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Isolation of Keratanase-Producing Bacteria from Natural Habitats

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A study was undertaken to isolate new strains of bacteria that produce keratan sulfate-hydrolyzing endoglycosidase (keratanase) at reasonable high rate in usual culture medium. Firstly, screening was made on all the bacteria capable of growing on the synthetic medium containing keratan sulfate as the sole carbon source, and 658 isolates were thus obtained from marine, freshwater and land environments in Japan. These isolates were cultured in keratan sulfate-containing peptone medium, and the cultures obtained were assayed for keratanase activity. Out of 658 isolates, only 22 isolates were found to produce appreciable amounts of keratanase in the presence of keratan sulfate. The chosen bacteria were transferred to the peptone medium without any supply of keratan sulfate, and the cultures obtained were then assayed for keratanase activity. It was found that in the absence of keratan sulfate some bacteria produced more whereas others produced less keratanase. Strain no. F-179 from *Flavobacterium* sp. possessed the highest productivity of keratanase among all the isolates. Finally, the keratanase from strain no. F-179 was purified, and its enzymatic properties were examined. It was concluded that this organism may be a good source of keratanase.

INTRODUCTION

The acidic glycosaminoglycan, keratan sulfate, is widely distributed in animal connective tissues. The main structure is a linear molecule made up of the repeating unit (1→3)-β-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-6-O-sulfate. Keratan sulfates from different sources show heterogeneity in their structures. The variations occur in the chain length, the degree of sulfation, the content of minor sugars (fucose, sialic acid, mannose, and N-acetylgalactosamine), and the oligosaccharide structure in the core region of the keratan sulfate molecule (Muir, 1973).

There have been reports on the occurrence of keratan sulfate-hydrolyzing endoglycosidase, keratanase, in several bacteria : *Coccobacillus chase* (Rosen *et al.*, 1960; Hirano and Meyer, 1971), *Escherichia freundii* (Kitamikado *et al.*, 1970a, b), and *Pseudomonas* sp. (Nakazawa *et al.*, 1975a, b). The keratanase from *E. freundii* was first reported by our laboratory, and found to be an endo-β-galactosidase after exhaustive purification (Kitamikado and Ueno, 1970, 1972). The enzyme has been demonstrated to be a useful tool in the structural analysis of keratan sulfate as well as many similarly related sugar sequences in glycoproteins and glycolipids (Ueno and Kitamikado, 1972; Tsay

et al., 1975 ; Fukuda *et al.*, 1975, 1976, 1978).

However, there are some problems in using the existing bacteria as sources for producing keratanase. The production of keratanase by *E. freundii* is not consistent. As slant culture this organism can lose the ability to produce keratanase during storage in a few days regardless of the presence or absence of keratan sulfate. The liquid culture containing keratan sulfate sustains the productivity for a somewhat longer time, but the irretrievable loss of enzyme productivity occasionally occurs during storage (Kitamikado *et al.*, 1970a). In addition, the keratanases from *E. freundii* and all other bacteria are induced enzymes, that is, these bacteria are capable of producing keratanase only in the presence of keratan sulfate. Furthermore, there is great difficulty in obtaining adequate amounts of keratan sulfate to supplement the culture medium (its concentration is 0. 2-l. 0 % in the culture medium).

The purpose of this study is to isolate new strains of bacteria that consistently produce large amounts of keratanase in usual culture medium without adding keratan sulfate.

