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NONSPECIFIC COLLAGENOLYTIC ACTIVITY HYDROLYSIS OF NATIVE COLLAGEN BY A MOLD PROTEASE

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SUMMARY

A proteolytic enzyme from culture fluids of *Aspergillus oryzae* was purified by a multistep procedure including carrier-free electrophoresis and DEAE-Sephadex chromatography It was a homogenous protein, judging from results obtained by disc electrophoresis, immunoelectrophoresis and end-group determination

The enzyme hydrolysed native collagen at a slow rate Electron microscopic investigations of the digest revealed triple-helical fragments remaining after degradation of the collagen molecule at its *N*-terminal end The results thus contribute to a current discussion on collagenolysis by proteolytic enzymes other than specific collagenases

INTRODUCTION

A striking property of a highly purified proteolytic enzyme from culture filtrates of *Aspergillus oryzae* was described in previous publications[1,2] Even though its action towards peptide and protein substrates was rather unspecific, this enzyme was also able to hydrolyse native collagen and certain model peptides of collagenlike structure This finding was rather unexpected since collagen and related model substrates were believed for a long time to be digested only by a special class of proteases — collagenases — which are characterized by very restricted specificities (for reviews on bacterial and animal collagenases, respectively, see refs 3–5) However, slow breakdown of the triple-helical body of dissolved native collagen was also observed with trypsin and even with crude neutral proteases of poor specificity such as pronase[6–9] (see ref 5) Thus, we felt our earlier results might be of relevance to a common concept of collagen breakdown

The present paper describes an improved procedure for purification of the aspergillopeptidase which we termed aspergillopeptidase C formerly[2], evidence for homogeneity of the preparation and electron-microscopic investigations of collagen digests obtained with this enzyme

MATERIALS AND METHODS

Enzyme purification

Concentrated culture fluids of *A oryzae* ("Luitase", Luitpoldwerk, Munchen) were ultrafiltrated in the cold room using the apparatus Type SM 16 218 of Sartorius-Membranfilter, Gottingen, and a filter No SM 12 136 The solution thus obtained was centrifuged for 30 min at 4° and 27 000 × *g* before being fractionated by carrier-free electrophoresis (model VAP-I, Bender and Hobein, Munchen) The procedure used was essentially the same as described in detail previously[2], but Tris–acetate buffer was used for the separation (buffer in the separation unit 3 5 g Tris per l, electrode buffer 14 g Tris per l, both adjusted with glacial acetic acid to pH 6 0) Proteolytic activity was monitored by hemoglobin digestion and Fractions 35–41 were pooled accordingly

Pooled fractions (approx 500 ml) were mixed with 60% (v/v) acetone (for procedure, see ref 2) The precipitate collected by centrifugation was dissolved in 4 ml 0 02 M sodium phosphate buffer, pH 6 0, and applied to a DEAE-Sephadex A-50 column (2 5 cm × 80 cm) that was cooled by running tap water The ion exchange material was swollen, washed, freed from fines and equilibrated as described by the manufacturers (Pharmacia, Uppsala, Sweden) Elution was performed with the buffer mentioned above from bottom to top at a rate of 15 ml/h Fractions of 5 ml were collected The fractions constituting the left hand peak of Fig 1 were pooled (60 ml) and the enzyme was precipitated with acetone and collected as above

The following analytical methods were used during purification of the enzyme and to ascertain its homogeneity Proteolytic activity was measured according to ANSON[10] and protein concentration by the procedure of LOWRY *et al* [11] as indicated earlier[2] The cleavage of model peptides was followed by the method of GRASSMANN AND NORDWIG[12] or by thin-layer chromatography (*cf* ref 2) Endgroup determina-

