Chondroitinase-Producing Bacteria in Natural Habitats

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A search was undertaken for bacteria which degrade chondroitin sulfate in nature and to find bacteria with a usefully high rate of chondroitinàse (ChSase) productivity. First, 253 ChSase-producing bacteria were obtained from aquatic and land environments in Japan by aerobic and anaerobic screening methods. Identification according to *Bergey's Manual of Determinative Bacteriology* or Bain and Shewan (1968) permitted assignment of the majority of the isolates to seven genera, *Aeromonas, Vibrio, Flavobacterium, Beneckea, Proteus, Micrococcus,* and *Arthrobacter.* Next, ChSase productivities of all the isolates were compared with those of two established ChSase-producing stock strains, *Proteus vulgaris* NCTC 4636 and *Flavobacterium heparinum* ATCC 13125. As a result, special attention was given to production by a strain of *Aeromonas* sp. of large quantities of extracellular ChSase-AC. None of the isolates from the current study displayed significant ChSase-ABC productivity. Finally, ChSase-AC was prepared from the culture fluid of the *Aeromonas* strain by fractional precipitation with ammonium sulfate, chromatography on phospho-cellulose and diethylaminoethyl-cellulose, and gel filtration on Sephadex G-200. It was concluded that the *Aeromonas* strain may represent a profitable source of the enzyme ChSase-AC.

The acid mucopolysaccharide, chondroitin sulfate (ChS), is widely distributed in animal connective tissues. It is comprised of uronic acid, hexosamine, acetyl, and sulfate units. There are several isomers designated ChS-A, ChS-B, or ChS-C, according to the position in which sulfate is bound or the identity of uronic acid component (5). Bacterial chondroitinase (ChSase; condroitin sulfate lyase, EC 4.2.99.6) may be associated with bacterial pathogenicity and degradation of animal residue in nature and may serve in addition as a tool in medical and biochemical studies on connective tissues. Neuberg and Rubin (16) described bacterial degradation of ChS as early as 1914, and Neuberg and Hofmann (15) demonstrated that extracts of the cells of *Pseudomonas fluorescens, Pseudomonas aeruginosa,* and *Proteus vulgaris* degrade ChS with the simultaneous release of a product having reducing power and of sulfuric acid. Several workers reported that enzymes extracted from the cells of *P. vulgaris* and *Flavobacterium heparinum* degrade ChS by an elimination mechanism rather than by hydrolysis (4, 12, 13). Yamagata et al. (22) purified enzymes from the cells of *P. vulgaris* NCTC 4636 and *F. heparinum* ATCC 13125. They indicated that *P. vulgaris* produces only one kind of ChSase, which attacks ChS-A, ChS-B, and ChS-C. *F.*

heparinum repeatedly produces two kinds of ChSase; one attacks ChS-A, ChS-B, and ChS-C, and the other attacks ChS-A and ChS-C but not ChS-B. The enzyme of *P. vulgaris* and one enzyme of *F. heparinum* was designated ChSase-ABC, and the other enzyme of *F. heparinum* was designated ChSase-AC. Saito et al. (18) also utilized ChSase-ABC and ChSase-AC for the analysis of ChS isomers. Recently, Makarem and Berk (11) and Smith and Willett (21) reported that *Proteus mirabilis* and *Corynebacterium acnes* produce ChSase, but the range of isomers degraded was not determined.

Previous investigations of ChSase-producing bacteria have dealt almost exclusively with stock laboratory strains, and little is known about the distribution of such bacteria in natural habitats. The purpose of this study was therefore to search for ChSase-producing bacteria in various environments and to isolate strains having high ChSase productivity.

MATERIALS AND METHODS

Sources of ChSase-producing bacteria. Three hundred and ninety-four specimens, collected during 1971, 1972, and 1973 in the Kyushu and Hokkaido areas of Japan, were examined. Those of marine origin included: 24 from coastal sea water, 33 from bottom

mud of the coastal sea (depth, 1 to 18 m), 61 from marine algae (green, brown, and red algae), 18 from marine plankton (mixture of zoo- and phytoplankton collected in coastal seas), and 36 each from gills, stomach, and intestinal· contents of marine fish (108 specimens in all). Those from land and freshwater origin included: 47 soil specimens, 28 from feces of land animals bred in a zoo (carnivorous and graminivorous beasts, fowls, etc.), 32 from river water, and 16 from gills, 11 from stomach contents, and 16 from intestinal contents of freshwater fish.

