83, 65.
Lindskog, S. (1970) *Struct. Bond. (Berlin) 8,* 153-196.
Margerum, D. W., & Bydalek, T. J. (1963) *Inorg. Chem. 2,* 683.
Margerum, D. W., Cayley, G. R., Weatherburn, B. C., & Pagenkopf, G. K. (1978) *Coord. Chem.* (in press).
Shore, J. D., & Santiago, D. (1975) *J. Biol. Chem. 250,* 2008.
Simpson, R. T., & Vallee, B. L. (1968) *Biochemistry 7,* 4343.
Sloan, D. L., Young, J. M., & Mildvan, A. S. (1975) *Biochemistry 14,* 1998.
Sytkowski, A. J. (1978) *Arch. Biochem. Biophys. 184,* 505.
Sytkowski, A. J., & Vallee, B. L. (1975) *Biochem. Biophys. Res. Commun. 67,* 1488.
Sytkowski, A. J., & Vallee, B.L. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73,* 344.
Sytkowski, A. J., & Vallee, B. L. (1978) in *Pharmacology of Ethanol* (Majchrowicz, E., & Noble, E., Eds.) Plenum Press, New York, N.Y. (in press).
Takahashi, M., & Harvey, R. A. (1973) *Biochemistry 12,* 4743.
Theorell, H., Yonetani, T., & Sjöberg, B. (1969) *Acta Chem. Scand. 23,* 255.
Thiers, R. (1957) *Methods Biochem. Anal.* 273-335.
Thompson, G. A., & Carpenter, F. H. (1976) *J. Biol. Chem. 251,* 1618.
Ulmer, D. D., & Vallee, B. L. (1965) *Adv. Enzymol.,* 37-104.
Vallee, B. L., & Wacker, W. E. C. (1970) *Proteins, 2nd Ed.,* 5.
von Hippel, P. H., & Schleich, T. (1969) in *Structure and Stability of Biological Macromolecules,* (Timasheff, S. N., & Fasman, G. D., Eds.) pp 417-574, Marcel Dekker, New York, N.Y.
Wilkins, R. G. (1974) *The Study of Kinetics and Mechanism of Reactions of Transition Metal Complexes,* Allyn and Bacon, Boston, Mass.
Young, J. M., & Wang, J. H. (1971) *J. Biol. Chem. 246,* 2815.

Purification and Characterization of a Marine Bacterial Collagenase†

Joseph R. Merkel* and Joseph H. Dreisbach‡

ABSTRACT: A true collagenase was isolated from the culture fluid of a marine bacterium which has been designated *Vibrio* B-30 (ATCC 21250). Collagenase production was obtained only in media containing collagen or certain degradation products of collagen. Partial purification on DEAE-cellulose and Sephadex G-200 columns produced active enzyme which was free of nonspecific proteases but which contained two collagenases. The two collagenases have the same apparent molecular size, and evidence is presented to support the theory that one collagenase is derived from the other. *Vibrio* B-30 collagenase appears to be a tetramer with a molecular weight of about 105 000 composed of two different subunits (mol wt 24 000 and 28 000). Some of the properties of the *Vibrio* collagenase are compared with those of *Clostridium histolyticum* collagenase. Molecular weights, subunit structures, specificity and mode of collagen hydrolysis, insensitivity to diisopropyl fluorophosphate and calf serum, and sensitivity to certain metal ion complexing agents and isopropyl alcohol are similar for the collagenases from both organisms. However, *Vibrio* B-30 collagenase and *Clostridium* collagenase differ immunologically and electrophoretically.

We recently reported on the collagenolytic activity of certain marine bacteria (Merkel et al., 1975). Reconstituted, acid-extracted collagen was used to prepare a medium to screen proteolytic marine bacteria for their ability to elaborate collagenolytic enzymes. Approximately 44% of the proteolytic marine isolates were capable of producing collagenases when they were grown in the presence of collagen or degradation products of collagen. One of the most active isolates, designated *Vibrio* B-30,[1] was selected for further studies. The production, purification, and partial characterization of a true collagenase of *Vibrio* B-30 is the subject of this report. Attention is drawn to the similarities and differences between the collagenase produced by *Vibrio* B-30 and that produced by *Clostridium histolyticum* (Keller & Mandl, 1963; Yoshida & Noda, 1965; Lee-Own & Anderson, 1975).

Materials and Methods

Collagenase Production. Two collagen-containing media were used in enzyme production studies and these had the following compositions: (1) CHD½SW contained 2 g of NZ-amine, type HD, hydrolyzed casein (Sheffield Chem. Co.), 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 L, adjusted to pH 7.0-7.2; and (2) CHC½SW was similar to CHD½SW except that 40 mL of pancreatin-digested casein (Prescott & Wilkes, 1966) was used in place of the commercial hydrolyzed casein.

Medium was also prepared using commercial gelatin as a substitute for acid-extracted calfskin collagen. The gelatin medium (referred to as HDG½SW) contained 10 g of NZ-amine, type HD hydrolyzed casein, 20 g of gelatin (Fisher Sc.

† From the Department of Chemistry, Lehigh University, Bethlehem, Pennsylvania 18015. *Received September 27, 1977.*

‡ Supported in part by a graduate fellowship from the Jesse Noyes Foundation through the Wetlands Institute of Lehigh University. Part of the data used in the report was abstracted from the doctoral thesis of J.H.D.

[1] Details on the isolation, cultivation, and characterization of this marine bacterium can be obtained from the senior author.

