.642 μkat/mg.

Pure collagenase could be stored for 2 weeks at 4° C in a solution of 1 M NaCl without any change of the electrophoretic pattern or activity. A longer

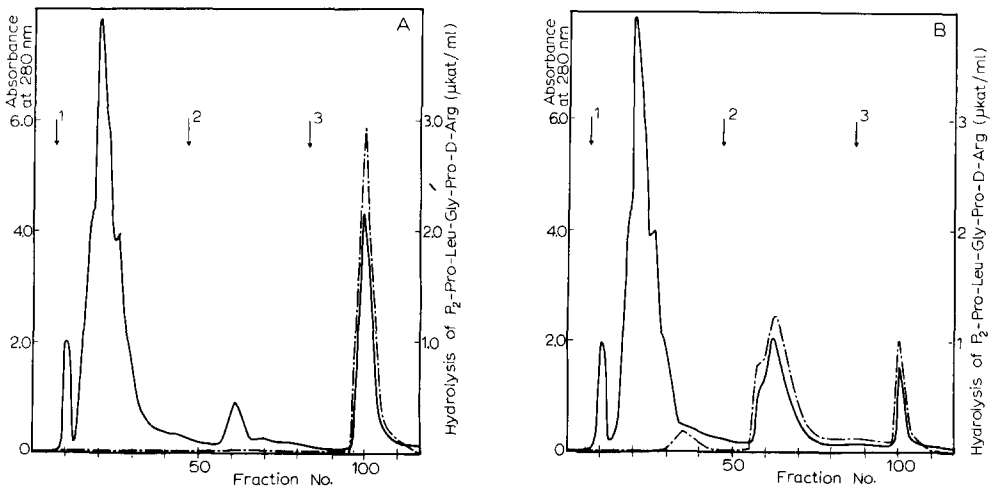

Fig. 1. Chromatography on DE-32 cellulose of crude *A. iophagus* collagenase. A column (15 cm × 4 cm) was equilibrated with 50 mM Tris · HCl/10 mM $CaCl_2$ buffer (pH 7.0). A stepwise elution was made at pH 7.0 by subsequent application of three Tris · HCl buffers: (1), 0.2 M, (2), 0.3 M and (3), 0.3 M brought to 1 M NaCl. All buffers contained 10 mM $CaCl_2$. The flow rate was 50 ml/h, fraction volumes, were 10 ml. 1 g of crude collagenase was dissolved in 50 ml of stabilizing buffer, containing 0.1 mM *p*-chloromercuribenzoate. (a) The sample was immediately applied on the column. (b) The sample was incubated 10 days at 4°C and then applied on the column. ———, absorbance at 280 nm; - - - - -, collagenase activity on Pz-Pro-Leu-Gly-Pro-D-Arg (μkat/ml).

storage resulted in the appearance of additional bands (Fig. 2d).

On the other hand no traces of decomposition were found after 2 months of storage at 4°C and pH 7.0, in the presence of 0.1 M histidine. As we have already mentioned [2], histidine is a reversible inhibitor of the collagenolytic activity.

Amino acid composition and the metal content of the Achromobacter iophagus collagenase

The amino acid composition of the pure collagenase is shown in Table I. The high content of aspartic and glutamic acid explains the acidic properties of this protein. It is important to mention the presence of cyst(e)ine and methionine, which are absent in the *Cl. histolyticum* collagenases.

1 mol of zinc was found per molecular weight unit of 104 000.

Electrophoretic separation of the products of the collagenase decomposition

A gel filtration on Sephadex G-100 in diluted buffers of pure collagenase from Fig. 1a resulted in the formation of an electrophoretically heterogeneous mixture of several collagenolytically active compounds (Fig. 2b). An Edman degradation of this mixture revealed in the first step only one N-terminal residue, glycine. Alanine was determined as a second residue. This seems to indicate that the predominant N-terminal sequence of degradation products is Gly-Ala.