Method of screening for ChSase-producing bacteria. The specimens were assayed for ChSase-producing bacteria by methods i, ii, and iii as described below. The incubation temperature was 25 C. Before the assay, each solid specimen was blended with a small volume of either sterilized sea water (for specimens of marine origin) or physiological saline solution (for those of land and freshwater origin). (i) A small quantity of the specimen was streaked on the surface of agar medium in a petri dish or mixed with melted agar medium at about 50 C. The seeded medium was incubated aerobically for 3 days. The basal medium used for assaying marine specimens comprised 0.5% peptone, 0.1% yeast extract, and 0.1% ChS-A in sea water. The basal medium for assaying land and freshwater specimens was nutrient broth containing 0.1% ChS-A. These media were solidified with 1.5% agar where necessary. Inocula from 10 to 20 colonies originating from each specimen were transferred aseptically to 2 ml of the appropriate liquid medium and incubated aerobically for 3 days. A volume of 0.5 ml of each culture grown was mixed with 2 ml of acid albumin solution (12) (3.26 g of sodium acetate, 4.56 ml of glacial acetic acid, and 1.0 g of bovine albumin fraction V dissolved in 1 liter of water, pH adjusted to 3.72 to 3.78 with 1:1 concentration of HCl). Failure of mixtures to develop white turbidity due to the combination of ChS and albumin identified the cultures as ChSase producers. Other cultures were discarded. The purity of ChSase-producing cultures was then assured by repeated single-colony isolations. Finally, isolated ChSase-producing bacteria were grown on agar slants, stored in a refrigerator, and transferred monthly in homologous agar media to maintain viability. (ii) Each specimen was streaked on or mixed with basal agar medium supplemented with 0.1% sodium formaldehydesulfoxylate and incubated anaerobically in an N_2 atmosphere for 3 to 7 days. (iii) Specimens from which no ChSase-producing bacterium was obtained by either method were subjected to further examination. The basal medium (pH 7.0) employed was comprised of 0.1% K_2HPO_4, 0.05% $MgSO_4·7H_2O$, 3.0% NaCl for marine specimens or 0.05% for land and freshwater specimens, 0.01% $CaCl_2·2H_2O$, 0.0001% $FePO_4$, 0.1% NH_4Cl, and 0.1% ChS-A as a sole source of carbon. Either 1 g or 1 ml of each specimen was mixed with 5 ml of the liquid medium and incubated aerobically for 4 days. A small quantity of the culture was then streaked on the agar medium and incubated for 3 days. Inocula from 10 colonies originating from each specimen were transferred separately to homologous liquid media. After incubation for 3 days, 0.5 ml of

each culture was tested with 2 ml of the acid albumin solution. Material from ChSase-producing cultures was streaked several times for single-colony isolation to insure pure cultures. Then, isolated ChSase-producing bacteria were transferred to the agar media used in method (i) and stored in a refrigerator.

Identification methods employed. Identification of ChSase-producing bacteria was made according to the criteria described in *Bergey's Manual of Determinative Bacteriology* (2), except that assignment of isolates to the genera *Aeromonas* and *Vibrio* was based on the criteria proposed by Bain and Shewan (1). The following methods were used. The media consisted of 0.5% peptone, 0.1% yeast extract, and sea water for marine bacteria, and nutrient broth for land and freshwater bacteria. The incubation temperature was 25 C. Motility, morphology, and gram-staining characteristics were determined by light microscopy. Arrangement of flagella was investigated by transmission electron microscopy. The oxidase test of Kovacs was used (8), and attack on glucose (OF test) was investigated by the method of Hugh and Leifson (6). Chitin digestion was measured by development of the clear zone around the colony on chitin agar (20), and sensitivity to vibriostatic agent 0/129 was measured by the method of Shewan et al. (19). Other usual identification methods were employed as required.