MATERIALS AND METHODS

Keratan sulfate and other chemicals

Bovine cornea1 keratan sulfate (Ca-salt) and whale nasal cartilage keratan sulfate (Na-salt) were prepared and analyzed by the methods as described in the previous paper (Kitamikado *et al.*, 1970a). The following analytical values were obtained on a dry weight basis. The bovine cornea1 keratan sulfate contained hexose (as galactose), 35.9 %; hexosamine (as glucosamine), 32.7 %; sulfate, 17.5 %; sialic acid (as N-acetylneuraminic acid), 1.0 %; uronic acid (as glucuronic acid), 1.0 %; and total nitrogen, 2.7 %. The whale nasal cartilage keratan sulfate contained hexose, 28.2 %; hexosamine, 30.8 %; sulfate, 15. 0 %; sialic acid, 3. 3 %; uronic acid, null ; and protein (as Lowry protein), **11.0 %.**

Peptone and yeast extract used for the preparation of media B and C were obtained from Daigo Eiyokagaku Co. (commercial name of peptone, Polypeptone). Dry media and other chemicals were purchased from Difco Laboratories (U.S.A.), Eiken Chemical Co., Sigma Chemical Co. (U.S.A.), Wako Chemical Co. and other commercial sources.

Sources of keratanase-producing bacteria

Five hundred samples, collected from 1976 through 1977 in the Kyushu and Hokkaido areas in Japan, were examined. The samples of marine origin included 24 from coastal sea water, 38 from bottom mud of coastal sea (depth 0–20 m), 50 from seaweeds (green, brown and red weed), 8 from marine planktons (mixture of zoo- and phytoplankton), 38 from gills, 39 from intestinal contents, 3 from scales of marine fish, and 19 from the contents in the alimentary canal of marine invertebrates. Those from land and freshwater origin included 157 from land soil, 53 from river bottom mud, 15 from river

water, 15 from gills, 14 from intestinal contents, 3 from scales of freshwater fish, 6 from water-weed, 5 from air dust, and 13 from feces of land animal bred in a zoo.

Preparation of culture media

Bacteria were cultivated in the following media. Medium A (synthetic medium containing keratan sulfate as the sole carbon source) contained 0.5 % whale cartilage keratan sulfate, 0.05 % NH_4Cl, and 0.05 % K_2HPO_4. Medium B (peptone medium containing keratan sulfate) contained 0.2 % whale cartilage keratan sulfate, 1.0 % peptone, 0.1 % yeast extract, and 0.2 % NaCl. Medium C (peptone medium without keratan sulfate) contained 1.0 % peptone, 0.1 % yeast extract, and 0.2 % NaCl. These media *were* adjusted to pH 7.0 for the bacteria originated from freshwater and land environments. For the bacteria from marine environment the media were adjusted to pH 7.5 and supplemented with 3.0% NaCl. These media were solidified with 1.8 % agar and used as slant medium in a test tube or as plate medium in a petri dish where necessary.

Isolation of keratanase-producing bacteria

Before the isolation, each sample was mixed with a small volume of either sterilized sea water (for samples of marine origin) or physiological saline solution (for those of freshwater and land origin). A small quantity of each sample was initially streaked on the surface of agar plate medium A, and the seeded medium was incubated aerobically at 25°C. After incubation for 3-7 days, the well-grown and apparently different colonies were selected. Usually 1-3 colonies were chosen from each sample, and transferred aseptically onto agar slant medium B, and then incubated for several days. Cells from the agar slant cultures were transferred into 20-ml lots of sterilized liquid medium B in 50-ml flasks with cotton wool plugs. After incubation at 25°C for 4 days, each culture was centrifuged, and the supernatant solution obtained was dialyzed against deionized water overnight. The extracellular keratanase in the supernatant was then assayed. The harvested cells were washed with physiological saline solution, resuspended in 10 ml of the same solution, and ruptured by sonication. The cell debris was then centrifuged off, and the supernatant solution obtained was assayed for intracellular keratanase. Then, the bacteria having appreciable keratanase-productivity were transferred to agar slant medium C. The cells from each cuture were cultivated in liquid medium C, and the extracellular and intracellular keratanases produced were assayed by the same procedure described above. Thus, the bacteria capable of producing keratanase in the absence of keratan sulfate were obtained. After the purity of bacteria was assured by repeated single colony isolations on agar medium C, they were stored in refrigerator as slant cultures, and transferred monthly in homologous agar media to maintain viability.