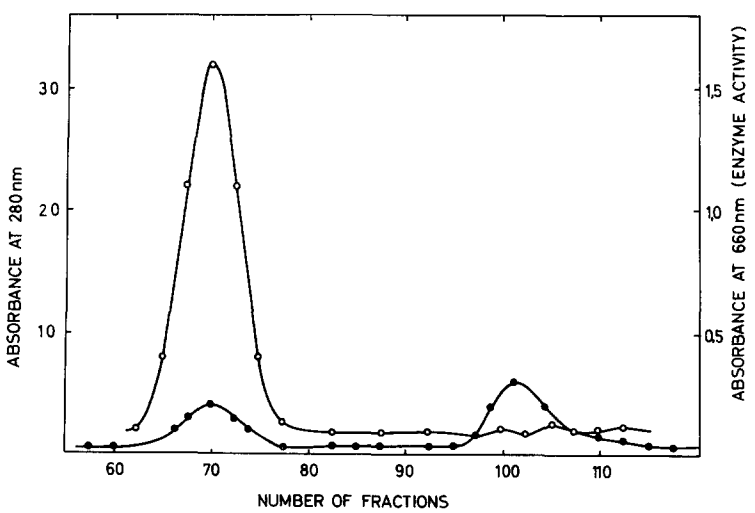

Fig 1 Purification of aspergillopeptidase C by DEAE-Sephadex chromatography For experimental conditions, see text Amount of protein applied, 25 mg ●—●, absorbance at 280 nm, ○—○, enzyme activity (Anson assay)

tions were performed by the dansyl method of GRAY AND HARTLEY[13] Disc electrophoresis (in a 15% gel) and immunoelectrophoresis were performed as already described[2] Dialysed crude culture fluids of *A oryzae*, precipitated with acetone and lyophilized subsequently, were used for preparation of rabbit antiserum

For isoelectric focusing[14,15] of pure enzyme (10 mg), a 1% (w/v) solution of Ampholine (LKB, Bromma, Sweden), was used to fill the separation column (LKB, type 8101) The procedure proposed by LKB (leaflet I-8100-T01) was closely followed (temperature, 5°) At least 65 h were necessary to reach equilibrium at a voltage of 300 V Absorption at 280 nm, proteolytic activity and pH were determined in 3-ml fractions after separation

Enzymatic treatment of collagen

Acid-soluble calf skin collagen was prepared according to a procedure described in detail by VON DER MARCK *et al* [16] A 0 25% (w/v) solution of calf skin collagen was treated with the pure Aspergillus protease (enzyme–substrate ratio, 1 10) in a 0 05 M Tris–HCl buffer, pH 7 0, containing 0 05 M $CaCl_2$ Hydrolysis at 30° was followed by a decrease of optical rotation (see ref 2) The mixture resulting after an incubation of 10 h was fractionated with $(NH_4)_2SO_4$ (11% saturation) The supernatant was treated with ATP to prepare "segment long spacing" crystallites for electron microscopic observation (see, *e g* ref 17) Disc electrophoretic investigations on unfractionated enzymatic digests or fractions obtained with $(NH_4)_2SO_4$ fractionation were carried out in 7 5% gels according to REISFELD *et al* [18]

Incubation of calf skin collagen with highly purified collagenase from *Clostridium histolyticum* (Worthington, Freehold, N J , U S A) at an enzyme–substrate ratio of 1 1000 was performed and followed as above

RESULTS

Purification, homogeneity and properties of aspergillopeptidase

The results of the modified purification procedure are shown in Table I With respect to protein and activity yields as well as to the purity achieved, the present scheme is very similar to our previous one[2] It is, however, superior in terms of separable amounts and of reproducibility since the material resulting from the most effective step, carrier-free electrophoresis, is further separated by column chromatography (Fig 1) Fractionation with acetone alone[2] turned out to be less reliable Further, dialysis of the starting material[2] to remove low molecular weight substances and the bulk of pigments was problematic due to the presence of cellulases Ultrafiltration at this stage is, therefore, a considerable improvement

Disc electrophoretic investigations indicated a single protein component, in agreement with previously reported results[2] Immunoelectrophoresis, using crude and purified enzyme as antigens (Fig 2), also showed a single sharp band N-terminal analysis revealed alanine as the only endgroup, and not even traces of impurities could be detected by the sensitive dansyl procedure To judge from these criteria, our preparation can be regarded as a pure substance This conclusion was supported by isoelectric focusing of the protease after the second acetone treatment Only one protein component was detected The isoelectric point of aspergillopeptidase C thus determined was $pI = 5\ 91$

TABLE I

PURIFICATION OF ASPERGILLOPEPTIDASE C

Fraction	*Vol (ml)*	*Protein (total) (mg)*	*Protein yield (%)*	*Activity (total) (units*)*	*Activity yield (%)*	*Purification factor (-fold)*
Crude	100	14 600	(100)	66 000	(100)	(1)
After ultrafiltration	100	3 440	23 5	26 800	41	1 7
Carrier-free electrophoresis**	500	66	0 45	5 700	8 7	19
Precipitate obtained with acetone (60%)	4	25	0 17	2 800	4 2	27
DEAE-Sephadex chromatography***	60	14	0 1	2 300	3 5	36
Precipitate obtained with acetone (60%)	2	11	0 08	1 900	2 9	38

