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Collagenolytic Activity of Some Marine Bacteria[1]

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Reconstituted, acid-extracted collagen was used to prepare a medium to screen proteolytic marine bacteria for their ability to elaborate collagenolytic enzymes. The medium was resistant to solubilization by trypsin, hyaluronidase, chondroitinase ABC, and various marine proteinases, but was readily hydrolyzed by commercial *Clostridium* collagenases. Eighty-seven marine isolates collected in the vicinity of Bermuda, Oahu (Hawaii), and Stone Harbor and Cape May, N.J., were screened. Approximately 44% of the isolates were capable of elaborating enzymes that hydrolyzed reconstituted collagen gels. Several cultures produced collagenolytic enzymes only when grown in the presence of collagen or degradation products of collagen, and with very few exceptions the presence of collagen in the medium greatly enhanced collagenolytic enzyme production. The enzymes from a collagenolytic Bermuda marine isolate were studied in more detail to illustrate that the enzymes capable of hydrolyzing reconstituted collagen were separable from nonspecific proteinases by zone electrophoresis and that these enzymes were true collagenases by virtue of their ability to hydrolyze native bovine Achilles' tendon obtained from three different sources.

Collagenases were defined by Mandl (6) as enzymes that are capable of hydrolyzing native collagen at or near physiological pH. Furthermore, Mandl (6) concluded that "true collagenases were extremely rare and have been confirmed only in culture filtrates of certain clostridia." Since then the existence of collagenolytic enzymes from other microbial sources has been demonstrated. Nordwig (12) summarized the advances made in the study of collagenolytic enzymes in the 10 years after Mandl's review. During the course of our studies, Hanada et al. (5) screened 2,100 marine bacteria by using an Azocoll medium and reported finding only one collagenolytic culture. They identified the culture as a strain of *Pseudomonas marinoglutinosa* (despite the fact that the organism produced H_2S and had a fermentative metabolism).

In 1969 we began a search for proteinases that could hydrolyze specific peptide bonds in native collagen, using the senior author's collection of marine bacteria. More recently we isolated collagenolytic bacteria in samples from the inshore area around Stone Harbor and Cape May, N.J. This report describes the screening procedures and culturing techniques used to demonstrate the production of collagenolytic enzymes by a relatively high percentage of marine isolates.

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MATERIALS AND METHODS

Maintenance and storage media. Marine bacteria were subcultured on PYSW agar, which had the following composition (percentages are grams per 100 ml of solution): 1% peptone, 0.1% yeast extract, 2% agar, and 4% Rila Marine Mix (Rila Products, Teaneck, N.J.) adjusted to pH 7.2 to 7.5 before autoclaving. Cultures were stored at −15 C in a solution composed of 15% glycerol and 4% Rila Marine Mix.

Isolation media. Proteolytically active isolates were selected from either chromoprotein plates prepared with PYSW medium and algal chromoproteins as described by Merkel (8) or from plates prepared with a slightly modified Smith and Goodner (14) gelatin medium. The latter medium had the following composition: 0.4% hydrolyzed casein (NZ-amine, type HD, Sheffield Chemical Co.), 0.1% yeast extract, 4% gelatin, 1.5% agar, and 4% Rila Marine Mix. Proteolytic activity of most isolates was also examined on casein plates prepared with 100 ml of a basal medium of 0.1% peptone, 2% agar, and 4% Rila Marine Mix adjusted to pH 7.2 to which 12 ml of separately autoclaved skimmed milk was added just before plates were poured.

Enzyme production media. Enzyme production studies were done in hydrolyzed casein-sea water

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media with or without collagen or collagen-derived substrate added to the media. Two types of casein hydrolyzate (enzymatic) media were used: one (referred to as HD½SW) was prepared with 1% NZ-amine, type HD, and 2% Rila Marine Mix, and the other was similar except that 200 ml of pancreatin-digested casein prepared in our laboratory according to the procedure of Prescott and Wilkes (13) was used per liter of medium in place of the commercial casein hydrolyzate. The latter medium is referred to as HC½SW.