Determination of enzyme activity

For the assay of keratanase the reaction mixture consisted of 0.25 ml of enzyme solution and 0.25 ml of 0.1 M sodium acetate buffer, pH 5.8, containing

2.5 mg of whale cartilage keratan sulfate. After incubation for 30 min or 3 hr at 37°C, the reducing power produced was measured by the method of Park and Johnson (1949). For the assays of exo-p-galactosidase and exo-β-N-acetylglucosaminidase the corresponding *p*-nitrophenyl glucosides were used as substrates (Conchie et al., 1959). Keratan sulfatase was assayed with whale cartilage keratan sulfate according to the method for glycosulfatase assay (Lloyd, 1959). For the controls, enzyme and substrate solutions were incubated separately, and they were mixed after heating the enzyme solution in boiling water bath for 3 min. One enzyme unit is defined as the amount of enzyme required to release 1 pmol of reducing sugar as galactose (for keratanase) or 1 μmol of *p*-nitrophenol (for exoglycosidase) or 1 μmol of sulfate ion (for keratan sulfatase) per min.

Identification of bacteria

The procedure for identification was carried out according to Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1974). The medium used here was medium C or nutrient broth, which was solidified with 1.8 % agar where necessary. The incubation temperature was 25°C except for special cases. Motility, morphology, and gram-staining characteristics were determined by light microscopy. The arrangement of flagella was investigated by transmission electron microscopy. The oxidase test of Kovacs (1956) was used, and the utilization of glucose (O-F test) was examined by the method of Hugh and Leifson (1953). Chitin digestion was measured by the development of a clear zone around the colony on chitin agar medium, and sensitivity to vibriostatic agent 0/129 was measured by the method of Shewan *et al.* (1954). Other usual identification methods were employed as required.

Purification of keratanase from *Flavobacterium* sp.

The method for the induced keratanase of ***E. freundii*** (Kitamikado and Ueno, 1970) was employed with minor modifications. Step 1. Culture of bacterium. Inocula from the agar slant culture of strain no. F-179 were transferred into cotton-plugged 50-ml flasks each containing 10 ml of sterilized liquid medium C. The flasks were then incubated without shaking at 25°C for 2 days. Each culture was then transferred to two cotton-plugged 2-liter flasks each containing 900 ml of the same medium, and incubated stationarily at the same temperature for 5 days. The cultures were combined and centrifuged at 17,000×g for 30 min. The supernatant obtained, 1,600 ml, was subjected to the following purification procedures. All the remaining steps were conducted between 0 and 5°C, and centrifugations were carried out at 17,000 ×g for 30 min. Step 2. Ammonium sulfate precipitation. The supernatant fluid from step 1 was adjusted to 75% saturation with solid ammonium sulfate, and allowed to stand overnight. The precipitate was collected by centrifugation, dissolved in 140ml of deionized water, and dialyzed against deionized water. Step 3. CM-Sephadex chromatography. The enzyme solution was dialyzed against 0.05 M sodium acetate buffer, pH 6.0, and then applied to a column of CM-Sephadex C-50 (1.6×50 cm) equilibrated with the same buffer. Almost all of the enzyme was unadsorbed and passed through the column. Step 4. DEAE-Sephadex

chromatography. The enzyme solution was dialyzed against 5mM sodium phosphate buffer, pH 7.2, and applied to a column of DEAE-Sephadex A-50 (1.6×50 cm) equilibrated with the same buffer. The column was eluted with the same buffer and fractions of 10 ml were collected. The fractions containing keratanase activity were pooled. Step 5. Gel filtration through Sephadex G-100. The pooled solution was lyophilized, and dissolved in 15ml of deionized water. The solution was divided into three 5-ml aliquots according to the capacity of the column used. Each aliquot was applied to a column of Sephadex G-100 (1.5~80 cm) which had been equilibrated with 0.2 M NaCl solution, and eluted with the same solution. Fractions of 5 ml were collected, and the fractions with major activities were pooled, and dialyzed against deionized water.