* By definition, one enzyme unit produces an absorption of 0 1 in the Anson assay (see ref 2)
** Pooled fractions as indicated in MATERIALS AND METHODS
*** Pooled fractions as indicated in Fig 1

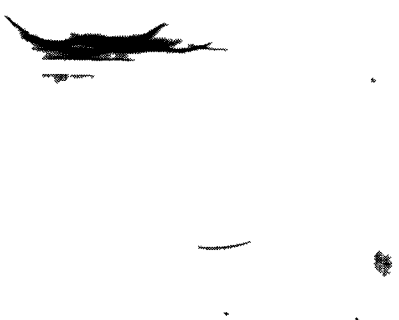

Fig 2 Immunoelectrophoresis of pure aspergillopeptidase C (lower part) and of a crude enzyme fraction (upper part) The latter was also used for the preparation of rabbit antiserum

Lyophylisation of pure enzyme produces at least three fragments, as found by disc electrophoresis and immunoelectrophoresis The specific activity is decreased by 40% On the contrary, storage of aspergillopeptidase C in the frozen state (−20°, 2 mg protein per ml distilled water) for at least 12 months neither affects its specific activity nor the molecular state of the enzyme

Pure enzyme was readily capable of cleaving the synthetic hexapeptide, benzyloxycarbonyl-Gly–Pro–Gly–Gly–Pro–Ala to benzyloxycarbonyl-Gly–Pro–Gly and Gly–Pro–Ala Thus, previously reported results[1,2] could be verified The digestion by aspergillopeptidase C of oxidised B-chain from insulin as followed by endgroup analysis, and further properties are reported in a separate paper[19]

Action of aspergillopeptidase C on collagen

Slow decrease of specific optical rotation was observed upon incubation of collagen solutions with the Aspergillus enzyme (Fig 3) Enzyme-free controls indi-

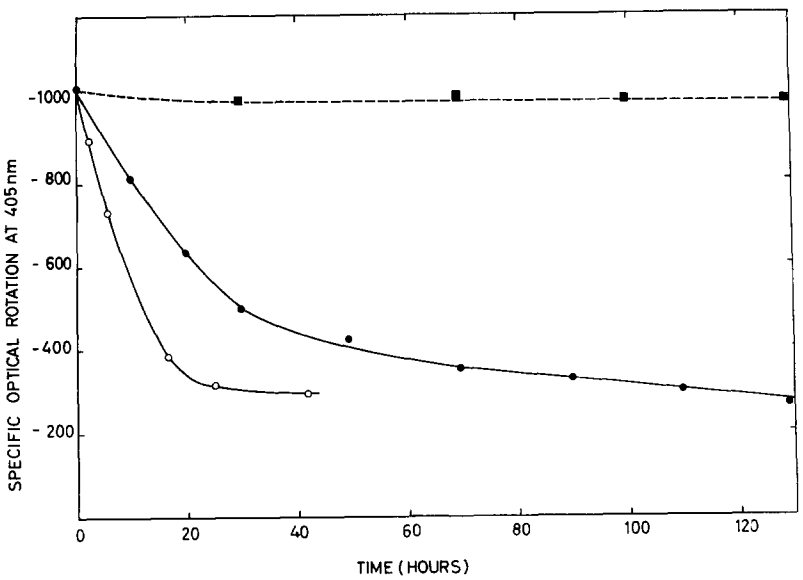

Fig 3 Degradation of calf skin collagen by aspergillopeptidase C (enzyme–substrate ratio, 1 10 ●—●) and bacterial collagenase (enzyme–substrate ratio, 1 1000 ○—○) Incubation medium Tris buffer–$CaCl_2$, pH 7 0 Temperature, 30° ■ — — — ■, control incubation

cated the suitability of the experimental conditions used which guarantee the native state of the substrate and, concomitantly, reasonable reaction rates The T_m value (midpoint denaturation temperature) of calf skin collagen under the conditions applied is 39° Disc electrophoretic investigations of the digest after varying incubation periods showed very complex mixtures of degradation products Fractionation with $(NH_4)_2SO_4$ was only partially successful (Fig 4) Likewise, molecular fragments of different length were seen as "segment long spacing" in the electron microscope when digests were fractionated with $(NH_4)_2SO_4$ The "segment long spacing" shown in Fig 5 serves only as an example It should be emphasized that in our experiments only fragments could be detected which were proteolytically attacked at the N-

