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Induction of Collagenase Production in *Vibrio* B-30

JOSEPH H. DREISBACH† AND JOSEPH R. MERKEL*

Department of Chemistry and The Center for Marine and Environmental Studies, Lehigh University, Bethlehem, Pennsylvania 18015

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The inducible nature of an extracellular collagenase produced by a marine *Vibrio* (*Vibrio* B-30, ATCC 21250) was demonstrated by observing the increase in extracellular collagenase activity after the addition of collagen to cell cultures in the latter part of the exponential growth phase. When collagenase-hydrolyzed collagen was added, the lag time required before collagenase production was detected decreased significantly compared with cultures receiving collagen. Cells preinduced to synthesize collagenase did not produce the enzyme when collagen was removed from the culture medium. Incorporation of penicillin G had no effect on final collagenase activity levels in suspensions of *Vibrio* B-30 in complete medium supplemented with collagen. However, chloramphenicol and tetracycline inhibited collagenase production, indicating that de novo protein synthesis was necessary for the appearance of activity. Attempts to isolate the inducing substance(s) involved filtering hydrolyzed collagen through a series of ultrafiltration membranes. The lowest-molecular-weight fraction of collagen hydrolysate with inducing ability was between 1,000 and 10,000. Gel filtration of this fraction on Sephadex G-50 resulted in the appearance of three protein peaks, two of which were capable of inducing collagenase production. Results from amino acid composition and *N*-terminal amino acid analysis suggest that the inducing substance originates from the polar helical portion of the collagen molecule.

Collagenases are proteolytic enzymes capable of hydrolyzing collagen in the undenatured state (12). Since the original demonstration of collagenase activity in the culture filtrates of certain species of *Clostridium* by Mandl (12), a number of other microbial species have been shown to elaborate enzymes which can be classified as true collagenases (16, 21). Studies in our laboratory concentrated on the isolation of marine organisms capable of producing collagenases. In the course of this work, it was shown that approximately 44% of the marine isolates were capable of elaborating enzymes which hydrolyzed reconstituted collagen gels (14). In this search for collagenolytic marine bacteria, the existence of apparently inducible collagenases was observed.

Rose (18) defines an inducible enzyme as one that is "synthesized by a microorganism only in response to the presence in the environment of an inducer, which is usually the substrate for the enzyme or some structurally related compound." The inducible β-galactosidase system of *Escherichia coli* is the classic example of this phenomenon (1, 8). More recently, reports on inducible proteolytic enzymes from microorganisms have appeared in the literature (2, 3, 11). However, information on induction of bacterial proteases remains scarce. Although extracellular collagenases have been reported for a number of microorganisms, no investigations on the possible inducible nature of these enzymes have been undertaken before this study, which describes the induction of collagenase production in one marine bacterium, *Vibrio* B-30 (ATCC 21250), by collagen or collagen degradation products. The inducing substance(s) was partially purified, and some initial characterizations are also described herein.

(A preliminary report of these results was presented at the 77th Annual Meeting of the American Society for Microbiology, New Orleans, La., 8-13 May 1977.)

MATERIALS AND METHODS

Organism and cultural conditions. *Vibrio* B-30 is a proteolytically active marine bacterium that was isolated from a seawater sample collected at a depth of 3 m in Harrington Sound, Bermuda. A culture was deposited with the American Type Culture Collection (ATCC 21250).

Cultures used in the current study were maintained on medium containing 1% peptone, 0.1% yeast extract, 4% synthetic sea salts (Rila Marine Mix [RMM]; Rila Products, Teaneck, N.J.), and 2% agar. We refer to this medium as PYSW agar. Induction experiments were performed in a medium containing 1% hydrolyzed casein (NZ Amine, type HD; Humko Sheffield Chemical Div., Memphis, Tenn.) and 2% RMM and adjusted

† Present address: Department of Chemistry, University of Scranton, Scranton, PA 18510.

to pH 7.0 to 7.2. This medium is referred to as HD½SW. Other details of culture maintenance and storage were described previously (14).