Two collagen-containing media were also used in production studies, and these had the following compositions. CHD½SW contained 2 g of NZ-amine, type HD, hydrolyzed casein, 400 ml of a 1:3 dilution of acid-extracted, undialyzed calfskin collagen, 20 g of Rila Marine Mix, and distilled water to a total volume of 1 liter, adjusted to pH 7 to 7.2. CHC½SW was similar to CHD½SW except that 40 ml of pancreatic-digested casein (Schwarz-Mann) was used in place of Sheffield's hydrolyzed casein. Acid-extracted collagen was prepared from freshly slaughtered calves by the method described below.

Reconstituted collagen. Collagenolytic activity in crude-enzyme precipitates of culture filtrates was measured in assay dishes containing reconstituted, acid-solubilized calfskin collagen prepared basically according to the procedure of Gross and Kirk (3). A fresh calfskin was soaked in 5% NH_4Cl containing 1% NaCl and 1:10,000 merthiolate for 5 to 6 days at room temperature to loosen the epidermis. The epidermis, hair, and subcutaneous tissues were scraped away along with all fat. The dermis was coarsely ground in a hand-turned meat grinder, and the tissue was suspended in 10 volumes of 5% acetic acid and set aside in the cold room for 4 to 5 days. The swollen, softened mass was squeezed through several thicknesses of cheesecloth, and the viscous filtrate was centrifuged and then dialyzed at 2 to 4 C for at least 48 h against two changes of Sorensen phosphate buffer at pH 7.6 and an ionic strenth of 0.4. The dialyzed material was then poured (about 20 ml) into flat-bottom assay dishes that had adsorbent pads in aluminum tops, and placed in a 37 C incubator to harden. Well-dialyzed collagen forms into a rigid gel at 37 C in approximately 1 to 2 h, but generally our collagen plates were left in the incubator overnight to allow a slight amount of surface drying to occur before use.

Reconstituted collagen prepared by the above procedure was tested for its resistance to trypsin (Worthington Biochemical Corp., twice crystallized, lot 7JA), hyaluronidase (Mann Research Laboratories, ovine, lot U-3138), chondroitinase ABC (chondroitin sulfate lyase, EC 4.2.99.6, from *Proteus vulgaris*, Miles Research Laboratories, lot 901), and a crude proteolytic enzyme mixture from a non-collagenolytic marine bacterium (B-118). Commercial preparations of *Clostridium* collagenase (Calbiochem Corp. and Worthington Biochemical Corp.) were used to ascertain the substrate's susceptibility to hydrolysis by true collagenases.

Isolation procedures. Bacteria were isolated from Bermuda (1964 and 1966) and Hawaiian (1968) waters surrounding the island of Oahu by membrane (Millipore Corp.) filtration using field monitors (Millipore Corp., MABG 03700). Individual colonies having different growth characteristics (colony size, margin pattern, elevation, pigmentation, optical features, and consistency) were picked and streaked on the surface of PYSW agar to which algal chromoproteins had been added. Proteolytic colonies were detected by their decolorization zones (8). Transfers were made from active colonies to PYSW agar and to nutrient agar medium prepared with distilled water to verify purity of the cultures and their marine nature. Cultures were also obtained from a live sea cucumber (*Stichopus*) brought to the laboratory from Whalebone Bay, Bermuda, on 25 March 1968. The animal was sacrificed and dissected, and bacteriological samples were taken from various parts of the animal, including epidermis, dermis, visceral fluid, water canal scrapings, and small and large intestines.

The proteolytic potential of isolated cultures was next assessed by their digestion of casein, gelatin, and hemoglobin. Active cultures were stored at −15 C in 15% glycerol-seawater and transferred annually on PYSW agar medium. Forty-four proteolytically active cultures from this Bermuda and Hawaii collection were screened for collagenolytic activity.

A second set of marine bacterial isolates that was screened for collagenolytic activity was obtained in the vicinity of Stone Harbor and Cape May, N.J., during the spring of 1974. This set included 43 cultures that were isolated by diluting and plating water samples on PYSW agar, selecting colonies, and transferring them to Smith and Goodner's gelatin medium (14). The colonies with gelatinase activity were tested for collagenase activity. Additional isolates were obtained from fish scales that were suspended in sterile seawater and incubated for 2 days.

