EVIDENCE FOR SUBUNITS IN BACTERIAL COLLAGENASE*

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The collagenase of Clostridium histolyticum acts on undenatured collagens, denatured collagens, and on many synthetic peptides which contain a well-defined sequence, -P-X-Gly-P-X-. (P is either Pro or Hypro, and X is any one of many amino acid residues.) The enzyme splits the X-Gly bond and produces peptides with N-terminal glycine. The collagenase of the resorbing tail of the tadpole acts on both undenatured and heat-denatured collagens but not on Cbz-Gly-Pro-Leu-Gly-Pro-OH, a substrate for the bacterial enzyme (Gross and Lapiere, 1962; Nagai, Lapiere and Gross, 1964). The tadpole enzyme, of specificity different from that of bacterial collagenase, catalyzes only a few scissions of the 3 chains of the collagen molecule. Thus, although both enzymes degrade collagen, the extent of proteolysis produced by each is considerably different.

The heterogeneity of clostridial collagenase preparations in column chromatography was first noted by Grant and Alburn (1959). More recently, Noda (1963) and Mandl et al. (1963, 1964) described the separation of 2 collagenases of seemingly different activities against collagen, denatured collagen or synthetic substrates. Seifter, Gallop et al. (1959) had established the molecular weight of

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the enzyme, purified by treatment with alumina Cγ gel, to be 109,000, and demonstrated inhibition of the enzyme by o-phenanthroline or EDTA. Levdikova et al. (1963, 1964) demonstrated the dissociation of collagenase into 4 inactive subunits of 25,000 molecular weight by treatment with o-phenanthroline or EDTA at pH 11 or with alkali alone at pH 12.

The present study was initiated to determine whether the reported heterogeneity or multiplicity of collagenases reflects a dissociation into subunits with some limited changes in specificity or whether different collagenases indeed are present even in purified preparations. In this communication we report the molecular weights, amino acid compositions, and aspects of substrate specificity for 2 enzymatic fractions from Clostridium histolyticum.

MATERIALS AND METHODS.

Collagenase was prepared by the method of Hospelhorn (1964) using successive fractionation on Sephadex G-200 and DEAE-cellulose. This method results in 2 fractions labeled here A and B. Beef tendon collagen was prepared by the method of Einbinder and Schubert (1951). Protein was measured by the procedure of Lowry et al. (1951), and amino groups by the ninhydrin method of Rosen (1957). Amino acids were determined by the single column technique of Piez and Morris (1960). Sedimentation equilibrium analyses were by the method of Yphantis (1964). Activity of collagenase preparations was assayed viscometrically using as substrate the soluble collagen of carp swim bladder (ichthyocol) (Gallop, Seifter and Meilman, 1957).

EXPERIMENTAL AND RESULTS.

Molecular Weights. Sedimentation equilibrium studies on solutions of enzyme (0.04 per cent) in 0.1 M sodium phosphate buffer pH 7.0 showed molecular weights of 105,000 for collagenase A and 57,400 for B. Experiments at half this concentration of enzyme did not markedly affect the determined molecu-

lar weights. Thus the weight of A agrees well with published values for bacterial collagenase, and that for B is approximately half as large.

TABLE I

AMINO ACID CONTENTS

(residues/1000 residues)
(uncorrected for losses during hydrolysis)

	A	B
Aspartic acid	159	170
Threonine	54	60
Serine	27	32
Glutamic acid	85	93
Proline	56	40
Glycine	96	96
Alanine	73	68
Valine	66	60
Isoleucine	56	61
Leucine	73	74
Tyrosine	50	48
Phenylalanine	50	49
Lysine	103	103
Histidine	15	18
Arginine	37	29

Amino acid compositions. Table I shows that the amino acid compositions of A and B are similar with some small differences. These analyses bear strong resemblance to those of collagenase purified by the method of Seifter, Gallop et al. (1959); there are, however, some differences which may be significant.

Enzyme activity. By the viscometric method, A was 1.6 times more active per mg. of enzyme than B. The enzymes were assayed also by the method of Grassmann and Nordwig (1960) using Cbz-Gly-Pro-Gly-Gly-Pro-Ala-OH. In the original procedure, 0.08 M citrate was used with 0.01 M Ca acetate as activator. Since this amount of citrate would bind Ca^{++}, the assay was modified to employ maleate instead. In 0.05 M sodium maleate pH 7.0, with and without 0.05 M $CaCl_2$, the results shown in Table II were obtained.

TABLE II

Enzymatic Activity

	initial rate	at 15 min.
	μmoles/min/per visc. unit	total μmoles per visc. unit
A (no added Ca^{++})	0.13	1.2
A (with added Ca^{++})	0.26	1.5
B (no added Ca^{++})	0.05	0.60
B (with added Ca^{++})	0.10	0.90

Thus, when the 2 enzymes were used in amounts corresponding to the same number of viscometric units, A had an initial rate with the synthetic substrate which was more than twice that of B. Addition of Ca^{++} in both cases caused doubling of activity. The Ca effect showed that the difference in initial rates of A and B could not be ascribed to trace amounts of Ca in A, since an excess still did not bring B to the level of activity of A. In 90 minutes, however, B with added Ca^{++} was able to produce almost an equal number of peptide scissions as A.

That both A and B in the presence of Ca^{++} cleaved the same number of total peptide bonds with the hexapeptide is consistent with results obtained when the enzymes acted to completion with ichthyocol as substrate. A or B, acting for 3.5 hours on ichthyocol in solution in 0.5 M $CaCl_2$, gave a 7-fold increase over initial leucine equivalents as determined by ninhydrin reaction.

Action on beef tendon collagen. A quantity of A corresponding to 2 viscometric units, completely dissolved in 4 hours 5 mg of collagen suspended in 0.05 M maleate buffer pH 7.0 containing 0.005 M $CaCl_2$. An approximate 7-fold increase in ninhydrin reaction occurred. B, under the same conditions, dissolved only a portion of the collagen. B was then inhibited by addition of EDTA. The tube, containing dissolved collagen and undissolved residue, was then autoclaved for 17 hours. Complete solution of the contents occurred. A ninhydrin determina-

tion gave the surprising result that no increase in leucine equivalents occurred as compared with a control carried through the whole procedure in absence of enzyme. Apparently, the partial solution of tendon collagen by B occurred without proteolytic scissions sufficient in number to be detected.

DISCUSSION AND SUMMARY.

Two collagenases of Clostridium histolyticum separated by column chromatography have an approximate 2:1 ratio in molecular weight. They have similar amino acid compositions. Enzyme A, in the viscometric assay, is more than twice as active as any preparation heretofore tested in this laboratory. The B enzyme has more than one-half the specific activity of the A enzyme in the viscometric assay, and less than one-fourth the specific activity of A in the assay with a synthetic substrate. An equal number of bonds are cleaved when A or B acts to completion on ichthyocol in the presence of Ca^{++}. The same is true when A or B acts on the synthetic hexapeptide.

The evidence indicates that A is a dimer of B, and that the molecular state of collagenase affects its rate of proteolysis on a given substrate. A is 4 times, and B is twice, the molecular weight of the inactive subunit described by Levdikova et al. (1964). Like these authors, we observed that o-phenanthroline at pH 7 does not dissociate the enzyme.

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