The separation of this mixture on the preparative polyacrylamide gel electrophoresis is shown in Fig. 3a. A second run of peak I from Fig. 3a under the

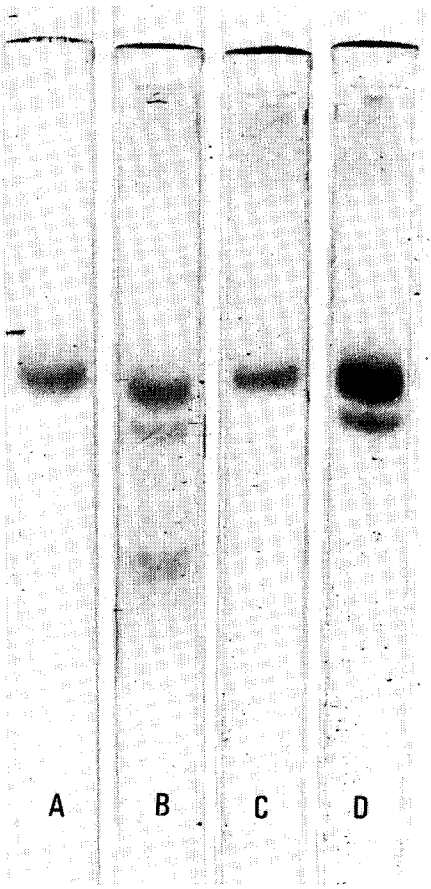

Fig. 2. Analytical polyacrylamide gel electrophoresis. (A) *A. iophagus* collagenase after purification on DE-32 cellulose (Fig. 1a last peak). (B) The same collagenase after gel filtration in the absence of NaCl and (C) after gel filtration in the presence of 1 M NaCl. (D) Pure collagenase after three weeks of storage in the solution of 1 M NaCl at 4°C.

same conditions showed that its position on the elution diagram was not changed (Fig. 3b). On the other hand, an identical separation of the more acidic peak IV from Fig. 3a gave the same elution pattern as the original mixture (Fig. 3c). Therefore all the products of the decomposed collagenase originated from the more acidic fraction IV, which corresponds to the pure collagenase on the analytical polyacrylamide gel electrophoresis.

Characterization of the isolated fractions

The results of analytical polyacrylamide gel electrophoresis (Fig. 4) indicate that only traces of protein are present in fraction I. Consequently, this fraction must be mostly composed of peptide fragments produced during the collagenase degradation, which are not susceptible to staining. This explains that fraction I has no activity with synthetic substrate and native collagen (Table II).

Three other fractions (II, III and IV in Table II) were active with synthetic

TABLE I

AMINO ACID COMPOSITION OF *A. IOPHAGUS* COLLAGENASE

(residues per mol)

Amino acid	*A. iophagus* collagenase [c]	*Cl. histolyticum* collagenase [13] [d]
Half-cystine [a]	10.4 ± 0.3	0
Aspartic acid	116.4 ± 0.6	149
Methionine [b]	7.6 ± 0.1	0
Threonine	68.9 ± 0.2	51
Serine	81.4 ± 0.1	25
Glutamic acid	117.3 ± 0.3	80
Proline	21.4 ± 0.6	53
Glycine	84.5 ± 0.9	90
Alanine	72.6 ± 0.4	69
Valine	60.5 ± 0.2	62
Isoleucine	47.5 ± 0.1	53
Leucine	65.6 ± 0.6	69
Tyrosine	44.4 ± 0.2	47
Phenylalanine	39.3 ± 0.4	47
Lysine	34.2 ± 0.7	97
Histidine	22.6 ± 0.6	14
Arginine	27.2 ± 0.8	35
Tryptophane	17.4	7
Molecular weight	104 000	105 000

[a] Determined as cysteic acid.
[b] Determined as methionine sulphone.
[c] Average values of six parallel runs are given.
[d] The results of Harper et al. were recalculated for the molecular weight 105 000.

peptide as well as with native collagen, and the ratio between those activities was of the same order.