Detection of collagenolytic activity. Two methods were used to demonstrate collagenase production by marine bacteria. Initially, cultures were spot inoculated (rather heavily) on the surface of reconstituted collagen medium. Our method was basically similar to one published by Waldvogel and Swartz (15) except that these authors transferred a portion of the solid medium on which their bacteria had grown overnight. Positive cultures in either case liquefy the collagen in a zone around the inoculum. In our procedure positive cultures also had to grow on the collagen by virtue of their collagenolytic activity. This method was unsuitable because of the inability of many cultures to initiate growth and the hazards of incubating an unsterile medium for extended periods even though uninoculated controls never showed signs of contaminant growth.

The second method used was to produce the enzymes by growing the bacteria in shaker flasks containing 50 ml of HC½SW, HD½SW, CHC½SW, or CHD½SW. After the flask cultures were incubated for 20 to 48 h, cells were removed by centrifugation and ammonium sulfate was added to the supernatant fluid to 70% of saturation. After sitting overnight at 4 C, the precipitate was collected by centrifugation and redissolved in 3 to 5 ml of phosphate or tris(hy-

droxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 8.0), and 0.15 to 0.2 ml of this enzyme solution was applied to Schleicher & Schuell penicillin assay disks (1.25-cm diameter). After the liquid had evaporated, the disks were placed on the surface of reconstituted collagen plates. By this latter procedure, active preparations could be detected within 1 h, and after 18 to 24 h large liquefied zones surrounded assay disks containing active enzymes.

Endopeptidase assay. Endopeptidase activity of culture filtrates was measured with urea-denatured hemoglobin (Worthington Biochemical Corp.) by a procedure described previously (10).

Digestion of native collagen. True collagenases were demonstrated by assessing the ability of enzyme preparations to catalyze the hydrolysis of native bovine Achilles' tendon. The enzyme preparations used were obtained from a marine bacterium that we designated *Vibrio* B-30 (10). The organism was grown in 5 liters of CHC ½SW medium in a New Brunswick fermentor for 24 h, the cells were removed by centrifugation, and the enzymes in the supernatant were precipitated with 70% of saturated ammonium sulfate. Precipitation was allowed to continue for 24 to 48 h, after which it was collected by centrifugation, dissolved in a minimum of 1% NaCl, dialyzed for 24 to 48 h against 0.05 M Tris-hydrochloride buffer (pH 8), and then lyophilized. The freeze-dried preparation is referred to as the crude enzyme. Partial purification to remove nonspecific proteinases was effected by diethylaminoethyl (DEAE)-cellulose chromatography (described below).

Collagen hydrolysis was followed by two procedures: the release of hydroxyproline (2, 11) and the increase in ninhydrin-positive material (7) during the incubation period of 1 or 2 h. Each test solution contained 20 mg of shredded collagen, 1 ml of 0.05 M $CaCl_2$-0.5 M Tris-hydrochloride at pH 7.4, 4 ml of water, and 1 ml of enzyme (concentrations varied as noted in Table 2). Controls received 1 ml of boiled enzyme. Incubation was carried out in 24-ml flasks attached to a shaker and immersed in a 38 C bath. At the end of the incubation period, each flask was filtered to remove undigested collagen and the filtrate was immediately assayed for hydroxyproline and ninhydrin-positive material. Native bovine Achilles' tendon used in these studies were either commercial preparations from Mann Research Laboratories (undenatured bovine Achilles' tendon, lot 2717), Worthington Biochemical Corp. (shredded and graded bovine Achilles' tendon lot CL-7GA), or our own preparations using Achilles' tendons from freshly slaughtered steers and the method of Einbinder and Schubert (1) to purify the collagen. Every preparation of collagen used in our studies was shown to be resistant to tryptic digestion.