Assay of collagenase activity. Acid-extracted, dialyzed calfskin collagen (10 to 13 mg of protein per ml as determined by the method of Lowry et al. [10] or by the biuret method [5]) was prepared by the procedure of Gross and Kirk (7), as described previously (14). Dialysis of the extract against cold (0 to 4°C) Sorensen phosphate buffer (pH 7.6; $\mu = 0.4$) was continued until the pH of the extract was between 7.0 and 7.6. If not used immediately, the extract was stored at 0 to 4°C for periods of up to 2 weeks. Neutralized, acid-extracted, calfskin collagen must be kept cold to prevent it from forming a rigid gel. Collagen prepared in the above manner was shown to be resistant to hydrolysis by noncollagenous enzymes (14). Immediately before use in the assay, dialyzed calfskin extract was diluted with ice-cold 0.02 M tris(hydroxymethyl)aminomethane (Tris; Chemzymes, Inc., East Stroudsburg, Pa.) containing 0.001 M $CaCl_2$ at pH 8.4. A 2-ml quantity of this diluted collagen solution was preincubated at 37°C for 15 min to allow gelling to occur. Then 0.1 ml of enzyme solution was added, and the mixture was shaken to suspend the collagen fibrils. After 1 h the reaction was stopped by addition of 0.5 ml of 30% trichloroacetic acid. The mixture was kept at 37°C for 15 min before being filtered through Whatman no. 2 filter paper. Peptides and amino acids released by this method were determined by the ninhydrin procedure of Rosen (19). Standard leucine curves were prepared with each determination, and units of activity are expressed as micromoles of leucine equivalents released per minute. Controls consisted of enzyme solutions heated in a boiling water bath for 5 min before being treated as described above.

Induction experiments. Growth from a 24-h PYSW agar slant culture of *Vibrio* B-30 was suspended in 5 ml of sterile 4% RMM. A 0.1-ml quantity of this growth suspension was used to inoculate 50 ml of HD½SW medium in a 250-ml shaker flask. Inoculated medium was incubated at 23°C on a New Brunswick model V rotary shaker at a setting of 16. After 12 h (latter portion of the exponential growth phase) the flask was removed from the shaker, 10 ml of the inducer was added, and the flask was immediately returned to the shaker. Samples (3 ml) were withdrawn periodically. Growth was measured by reading the optical density of the sample at 660 nm, and the bacteria were removed by centrifugation. Ammonium sulfate (1.3 g) was added to 2 ml of the cell-free culture supernatant (70% saturation). Proteins were allowed to precipitate overnight and were collected by centrifugation at 10,000 rpm for 15 min. The precipitate was dissolved in 1 ml of Tris buffer and dialyzed for 18 h against two changes of the same buffer at 4°C. Collagenase activity was determined on these protein solutions as described above. Controls in the induction experiments consisted of identical *Vibrio* B-30 cultures to which 10 ml of sterile 0.146 M Sorensen phosphate buffer was added in place of the potential inducer.

Acid-extracted, reconstituted calfskin collagen was used in all of the experiments requiring collagen. The soluble nature of this material made the preparation of solutions easier than when insoluble, bovine Achilles tendon collagen was used. The latter material also induced collagenase production in *Vibrio* B-30 (data not shown).

Elimination of the enzyme inducer(s) from the culture medium used to grow adapted cells. A flask containing 50 ml of HD½SW and 10 ml of collagenase-hydrolyzed collagen was inoculated as described above. Exponentially growing cells were removed from the growth medium by centrifuging at 5,000 rpm for 10 min under aseptic conditions. Cells were suspended in 15 ml of sterile 4% RMM, and 6 ml of this suspension was added to a shaker flask containing medium identical to that used to induce the cells. A separate 6-ml sample was used to inoculate a second shaker flask containing 10 ml of 0.146 M Sorensen buffer and 50 ml of HD½SW. Samples were removed periodically for turbidity and collagenase activity measurements.