The difference in the specific activity of isolated fractions (Table II) could be explained by their different degree of heterogeneity, as shown in Fig. 4. This heterogeneity could be due either to incomplete separation or to decomposition during the separation which was performed at pH 8.9, close to the pH optimum of the enzyme (8.5). The appearance of basic zones in the fractions II and III which are not present in the applied sample also proves that some

TABLE II

SPECIFIC ACTIVITY OF THE FRACTIONS RESULTING FROM SEPARATION OF DEGRADED COLLAGENASE BY PREPARATIVE POLYACRYLAMIDE GEL ELECTROPHORESIS

(Fig. 3a)

Fractions	Specific activity (colorimetry) (μkat/mg)			Helix degradation
	Synthetic peptide	Native collagen	Ratio of two activities	% per μg enzyme per min.
I	0.001	0	—	0
II	0.990	0.112	8.83	0.69
III	1.277	0.149	8.57	1.04
IV	1.611	0.172	9.36	1.78

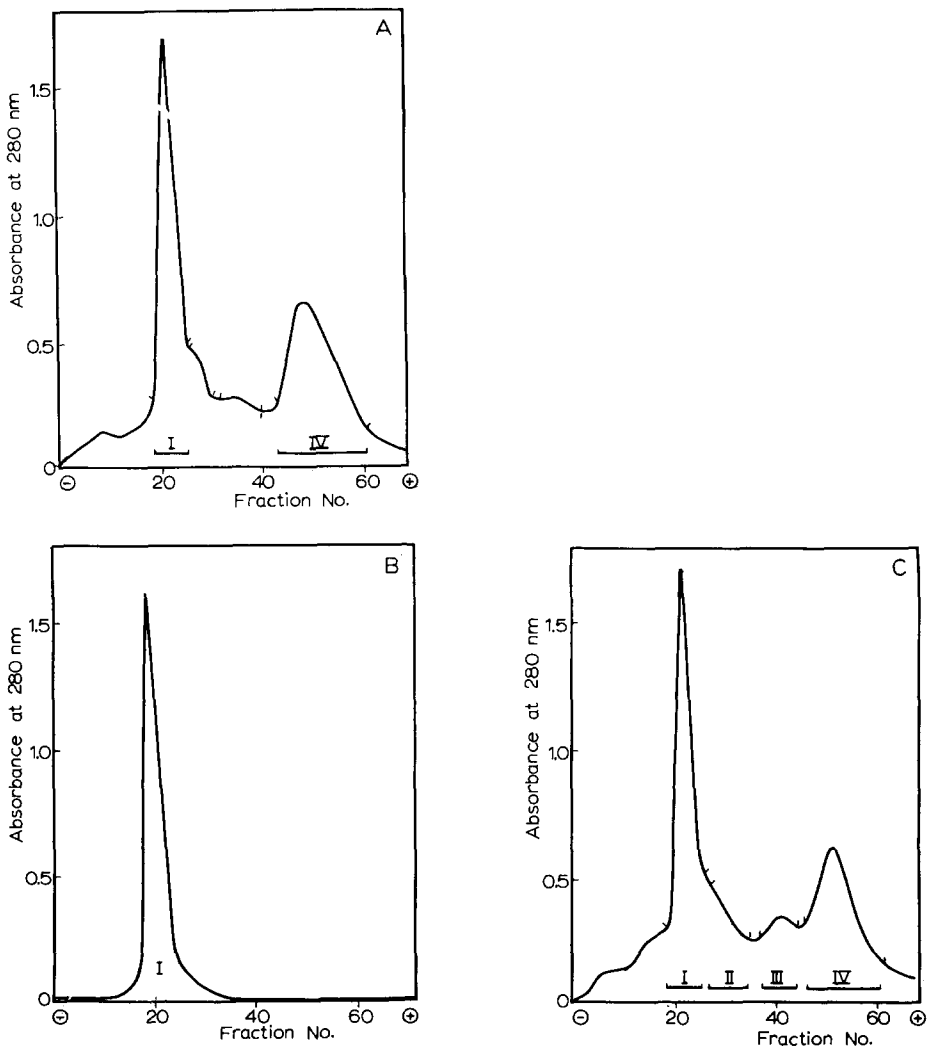

Fig. 3. Elution patterns of the decomposed collagenase after preparative polyacrylamide gel electrophoresis. Electrophoresis was carried out in a Shandon column (12 cm × 2 cm) containing 7.5% gel with glycine/Tris buffer system at a final pH of 8.9. Electrophoresis was performed at 30 mA for 1 h, followed by 80 mA for 12 h. The elution buffer was 0.6 M Tris · HCl/20 mM $CaCl_2$ (pH 7.0). The flow rate of elution was 12 ml per h. ———, protein concentration absorbance at 280 nm. (A) Separation of 25 mg of the preparation obtained from gel filtration on G-100 in the absence of NaCl. (B) Rechromatography of the fraction I from 3a. (C) Rechromatography of the fraction IV from 3a. In B and C the sample originated from two preparative runs (A).