Partial purification of Vibrio B-30 collagenase. Crude enzyme (500 mg) was dissolved in 20 ml of distilled water and added to a slurry of regenerated DEAE-cellulose (about 60 g of wet filter cake) that had been equilibrated with 0.02 M Tris-hydrochloride buffer (pH 8.5) containing 0.1 M NaCl. The enzyme-DEAE-cellulose slurry was kept at 4 C with intermittent stirring for 20 min to allow for complete adsorption of the enzymes. The enzyme-DEAE-cellulose was next washed three times with 0.02 M Tris-hydrochloride buffer (pH 8.5) containing 0.1 M NaCl, and then the enzyme-DEAE-cellulose was added to the top of a column (4.7 by 100 cm) previously packed (gravity) with about 60 g (wet) of DEAE-cellulose that had been equilibrated with 0.02 M Tris-hydrochloride buffer (pH 8.5). This gave a DEAE-cellulose column with a height of approximately 40 cm. The column was washed with the equilibrating mixture (about 300 ml) and then elution of the enzymes was effected with a continuous NaCl gradient (0.1 to 1.0 M). Protein fractions (5 ml) eluted from the column with the NaCl gradient were assayed for their ability to digest reconstituted collagen, and those with activity were pooled and concentrated approximately 12-fold by ultrafiltration. These concentrates are referred to as partially pure preparations. They were not homogeneous but were virtually free of nonspecific proteinase activity.

Zone electrophoresis. Collagenases were separated from crude enzyme preparations by electrophoresis on cellulose acetate strips (Sepraphore III) by using a Gelman Instruments electrophoresis chamber. Collagenolytic bands were located by applying one-half of an electrophoresis strip to the surface of reconstituted collagen medium. The other half was laid on the surface of chromoprotein medium. The latter medium was used to locate nonspecific proteinase activity (9). Alternatively, one-half of an electrophoresis strip was stained with a nonspecific protein stain (Ponceau S).

RESULTS AND DISCUSSION

Reconstituted collagen medium. Although reconstituted collagen gels were shown by Gross and Kirk (3) to contain fibrils with the characteristic axial periodicity of native collagen, they cannot be considered truly native because they lack the interchain cross-linkages and the more complex quaternary structure of the natural material. However, Gross and Kirk (3), Gross and Lapiere (4), and Waldvogel and Swartz (15) have shown that reconstituted collagen can serve as an excellent primary screening medium. In general, organisms that hydrolyze properly reconstituted collagen will also hydrolyze native collagen.

Before using reconstituted collagen gels for screening, each batch of gel was tested for its resistance to trypsin, certain crude enzyme mixtures from proteolytic marine bacteria, hyaluronidase, and bacterial chondroitinases. On the other hand, reconstituted collagen gels had to be susceptible to the action of *Clostridium* collagenases.

Figure 1 illustrates the above-mentioned characteristics of a good reconstituted collagen gel. The medium was not liquefied by trypsin,