Assay of chondroitinase. (i) The conversion of ChS-C to Morgan-Elson reactive N-acetylhexosamine-containing oligomers was measured. A mixture of 0.25 ml of suitably diluted enzyme solution and 0.25 ml of substrate solution [2 mg of ChS-C per ml in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 7.2] was incubated at 37 C for 30 min and heated at 100 C for 1.5 min to stop the reaction. The N-acetylhexosamine end group produced in the mixture was analyzed by the method of Reissig et al. (17). One unit of enzyme was defined as the quantity that catalyzed the release of 1 μmol of product (as Δ4,5-glucuronosyl-N-acetylgalactosamine-6-sulfate) per min under the assay conditions. Specific activity is reported as units per milligram of enzyme protein. (ii) Unsaturated uronides with a marked absorption in the ultraviolet region (9) were measured. A mixture of 0.25 ml of enzyme solution and 0.25 ml of ChS-A solution (2 mg of ChS-A per ml in 0.1 M Tris-hydrochloride buffer, pH 7.2) was incubated at 37 C for 10 min. The reaction was stopped by adding 4.0 ml of 0.033 M HCl solution to the mixture, and the absorbance was measured at 235 nm. The amount of unsaturated uronides was calculated from the response curve previously obtained by using Δ4,5-glucuronosyl-N-acetylgalactosamine-4-sulfate as standard. In the case of a fairly crude enzyme solution, 3% perchloric acid was used instead of 0.033 M HCl. The absorbancy was measured in the supernatant solution obtained by centrifugation of the mixture. One unit of enzyme was defined as the quantity that catalyzed the release of 1 μmol of the unsaturated disaccharide per minute under the assay conditions. This method was also employed to assay for the occurrence of ChSase-ABC by using ChS-B as a substrate.

Comparison of ChSase productivity of isolated

bacteria. Cells taken from agar slant cultures of each isolate were transferred to 20-ml lots of sterile alternative medium in 50-ml, cotton-plugged flasks which were then incubated at 25 C for 3 days. Each culture was centrifuged, and the harvested cells were washed with 0.02 M Tris-hydrochloride buffer, pH 7.2, suspended again in 5 ml of fresh buffer, and disrupted by sonication. The cell debris was then centrifuged off, and the supernatant obtained was assayed for intracellular ChSase activity. After dialysis against several changes of buffer, the culture supernatant was tested for extracellular ChSase activity. Analytical method (i) was used for these assays. Finally, the intracellular and extracellular ChSase activity in the 20-ml cultures of all isolates was calculated.

Purification of ChSase-AC. Inocula from the agar slant culture of *Aeromonas* sp. 83 were transferred to two 20-ml lots of sterilized nutrient broth containing 0.1% ChS-A in 50-ml, cotton-plugged flasks, and the flasks were then incubated at 25 C for 2 days. Each culture was transferred to 2 liters of homologous medium in 3-liter, cotton-plugged flasks and incubated at the same temperature for 4 days. The cultures were combined and centrifuged at 17,000 × g for 30 min. The 3,905-ml volume of supernatant obtained was subjected to the following purification procedures. All the operations were conducted between 0 and 5 C, and centrifugations were at 17,000 × g for 30 min. For ammonium sulfate precipitation, solid ammonium sulfate was added to the supernatant up to a final concentration of 75% saturation. The mixture was kept overnight and centrifuged. The precipitate obtained was dissolved in about 50 ml of 0.02 M Tris-hydrochloride buffer, pH 7.2, and the solution was poured into a cellulose tube and dialyzed overnight against several changes of homologous buffer. For chromatography on phospho-cellulose, the dialyzed solution was applied to a column of phospho-cellulose (1.5 by 20 cm) equilibrated with 0.02 M Tris-hydrochloride buffer, pH 7.2, and the column was washed with homologous buffer. ChSase-AC passed through the column without adsorption. A washing volume of 75 ml contained the active fraction. For chromatography on diethylaminoethyl (DEAE)-cellulose, a 25-ml portion of the active fraction was applied to a DEAE-cellulose column (1.0 by 10 cm) equilibrated with 0.02 M Tris-hydrochloride buffer, pH 7.2, and the column was washed with homologous buffer. Then, ChSase adsorbed on the column was eluted with a continuous gradient of NaCl in buffer. The active fractions (see Fig. 2, tube no. 38-46) were pooled. For gel filtration, the pooled solution was applied to a Sephadex G-200 column (1.5 by 102 cm) after concentration to about 2 ml by dialysis against polyethylene glycol solution, and the column was washed with 0.02 M Tris-hydrochloride buffer containing 0.1 M NaCl, pH 7.2. The active fractions (see Fig. 3, tube no. 27-39) were collected and kept in a refrigerator. The portion remaining after phospho-cellulose chromatography was subjected to DEAE-cellulose chromatography and Sephadex filtration as well, and the active fractions were collected and combined with the above fractions. The combined enzyme solution was concentrated to about 5 ml

by dialysis against polyethylene glycol solution and stored in a refrigerator until used.