Preparation of collagenase inducers by enzymatic hydrolysis of collagen. Hydrolyzed collagen solutions were prepared by dissolving either 1 mg of partially purified (14) *Vibrio* B-30 collagenase or clostridial collagenase (CLSPA; Worthington Biochemicals Corp., Freehold, N.J.) in 1 ml of Tris buffer. A 1-ml quantity of the solution to be used was added to 50 ml of dialyzed, acid-extracted calfskin collagen, and the mixture was incubated at 37°C for 24 h. The mixture was then autoclaved at 15 lb/in^2 for 20 min and centrifuged at 8,000 rpm for 10 min. The supernatant was used in subsequent induction studies.

Isolation of an inducing fraction using ultrafiltration. A 200-ml quantity of collagenase-hydrolyzed collagen was filtered through an Amicon UM-10 membrane (10,000-dalton retention limit; Amicon Corp., Lexington, Mass.) using 50-lb/in^2 nitrogen pressure in a 150-ml Amicon Diaflo apparatus. The filtrate obtained from UM-10 filtration (UM-10 filtrate; approximately 195 ml) was then passed through an Amicon UM-2 membrane (1,000-dalton retention limit) using the same conditions as described above. Ultrafiltration was allowed to proceed until approximately 95% of the sample had been collected. Nonfiltered material (UM-2 retained) was removed from the membrane by successive rinsings with distilled water. A final volume of 19.5 ml (10-fold concentration of the UM-10 filtrate) of the UM-2-retained fraction was membrane filtered (Millipore Corp., Bedford, Mass.) and stored in a sterile, capped culture tube at 4°C.

Gel filtration of the inducing fraction. Further fractionation of the inducing substance was performed by gel filtration on Sephadex G-50. Final column dimensions were 2.8 by 120 cm. A typical experiment involved the application of 4 ml of the UM-2-retained fraction (32 to 35 mg of protein) to which was added 0.5 ml of blue dextran solution (1.7 mg/ml). Fractions of 3.5-ml volume were eluted with distilled water at a rate of 36 ml/h at 4°C. Protein measurements were taken on every second fraction by reading the absorbance at 280 nm with a Perkin Elmer model 200 UV-visible spectrophotometer. Fractions under each protein peak were pooled, and the inducing abilities were determined as described above.

Characterization of the inducing fraction. The amino acid composition of the inducer substance was

determined as described below. A 2-ml quantity of 6 N HCl was added to 1 ml of protein sample (10 to 50 mg) in a 5-ml vial. The tube was evacuated, sealed, and placed in a 110°C oven for 48 h. HCl was removed by evaporation on a rotary flash evaporator (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N.J.), and amino acids were determined by using two-dimensional thin-layer chromatography. A sample of the hydrolysate (10 to 20 μl) was applied to a Silica Gel G (EM Laboratories Inc., Elmsford, N.Y.) thin-layer chromatogaphy plate. The first dimension was developed with ethanol-concentrated ammonium hydroxide-distilled water (16:1:3), and the second dimension was developed with butanol-acetic acid-distilled water (6:2:2). Spots were detected by spraying with a ninhydrin-butanol mixture, drying at room temperature, and heating at 70°C. Observed spots were identified by comparing migration positions with those of a number of amino acid standards run in an identical manner.

Determination of amino-terminal amino acids of the inducing fractions was performed by the method of Sanger (20). Preparation of the dinitrophenylated proteins and amino acids was accomplished by the procedure of Fraenkel-Conrat et al. (4). Dinitrophenylated amino acids were identified by thin-layer chromatography on Silica Gel G. The solvent system used was that of Pataki (17) and consisted of chloroform-benzyl alcohol-acetic acid (70:30:3).

RESULTS

Effect of collagen and hydrolyzed collagen on collagenase production. Initial investigations on the production of collagenase by *Vibrio* B-30 cultures involved the addition of acid-extracted and reconstituted, autoclaved collagen to the growth medium. (Although it is referred to as "collagen" in this report, the preparation used was autoclaved before its addition to the medium. Thus, the protein no longer could be considered to possess the specific conformational structure of native collagen.) It was decided to add collagen in the latter stages of the exponential phase of growth so that a maximum number of actively growing cells could be induced. In this way adaptation to produce collagenase would result in a burst of activity, negating any sensitivity problems encountered with the assay procedures. Early-log-phase cells were induced in several experiments (data not shown) with results that were similar to those obtained with late-log-phase cells. All results presented are reproducible and are representative data from at least two and in most cases six to eight repetitions of the individual experiments.

