EVIDENCE FOR A NEW BACTERIAL COLLAGENOLYTIC ENZYME

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Received August 26, 1965

The commercial collagenase from Clostridium histolyticum represents a complex enzymic mixture. Among these enzymes as yet only the clostridiopeptidase A (E.C. 3.4.4.19) acts at physiological pH on native collagen as well as on synthetic peptides containing the typical sequence -P-X-Gly-P-X- (P is either Pro or Hypro, and X may be any kind of amino acid residue). In this laboratory we recently isolated a new collagenolytic enzyme, which degrades native collagen but does not split the synthetic collagenase substrate Z-Gly-Pro-Gly-Gly-Pro-Ala-OH.

Materials and Methods

The starting material for the enzymic preparation was a crude Clostridium histolyticum collagenase (Worthington Biochem. Corp. Freehold, N.Y. USA). By continuous carrierfree electrophoresis, chromatography on polyamide, and gel filtration on Sephadex G-100 we isolated the fraction of the amidase-esterase (Grassmann, Strauch and

* Supported by a biological-medical scholarship of the Swiss Academy of Medical Sciences.

** Supported by the Helen Hay Whitney Foundation, New York, USA.

Nordwig 1963, Strauch and Grassmann 1965). This fraction, which was free of any detectable clostridiopeptidase A contamination, also contains the new collagenolytic enzyme. Since the amidase-esterase can be completely inhibited by oxidizing agents (Nordwig and Strauch 1963), in all experiments reported here, 0,02 - 0,05 M hydrogen peroxide was added. The enzymic activity was measured on different substrates under identical conditions of incubation at pH 7. Trichloroacetic acid (usually 5 %, but 15 % for precipitation of collagen) was added to stop the reaction. After centrifuging the amount of nitrogen (Strauch 1965), tyrosine (Folin and Ciocalteu 1927), or hydroxyproline (Stegemann 1958, Woessner 1961) was measured in the supernatant. The liberated amino groups were determined by the ninhydrin method (Moore and Stein 1948). As substrate we used acid soluble calf skin collagen (Orekhovich et al. 1948), insoluble beef tendon collagen (Einbinder and Schubert 1951), denatured casein and egg albumin (Kunitz 1947), and denatured haemoglobin (Anson and Mirsky 1932).

Experimental and Results

Table I shows in terms of relative activities on different substrates that the new enzyme preferentially attacks collagen and does not split the synthetic hexapeptide at all.

The clostridiopeptidase A is known to lose rapidly its activity at acid pH especially in calcium free solutions (Mandl et al. 1958, Nordwig and Strauch 1963). We therefore tested our new enzyme on reconstituted calf skin collagen after pretreatment at different pH's in 0.05 M sodium maleate buffer without calcium and compared the results with those obtained from the clostridiopeptidase A treated in the same manner. Table II shows that our enzyme is independent of calcium and hardly damaged by high concentrations of hydrogen ions.

Studies were also made in which collagen was digested successively with highly purified clostridio-

Table I

Relative Activities of the New Collagenolytic Enzyme on Different Substrates in %

Casein denatured	2.9
Haemoglobin denatured	2.6
Egg albumin denatured	1.6
Soluble collagen denatured	100
Soluble collagen native in 0.5 M calcium chloride	50
Reconstituted collagen native (in solid state)	11.3
Insoluble collagen native	6.8
Z-Gly-Pro-Gly-Gly-Pro-Ala-OH in 0.02 M calcium chloride	0

5 mg substrate incubated with 50 µg enzyme for 3 hrs at 30° in 0.05 M Tris buffer pH 7 and 0.05 M hydrogen peroxide. The hexapeptide was tested according to the standard procedure of Grassmann and Nordwig (1960).

Table II

Relative Activities in % on Reconstituted Collagen after Preincubation of the Enzymes for 2 hours at 37° at Different pH without Calcium Chloride

pH	Clostridiopeptidase A	New Enzyme
7	42	100
4	7	67
3	3	72

Clostridiopeptidase A (Worthington, lot number CLS-64 88) was incubated with reconstituted native collagen in the ratio 1 : 50 for 15 min at 37° and pH 7. The new enzyme was incubated with the same substrate in the ratio 1 : 10 for 2 hrs under the same conditions.

peptidase A and our new enzyme or by the same enzymes in reverse order. 10 - 18 mg of native calf skin collagen soluble in 0.1 M sodium maleate and 0.5 M calcium chloride

were incubated at 30° with the corresponding enzyme in the ratio 10 : 1. The enzymic reactions were followed to completion, withdrawing samples at appropriate intervals for ninhydrin determinations. Fig. 1 shows that the action of clostridiopeptidase A was complete in a few hours and that its products were not affected further by subsequent incubation with the new enzyme. On the other hand the new enzyme acts at a ten fold

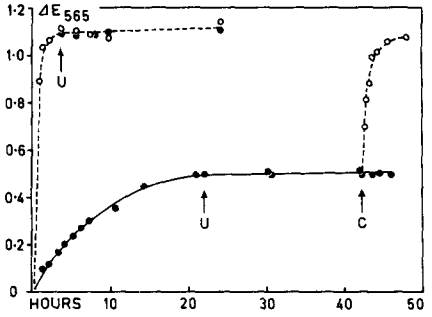

Fig. 1. Hydrolysis of collagen in terms of ninhydrin values by clostridiopeptidase A (broken lines and open symbols) and the new enzyme (black line and symbols). Arrows indicate addition of the same amount of enzyme as at time 0 (C = clostridiopeptidase A, U = new enzyme).

slower rate and the curve levels off at about half of the ninhydrin values found after digestion with the clostridiopeptidase A. With addition of clostridiopeptidase A the degradation proceeds to the expected endpoint. On the basis of free amino groups determined by the ninhydrin method, we calculated the average molecular weight of peptides released from native collagen by the new enzyme to be about 1100. That is twice the molecular weight of the fragments released by the clostridiopeptidase A (Seifter et al. 1959).

In immunoelectrophoretic assays we found that our new enzyme has its own distinct precipitation line in the crude preparation as well as in the purified state and shows no cross reaction with the clostridiopeptidase A.

In contrast to the clostridiopeptidase A the new collagenolytic enzyme is much more stable against acid denaturation, independent of calcium, and not inhibited by mercaptoethanol.

Discussion

The new enzyme degrades native collagen at physiological conditions and thus fits the term "collagenase", but it does not split the synthetic low molecular collagenase substrate. It does not further degrade the peptides released from collagen by clostridiopeptidase A. On the other hand it is able to split only about half of the bonds which can be attacked by the clostridiopeptidase A. Therefore we conclude that this collagenolytic enzyme should be more specific.

Grant and Alburn (1959) first noted the heterogeneity of collagenase preparations by column chromatography. Later Noda et al. (1963) and Mandl et al. (1964) described the separation of 2 collagenase fractions of different activities against collagen and synthetic peptides. The results of Harper, Seifter and Hospelhorn (1965) support the idea, that this may reflect a dissociation of the collagenase into subunits with some limited changes in specificity. These differences may be caused by the existence of different forms of the clostridiopeptidase A, but they may as well be caused by mixtures to a different extent of the clostridiopeptidase A with this second collagenolytic enzyme.

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