Assay for β-glucuronidase activity. A mixture of 0.25 ml of enzyme solution and 0.25 ml of Δ4,5-glucuronosyl-*N*-acetylgalactosamine solution (1 mg/ml in 0.1 M Tris-hydrochloride buffer, pH 7.2) was incubated at 37 C for 1 h. After the addition of 4.0 ml of 0.033 M HCl, the decrease in absorbancy at 235 nm was determined as an indication of β-glucuronidase activity.

Protein content. Protein content of the enzyme preparations was determined by the method of Lowry et al. (10).

Disc electrophoresis. To examine the enzyme preparations for homogeneity, samples were subjected to acrylamide gel electrophoresis by the method of Davis (3).

Chemicals. Dry media and materials for the preparation of various media were obtained commercially from Difco Laboratories (Detroit, Mich.), Eiken Chemical Co. (Japan), and Daigo Eiyokagaku Co. (Japan). ChS-A (sodium salt) from nasal cartilage of

TABLE 1. *Genera of ChSase-producing bacteria isolated from natural habitats*

Genera	Screening method[a]			Main sources of isolates
	i	ii	iii	
Aeromonas	15[b]	3	0	Stomach, intestine and gills of freshwater fish Marine plankton Feces of land animals
Vibrio	114	14	0	Stomach, intestine and gills of marine fish Marine plankton Intestine of freshwater fish
Flavobacterium	17	0	0	Marine mud Marine plankton Stomach of marine fish
Beneckea	27	10	10	Marine mud Marine plankton Coastal sea and river water
Proteus	8	0	0	Feces of land animals Stomach, intestine and gills of marine fish
Micrococcus	2	0	0	Intestine of freshwater fish
Arthrobacter	0	0	4	Land soil
Unknown	19	2	8	River water Land soil Marine algae

[a] See text.
[b] Number of isolates.

the whale was obtained from Usizu Seiyaku Co. (Japan), and sodium salts of ChS-B, ChS-C and heparin, phospho-cellulose, DEAE-cellulose, and the disaccharides used in the assay of ChSase and β-glucuronidase were obtained from Seikagaku Kogyo Co. (Japan). Hyaluronic acid (sodium salt) was purchased from Sigma Chemical Co. (St. Louis, Mo.), and Sephadex was obtained from Pharmacia (Sweden). Keratosulfate (calcium salt) was prepared from bovine cornea by the procedure of Meyer et al. (14). ChS-A was chemically desulfated by the procedure of Kantor and Schubert (7). Chemicals other than those listed were obtained from Sigma Chemical Co., Wako Chemical Co. (Japan), and other commercial sources.

RESULTS

Screening of ChSase-producing bacteria. From 289 specimens examined by method (i), 202 ChSase-producing isolates were obtained. From 89 specimens examined by method (ii), 29 ChSase-producing isolates were obtained. All isolates obtained by method (ii) were facultative anaerobes; no obligate anaerobe was isolated. From river water and soil, ChSase-producing bacteria were isolated only by method (iii), by which ChSase-producing bacteria in specimens would be selectively grown prior to the screening procedure. In total, 6,080 isolates from 394 specimens were subjected to screening, and 253 ChSase-producing isolates were selected by the three methods.