0006-2960/78/0417-2857$01.00/0

Co., Certified), and 20 g of Rila Marine Mix in 1 L of distilled water.

Acid-extracted, calfskin collagen was prepared from freshly slaughtered calves by a modification (Merkel et al., 1975) of the procedure of Gross & Kirk (1958).

Laboratory scale enzyme production was accomplished in 7.5-L fermentors (New Brunswick Sc. Co.). In a typical fermentation 5 L of HDG½SW medium was inoculated with 50 mL of a 20–24-h culture of *Vibrio* B-30 that had been incubated on a shaker (New Brunswick, Model V) at 23 °C. Aerobic growth was allowed to proceed for 23–26 h after which the culture medium was collected by centrifugation. The culture supernatant prepared in the above manner was used as the source of crude collagenase.

Isolation and Purification of Vibrio B-30 Collagenase. The supernatant culture fluid was brought to 70% saturation by the addition of solid ammonium sulfate at 4 °C. Precipitation was allowed to proceed for 24 to 72 h. The protein precipitate was collected by centrifugation, redissolved in a minimum (200 mL) of 0.02 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer,[2] pH 8.5, and dialyzed at 4 °C for 24 h against two changes of the buffer.

The redissolved, dialyzed crude enzyme was applied to a DEAE-cellulose column (usually 4.5 × 25 cm) that had been equilibrated with 0.02 M Tris-HCl, pH 8.5. Elution of collagenase was accomplished with a linear gradient of increasing ionic strength and decreasing pH. Protein elution was followed by monitoring 280-nm absorbances, and collagenase activity was assayed by the procedure described below using acid-extracted calf-skin collagen as the substrate.

Active fractions from DEAE-cellulose chromatography were pooled, concentrated by ultrafiltration (Amicon UM-10 filter), dialyzed overnight against distilled water at 4 °C, and, if the sample was not immediately applied to a Sephadex column, it was lyophilized. The active material taken from DEAE-cellulose columns was essentially free of nonspecific endopeptidase activity. These preparations were used in some of the characterization studies and are referred to as partially pure *Vibrio* B-30 collagenase.

Further purification of the active fractions was accomplished by gel filtration using Sephadex G-200 (Pharmacia Fine Chem.) in a 1.9 × 118 cm column and eluting with 0.02 M Tris-HCl, pH 8.5.

Protein Determinations. Absorbance at 280 nm was used to monitor the purification steps. The method of Lowry et al. (1951), using a bovine serum albumin standard, was also employed to determine the protein content of some pooled fractions of enzymes, and of collagen substrates.

Carbohydrate Determination. The phenol–sulfuric acid method of Dubois et al. (1956) was used to determine the carbohydrate content of purified *Vibrio* B-30 collagenase.

Endopeptidase Activity Determination. Prescott & Willms' (1960) modification of Anson's (1938) method was used to determine nonspecific protease activity. Activity is expressed in the arbitrary units used by Prescott & Willms (1960) which were defined as the amount of enzyme that produced a change of 1.0 in the absorbance at 280 nm of the hemoglobin hydrolyzate obtained after 5 min of digestion.

Assays for Collagenase Activity. (1) Reconstituted collagen plate method (Merkel et al., 1975) was used as a semiquantitative measure of collagenase activity in various enzyme preparations.

(2) Quantitative Determination Using Acid-Extracted Collagen as the Substrate. Acid-extracted, dialyzed calf-skin collagen (16 mg of protein per mL) was diluted at 4 °C with an equal volume of 0.02 M Tris-HCl, pH 8.4, buffer. Two milliliters of the diluted collagen solution was preincubated at 37 °C for 15 min (fibrils form during this process). Then 0.1 mL of enzyme solution or boiled enzyme solution (control) was added and incubation was continued with intermittent shaking for 1 h. The reaction was stopped with 0.5 mL of 30% trichloroacetic acid solution. The mixture was held at 37 °C for another 15 min and was then filtered through Whatman No. 2 filter paper. Peptides and amino acids released during hydrolysis were determined using the ninhydrin procedure of Rosen (1957). Standard leucine curves were used to determine the amount of collagen hydrolyzed. A unit of collagenase is defined as the amount of enzyme that liberates 1 μmol of leucine equiv per min per mg of protein in the sample.

(3) Quantitative Determination Using Insoluble, Bovine Achilles' Tendon Collagen as the Substrate (Mandl Assay). The procedure of Mandl et al. (1953) was employed except that 0.02 M Tris-HCl, pH 8.4, buffer containing 0.001 M $CaCl_2$ was used in place of phosphate buffer, and soluble peptides released by the action of collagenases were measured by the method of Rosen (1957). Native bovine Achilles' tendon collagen used in this procedure was either a commercial preparation from Worthington Biochemical Corp. or a laboratory preparation (Einbinder & Schubert, 1951). Boiled enzyme controls were used as blanks. A Mandl unit is defined as the amount of enzyme that releases 1 μmol of leucine equiv per mg of protein after 18-h incubation.

(4) Quantitative Determination Using Z-Gly-L-Pro-Gly-Gly-L-Pro-L-Ala as the Substrate. The method of Grassman & Nordwig (1960) was modified slightly to accommodate our 0.02 M Tris-HCl buffer systems, and peptides released during hydrolysis were measured by Rosen's procedure. A unit of enzyme activity as defined by Grassman & Nordwig (1960) is the amount of enzyme required to liberate 1 μmol of leucine equiv per mg of protein during 15 min of incubation.