Lyophilization of bacterial cells for storage

Bacterial cells taken from agar slant cultures were suspended in 5 % aqueous lactose solution, and they were lyophilized according to the usual bacteriological methods.

Reduction of oligosaccharides

Sodium borohydride, 10 mg, was added to 2. Oml of oligosaccharide solution. After standing at 4°C for 20 hr, the reaction was stopped by adding 0.8 ml of glacial acetic acid. The reaction mixture was evaporated with successive addition of methanol to remove methyl borate. The reduced oligosaccharides were dissolved in 2. Oml of deionized water, and subjected to chemical analysis.

RESULTS

Isolation of keratanase-producing bacteria

From 500 samples collected from marine, freshwater and land environments, a large number of isolates (658 strains) capable of growing on the synthetic medium (medium A) were obtained. However, only 22 isolates showed appreciable keratanase-productivity, and they were selected due to their ability to produce keratanase after growing in peptone medium containing keratan sulfate (medium B). Keratanase activity was detected either in the culture medium or in the cells. Then, the keratanase-productivity of the isolates was determined by cultivation in peptone medium without keratan sulfate (medium C). The results showed that 18 strains of the isolates were able to produce keratanase in the absence of keratan sulfate. Table 1 shows the range of keratanase-productivity in the 22 isolates cultivated in media B and C. For the purpose of comparison, *E. freundii* is also included in the Table, as it is an established keratanase-producing stock strain. It was found that four strains from land soil and air dust possessed high keratanase-productivity in medium C, and they released a large amount of keratanase into their culture fluid.

Table 1. Keratanase-productivity of isolated bacteria.

Strain No.	Keratanase-productivity (m units /20 ml culture) in Medium B		in Medium C		Conceivable genus	Source
	Extra-cellular	Intra-cellular	Extra-cellular	Intra-cellular		
F-179	**690**	169	352	70. 2	*Flavobacterium*	Land soil
F-167	580	*140*	270	26.8	*Flavobacterium*	Land soil
F-127	350	*124*	114	25.0	*Flavobacterium*	Land soil
F-197	216	*128*	62. 4	50.0	*Flavobacterium*	Air dust
s-103	38. 4	*0*	3.8	4. 6	*Vibrio*	Intestine of marine fish *Tracurus japonicus*
s- 37	32. 6	17. 2	7. 6	5. 6	*Vibrio*	Seaweed *Gelidium divaricatum*
F-186	32. 6	14. 4	0	9.8	*Micrococcus*	Land soil
s- 90	32. 6	0	0	0	*Vibrio*	Marine mud
F-169	30. 6	0	0	5. 8	*Micrococcus*	Air dust
S- 80	26. 8	13. 4	23. 4	0	*Vibrio*	Scales of marine fish *Ditrema temmincki*
s- 19	26. 8	0	15. 2	0	*Vibrio*	Sea weed *Gelidium divaricatum*
F-188	26. 8	0	5. 8	0	*Micrococcus*	Land soil
F-166	26.8	0	0	0	*Micrococcus*	Land soil
s -146	25. 0	7. 6	10. 4	0	*Vibrio*	Gills of marine fish *Pterogobius elepodes*
S -142	25. 0	0	7. 6	0	*Vibrio*	Gills of marine fish *Tracurus japonicus*
F-192	21.2	0	0	0	*Aeromonas*	River mud
s -139	19. 2	17. 2	13. 5	0	*Vibrio*	Intestine of marine fish *Sebasticus marmoratus*
F -267	9. 6	26. 8	11. 6	11.6	*Micrococcus*	Land soil
F-. 2	7. 6	25. 0	5. 8	9. 6	*Micrococcus*	Land soil
F-281	0	26. 8	9. 6	0	*Micrococcus*	Land soil
F -280	0	23.0	0	0	*Micrococcus*	Land soil
F- 8	0	21. 2	5. a	0	*Flavobacterium*	River mud
E. freundii	192	550	0	20. 8		Stock strain

F mark was used for the isolates from freshwater and land environments, and S mark was for the isolates from marine environment.