Fig 4 Disc electrophoretic analysis of calf skin collagen before (a) and after incubation (b) with aspergillopeptidase C for 10 h (see Fig 3) The disc patterns of precipitates obtained by $(NH_4)_2SO_4$ fractionation at 8, 10, 12, 14 and 16% saturation are shown in c–g

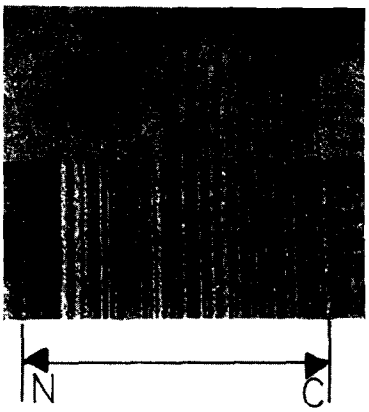

Fig 5 "Segment long spacing" crystallites of collagen molecules (lower part) and collagen molecule fragments obtained by treatment with aspergillopeptidase C (upper part) Positive staining with phosphotungstic acid and uranylacetate N,N-terminus of the collagen molecule C,C-terminus Length of the original molecule as indicated by the arrows, 3000 Å

terminus of the molecule whereas the C-terminal end appeared to be resistant Our results are thus slightly different from those obtained with clostridial collagenase, which removes about 10% of the total molecule length from the C-terminus and which produces only few molecule fragments of well-defined length[17,20]

Partially purified enzyme (purification factor 27, see Table I) was also used for these types of experiments with practically identical results

DISCUSSION

Direct evidence is presented in the present paper for hydrolysis of the triple-helical structure of collagen by aspergillopeptidase C Even though, we do not consider the mold protease a collagenase Two major parameters of its action, *i e* the specificity requirements and the rate of attacking collagen, are markedly different from those of specific collagenases

Aspergillopeptidase C is a protease of rather poor specificity, digesting proteins such as hemoglobin, casein, serum albumin and others, as well as a variety of peptide bonds in polypeptide substrates, *e g* insulin or glucagon[2,19] This property, combined with the inability to cleave amino acid peptide bonds[1,2], accounts for casual and, therefore, slow cleavage of sequences frequently occurring in collagen, ↓ Gly–Pro–R ↓ Gly–Pro–R ↓, at the sites indicated (R represents amino acid residues) In contrast, collagenase from *Cl histolyticum* (clostridiopeptidase A, EC 3 4 4 19) hydrolyses exclusively these types of sequences, including bonds with proline or hydroxyproline in position R

These narrow specificity requirements, unique in protease chemistry but met by a likewise unique primary structure of collagen, are reflected by reaction rates which are faster by orders of magnitude as compared with nonspecific proteases Under identical conditions of incubation, pure collagenase cleaves native collagen at a rate 250 times faster than pure aspergillopeptidase (Fig 3) As already reported[2], the respective factor for the hydrolysis of the model peptide, benzyloxycarbonyl-Gly–Pro–Gly–Gly–Pro–Ala, is 800

It should be mentioned that collagenases from animal sources also exhibit an extremely specific mode of attacking collagen (though different from clostridiopeptidase A, see refs 4 and 5), and there are good reasons to assume that the corresponding reaction rates are also very high

Therefore, we suggest[5] the reaction rates for the digestion of native collagen be used to differentiate, if necessary, between specific and nonspecific collagenolytic enzymes, since there are no suitable methods currently available for measuring the binding constants of macromolecular substrates

The question arises as to how nonspecific proteolysis of the native collagen molecule is possible at all In this context, one has to consider the interpretation of KUHN AND EGGL[9,20] explaining the exopeptidase-like action of clostridial collagenase and other proteases These authors suggested that, in a temperature-dependent reaction, slow continuous unfolding at the ends of the triple-helical molecule would expose short regions to proteolytic attack In other words, special features of the collagen triple-helix appear to play a decisive role Our findings are entirely compatible with these ideas One consequence of the above hypothesis, slow degradation of native collagen by otherwise quite unspecific proteases in a manner resembling the action of exopeptidases, could be verified by our results One is led to assume that nonspecific collagenolytic activity is not at all a rare event Preliminary results obtained with proteases from *Bacillus subtilis* and *Bacillus pumilus* (FABIÁN AND NORDWIG, unpublished) appear to favour this assumption

The relevance of nonspecific collagenolytic activity to *in vivo* processes is a very open question But there can be no doubt that breakdown of collagen, whether soluble or insoluble, in animal organisms is initiated by a specific enzyme, collagenase[3,4]

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