In all of the above procedures the reactions were linear within the prescribed incubation periods.

Molecular Weight and Subunit Determinations. The gel filtration method of Andrews (1965) was employed using a 1.9 × 118 cm Sephadex G-200 column, and eluting with 0.02 M Tris-HCl buffer (pH 8.4). Subunit molecular weights were estimated by sodium dodecyl sulfate–polyacrylamide gel (7.5%) electrophoresis according to the procedure of Weber et al. (1972). Homogeneity of purified enzymes was verified by polyacrylamide gel electrophoresis according to the method of Ornstein (1964) and Davis (1964) as modified by Clarke (1964).

Optimum pH and pH Stability. The optimum pH for activity measured against native Achilles' tendon collagen by the method of Mandl et al. (1953) was determined with a DEAE-cellulose purified fraction that had a specific activity of 637 units per mg of protein. Five milliliter quantities of Tris-HCl buffer at various pHs (5.7–9.0) adjusted to ionic strengths of 0.205 were used in place of the 0.067 M phosphate buffer (pH 7.4) called for in the Mandl assay. Enzyme activity was measured after 18 h of incubation. The pH of each reaction mixture was measured before addition of the enzyme and at the end of 18 h of incubation. The values differed little (±0.8 at pH 5.7 and 9.6). pH curves were plotted from the average values.

The stability of the collagenase preparation used above for pH optimum determination was tested by holding the enzyme solutions at 4 °C. Collagenase activity was then measured by

[2] Unless specifically noted, all Tris-HCl buffers used in these studies contained 0.001 M $CaCl_2$.

the Mandl procedure immediately after the enzyme solutions were prepared and after 24 h of storage at 4 °C.

Optimum Temperature. Optimum temperature for activity as measured by the Mandl assay was determined with bovine Achilles' tendon collagen and the same enzyme fraction used above for pH activity determination (specific activity 637 units/mg). Incubation was for 18 h at a given temperature, the solution volumes were readjusted with distilled water where evaporation had occurred, and then the extent of collagen hydrolysis was determined by the Mandl procedure. Appropriate controls without enzyme and without collagen were incubated along with the reaction tubes.

Inhibition Studies. The effect of disodium ethylenediaminetetraacetate (Na_2EDTA), iodoacetamide, *p*-chloromercuribenzoate, and cysteine were tested by incorporating various concentrations of these materials into the phosphate buffer (pH 7.4) used to suspend insoluble Achilles' tendon collagen in the Mandl assay procedure. Controls received distilled water in place of the inhibitor. A purified collagenase preparation was used at a final concentration of 1 mg/mL and a specific activity of 128 units/mg by the Mandl assay.

The effects of diisopropyl fluorophosphate (DFP) and of isopropyl alcohol were tested by incubating the enzyme at room temperature with 10^{-3} M DFP or different concentrations of isopropyl alcohol in water. Twenty microliters of stock DFP (0.05 M) or isopropyl alcohol was mixed with 1.0 mL of enzyme solution containing 1.2 mg of purified enzyme. At various intervals of time, a 0.1-mL sample was removed from the reaction mixture and added to 4.9 mL of 0.067 M phosphate buffer (pH 7.4) containing 25 mg of Achilles' tendon collagen, and the activity of the enzyme was assessed as described above in the Mandl assay. Potency of the DFP (Mann Research Labs, lot R 1534) was tested against trypsin (Worthington, lot OEB). Controls containing enzyme plus distilled H_2O or appropriately diluted isopropyl alcohol (the DFP diluent) were carried along in each experiment. DFP inhibition results were repeated in three separate experiments and in each experiment commercial clostridial collagenase was treated similarly.

The possible effect of fetal calf serum (Grand Island Biol. Co.) was checked by mixing crude or purified B-30 collagenase preparations at concentrations of 2 mg/mL with equal amounts of calf serum and incubating the mixtures at room temperature for 10 min. Then 0.2 mL of each incubation mixture was applied to a penicillin assay disk (13 mm). The disks were air dried and placed on the surface of reconstituted collagen plates. Zones of liquefaction surrounding the disks were measured after 18 h of incubation at 37 °C.

Specificity of Vibrio B-30 Collagenase. Peptide specificity was determined with native Achilles' tendon collagen and with Grassman & Nordwig's (1960) hexapeptide, Z-Gly-L-Pro-Gly-Gly-L-Pro-L-Ala. Several different partially purified *Vibrio* B-30 collagenases were used at 1 mg/mL concentrations and the products of hydrolysis were compared with those obtained after *Clostridium histolyticum* collagenase (Worthington Biochemical Corp., CLSPA, and Calbiochem Corp.) digestion. The collagenase preparations used in these studies had virtually no nonspecific endopeptidase activity and good (3–5 mm digestion zones) collagenase activity as measured by the reconstituted collagen plate method. *Clostridium* and *Vibrio* B-30 collagenases were compared at equivalent protein concentrations.

Hexapeptide hydrolysis was accomplished by the procedure of Nagai et al. (1960) using 1.5 mL of a 1.25 mg/mL solution of hexapeptide and 0.5 mL of enzyme or boiled enzyme (approximately 0.1 mg/mL). Thin-layer chromatography on silica gel plates was used to detect the hydrolysis products. Dinitrophenylation (Fraenkel-Conrat et al., 1955) followed by thin-layer chromatography was used to determine the N-terminal amino acids of the hydrolysis products.