Identification of keratanase-producing isolates

Morphological and biochemical characteristics of the **22** isolates were examined. The genera assignments are shown in Table 1. The 22 isolates could be classified into four genera, *Flavobacterium*, *Micrococcus*, *Aeromonas* and *Vibrio*, and the four isolates having high keratanase-productivity belonged to

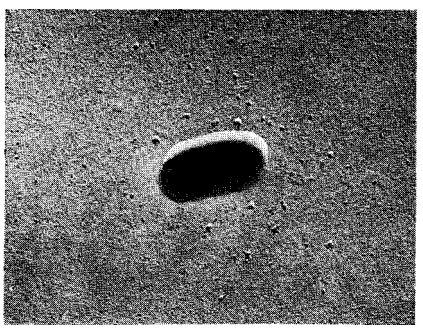

Fig. 1. Electron micrograph of strain no. F-179. Cr shadowing. ~12,000

Table 2. Morphological, cultural, and biochemical characteristics of strain no. F-179.

Morphology		Culture		Biochemistry			
						Fermentation.	
Trait	Type	Method	Appearance	Test for	Reaction	Carbohydrate	Reaction: Acid / Gas
Form	Rod with rounded ends'	Agar colony	Yellow, slightly viscid, entire, circular	Indole production	—	Glucose	+ / —
Size	0.5 to 0.8 by 1.3 to 2.6 μ	Agar slant	Yellow, spreading medium unchanged	Nitrate reduction	−	'Lactose	—
Motility	Non-motile			Methylred reaction	—	Sucrose	i +
Spores	Non-forming	Gelatin colony	Yellow, slow liquefaction	Voges-Proskauer reaction		Maltose	+
Gram strain	#Negative	Gelatin stab	Slow liquefaction	Citrate utilization			
		Broth	Turbid with no pellicle	H_2S production			
		/Litmus milk	Coagulation, reduced	Hugh-Leifson	0		
		Potato	/Luxuriant yellow colonies	Kovacs' oxidase			
		Optimum temperature	25-30°C	Urease	+		
		Growth for 37°C	+	Catalase	+		
		5% NaCl broth	—	Hydrolysis	Reaction		
				Gelatin	+		
				Casein	+		
				Starch	+		
				Agar	—		
				Cellulose	—		
				Chitin			

the genus *Flavobacterium*. Table 2 shows the detail characteristics of strain no. F-179 which possessed the highest keratanase-productivity in the absence of keratan sulfate, and its electron micrograph is shown in Fig. 1.

Factors affecting keratanase-productivity of strain no. F-179 from *Flavobacterium* sp.

The effects of medium composition and culture conditions on the keratanase-productivity of the organism were examined. From the results, it became clear that this strain secreted a large amount of keratanase into the culture fluid when cultivated stationary at 25°C for 2-6 days in the medium containing 1-2 % peptone, 0.1-O. 2 % yeast extract or 0.5 % meat extract and O-O. 5 % NaCl at pH 7.0-7.2. The source of peptone affected the keratanase-

productivity of the strain. Peptones obtained from casein or mixture of casein and animal meat such as Polypeptone (Daigo Eiyokagaku), Casitone (Difco) and Tryptone (Difco) were suitable for the enzyme production, but the use of peptones obtained from animal meat such as Proteose peptone (Difco) reduced the enzyme productivity to about one fourth. Addition of keratan sulfate into the culture medium increased the keratanase production (Table 1), but chondroitin 4-sulfate, chondroitin 6-sulfate and hyaluronic acid had no effect on enzyme production.

Keratanase-productivity of this strain is very consistent, and can be maintained for more than 20 months by cell lyophilization method or by successive culture on agar slant medium C.

Purification of keratanase from strain no. F-179 from *Flavobacterium* sp.

After the organism had grown in liquid medium C for 5 days at 25°C, the distribution of the enzymes involved in degrading keratan sulfate was examined in the culture fluid and cells. The result is shown in Table 3. The culture fluid contained a large amount of keratanase but little exoglycosidases. Therefore, the culture fluid was chosen as the starting material for the isolation of keratanase.