Identification of isolates. Genera assignments, numbers of isolates, and their main sources are summarized in Table 1. In total, 224 of 253 isolates were assigned to seven genera, but the genus of 29 isolates was unknown. Assignments were as follows. For *Aeromonas* spp., all 18 isolates belonged to Bain and Shewan's group, i.e., bacteria which produce gas from glucose and grow at 37 C (1). For *Vibrio* spp., among 128 isolates, 31 belonged to Bain and Shewan's group, luminous bacteria; 26 belonged to the group of arginine-negative bacteria unable to tolerate 7.5% NaCl; two belonged to the group of arginine-negative bacteria able to tolerate 7.5% NaCl; and 69 belonged to the group of arginine-positive bacteria (1). For *Flavobacterium* spp., among 17 isolates, nine were assigned to *F. balustinum*, five to *F.*

TABLE 2. *ChSase productivity of a number of isolates*

Strain no.	Bacteria	ChSase activity (units/20 ml)		Sources
		Intra-cellular	Extra-cellular	
118	*Proteus vulgaris*	2.83[a]	0.02	Gills of flat fish *Limanda herzensteini*
127	*P. vulgaris*	2.38[a]	0.01	Intestine of mackerel *Scomber japonicus*
129	*P. vulgaris*	2.06[a]	0.02	Stomach of mackerel *Scomber japonicus*
126	*P. vulgaris*	1.57[a]	0.01	Intestine of mackerel *Scomber japonicus*
105	*Flavobacterium balustinum*	1.15[b]	0.13	Marine mud
145	*P. vulgaris*	0.88[a]	0.02	Feces of raccoon dog *Nyctereutes procyonoides*
83	*Aeromonas* sp.	0.75[b]	16.68[b]	Intestine of carp *Cyprinus carpio*
137	*Flavobacterium diffusum*	0.39[b]	0.01	Marine mud
139	*Beneckea labra*	0.27[b]	0.01	Marine mud
325	*Beneckea chitinovora*	·0.24[b]	0.17	Green alga *Codium fragile*
83	*Aeromonas* sp.	0.75[b]	16.68[b]	Intestine of carp *Cyprinus carpio*
326	*Aeromonas* sp.	0.11	14.70[b]	Feces of coatimundi *Nasua narica*
123	*Aeromonas* sp.	0.13	6.61[b]	Intestine of carp *Cyprinus carpio*
302	*Aeromonas* sp.	0.06	5.91[b]	Intestine of carp *Cyprinus carpio*
144	*Aeromonas* sp.	0.05	5.40[b]	Feces of raccoon dog *Nyctereutes procyonoides*
84	*Aeromonas* sp.	0.02	4.29[b]	Intestine of carp *Cyprinus carpio*
303	*Aeromonas* sp.	0.06	3.85[b]	Intestine of carp *Cyprinus carpio*
125	*Micrococcus varians*	0.10	2.94[b]	Intestine of eel *Anguilla japonica*
14	*Vibrio* sp.	0.05	2.58[b]	Intestine of mackerel *Scomber japonicus*
6	*Vibrio* sp.	0.01	2.52[b]	Intestine of mackerel *Scomber japonicus*
	Flavobacterium heparinum	0.52[a]	0.06	ATCC 13125
	Proteus vulgaris	1.22[a]	0.02	NCTC 4636

[a] Enzyme capable of degrading ChS-B (ChSase-ABC or mixture of ChSase-ABC and ChSase-AC).
[b] Enzyme not capable of degrading ChS-B (ChSase-AC).

TABLE 3. *Morphological, cultural, and biochemical characteristics of no. 83 strain of Aeromonas sp.*

Morphology

Trait	Type
Form	Rod with rounded ends
Size	0.5 to 0.6 by 1.3 to 2.2 μm
Motility	Motile
Flagella	Single polar flagellum
Spores	Non-forming
Gram stain	Negative

Culture

Method	Appearance
Agar colony	Grayish white, flat, entire
Agar slant	Grayish white, filiform
Gelatin stab	Crateriform liquefaction
Broth	Pellicle, turbid, sediment
Litmus milk	Acid, coagulation, peptonization
Potato	Brownish yellow to brownish red

Biochemistry

Test for:	Reaction
Indole production	+
Nitrate production	+
Ammonia production (from peptone)	-
Methyl red reaction	-
Voges-Proskauer reaction	+
Citrate utilization	+
Simons' method	+
Christiansen's method	+
H$_2$S production	F
Hugh-Leifson (glucose)	+
Kovacs' oxidase	+
Cytochrome oxidase	+
Arginine	-
Lysine decarboxylase	-
Alginate utilization	-
Agar digestion	-
Urease	+
Catalase	+
Starch hydrolysis	+
Malonate utilization	-
D-Tartarate utilization	-
Cellulose hydrolysis	-
Blood hemolysis	-
Growth on SS agar	+
KCN resistance	+
Luminosity	-