Similar analyses were performed on the solubilized products resulting from the hydrolysis of bovine Achilles' tendon collagen (lab. prep.) in the same manner as described above for the hexapeptide except that 40 mg of finely chopped collagen was used in 4 mL of buffer containing 1 mL of enzyme.

DNP-amino acids were chromatographed on silica gel G plates using $CHCL_3$:benzyl alcohol:acetic acid (70:30:3) as the solvent. Standard DNP-amino acids were used to identify the N-terminal amino acids. The unmodified hydrolyzate was chromatographed on silica gel G plates using 1-propanol:water (70:30) as the solvent and the synthetic tripeptide Gly-L-Pro-L-Ala as the standard.

Immunological Comparison of Vibrio B-30 and Clostridium histolyticum Collagenases. Antigens were prepared by dissolving 57.5 mg of thoroughly dialyzed, partially pure *Vibrio* B-30 collagenase in 5 mL of 0.85% NaCl solution and 40.2 mg of CLSPA grade clostridial collagenase (Worthington) in 5 mL of 0.85% NaCl solution. These amounts of enzyme contained the same amount of protein. Each solution was sterilized by Millipore filtration and aseptically mixed with an equal volume of sterile Freund's complete adjuvant (Calbiochem). The mixtures were then used to prepare antisera in rabbits.

Serological comparisons were performed by the slide method of Ouchterlony (1968). After incubation of the antigens and antisera in a humid chamber at 23 °C overnight, the slides were stained with Thiazine Red R according to the instructions of Crowle (1958) and precipitin bands were photographically recorded.

Results

Enzyme Production and Purification. The bacterium grows well and produces proteolytic enzymes on a variety of hydrolyzed casein media, but collagenase production occurs only when induced by the addition of collagen or certain collagen-derived products to the media (Merkel et al., 1975). Maximum collagenase production in collagen or gelatin-containing media occurred in 23–24 h of incubation at 25 °C in shaker flasks and in 10–12 h of incubation in a New Brunswick Scientific 7.5-L fermentor.

Figure 1 shows a typical separation of crude *Vibrio* B-30 enzymes (70% ammonium sulfate precipitate of culture fluid from a 5-L HDG½SW, 23-h fermentation) on a DEAE-cellulose column. This pattern of separation has been repeated many times. The separation can be improved by using a longer column or smaller amounts of crude enzyme. The active eluent from the DEAE-cellulose column was divided into three fractions as indicated in the insert (I, II, III). Each pooled fraction was concentrated by ultrafiltration, dialyzed against distilled water (4 °C), and lyophilized. Small samples of the dry fractions were removed for assays and the remainder was applied to a Sephadex G-200 column.

Figure 2 shows the elution pattern obtained when fraction I was passed through a Sephadex G-200 column. Identical results were obtained when fractions II and III were passed through the same column, i.e., the active materials were eluted with the same volume as fraction I.

Polyacrylamide gel electrophoresis of the three fractions separated on a Sephadex G-200 column is illustrated in Figure 3. The lowest band on the gels locates the tracking dye. The two heavier bands slightly below center are collagenolytically active.

Table I summarizes the purification results from the above procedures.

TABLE I: Isolation and Purification of *Vibrio* B-30 Collagenases.

procedure	total col'ase (units)[a]	total endopeptidase (units)	total protein[b] (mg)	spec act. col'ase
1. culture supernatant	7367[c]	4900	46 000	1.16[c]
2. dialyzed 70% ammonium sulfate precipitate	1941[c]	4255	2 296	0.85[c]
3. DEAE-cellulose				
fraction I	271	90.7	101.5	2.7
fraction III	349	16.6	31.3	11.2
4. lyophilized				
fraction I	168	21.9	52.8	3.2
fraction III	127	0.2	16.4	7.8
5. applied to Sephadex G-200[d]				
fraction I	131	16.7	41.2	3.2
fraction III	89	0.15	11.4	7.8
6. pooled, dialyzed active fractions				
fraction I	104	1.5	7.0	14.8
fraction III	20	0.03	1.95	10.3

[a] Units of collagenase (col'ase) were determined using acid-extracted, reconstituted calfskin collagen as substrate. Details are described in Materials and Methods. [b] Approximate protein concentrations were determined by recording the absorbance of the protein solution at 280 nm and making the assumption that a concentration of 1 mg per mL results in an absorbance of 1.0 (Dixon & Webb, 1964). [c] High endopeptidase activity at this stage of purification probably contributes to greater activity than would be observed with collagenases alone. [d] Portions of the lyophilized fractions I and III were applied to a Sephadex G-200 column.