changes occur in the sample during the separation procedure. On the other hand, no change in the pattern of analytical polyacrylamide gel electrophoresis was observed during the storage of isolated fractions at pH 7.0 at 4°C.

In order to know whether all isolated degradation products of the collagenase still split the collagen specifically in its helical regions, the ORD method was used.

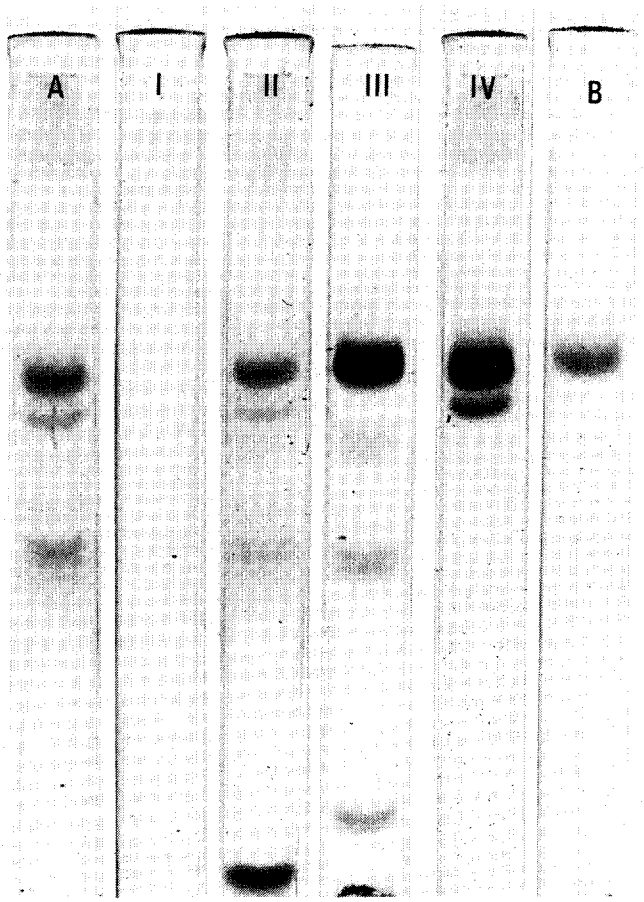

Fig. 4. Analytical polyacrylamide gel electrophoresis. (A) Collagenase obtained by gel filtration in the absence of NaCl. Fractions I, II, III, IV are from the separation represented in Figure 3c. (B) Pure collagenase purified and stored at 4°C in the presence of 1 M NaCl and 0.1 M His.

Fig. 5a shows the optical rotary dispersion recording of native and denatured collagen.

Fig. 5b shows the disappearance of the negative peak at 215 nm during the digestion of native collagen by all the active fractions which were isolated by preparative polyacrylamide electrophoresis. The negative value for non-digested collagen at 215 nm was considered as 100% of helical structure. The extent of disappearance of helical structure in per cent per min could be calculated on the basis of data from Fig. 5b. These data are included in Table II. They show that all degraded forms digest collagen in its helical parts.