Table 3. Distribution of keratanase and related enzymes in the culture of strain no. F-179.

Fraction	Enzymes (units/l, 600 ml culture)			
	Keratanase	Exo-β-galactosidase	Exo-β-N-acetyl-glucosaminidase	keratan sulfates
Culture fluid	17.8	0.56	3.44	0
Cell extract	5.6	2.04	139	0

The recovery of protein and enzyme activity and the degree of purification throughout the procedures are summarized in Table 4. After completion of the procedures, the keratanase was purified 1,380-fold from culture fluid with 19.4 % of activity recovered. The purified enzyme preparation was free of exo-p-galactosidase, exo-p-N-acetylglucosaminidase and keratan sulfatase.

Table 4. Purification of keratanase from strain no. F-179

Purification step	Volume (ml)	Total activity (units)	Protein* (mg)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
1. Culture fluid	1,600	17.8	5168	0.0034	100	1
2. Ammonium sulfate	140	15.7	60.2	0.261	88.2	76.8
3. CM-Sephadex	170	14.8	22.1	0.670	83.1	8127
4. DEAE-Sephadex	380	10.5	3.80	2.76	59.0	13806
5. Sephadex G-100	60	3.46	0.737	4.69	19.4	

* Protein was determined by the method of Lowry *et al. (1951).*

The properties of keratanase from strain no. F-179 of *Flavobacterium* sp.

This enzyme showed fairly high activity toward cornea1 keratan sulfate and whale cartilage keratan sulfate, but it did not act on chondroitin 4-sul-

fate, chondroitin 6-sulfate, hyaluronic acid, dermatan sulfate, and heparine. The optimal pH for this enzyme was between 5.0 and 6.0, and the optimal temperature for activity was approximately 50°C. Cations, Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+}, had little effect on the enzyme activity when tested as chloride salts at 10^{-2} M concentration.