Collagenase activity in the medium was significantly increased at 1.5 h after addition of the collagen, and the maximum rate of collagenase production was attained within 3.5 to 4 h after addition (Fig. 1). A control experiment using

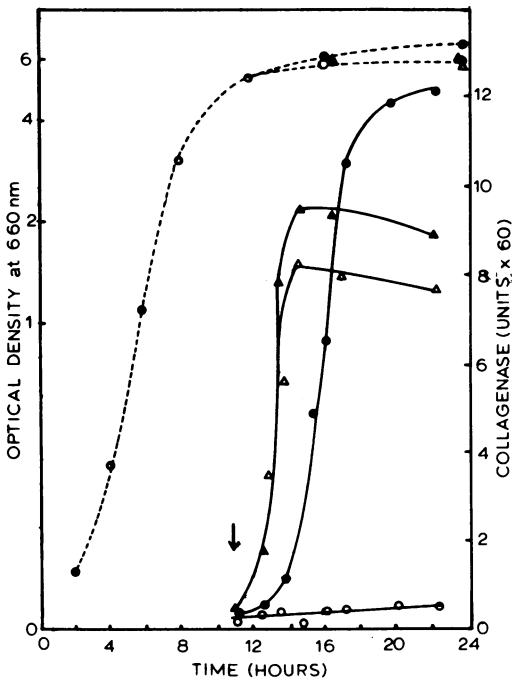

FIG. 1. *Effects of collagen and hydrolyzed collagen preparations on the induction of collagenase in Vibrio B-30. A 10-ml quantity of the solution to be examined for inducing ability was added to a shaker flask containing 50 ml of Vibrio B-30 near the end of the exponential phase of growth (inducer added at the arrow). Samples were periodically removed and assayed for collagenase activity* (—) *and turbidity* (---) *as described in the text. Symbols:* ●, *unhydrolyzed collagen added as the inducer;* △, *Vibrio B-30 collagenase-hydrolyzed collagen added;* ▲, *C. histolyticum collagenase-hydrolyzed collagen added;* ○, *0.146 M Sorensen buffer added in place of an inducing solution. All culture flasks had the same amount of cells (turbidity) at the time that the inducer substances were added. All shaker flasks were incubated under the same environmental conditions.*

0.146 M Sorensen phosphate buffer in place of collagen showed only a very slight increase in apparent collagenase activity through the course of the experiment. At 11 h after addition, activity in the control was only 4.1% of that in the collagen-containing medium and could be considered negligible. This low amount of activity was probably the result of non-collagenolytic proteases which this organism produces.

When collagen hydrolyzed with either *Vibrio* B-30 or *Clostridium histolyticum* collagenase was added to the *Vibrio* B-30 culture, maximum collagenase production occurred at 1.5 h after addition (Fig. 1). Growth curves for the cultures receiving collagen and hydrolyzed collagen were similar to that of the control. Slight increases in

the final growth levels can be attributed to additional nutrients provided by the added collagen preparations.

Effect of removal of collagen from the culture medium on collagenase production. One might propose a mutant-selection explanation for the increase of collagenase activity in response to the addition of collagen to the growth medium. That is, it may be the case that a low percentage of cells are constitutively endowed with the capability to produce collagenase. Growth in a collagen-containing medium may result in selection for these collagenase-active mutants. If this were the case, one would expect a culture producing collagenase to continue to do so after the collagen is removed from the medium.

Washed, collagenase-producing cells which were placed into a collagen-free medium showed only a slow and negligible increase in activity (Fig. 2). An identical culture preparation, when added to a collagen-containing medium, displayed maximal production of collagenase within 1 to 2 h. Growth curves in both media were similar.