Purification of the partially degraded active forms of the collagenase on DE-32 cellulose

In order to obtain the products of the collagenase degradation in pure form, the same sample as for preparative polyacrylamide gel electrophoresis was submitted to rechromatography on DE-32 cellulose. The elution pattern of this

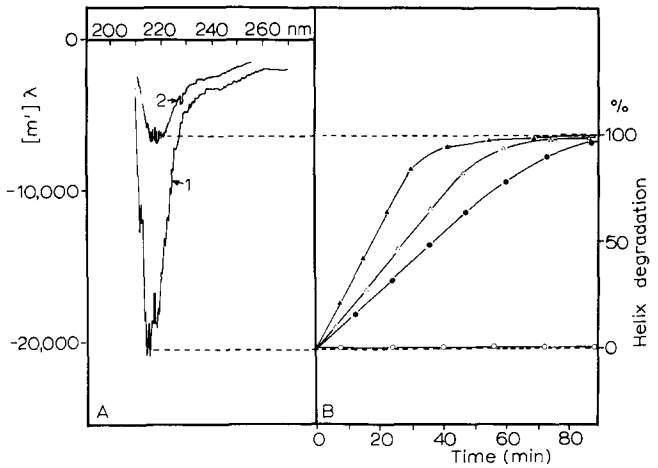

Fig. 5. A, Rotatory dispersion of native skin collagen (1) and gelatin (2). B, Disappearance of the negative Cotton effect during the digestion of the native collagen by the fractions I, II, III and IV from preparative polyacrylamide gel electrophoresis, as monitored at 215 nm. The native collagen and gelatin were dissolved in 0.1 M Tris · HCl/1 mM $CaCl_2$/0.4 M NaCl (pH 7.2) buffer; the concentration was 0.01%. 10 µl of the enzyme solution which contained 1.8 µg of protein from the fractions II, III and IV was injected to the cuvette containing 3 ml of substrate solution. 3.0 µg of protein were injected in the case of the fraction I. Digestion by enzyme from fraction I (○———○), II (●———●), III (△———△), IV (▲———▲).

separation is shown in Fig. 6. The distribution of the collagenase activity is similar to that shown in Fig. 1b.

The results of the analytical polyacrylamide gel electrophoresis (Fig. 7) show

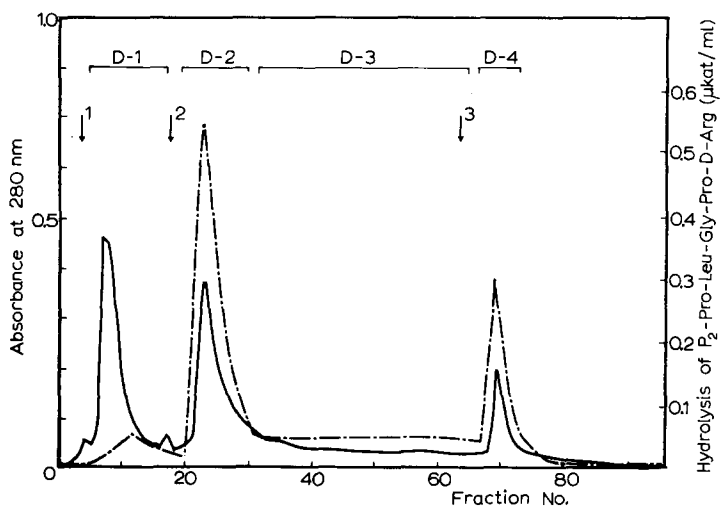

Fig. 6. Rechromatography of collagenase which was obtained by gel filtration in the absence of NaCl, on DE-32 cellulose column (10 cm × 1 cm). The flow rate was 15 ml/h, the fraction volume was 2.5 ml. A 2-ml sample containing 10 mg of protein was applied on the column which was equilibrated with 0.1 M Tris · HCl/10 mM $CaCl_2$ buffer (pH 7.0). A stepwise elution was made at pH 7.0 by subsequent application of three Tris · HCl buffers: 0.2 M, 0.3 M and 0.3 M made 1 M in NaCl. All buffers contained 10 mM $CaCl_2$. ———, absorbance at 280 nm; - - - - -, hydrolysis of Pz-Pro-Leu-Gly-Pro-D-Arg (µkat/ml).