Fermentation

Carbohydrate	Acid	Gas
Adonitol	-	-
Arabinose	+	+
Cellobiose	-	-
Dextrin	+	+
Dulcitol	-	-
Fructose	+	+
Galactose	+	+
Glucose	+	+
Glycerin	+	+
Inulin	-	-
Inositol	-	-
Lactose	+	+
Maltose	+	+
Mannitol	+	+
Mannose	+	+
Raffinose	-	-
Rhamnose	-	-
Saccharose	+	+
Salicin	-	+
Sorbitol	-	-
Trehalose	+	+
Xylose	-	-

Sensitivity to: Antimicrobial agent

Antimicrobial agent	Reaction
Vibrostatic agent (saturated)	-
Novobiocin (2 μg)	-
Penicillin (2 U)	-
Chloramphenicol (5 μg)	+
Dihydrostreptomycin (2 μg)	+
Erythromycin (10 μg)	-
Tetracyclin (5 μg)	+
Kanamycin (5 μg)	+
Sulfisoxazole (50 μg)	+
Colistin (50 U)	+

lutescens, one to *F. dormitator*, one to *F. diffusum*, and one was not assigned a specific epithet (2). For *Beneckea* spp., 19 of 47 isolates were identified as *B. labra*, 24 as *B. chitinovora*, and four as *B. hyperoptica* (2). For *Proteus* spp., all eight isolates were identified as *P. vulgaris* (2). For *Micrococcus* spp., both isolates were assigned to *M. varians* (2). For *Arthrobacter* spp., two of four isolates were identified as *A. ureafaciens*, and two were not assigned specific epithets (2). Of unknown species, 23 of 29 additional isolates were not identified (1, 2), and six died before identification could be completed.

Bacteria with high ChSase productivity. The range of productivity of either intracellular or extracellular ChSase for 20 isolates and an indication of their ability for ChS-B degradation are shown in Table 2. These highly productive isolates were obtained by methods (i) and (ii) but not (iii). For comparison, enzymatic activity of established ChSase-producing stock strains, *P. vulgaris* NCTC 4636 and *F. heparinum* ATCC 13125, was determined. The culture fluid of *Aeromonas* sp. 83 yielded the highest ChSase-AC activity, whereas none of the isolates from the current study displayed significant ChSase-ABC productivity.

Characteristics of *Aeromonas* sp. 83, isolated from the intestinal contents of a carp *Cyprinus carpio* by screening method (i), are shown in Table 3. Polar flagellation is revealed by electron microscopy (Fig. 1).

Purification of ChSase-AC from culture fluid of *Aeromonas* sp. 83. The representative results obtained from DEAE-cellulose chromatography and Sephadex filtration are shown in Fig. 2 and 3. The changes of unit, yield, specific activity, and degree of purification throughout the procedures are summarized in Table 4.

Barely detectable β-glucuronidase observed in the culture fluid was not discerned in the final enzyme preparation. Gel electrophoresis of the preparation gave a single band of protein.

Some properties of ChSase-AC from *Aeromonas* sp. 83. For study of the enzyme's substrate, ChS-A, ChS-C, and desulfated ChS were rapidly degraded to unsaturated uronide (Fig. 4). After prolonged incubation, the level of the final product determined as unsaturated disaccharide rose to nearly 100% of the total glucuronic acid in the substrates. Hyaluronic acid was degraded less rapidly, and ChS-B, keratosulfate, and heparin were not degraded by the enzyme. The effect of pH was seen when active degradation of the substrates was observed in reaction mixtures with pH values near 6.6 (Fig. 5). The effect of inorganic metal ions was observed when Mn^{2+}, Mg^{2+}, Ca^{2+}, and Ba^{2+} activated the enzyme (approximately 1.3- to 1.5-fold at 0.001 M), whereas Zn^{2+}, Fe^{2+}, and
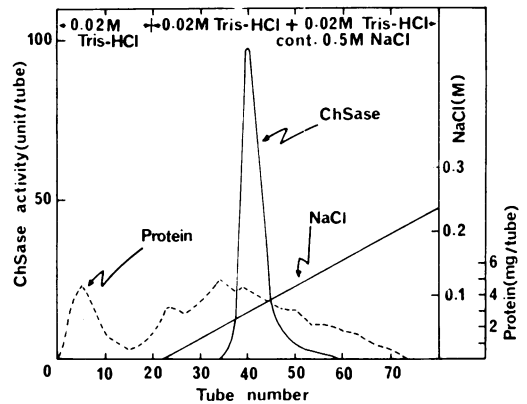