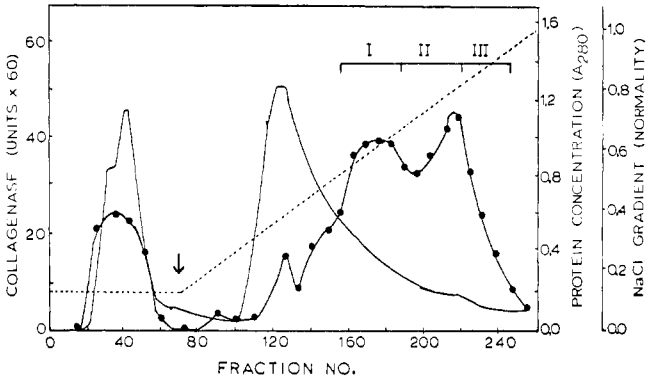

FIGURE 1: DEAE-cellulose chromatography elution pattern of crude B-30 collagenase using a linear NaCl gradient. The crude preparation of B-30 collagenase obtained from the 70% ammonium sulfate-saturated, cell-free, growth medium was dialyzed and layered onto the column surface. A total volume of 280 mL which had a collagenase activity (soluble collagen substrate) of 6.8 units per mL and a protein content (A_{280nm}) of 8.2 mg per mL was applied. Elution was performed using two buffer systems arranged to give a linear gradient. Initial buffer was 0.02 M Tris + 0.001 M $CaCl_2$ + 0.1 N NaCl, pH 8.5; the final buffer was 0.02 M Tris + 0.001 M $CaCl_2$ + 1.0 N NaCl, pH 6.5. The gradient was started at fraction 60 (arrow) after eluting with 450 mL of initial buffer. Fractions of 7.5 mL were collected at a rate of 1.5 mL per min. Symbols: (●—●) collagenase activity, μmol of leucine equivalents released per min per mL of enzyme solution; (—) protein concentration; absorbance at 280 nm; (- - -) normality of NaCl.

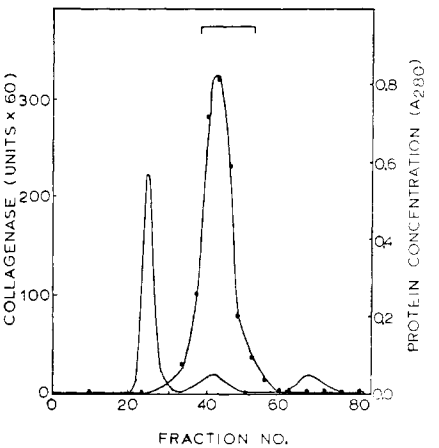

FIGURE 2: Sephadex G-200 elution pattern of fraction I from DEAE-cellulose ion-exchange chromatography. A solution containing 500 mg of lyophilized fraction I from the DEAE-cellulose ion exchange procedure was layered on the surface of the Sephadex G-200 column. The preparation had a collagenase activity (soluble collagen substrate) of 0.25 units per mg dry weight and a protein content (A_{280nm}) of 0.085 mg per mg of sample. Fractions of 5.0 mL were collected at a rate of 15 mL per h. Symbols: (●—●) collagenase activity, μmol of leucine equivalents released per min per mL of enzyme solution; (—) protein concentration, absorbance at 280 nm.

We were not successful at separating the two active collagenases (designated I and II on the basis of their location in the electrophoresis gels) by repassage through Sephadex G-100, DEAE-cellulose, or agarose columns. From the electrophoresis patterns in Figure 3, it appears that fraction I (Figure 1) possesses a higher concentration of collagenase I relative to collagenase II. A portion of lyophilized fraction I was dissolved in cold 0.02 M Tris-HCl, pH 8.4, at a protein concentration of 1.15 mg/mL. A portion of the solution was immediately frozen as a control, and the remainder was incubated at 37 °C for 3 h. Both samples were subjected to analytical polyacrylamide gel electrophoresis. The results are shown in Figure 4. The increase in concentration of collagenase II (the lower band) along with the decrease in band intensity of collagenase I after incubation seems to indicate a conversion of some I into II.

Both bands were shown to have collagenolytic activity by laying an unfixed and unstained gel column on the surface of a reconstituted calfskin collagen plate. After incubation for about 2 h at 37 °C, two distinct zones of digestion appeared which coincided with the two stained bands. When similar gel columns were placed on an algal chromoprotein plate (Merkel, 1966), these two bands had no activity against the chromoproteins which ruled out their being non-specific proteinases. Purified *Vibrio* B-30 collagenase has no or negligible hydrolytic activity against algal chromoproteins, urea-denatured hemoglobin, or casein.

If carbohydrates were present in purified *Vibrio* B-30 collagenase preparations, they were below the levels detectable by the phenol-sulfuric acid procedure.

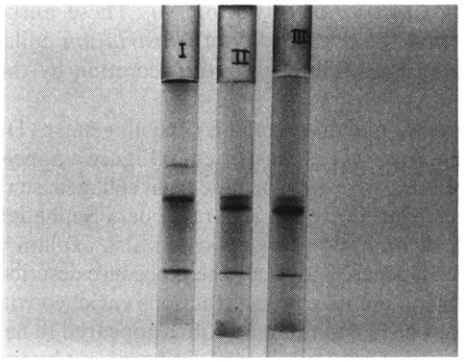

FIGURE 3: Analytical polyacrylamide gel electrophoresis of DEAE-cellulose fractions I, II, and III after gel filtration on Sephadex G-200. Gel electrophoresis, staining, and destaining were performed as described in Materials and Methods. (I) Lyophilized G-200 preparation of DEAE I, 32 μg protein applied. (II) Lyophilized G-200 preparation of DEAE II, 42 μg protein applied. (III) Lyophilized G-200 preparation of DEAE III, 27 μg protein applied.