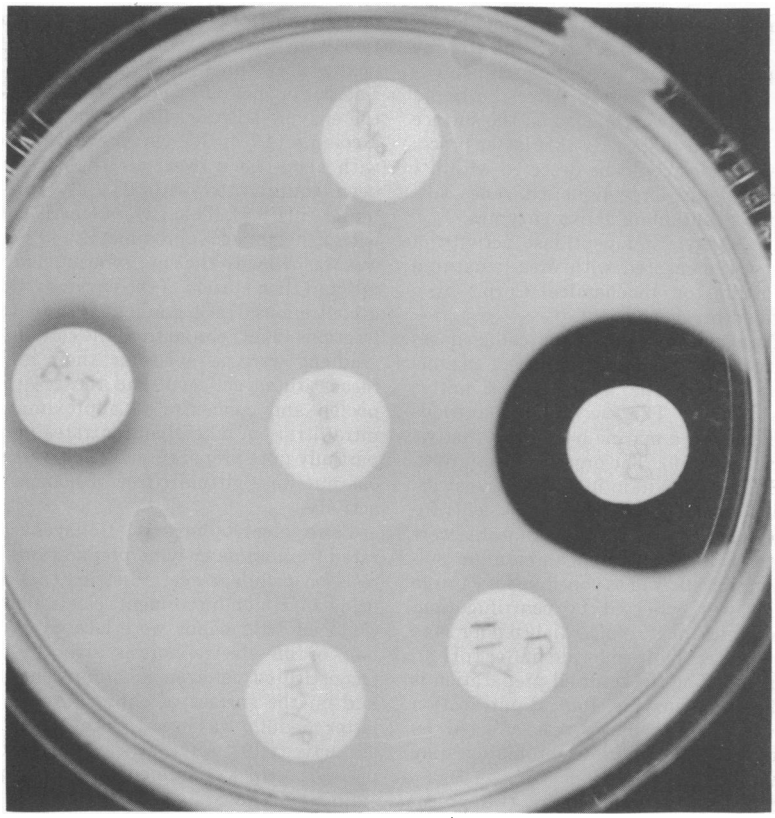

FIG. 1. *Specific digestion of reconstituted collagen by crude bacterial enzymes. Tryp, trypsin (2 mg/ml); Chon, chondroitinase ABC (5 U/2 ml); Hyal, ovine hyaluronidase (2 mg/ml); B-118, dialyzed and freeze-dried 70% saturated ammonium sulfate precipitate of the cell-free HD½SW medium of marine isolate B-118 (10 mg/ml); B-30 and B-51 are also 70% saturated ammonium sulfate precipitates of cell-free CHC½SW growth medium of two proteolytic marine bacteria (about 1 mg/ml). Each penicillin assay disk received 3 drops of enzyme solution. The plate was incubated at 37 C for 26 h with the disks before the photograph was taken.*

hyaluronidase, chondroitinase ABC, or a highly proteolytic preparation obtained from a marine bacterial isolate (B-118). However, crude enzyme preparations from two other marine organisms, B-30 and B-51, contained collagenases that hydrolyzed the collagen in zones around the disks. Digestion by B-30 was similar to that observed with commercial *Clostridium* collagenase preparations.

Screening for collagenolytic bacteria. Seventeen of the first 37 cultures screened by spot inoculating reconstituted collagen plates showed collagen digestion zones around the inoculum after 96 h of incubation. Some of the cultures did not show signs of digesting the collagen during the first 24 to 48 h of incubation, and then a rapid digestion ensued. This was partly due to the slow growth of some cultures, but it also suggested the possibility of induced collagenase production, alteration of the collagen followed by nonspecific proteolysis, or the growth of collagenolytic contaminants.

To avoid some of the above problems and to see whether collagen stimulated the production of collagenases, the bacteria were grown in shaker flasks containing hydrolyzed casein and in flasks containing collagen plus a small amount of hydrolyzed casein. The flask cultures were incubated at room temperature, and the crude enzymes were harvested and assayed as described in Materials and Methods and illustrated in Fig. 1. The method was not quantitative because of the differences in growth rates and enzyme production by each culture, but the size of the clearing zone and the speed with which the zone appeared provided an estimate of the relative activities of different cultures. Evidence of collagen digestion was often visible within 1 h of incubation at 37 C.

All of the cultures reported as collagenolytic

by the spot inoculation procedure were verified by using the liquid medium (CHC½SW). A total of 44 proteolytic cultures were included in the initial set that was screened, and 20 produced collagenolytic enzymes in CHC½SW medium.

Forty-three marine proteolytic isolates recently obtained in the vicinity of Stone Harbor and Cape May, N.J., were screened by the assay disk method. The cultures were all selected on the basis of their ability to hydrolyze gelatin. After purification, 24-h cultures were prepared on PYSW agar slants and used to inoculate 50 ml of HD½SW and CHD½SW media in shaker flasks. The cultures were incubated for 48 h with continuous shaking, and then the crude enzymes were collected and assayed as described. Eighteen of the 43 cultures produced enzymes that liquefied the reconstituted collagen.

With the exception of three highly proteolytic cultures, which grew much more abundantly in HD½SW than in collagen-containing media, collagenolytic enzyme production was greatly enhanced by the presence of collagen in the growth medium. Several cultures grown in hydrolyzed casein had no detectable collagenolytic activity, but when these same cultures were grown in collagen-containing medium, good collagenase production occurred. Table 1 illustrates the influence of collagen medium on the production of collagenase and endopeptidases (hemoglobin digestion) by several proteolytic marine bacterial isolates from Bermuda. The data included in Table 1 are from two different experiments (21- and 47-h incubations), but in many different experiments this same pattern was observed. Endopeptidase activity was high in HC½SW medium, and collagenase activity was frequently absent or low.