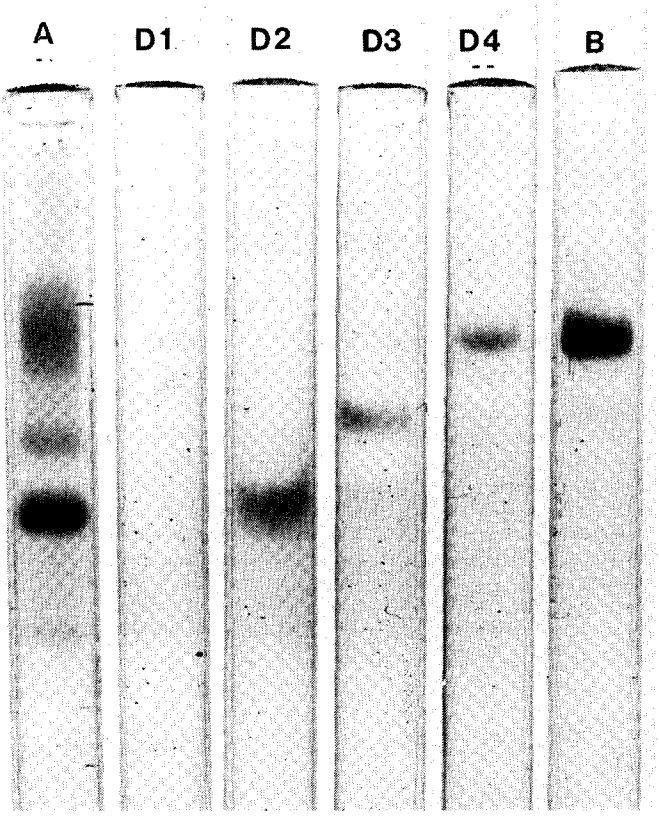

Fig. 7. Analytical polyacrylamide gel electrophoresis of the degraded sample before rechromatography on DE-32 cellulose (A) and of the fractions obtained after rechromatography (D-1, D-2, D-3, D-4); (B) pure collagenase purified and stored at 4°C in the presence of 1 M NaCl and 0.1 M His.

that the main products of the collagenase degradation (D-2, D-3, D-4) were isolated in an almost homogeneous form. The traces of impurities in the fractions D-2 and D-3 could be explained by further degradation during elution, because the eluant buffers contained lower concentration of salts than is neces-

TABLE III

SPECIFIC ACTIVITY OF THE FRACTIONS RESULTING FROM RECHROMATOGRAPHY OF DEGRADED COLLAGENASE ON DE-32 CELLULOSE

(Fig. 6)

Fractions	% of total activity recovered in each fraction	Specific activity (μkat/mg)		Ratio of two activities
		Synthetic peptide	Native collagen	
D-1	7.0	0.874	0.101	8.6
D-2	51.0	1.522	0.167	9.1
D-3	31.4	1.466	0.166	8.8
D-4	15.0	1.488	0.160	9.3
Pure collagenase		1.642	0.165	9.95

TABLE IV

COMPARISON OF BACTERIAL COLLAGENASES

	Collagenase	Specific activity μkat/mg (synth. peptide)	Homogeneity	Ref.
Kono	*Clostridium*	0.270	+	[14]
Welton and Woods	*Achromobacter*	0.107	+	[3]
Lecroisey et al.	*Achromobacter*	1.587	—	[2]
This paper	*Achromobacter*	1.642	+	

sary for the stabilization of collagenase. Nevertheless, the decomposition at pH 7.0 is less pronounced than in the case of preparative electrophoresis at pH 8.9. The fractions D-2, D-3 and D-4 were active with synthetic peptide as well as with native collagen (Table III). Half of the original collagenase activity was eluted in the fraction D-2.

A comparison of the diagrams of the elution from DE-32 cellulose, of the original collagenase used in this study (Fig. 1a) and of the decomposed collagenase (Fig. 6), shows that fraction D-4 corresponds to the pure collagenase. Their mobility on the analytical polyacrylamide gel electrophoresis is also similar (Fig. 7). Therefore, active fractions D-1, D-2 and D-3 originate from D-4 and are the products of its partial degradation. D-4 is identical with the pure enzyme both in electrophoretic mobility (Fig. 7) and specific activity (Table III).