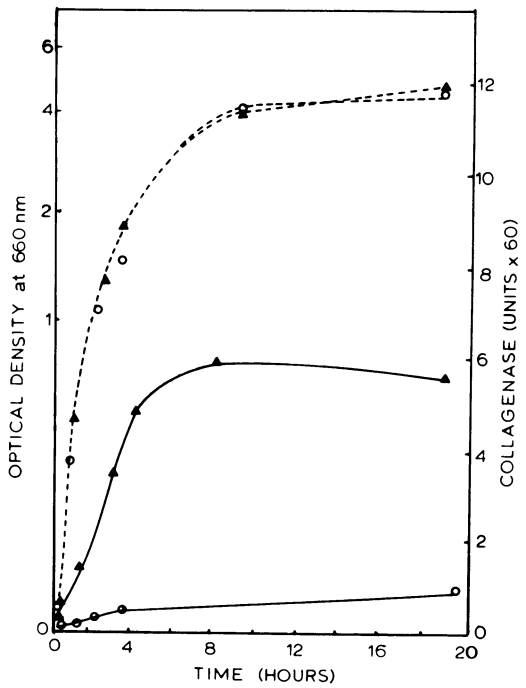

FIG. 2. *Elimination of the enzyme inducer(s) from culture medium used to grow adapted cells. Cells from a Vibrio B-30 culture induced to produce collagenase were removed by centrifugation, washed one time with sterile 4% RMM, suspended in 4% RMM, and used to inoculate two shaker flasks. One flask contained HD½SW + hydrolyzed collagen (identical to the induction medium) (▲); the other flask contained HD½SW + 0.146 M Sorensen buffer (○). Samples were periodically removed and assayed for collagenase activity (—) and turbidity (---) as described in the text.*

Effect of selected antibiotics on collagenase production. Further support for an actual induction phenomenon is provided by the observed effects of various antibiotics on collagenase production by *Vibrio* B-30 (Table 1). Inhibitors of protein synthesis, tetracycline and chloramphenicol, inhibited production of collagenase. Penicillin G, which presumably has its effect on peptidoglycan cross-linking rather than on protein or nucleic acid synthesis, had no effect on the final levels of collagenase which were produced. Culture tubes containing antibiotics displayed only slight increases in turbidity as compared with the control containing no antibiotics.

Various proteins and protein hydrolysates as collagenase inducers. As described previously, collagen hydrolyzed with either *Vibrio* B-30 or clostridial collagenase before autoclaving decreased the lag time required for maximum rate of extracellular collagenase production. Final levels of activity induced by hydrolyzed collagen preparations were slightly lower than those induced by unhydrolyzed collagen (Fig. 1 and Table 2). A number of other collagen hydrolysate preparations were examined for their ability to induce collagenase production (Table 2). Collagen pretreated with trypsin retained inducing capabilities, although the final activity was slightly lower than the activity induced when the hydrolysate was prepared with one of the collagenases. Initial treatment of collagen with collagenase followed by trypsin hy-

TABLE 1. *Effect of antibiotics on Vibrio B-30 collagenase production*[a]

Sample added	Collagenase [(units × 60)/ml]	Final turbidity (A_{660})
Hydrolyzed collagen + tetracycline	4.2	0.240
Hydrolyzed collagen + chloramphenicol	3.4	0.282
Hydrolyzed collagen + penicillin G	72.1	0.325
Hydrolyzed collagen + buffer	70.5	0.730
Buffer only	1.3	0.495

[a] Collagenase production was determined as described in the text. Each culture tube contained 3 ml of HD½SW medium, 1 ml of hydrolyzed collagen inducer, 0.3 ml of antibiotic solution (100-μg/ml final concentration), and 0.5 ml of inoculum which consisted of washed cells from a 12-h culture of *Vibrio* B-30. Initial turbidity (absorbance at 660 nm [A_{660}]) was 0.118. Final activity and growth measurements were taken after 24 h of incubation at 23°C.