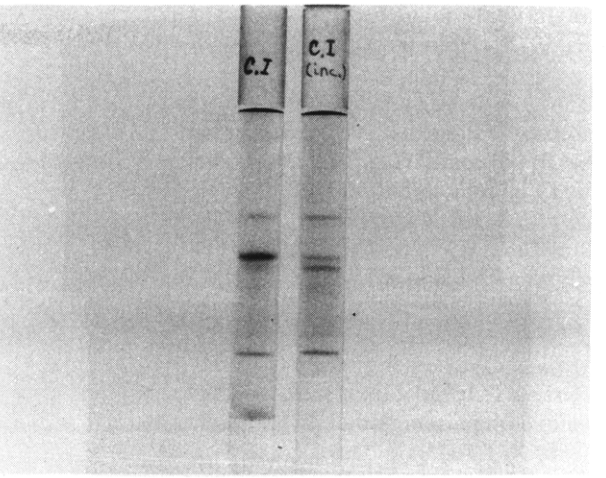

FIGURE 4: Analytical polyacrylamide gel electrophoresis showing the conversion of collagenase I to collagenase II. Gel electrophoresis, staining, and destaining were performed as described in Materials and Methods. (C.I) *Vibrio* B-30 collagenase I preparation (1.15 mg of protein per mL) stored in the frozen state before electrophoresis, 23 μg applied. (C.I inc.) Identical *Vibrio* B-30 collagenase preparation (1.15 mg of protein per mL) incubated at 37 °C for 3 h before electrophoresis, 23 μg applied.

Molecular Weight and Subunit Structure. Since all three fractions from DEAE-cellulose separated, crude enzymes eluted at the same volume from Sephadex G-200 columns, molecular weight determinations were done with an enzyme preparation that contained both collagenase I and II (fractions II and III from DEAE-cellulose columns). The ratio of elution volume to void volume of this enzyme preparation was determined on a Sephadex G-200 column that had been calibrated with RNase A, trypsin, hemoglobin, bovine serum albumin, and γ-globulin. On the basis of this calibration curve the molecular weight of *Vibrio* B-30 collagenase was calculated to be approximately 105 000.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed on a purified enzyme preparation that contained mostly collagenase I (fraction I, Figure 1, further purified on a Sephadex G-200 column). Similar results were obtained with enzyme preparations containing a mixture of collagenases I and II. The collagenase was denatured using 0.01 M phosphate buffer, pH 7.0, containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol, and incubating for 5 h at 50 °C. Under these conditions two protein bands were detected on the gels. Their mobilities were compared with those of the following protein standards: hemoglobin, trypsin, pepsin, aldolase, catalase, and bovine serum albumin. The mobilities of the *Vibrio* B-30 collagenase bands correspond to molecular weights of 24 000 and 28 000. Under milder denaturation conditions (0.1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol) *Vibrio* B-30 collagenase produced a single band on gel electrophoresis that corresponded to a molecular weight of 55 600, or approximately what would be expected from a dimeric form of the enzyme.

pH Optimum and Stability. The pH optimum for hydrolysis of native Achilles' tendon collagen under the conditions described in the Materials and Methods section was found to be 7.6. pH curves were reproduced with different enzyme preparations and by using phosphate buffer for low pH values (4–7) and Tris-HCl for high pH buffers (8–10) with basically the same results as those obtained with Tris-HCl alone. Phosphate buffers could not be used above pH 7.0 because enzyme inhibition was noted. A slight shoulder appeared at pH 7 in the pH curves which was reproducible and may reflect the presence of the two collagenase forms in the enzyme preparations.

Enzyme solutions were stable for at least 24 h at 4 °C in Tris-HCl and phosphate buffers between the pH range 4.4 to 7.4.

Temperature Optimum. Collagenase activity, as determined by the Mandl procedure, was measured at 5, 25, 37, 45, 60, and 70 °C. The optimum temperature was 37 °C. Activity was virtually eliminated (94%) at 45 °C. Measurements were not meaningful above 50 °C because the collagen substrate denatured too rapidly.

Enzyme Inhibitors. Na_2EDTA inhibited the enzyme at 10^{-5} M concentrations. *p*-Chloromercuribenzoate inhibited partially (58%) at 10^{-3} M concentration, but the enzyme was not affected by iodocaetamide (10^{-3} M), cysteine (10^{-3} M), or diisopropyl fluorophosphate (10^{-3} M for 60 min). The effect of isopropyl alcohol was tested because this was used as the solvent for DFP. A 20% (v/v) solution of isopropyl alcohol irreversibly inhibited *Vibrio* B-30 collagenase in less than 10 min at 21–23 °C. Inhibition of *Clostridium histolyticum* collagenase was observed at similar concentrations of isopropyl alcohol. Trypsin, which was used to determine the potency of our DFP solutions, was activated (45%) by incubation at 21–23 °C over a period of 30 min with 20% isopropyl alcohol. Fetal calf serum at a concentration of 50% (1 part calf serum to 1 part of 2 mg/mL enzyme solution) had no apparent effect on collagenase activity as measured by the disk assay method.

Specificity. The specificity of *Vibrio* B-30 collagenase was compared with that of commercial *Clostridium histolyticum* collagenase (see Nordwig, 1971). Specificities were determined with native Achilles' tendon collagen and with the protected hexapeptide, Z-Gly-L-Pro-Gly-Gly-L-Pro-L-Ala, as substrates.

Thin-layer chromatographic patterns were similar for the hydrolyzates produced by either *Clostridium histolyticum* or *Vibrio* B-30 collagenases. The major component in the hydrolyzates of Z-Gly-L-Pro-Gly-Gly-L-Pro-L-Ala was the expected peptide Gly-L-Pro-L-Ala. A trace of L-alanine also appeared in the hexapeptide hydrolyzates from each enzyme.