Since collagen digestion (Table 1 and Fig. 1) could result from the concerted action of mixtures of proteolytic enzymes, active collagenases were separated from the other proteinases by zone electrophoresis and demonstrated as separate protein bands capable of liquefying reconstituted collagen but not algal chromoproteins. Figure 2 contains exact tracings of

TABLE 1. *Collagenase and endopeptidase activity of certain marine bacteria grown in the presence and absence of collagen*

Culture	Medium	Incubation period (h)	Relative culture density[a]	Proteolytic activity	
				Endopeptidase[b]	Collagenase[c]
B 30	HC½SW	47	5+	1.7	1
B 30	CHC½SW	21	2+	0.352	7
B 51	HC½SW	47	5+	1.03	½
B 51	CHC½SW	21	3+	0.590	8
B 118	HC½SW	47	5+	0.980	0
B 118	CHC½SW	47	3+	1.27	0
B 207	HC½SW	21	3+	0.765	0
B 207	CHC½SW	21	3+	0.552	4
B 274	HC½SW	21	3+	1.0	1
B 274	CHC½SW	21	2+	0.43	3
B 277	HC½SW	21	6+	0.765	1
B 277	CHC½SW	21	3+	0.966	8
B 279	HC½SW	21	3+	0.951	½
B 279	CHC½SW	21	3+	1.419	2
B 280	HC½SW	21	3+	0.519	0
B 280	CHC½SW	21	3+	1.215	1
Trypsin (2 mg/ml)				2.0	0
Clostridium collagenase (1 mg/ml)				0.057	3

[a] Estimated from the turbidity.

[b] Units of activity in 1 ml of the cell-free growth medium as measured by a modification of Anson's procedure (10) using urea-denatured hemoglobin as the substrate. One unit is arbitrarily taken as the amount of enzyme required to produce an increase of 1 absorbance unit at 280 nm when the enzyme is incubated with urea-denatured hemoglobin at 37 C for 5 min, stopped with 5% trichloroacetic acid and filtered, and the absorbance of the filtrate measured.

[c] 70% ammonium sulfate precipitate of the growth medium (50 ml) redissolved in a minimum of water and 4 drops added to penicillin assay disks. Collagen digestion was assessed after 48 h incubation. Numbers are millimeters of digestion beyond the edge of the disk.

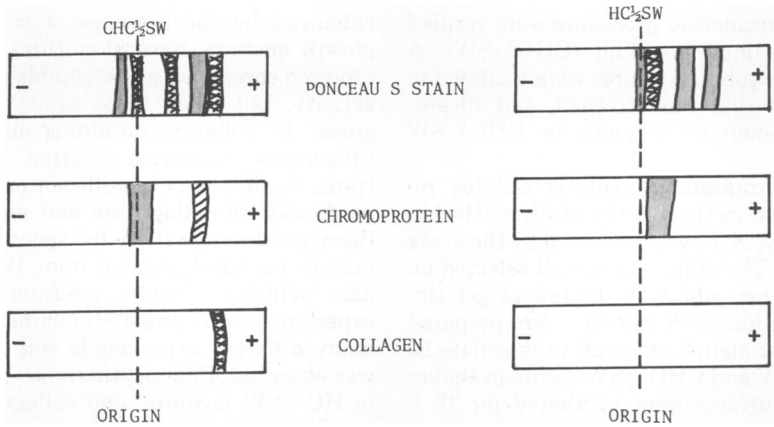

FIG. 2. *Electrophoretic comparison of enzymes produced by isolate B-30 in two different media. Electrophoresis on cellulose acetate strips (Sepraphore III) in 0.2 M Tris-hydrochloride at pH 8.0, at 2 mA per strip, 185 V for 3 h at 3 C. Strips were cut longitudinally with a razor blade. One-half was stained and the other half was applied to either a chromoprotein plate or a reconstituted collagen plate. The cross-hatched bands were the most active or most heavily stained, whereas the gray bands had the lowest activity.*

typical cellulose acetate electrophoresis patterns that were obtained with the crude enzymes from isolate B-30 grown in HC ½SW and CHC ½SW media. The crude enzymes produced by several other collagenolytic bacteria have been examined by this means with similar results indicating, at least in those preparations that were examined, that the collagenolytic activity is associated with a single protein band separable from other proteinases in the preparation.

Hydrolysis of native collagen. To prove that a protease is a true collagenase, it is essential to have a source of native collagen as the substrate. We have used several different sources of bovine Achilles' tendon, including commercial and laboratory preparations. Our criteria for native conditions were that the tendon had to be resistant to hydrolysis by trypsin and susceptible to the action of *Clostridium* collagenase.