TABLE 2. *Ability of various proteins and peptides to induce collagenase production*

Sample	Protein concn (mg/ml of medium)	Collagenase activity (% of activity obtained with *Vibrio* B-30 collagenase-hydrolyzed collagen as inducer)
Collagen hydrolyzed with *Vibrio* B-30 collagenase	0.86[a]	100
Collagen autoclaved before addition	2.4[a]	128
Collagen hydrolyzed with *Vibrio* B-30 collagenase followed by trypsin	2.4[b]	97
Collagen hydrolyzed with trypsin	2.4[c]	86
Gelatin	1.9[a]	79
Gelatin hydrolyzed with trypsin	1.9[d]	6.8
BSA	1.9[e]	0.0
BSA hydrolyzed with trypsin	1.9[d]	0.0
Hexapeptide (Z-Gly-L-Pro-Gly-Gly-L-Pro-L-Ala)	1.0[e]	1.1
Tripeptide (Gly-L-Pro-L-Ala)	1.0[e]	1.8

[a] Concentration was determined by using the biuret method and BSA as a standard.

[b] Hydrolysis was performed by adding 0.25 ml of *Vibrio* B-30 collagenase solution (1-mg/ml concentration, partially purified) and incubating at 37°C for 24 h. After 24 h, 0.25 ml of trypsin solution was added, and the sample was subjected to the procedure described in footnote *c* below.

[c] Hydrolysis was performed by adding 0.25 ml of trypsin solution (1-mg/ml concentration) to 25 ml of acid-extracted, dialyzed collagen and incubating at 37°C for 24 h. Toluene was layered on the surface to prevent microbial growth. Samples were autoclaved before addition to the culture medium. Concentration is expressed as milligrams of protein per milliliter before trypsin addition.

[d] Hydrolysis was performed as described in footnote *c* above.

[e] Concentration was determined by weighing the given amount of sample protein or peptide.

drolysis resulted in a preparation which gave final activity levels essentially identical to the collagenase hydrolysate levels.

Gelatin, a denatured form of collagen, was also a good inducer. The inducing ability of gelatin was destroyed by trypsin treatment before addition to the culture. Neither bovine serum albumin (BSA) nor trypsin-hydrolyzed BSA had the ability to induce collagenase production. The tripeptide glycyl-L-prolyl-L-alanine, which is one of the major products of *C. histolyticum* (15) and *Vibrio* B-30 (unpublished data) collagenase hydrolysis of collagen, was examined for its ability to induce collagenase synthesis. Also, the hexapeptide of Grassman and Nordwig (6) was tested for its induction capabilities. Neither of the synthetic peptides had any inducing effects (Table 2).

Isolation of inducing substances. Because the appearance of maximal rates of collagenase production had a shorter lag time when collagenase-hydrolyzed collagen was added to the culture in place of unhydrolyzed collagen, it was assumed that the hydrolyzed preparation contained a protein or peptide which was either the actual inducing substance or required only a slight alteration to be converted into the inducing substance. Hydrolyzed collagen was used as the starting material in attempts to isolate the inducer(s). Collagen was normally hydrolyzed with clostridial collagenase because of its commercial availability. However, *Vibrio* B-30 collagenase-hydrolyzed collagen gave identical results throughout the procedure.

Initial steps in the isolation of the inducer substances involved a sequence of ultrafiltrations of collagenase-hydrolyzed collagen as described above. Active filtrates obtained by Amicon UM-10 membrane filtration were retained on UM-2 membranes. These results indicate a lower-molecular-weight range of 1,000 to 10,000. Material capable of causing induction which was retained on the UM-10 membrane (molecular weight, >10,000) was probably incompletely hydrolyzed collagen. Fractionation of the material retained on the UM-2 membrane was performed by gel filtration on Sephadex G-50 (Fig. 3). Frac-

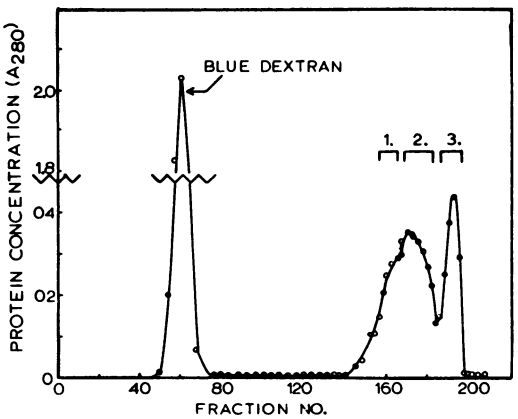

FIG. 3. *Gel filtration on Sephadex G-50 of the collagen hydrolysate retained on a UM-2 filter. A solution of the UM-2-retained fraction of the collagen hydrolysate (32 to 35 mg of protein) containing 0.75 mg of blue dextran was layered on the surface of the column. Elution was performed as described in the text. Fractions of 3.5 ml were collected, and fractions under each protein peak were pooled as shown in the figure (brackets).*