Thin-layer chromatographic patterns obtained with the hydrolyzate produced by the action of *Clostridium* and *Vibrio* B-30 collagenases on native Achilles' tendon collagen were also similar. Dinitrophenylation of the hydrolysis products of either the hexapeptide substrate or native Achilles' tendon collagen yielded DNP-Gly with traces of DNP-Ala and DNP-Pro.

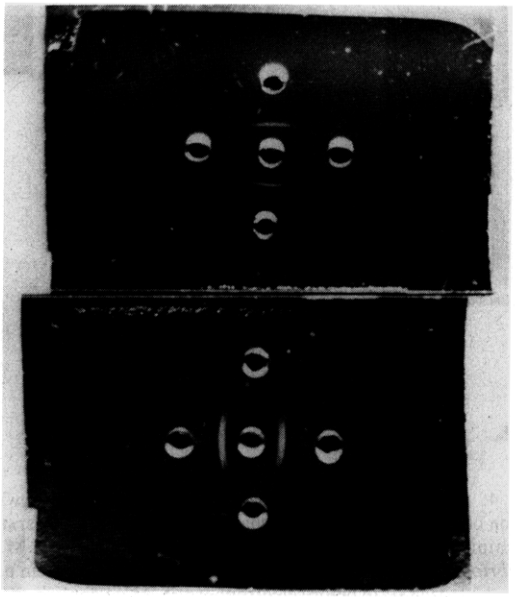

FIGURE 5: Immunological comparison of *Vibrio* B-30 and *Clostridium histolyticum* collagenases. Preparation of gels and antisera is described in Materials and Methods. In each case the upper and lower wells contain partially pure B-30 collagenase (9 μg); the right and left wells contain *Clostridium* collagenase, CLSPA grade, Worthington Biochemical Corp. (7 μg). The center well in the upper slide contains 1.0 μL of antiserum prepared against B-30 collagenase. The center well in the lower slide contains 1.0 μL of antiserum prepared against *Clostridium* collagenase. Control experiments using serum from the same rabbits before injection of the antigen gave no cross-reaction (not shown).

Serological Comparison of Vibrio B-30 and Clostridium histolyticum Collagenases. Figure 5 shows the results obtained in serological comparisons of the two collagenases. Each antigen is seen to react specifically with the antiserum prepared against that antigen and there are no visible cross reactions. *Vibrio* B-30 collagenase produces a single sharp precipitin band, whereas the *Clostridium* enzyme produced two precipitin bands.

Discussion

The marine isolate used in these studies was obtained from a sample of seawater collected in Harrington Sound, Bermuda. It required seawater for growth on initial isolation, and its characteristics best fit those of the genus *Vibrio*. A culture was deposited with the American Type Culture Collection (ATCC 21250).

Vibrio B-30 produces collagenolytic activity in measurable amounts only when collagen or certain collagen degradation products are included in the medium. By choosing the proper medium and cultural conditions, the bacterium can be induced to produce collagenase that is relatively free of nonspecific proteases. The small amount of nonspecific proteases contained in the crude enzyme (70% ammonium sulfate precipitate of the culture fluid) can be removed by passage sequentially through a DEAE-cellulose column (or careful batch extraction) and a Sephadex G-200 column. This treatment does not separate the two collagenases which are present in the preparations, but, as our gel filtration, electrophoresis, and serological results indicated, frees them from contaminating proteins. Observations of multiple collagenases produced by *Clostridium histolyticum* and subsequent attempts to separate the collagenases have been made by others. (For examples, see Grant & Alburn, 1959; Mandl et al., 1964; Harper et al., 1965; Kono, 1968). More recently Lwebuga-Mukasa et al. (1976) isolated and partially characterized four collagenases from extracts of *Clostridium histolyticum*. These authors also suggested that the multiplicity of *Clostridium* collagenases resulted from lysis following initial secretion by the bacteria.

The conversion of one *Vibrio* B-30 collagenase (I) into the other collagenase (II) as illustrated in Figure 4 appears to involve the removal of a small peptide from collagenase I because collagenase I and II are not separable on a Sephadex G-200 column. A deamination process could also explain the conversion of collagenase I to II. By the procedure described above we were able to obtain enzyme fractions enriched with either collagenase I or II and these fractions appeared to have identical properties. The similarity is also borne out by the results of our serological studies, i.e., a single, sharp precipitin band was obtained when antiserum to *Vibrio* B-30 collagenase was challenged with the purified collagenase (Figure 5).

Results from Sephadex G-200 chromatography and sodium dodecyl sulfate gel electrophoresis support a tetrameric model for *Vibrio* B-30 collagenase. Levdikova et al. (1963) found by ultracentrifugation that *Clostridium* collagenase has a molecular weight of about 100 000 (in agreement with Mandl (1961) and Kono (1968)), but in the presence of 0.01 M *o*-phenanthroline the apparent molecular weight was close to 25 000. They speculated that *Clostridium* collagenase is a tetramer composed of four equal subunits. Harper et al. (1965) found evidence to support a tetrameric model for *Clostridium* collagenase. However, on the basis of new studies (Lwebuga-Mukasa et al., 1976), Harper's group now believes that there are four distinct enzymes: three have molecular weights of about 81 000 and the third (IIIb) has a molecular weight of about 72 000.

Vibrio B-30 collagenase thus appears to fit the earlier models proposed for *Clostridium* collagenase, i.e., composed of four subunits. However, as Lwebuga-Mukasa et al. (1976) found for *Clostridium* collagenase, an autolytic or proteolytic process may be responsible for converting one enzyme into a slightly different form (collagenase I → II).

