CLEAVAGE OF β-CASEIN BY COLLAGENASES FROM *ACHROMOBACTER IOPHAGUS* AND *CLOSTRIDIUM HISTOLYTICUM*

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1. Introduction

It has been established that collagenase from *Clostridium histolyticum* (EC 3.4.24.3) is a proteolytic enzyme with a very high degree of substrate specificity which cleaves the helical region of native collagen, preferentially the bonds Y–Gly in sequence of the type Pro–Y–Gly–Pro–Y where Y is most frequently a neutral amino acid [1,2].

Recently, a new collagenolytic enzyme from a non-pathogenic aerobic *Achromobacter iophagus* strain described by Woods and Welton [3,4] has been obtained in the homogeneous state in our laboratory [5,6]. Its activity is many times higher than the purest samples of collagenase from *Clostridium histolyticum*. The mode of action of these two bacterial collagenases on native collagen and on a synthetic substrate has been compared in a previous study [7].

Even in the highly purified homogeneous *Achromobacter* collagenase we have found traces of caseinolytic activity [5,6]. On the other hand according to several authors the purified *Clostridium* collagenase is free of caseinolytic activity [8,9].

β-Casein is a proline-rich protein. When its primary structure is examined, several short sequences within the molecule resembling those in collagen can be found. In this connection it was of interest that some proteins with a relative high content of proline, like those from human parotid saliva [10] or immunoglobulin light chains [11] were susceptible to the cleavage by purified, but still not homogeneous *Clostridium* collagenase. It was therefore of interest to determine whether the residual caseinolytic activity in pure *Achromobacter* collagenase is due to another proteinase present in traces or if this collagenase can also selectively split bonds in proteins other than collagen. In this report we have studied the specificity of the proteolytic action of pure *Achromobacter* collagenase and of a commercial purified *Clostridium* collagenase on β-casein.

2. Experimental

2.1. *Materials and methods*

Crude collagenase from *Achromobacter iophagus* was a gift from Institut Pasteur Production. Homogeneous *Achromobacter* collagenase of specific activity 1.58 μkat per mg on the synthetic substrate 4-phenylazo-Cbz–Pro–Leu–Gly–Pro–D-Arg has been obtained as described [5,6]. Collagenase from *Clostridium histolyticum* type III fraction A, substantially free of protease and peptidase activity was a product from Sigma. Purified bovine β-casein was kindly provided by Dr B. Ribadeau-Dumas (CNRZ, Jouy-en-Josas).

2.2. *Collagenase digestion of β-casein*

The digestion of β-casein with collagenase was performed in 0.1 M $(NH_4)_2CO_3$ buffer, pH 7.8, at 30°C for different times. An enzyme: substrate molar ratio of 1:50 was used with *Clostridium* collagenase and 1:100 for *Achromobacter* enzyme. In each case, the reaction was stopped by lyophilisation.

2.3. *Fractionation of the enzyme digests*

The enzyme digests were subjected to gel filtration on a column (0.9 × 28 cm) of Sephadex G-25 equilibrated and eluted with H_2O. Purification of small fragments was achieved by descending paper chromato-

graphy on Whatman 1 MM paper in butanol–acetic acid–water pyridine (60:12:48:40) for 18 h.

2.4. *Edman degradation*

Automated Edman degradation of digestion mixture was performed with a Beckman Sequencer Model 890 C by the method of Edman and Begg [12], as modified by Hermodson et al. [13]. The resulting PTH amino acids were identified by gas chromatography and by thin-layer chromatography.

2.5. *Amino acid and end group analyses*

Peptides were hydrolyzed in 6 M HCl at 110°C for 24 h. The dried hydrolysate was examined on a Locarte analyser. N-terminal amino acids were determined by dansylation and thin layer chromatography on the DNS-derivatives [15].

3. Results and discussion

After incubation with *Achromobacter* collagenase for different times, β-casein yielded fragments which could be easily separated by gel filtration on a Sephadex G-25 column according to their size in two groups. From the low mol. wt. fraction three peptides could be purified by subsequent paper chromatography: Gly (Pro, Val, Arg); Gly (Pro_3, Val_2, Arg, Gly, Phe, Ile_2); Gly (Pro_2, Phe, Ile_2, Val). They correspond in β-casein unambiguously to the sequences 199–202, 199–209 and 203–209 respectively. Instead of attempting to separate the high mol. wt. fragments, we have subjected the whole β-casein digest to automatic Edman degradation. In each of the six steps the PTH-derivatives have been identified (table 1). One set of amino acids identified corresponds to the N-terminal sequence of β-casein, another to the fragment 199–209, the same as found independently by chromatography, the two remaining sets can unambiguously be attributed to sequences starting with Gly-64 and Ala-103 (fig.1).

These results indicate that in β-casein four bonds are cleaved by *Achromobacter* collagenase three of them involving the amino group of glycine, one of alanine; in each case the next residue is a proline. The rate of hydrolysis of the bond Pro-63–Gly-64 is relatively slower as it appears only after more extensive digestion.

It is evident that the usual routine assay of caseinolytic activity cannot reveal any significant degradation, even if four bonds have been cleaved: the high mol. wt. fragments are precipitated by trichloracetic acid and the small soluble fragment 199–209 has no significant absorption at 280 nm. On the other hand, the trace of caseinolytic activity which we have observed in the homogeneous *Achromobacter* collagenase [5,6] is now explained as inherent to the collagenase itself. The bonds Y–Gly cleaved correspond well to the specificity of *Achromobacter* collagenase on collagen and on synthetic substrates, as well as to the bonds cleaved during the autolysis of collagenase [6].