Data from three experiments in Table 2 compare the action of a commercial *Clostridium* collagenase on three different Achilles' tendon collagen substrates with the action of partially purified collagenase obtained from the marine isolate *Vibrio* B-30.

The partially purified collagenase obtained from the marine isolate *Vibrio* B-30 could hydrolyze native Achilles' tendon collagen much as the collagenase from *Clostridium histolyticum* did (Table 2). As a matter of fact, when our laboratory preparation of bovine Achilles' tendon was used as the substrate, the B-30 collagenase was almost three times more effective in hydrolyzing this collagen than was the commercial *Clostridium* enzyme (note last three lines in Table 2). This particular preparation of collagen was extremely resistant to the action of trypsin and other nonspecific proteinases.

The variation in amounts of hydrolysis with different collagen preparations can probably be attributed to (i) any partial denaturation of the collagen that may have occurred during its preparation, and (ii) differences in particle sizes of the several collagens used as substrates. It is well known that many proteinases can hydrolyze gelatin but few can hydrolyze native collagen. Consequently, if denaturation has occurred in the substrate, traces of nonspecific proteinases in the preparations along with the collagenases will contribute to the total digestion. We are currently investigating the effects of substrate particle size on its susceptibility to collagenases. In one experiment, Worthington collagen was shredded in a Waring blender packed with dry ice, and after the CO_2 was removed, the shredded collagen was graded through standard sieves. Thirty-five- to 60-mesh material was compared with the material that passed through the 60-mesh screen as a substrate for CalBiochem collagenase. The collagen that passed through the 60-mesh screen contributed a large amount of soluble, 280 nm-absorbing material to the solution, but the larger, 35- to 60-mesh collagen was more completely hydrolyzed by the collagenase. Under conditions similar to those used in Table 2, the smaller collagen particles (<60 mesh) yielded 0.014 μmol of hydroxyproline in 1 h of digestion, whereas the 35- to 60-mesh collagen yielded 0.022 μmol.

With the exception of one strain of *Staphylococcus aureus* (grown anaerobically), Waldvogel

TABLE 2. *Digestion of native bovine Achilles' tendon collagen*

Enzyme	Incubation period (h)	Collagen	Enzyme concn (A_{280})[a]	Collagen solubilized	
				μmol of Hyp/ml	μmol of Leu/ml
Clostridium[b]	1	Mann[c]	0.400	0.049	1.02
B-30 (fraction IV)	1	Mann	0.190	0.017	0.49
Clostridium	1	Worthington[d]	0.420	0.029	0.55
B-30 (fraction IV)	1	Worthington	0.217	0.010	0.75
Clostridium	2	Worthington	0.300	0.022	0.32
B-30 (fraction III)	2	Worthington	0.360	0.027	0.77
Clostridium	1	Laboratory prepn[e]	0.430	0.011	0.21
B-30 (pooled IV & III)	1	Laboratory prepn	0.225	0.032	0.51
Trypsin[f]	1	Laboratory prepn	0.610	0	0

[a] Absorbance at 280 nm of the filtrate of the control reaction solutions. (containing boiled enzyme).

[b] Calbiochem collagenase (bacterial), B grade, lot 801782, 1 mg/ml.

[c] Mann Research Laboratories, undenatured bovine Achilles' tendon, lot L2717, shredded in a Waring blender with dry ice.

[d] Worthington Biochemical Corp., bovine Achilles' tendon, lot CL-7GA, shredded in a Waring blender with dry ice and graded 35 to 60 mesh.

[e] Bovine Achilles' tendon prepared by the method of Einbinder and Schubert (1).

[f] Worthington Biochemical Corp., TRL 100S, twice crystallized, lot 7JA, 2 mg/ml.

and Swartz (15) were able to demonstrate collagenolytic activity only among anaerobic bacteria. Aerobic and facultatively anaerobic marine bacteria that we obtained from widely differing environments and geographic locations were shown to contain a relatively large percentage (~44%) of cultures capable of hydrolyzing reconstituted bovine Achilles' tendon collagen. Several cultures failed to produce collagenolytic enzymes unless they were grown in the presence of collagen, and production of collagenolytic enzymes by most of the cultures was greatly enhanced when they were provided with a collagen substrate.

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