FIG. 2. *Chromatography of ChSase-AC from no. 83 strain of Aeromonas sp. on DEAE-cellulose. Fraction volume, 5.0 ml.*
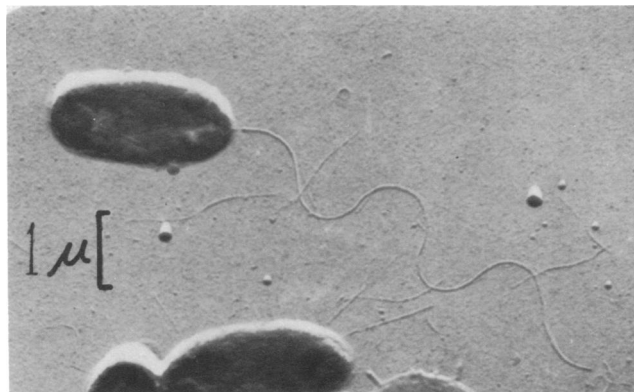

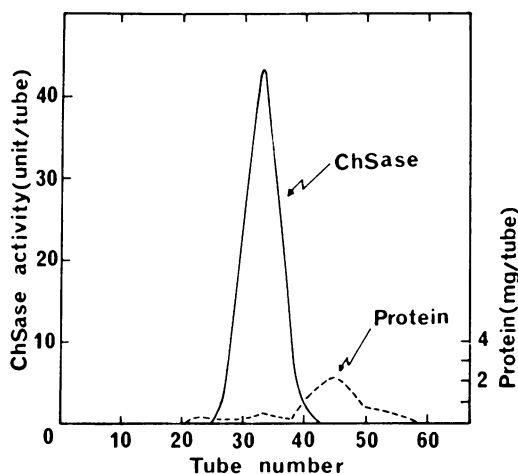FIG. 1. *Electron micrograph of no. 83 strain of Aeromonas sp. Cr shadowing.*

FIG. 3. *Gel filtration of ChSase-AC from no. 83 strain of Aeromonas sp. on Sephadex G-200. Fraction volume, 5.0 ml.*

TABLE 4. *Purification of ChSase-AC from culture fluid of Aeromonas sp. strain 83*

Step	Total units	Yield (%)	Sp act (μmol/ mg/ min)	Purification (#-fold)
Culture fluid	2,190	100.0	0.102	1.0
Ammonium sulfate, after dialysis	1,661	75.9	2.15	21.0
Phospho-cellulose column	1,624	74.2	3.20	31.2
DEAE-cellulose column	1,085	49.5	8.79	86.1
Sephadex G-200 column	934	42.7	99.7	977

Cu^{2+} inhibited the enzyme (half the inhibition being obtained at 0.005, 0.001, and 0.001 M, respectively). Other observations showed that the enzyme was stable in a pH region of 5.5 to 9.0 and at temperatures less than 45 C.

DISCUSSION

According to *Bergey's Manual* (2), the key to assignment of motile bacteria to the genus *Aeromonas* is their pathogenicity to fish and amphibians (2). *Aeromonas* sp. strain 83 was isolated from the intestinal contents of a fish, but its status as a pathogen was not determined. On morphological and biochemical bases, however, it is likely that the strain belongs to *A. liquefaciens*.