The lower part of table 1 shows the PTH-amino acids obtained by the stepwise degradation of the β-casein incubated with the collagenase from *Clostridium histolyticum*. As in the case of degradation with the *Achromobacter* enzyme, the analytical evaluation was very neat and no overlaps have been observed: as illustration of this the sixth step in the

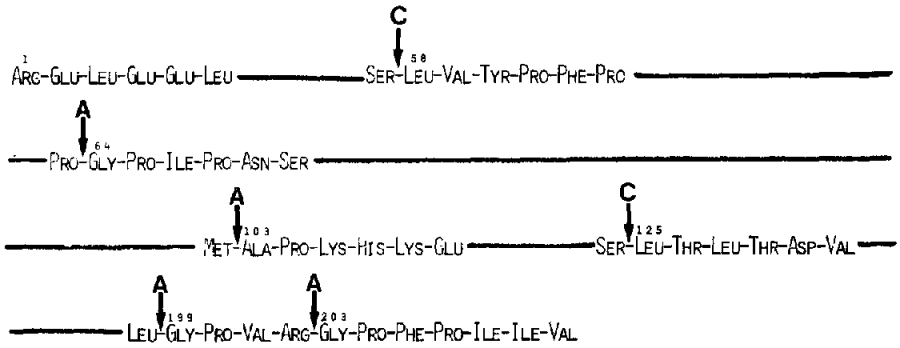

Fig.1. Bonds cleaved by collagenase from *Achromobacter iophagus* (A) and *Clostridium histolyticum* (C) in β-casein [14].

Table 1
Step-wise degradation of β-casein after incubation with collagenases from *A. iophagus* and *C. histolyticum*

		PTH-amino acids Step						Corresponding sequence [14]
		1	2	3	4	5	6	
β-Casein, zero time		Arg	Glu	Leu	Glu	Glu	Leu	1–6
β-Casein + collagenase *Achromobacter* (100:1)	1h	Arg Glu Ala	Glu Pro	Leu Val Lys	Glu Arg His	Glu Gly Lys	Leu Pro Glu	1–6 199–204 103–108
	15 h	Arg Gly Ala	Glu Pro	Leu Val Lys Ile	Glu Arg His Pro	Glu Gly Lys Asn	Leu Pro Glu Ser	1–6 199–204 103–108 64–69
β-Casein + collagenase *Clostridium* (50:1)	30 min	Arg Leu	Glu Val	Leu Tyr	Glu Pro	Glu Phe	Leu Pro	1–6 58–63
	60 min	Arg Leu	Glu Val Thr	Leu Tyr	Glu Pro Thr	Glu Phe Asp	Leu Pro Val	1–6 58–63 125–130

digest by *Clostridium* collagenase is reproduced in fig.2.

The appearance of additional PTH-amino acids to the N-terminal sequence after 30 and 60 min of incubation can be attributed to the cleavage of the bonds Ser-57–Leu-58 and Ser-124–Leu-125. The preferential release of two terminal leucine residues, Leu-58 and Leu-125 is in agreement with the observations made by Coletti-Previero [11] that the enzyme liberates N-terminal leucine from the immunoglobulin light chains. This action is close to the specificity of another bacterial proteinase, thermolysin. Miyoshi and Rosenbloom [16] found that *Clostridium* collagenase liberates in catalase, casein and cytochrome predominantly N-terminal glycine. The main difficulty in the interpretation of all these results is that the studies have been undertaken with enzyme preparations whose specific activities were far below the highest value described (Kono, [8]). Consequently the

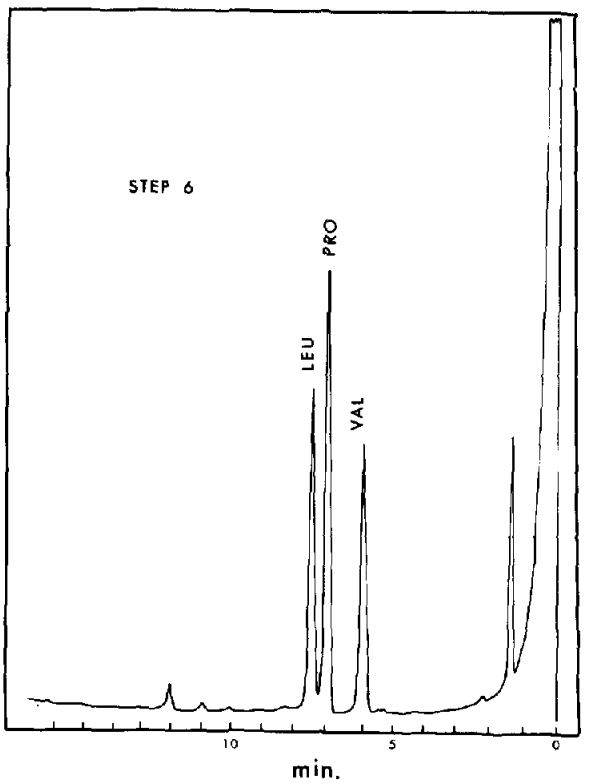

Fig.2. Gas chromatography of PTH-derivatives from digestion of β-casein by *Clostridium* collagenase. Ordinate is response of gas chromatographic detector.

presence in this *Clostridium* collagenase of contaminant proteinases of different specificity cannot be excluded.

The high specificity of *Achromobacter* collagenase and its ability to split bonds close to prolyl residues in proteins could be of help to obtain specific cleavages in regions resistant to other proteinases.

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