Discussion

The multiplicity of forms described in the last decade for the collagenase of *Cl. histolyticum* led us to the conclusion that the heterogeneity of bacterial collagenases could be provoked by proteolytic degradation during their purification. Indeed our results concerning the isolation and stabilization of the pure collagenase of *A. iophagus* indicate that the presence of NaCl at high concentrations or of reversible inhibitors like histidine, prevents the enzyme from further decomposition. The data of Table IV show that the method described in the present paper led for the first time to the isolation of a homogeneous bacterial collagenase with the highest specific activity.

Our results concerning the zinc content of the *A. iophagus* collagenase are 1 mol of zinc per molecular weight of 104 000. The value of the molecular weight agrees well with the values 111 700 ± 800 and 106 900 which were found earlier by Welton and Woods by electrophoresis in sodium dodecyl sulfate and gel filtration methods, respectively [3]. These data are close to those reported for the collagenase A of *Cl. histolyticum*. The difference between collagenase from *A. iophagus* and that from *Cl. histolyticum* is in the zinc content. The presence of 2 mol of zinc for a molecular weight of 105 000 was described for the latter [15,16].

As shown in Table I, the amino acid composition of *A. iophagus* collagenase is different from that of *Cl. histolyticum*. The most striking difference between the two extracellular collagenases is in the sulfur-containing amino acids. Harper et al. [13] did not find any traces of cysteine, cystine or methionine in

the clostridial collagenase. On the contrary, our results indicate the presence of 10 residues of half-cyst(e)ine and 8 residues of methionine in the molecule of the *A. iophagus* collagenase.

The gel filtration of pure *A. iophagus* collagenase in the absence of NaCl leads to the formation of electrophoretically different, active forms of the enzyme (Fig. 2). Our results indicate that all of them have the N-terminal Gly-Ala sequence.

In experiments which are still under way, we have so far failed to find any N-terminal group in the pure collagenase. On the contrary, the sequence Gly-Ala- was found in aliquots of the degraded enzyme of the same weight. This seems not to be in contradiction with the specificity of the enzyme. According to recent results obtained by our laboratory [1] the proline next to glycine in the typical substrate sequence -Pro-X-Gly-Pro- can be replaced by alanine or leucine. In view of this data, we can consider the decomposition of pure collagenase as an autodigestion.

Further indications of the collagenase autodigestion are provided by the results of rechromatography of the pure collagenase on the DE-32 cellulose column (Fig. 6), as well as by the results of preparative polyacrylamide gel electrophoresis (Fig. 3).

Several forms of *A. isophagus* collagenase which were isolated either by the preparative polyacrylamide gel electrophoresis or by rechromatography on DE-32 cellulose are active with the synthetic peptide and native collagen. The ratio between the two activities is of the same order. Moreover, the results of ORD have shown that different forms digest collagen in the helical parts. Therefore the autodigestion does not change the specificity of the original enzyme substantially. Nevertheless we could not consider the isolated active forms II, III and D-2, D-3, as final products of the decomposition. The results of the analytical polyacrylamide gel electrophoresis proved that under different conditions the degradation continued and resulted in the formation of weakly active fractions as well as in the formation of inactive peptide fragments.

The separation of the freshly prepared crude collagenase on DE-32 cellulose resulted in the appearance of one active peak only, which after further purification from peptide fragments was electrophoretically homogeneous and had a specific activity of 1.642 μkat/mg. Therefore, only one collagenase is produced by the bacterial cell. The multiplicity of the other collagenolytically active forms which has been described above and also in our earlier paper [2] must be attributed to enzyme degradation during the purification procedure.

The isolation of two collagenases from *A. iophagus* was also reported by Welton and Woods [3]. It is hardly possible to compare this result with those obtained in the present study. First of all the crude preparations originated from different culture conditions and they differed also in their specific activity. The low specific activity of the purified collagenase isolated by Welton and Woods (0.107 μkat/mg) indicates further that it was one of the degraded products of the original collagenase.

The formation of the partially digested forms of the proteolytic enzymes without significant change of activity is already described for α and β trypsins as well as for α, γ, δ and π chymotrypsins.

Further work is needed to clarify whether the multiplicity which was found

in the collagenases of *Cl. histolyticum* [14,17,18] or *Pseudomonas aeruginosa* [19] is not due to the same mechanism of enzymatic degradation which was reported in this paper for the collagenase of *Achromobacter.*

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