Among the bacteria capable of producing ChSase, *P. vulgaris* strains and *Aeromonas* spp. isolates were conspicuous (Table 2). The former produced the enzyme mainly within cells, whereas the latter released ChSase into the culture fluid. Further, it is of interest that the

enzyme of *P. vulgaris* degraded ChS-B, whereas the enzyme of the *Aeromonas* spp. did not. Therefore, it is natural to consider that the cells of *P. vulgaris* would be the preferred source of ChSase-ABC, and the culture fluids of *Aeromonas* spp. of ChSase-AC. Owing to its elevated ChSase productivity, use of the cells of *P. vulgaris* NCTC 4636 as the source of ChSase-ABC is quite rational. No more productive isolate was obtained from nature in the present survey. On the contrary, the cells of *F. heparinum* ATCC 13125 should be considered an unsuitable source of ChSase-AC inasmuch as their cell extracts yielded only a low ChSase-AC activity and were contaminated with ChSase-ABC (22). *Aeromonas* sp. strain 83 produced the greatest quantities of ChSase-AC observed. Properties of the enzyme from this strain are similar to those of ChSase-AC from *F. heparinum* ATCC 13125 previously reported (22). Therefore, *Aeromonas* sp. 83 should be used for production of the enzyme in place of *F. heparinum* ATCC 13125.
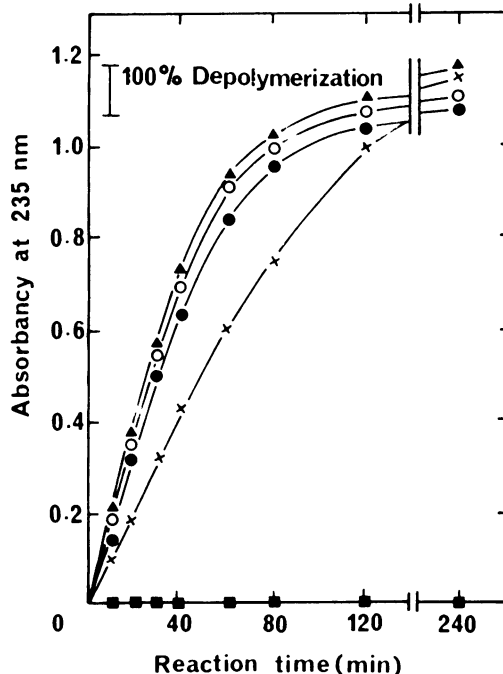

FIG. 4. *Rate and extent of degradation of various mucopolysaccharides with ChSase-AC from no. 83 strain of Aeromonas sp. The conditions of the experiments were those of assay method (ii), utilizing 0.016 U of enzyme and the following compounds as substrates:* O, *ChS-A;* ●, *ChS-C;* ▲, *desulfated ChS;* ×, *hyaluronic acid;* ■, *ChS-B, heparin, and keratosulfate.*
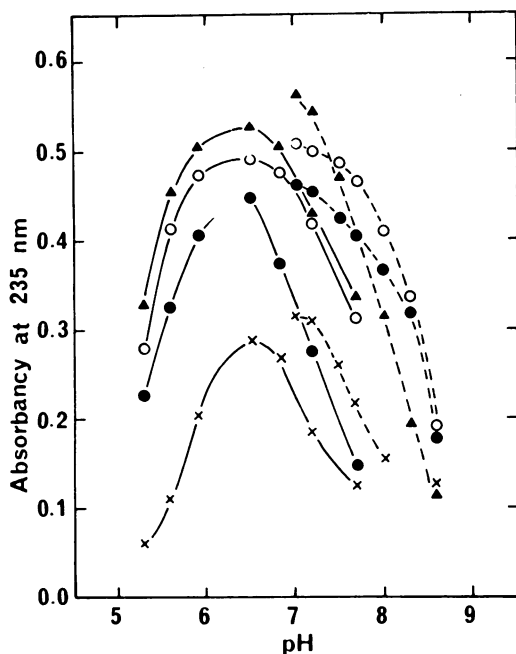
FIG. 5. *Effect of pH on the rate of degradation of various mucopolysaccharides with ChSase-AC from no. 83 strain of Aeromonas sp. The conditions of the experiments were those of assay method (ii) except Tris-hydrochloride buffer (pH 7.05 to 8.60) and potassium phosphate buffer (pH 5.29 to 7.73) were used at a concentration of 0.05 M in reaction mixture. Reaction mixtures contained 0.044 U of enzyme and the following substrates:* O, *ChS-A;* ●, *ChS-C;* ▲, *desulfated ChS;* +, *hyaluronic acid.*

LITERATURE CITED

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