Many other properties of *Vibrio* B-30 collagenase are similar to those reported for *Clostridium* collagenase (see Mandl, 1961): e.g., optimum pH (7.6); optimum temperature (37 °C); diisopropyl fluorophosphate does not inhibit either enzyme, but Na_2EDTA does; both enzymes are sensitive to isopropyl alcohol, but neither is inhibited by calf serum; and the peptide specificities of both enzymes are apparently identical (extensively degrading native collagen).

However, there are differences between the two enzymes: e.g., there is great difference in their electrophoretic mobilities at a given pH; possible differences in subunit makeup; and immunologically the two enzymes are very distinct (there is no cross reaction with the other's antiserum). Noncross reactivity has recently been demonstrated between a collagenase produced by *Achromobacter iophagus* and the clostridial enzyme (Welton & Woods, 1975).

The fact that *Vibrio* B-30 does not produce the toxins often encountered in *Clostridium* cultures makes its large scale production attractive for clinical and industrial uses (Mandl, 1972). *Vibrio* B-30 also has several advantages over clostridia in fermentation procedures, e.g., the *Vibrio* B-30 fermentation is aerobic, it is rapid (10–18 h), and by controlling the nutrients the production of nonspecific proteases can be kept to a minimum if this is desired.

Acknowledgments

The authors are indebted to Mr. Henry Ziegler of Chemzymes, Inc., for the generous gift of all of the UltraPure grades of ammonium sulfate, Tris-HCl, and urea, and to the Sheffield

Chemical Company for the NZ-amine, type HD hydrolyzed casein used in these studies. The able assistance of Mrs. Jo Gallagher and fermentation data provided by Linda Neiman are also gratefully acknowledged.

References

Andrews, P. (1965) *Biochem. J. 96,* 595.
Anson, M. L. (1938) *J. Gen. Physiol. 72,* 79.
Clarke, J. T. (1964) *Ann. N.Y. Acad. Sci. 121,* 428.
Crowle, A. J. (1958) *J. Lab. Clin. Med. 52,* 784.
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121,* 404.
Dixon, M., & Webb, E. C. (1964) *Enzymes,* p 30, Academic Press, New York, N.Y.
Dubois, M. K., Gilles, K. H., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28,* 350.
Einbinder, J., & Schubert, M. (1951) *J. Biol. Chem. 188,* 335.
Fraenkel-Conrat, H., Harris, J. I., & Levy, A. L. (1955) *Methods Biochem. Anal. 2,* 369.
Grant, N. H., & Alburn, H. E. (1959) *Arch. Biochem. Biophys. 82,* 245.
Grassman, W., & Nordwig, A. (1960) *Z. Physiol. Chem. 322,* 267.
Gross, J., & Kirk, D. (1958) *J. Biol. Chem. 233,* 355.
Harper, E., Seifter, S., & Hospelhorn, V. D. (1965) *Biochem. Biophys. Res. Commun. 18,* 627.
Keller, S., & Mandl, I. (1963) *Arch. Biochem. Biophys. 101,* 81.
Kono, T. (1968) *Biochemistry 7,* 1106.
Lee-Own, V., & Anderson, J. C. (1975) *Prep. Biochem. 5,* 229.
Levdikova, G. A., Orekhovich, V. N., Solov'eva, N. I., & Shpikiter, V. O. (1964) *Dokl. Akad. Nauk. SSSR (Engl. Transl.) 153,* 1429.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265.
Lwebuga-Mukasa, J. S., Harper, E., & Taylor, P. (1976) *Biochemistry 15,* 4736.
Mandl, I. (1961) *Adv. Enzymol. 23,* 163.
Mandl, I., Ed. (1972) *Collagenases,* Gordon and Breach, New York, N.Y.
Mandl, I., MacLennan, J. D., & Howes, E. L. (1953) *J. Clin. Invest. 32,* 1323.
Mandl, I., Keller, S., & Manahan, J. (1964) *Biochemistry 3,* 1737.
Merkel, J. R. (1966) *Anal. Biochem. 17,* 84.
Merkel, J. R., Dreisbach, J. H., & Ziegler, H. B. (1975) *Appl. Microbiol. 29,* 145.
Nagai, Y., Sakakibara, S., Noda, H., & Akabori, S. (1960) *Biochim. Biophys. Acta 37,* 567.
Nordwig, A. (1971) *Adv. Enzymol. 34,* 164.
Ornstein, L. (1964) *Ann. N.Y. Acad. Sci. 121,* 321.
Ouchterlony, O. (1968) *Handbook of Immunodiffusion and Immunoelectrophoresis,* Ann Arbor Publishers, Inc., Ann Arbor, Mich.
Prescott, J. M., & Wilkes, S. H. (1966) *Arch. Biochem. Biophys. 117,* 328.
Prescott, J. M., & Willms, C. R. (1960) *Proc. Exp. Biol. Med. 103,* 410.
Rosen, H. (1957) *Arch. Biochem. Biophys. 67,* 10.
Weber, J., Pringle, J. R., & Osborn, M. (1972) *Methods Enzymol. 26C,* 1.
Welton, R. L., & Woods, D. R. (1975) *Biochim. Biophys. Acta 384,* 228.
Yoshida, E., & Noda, H. (1965) *Biochim. Biophys. Acta 105,* 562.