tions under each protein peak were pooled, concentrated, and assayed for induction capabilities. Both fractions 1 and 2 were capable of inducing collagenase synthesis. Fraction 1 induced approximately 30% more production than fraction 2. The degree of overlap, however, prevents us from concluding that two different inducing proteins exist in the filtrate.

Partial characterization of the inducing substances. Dinitrophenylation of the material in fractions 1 and 2, which were obtained from Sephadex G-50 gel filtration, followed by acid hydrolysis and identification of the *N*-terminal amino acids yielded glycine in both fractions. Similar treatment of unchromatographed, UM-2-retained material also indicated that glycine was the major *N*-terminal amino acid of this material. Results were identical when either *Vibrio* B-30 or clostridial collagenase was used to prepare the collagen hydrolysate.

Qualitative amino acid analysis was performed on fractions 1 and 2 from Sephadex G-50 fractionation. Thin-layer chromatography patterns revealed only five to six different amino acids for each fraction. Three of the ninhydrin-positive spots were identified as proline, glycine, and alanine.

DISCUSSION

We have shown that production of extracellular collagenase by cultures of *Vibrio* B-30 in response to the addition of collagen, hydrolyzed collagen, or gelatin is an induction process. This is supported by the facts that (i) cells previously induced to produce collagenase did not produce the enzyme when the inducer was removed from the medium (Fig. 2), and (ii) collagenase production was a result of de novo protein synthesis as demonstrated in the antibiotics experiment (Table 1).

Decreased lag times required for collagenase production when hydrolyzed collagen was used as the inducing substance indicated that hydrolysis products of collagen were responsible for the actual induction. This was not an unexpected result because it is difficult to visualize a high-molecular-weight protein such as an α chain of collagen influencing an intracellular process.

Collagen consists of a nonpolar region which is characterized by the repetitive peptide sequence glycyl-L-prolyl-*R* or glycyl-*R*-L-proline, where *R* represents a number of different amino acids. This nonpolar region comprises about 95% of the tropocollagen molecule. The polar regions, located at the *N*-terminal portions of each chain, possess a globular conformation and are susceptible to attack by general proteases (9). Collagen treated with trypsin before autoclaving retained inducing ability, whereas this ability was lost when gelatin underwent the same treatment (Table 2). These results indicate that the nonpolar portion of the molecule or some part of the nonpolar region remaining after collagenase treatment must remain intact (although not necessarily in the native state because autoclaved collagen and gelatin act as inducers) to retain inducing ability. The polar region of collagen need not remain intact because trypsin, which is capable of attacking this region of the molecule (9), did not destroy the effectiveness of the preparation as an inducer.

Additional support for a nonpolar origin comes from the characterization studies on the inducing fractions from Sephadex G-50 filtration. Glycine was shown to be the major *N*-terminal amino acid of the peptides produced by hydrolysis of collagen with clostridial collagenase (15). Similarly, glycine was the only detectable *N*-terminal amino acid in the inducing fractions from Sephadex G-50 filtration. Collagen structural studies revealed that glycine and proline are the major amino acid constituents of the nonpolar region (13). These same residues were shown to predominate among a total of only six amino acids which were detected in acid hydrolysates of partially purified inducing fractions.

The exact nature of the proteases responsible for collagen hydrolysis by the organism leading to the production of an inducer molecule when it is presented with native collagen molecules is unknown. Because denatured forms of collagen act equally well as inducers, it is not mandatory that the protease be a true collagenase. However, because an inducible enzyme is present in a noninduced culture at certain basal levels (18), it may be collagenase which causes hydrolysis. Results from dinitrophenylated end group analysis indicated that the protease does have collagenase specificities.

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