Identification of enzymatic digestion products

The degradation products of cornea1 and cartilage keratan sulfates with

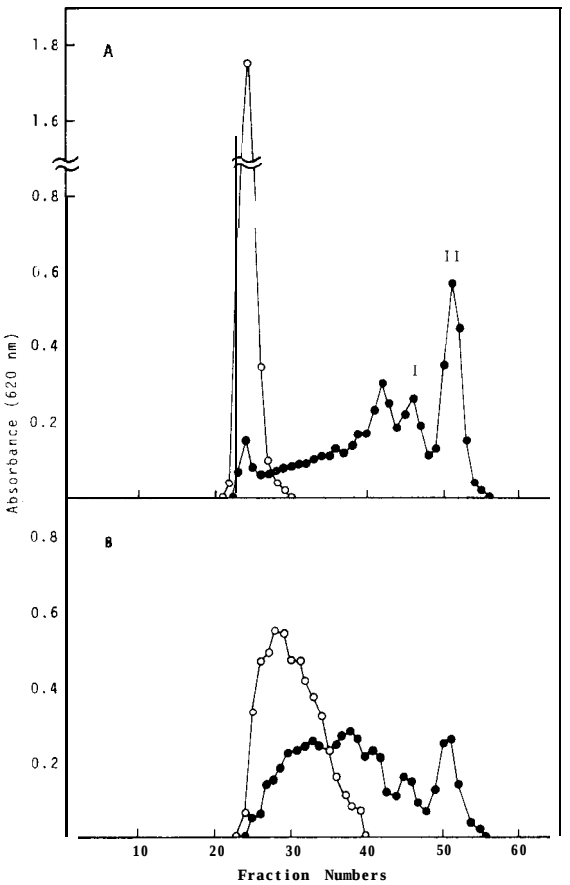

Fig. 2. Sephadex G-50 chornatograms of bovine corneal and whale nasal cartilngr keratan sulfates digested with keratanase from strain no. F-179. Keratan sulfate (15 mg), dissolved in 1. 0 ml of 0.1M sodium acetate buffer, pH 5.8, was incubated for 3 hr at 37°C with the purified enzyme (0.06 unit). Each aliquot of 1.0 ml of the reaction mixtures with (●——●) and without (○—○) enzyme was then applied to the column (1. 2×85 cm) of Sephadex G-50 equilibrated with 0.2 M NaCl solution and eluted with the same solution. Fractions of 2. 0 ml were collected and assayed for hexose by anthrone method. A. Bovine cornea1 keratan sulfate; B. Whale nasal cartilage keratan sulfatr

Table 5. Analysis of enzymatic digestion products of cornea1 keratan sulfate.

	Eefore reduction			After reduction	
	Glucosamine	Galactose	Reducing power	Glucosamine	Galactose
			Molar ratio		
Product I* (tetrasaccharide)	1.00	0.92	0. 52	1. 00	0. 53
Product II* (disaccharide)	1. 00	1.01	1.14	1.00	0.09

* Products I and II are shown in Fig. 2.

the purified keratanase from strain no. F-179 were chromatographed on Sephadex *G-50* column. The elution profiles are shown in Fig. 2. The results clearly showed that this enzyme hydrolyzed the keratan sulfates endoglycosidically at random to release oligosaccharides of various molecular sizes. The characterization of the peak products I and II from Sephadex G-50 column is shown in Table 5. Product I appeared to be a tetrasaccharide. Product II was appearently a disaccharide of smaller molecular size. The sugar analysis on borohydride-reduced oligosaccharides revealed that about one half of the galactose in the tetrasaccharide and almost all of the galactose in the disaccharide were destroyed, while glucosamine remained intact. These results demonstrated that the reducing terminal residue of both oligosaccharides was galactose, and the keratanase hydrolyzed the galactosidic bonds in keratan sulfate molecule, acting as an endo-β-galactosidase.

DISCUSSION

Since the large number of bacteria capable of growing on the synthetic, medium (Medium A) come from various sources, keratan sulfate-utilizing bacteria probably distribute widely in natural habitats. Yet the number of bacteria so far examined to have appreciable keratanase activity was few. In the present work it is interesting to find that the bacteria having high keratanase-productivity belong to the genus *Flavobacterium*, as the typical source for obtaining glycosaminoglycanases other than keratanase is also a bacterial strain of the same genus *(Flavobacterium heparinum)* (Linker, 1966).

Among the recognized bacteria, *Coccobacillus chase* (Hirano and Meyer, 1971) and *Pseudomonas* sp. (Nakazawa and Suzuki, 1975b) produced keratanase mainly in their cells. *E. freundii* produced the enzyme both in the cells and in the culture fluid (Kitamikado et *al.*, 1970a). Generally, there are some difficulties in purifying glycosaminoglycanases isolated from bacterial cells. In the case of *E. freundii* the intracellular keratanase was never separated from the contaminated exoglycosidases such as neuraminidase and exo-p-galactosidase, and thus the purified keratanase was obtained only from its culture fluid as reported in our previous paper. On the contrary, it is noteworthy that the four strains having high keratanase productivity secreted most of the enzyme into their culture fluid regardless of the presence or absence of keratan sulfate.

Especially strain no. F-179 showed the highest keratanase-productivity among all the isolates, and released large amount of keratanase into the culture fluid in the absence of keratan sulfate. On the other hand, there is little related exoglycosidases present in the culture fluid, which could interfere with the purification of keratanase. Thus, the keratanase was easily purified with the highest specific activity. Therefore, this strain is expected to be one of the profitable sources for producing keratanase.

The most profitable source for producing keratanase may be the microorganisms which produce constitutively the enzyme irrespective of medium compositions. However, the keratanase-productivity of strain no. F-179 from *Flavobacterium* sp. is dependent on the source of peptones used. It may be possible that the peptones from casein contain some substances which induce keratanase production on the organism, although the substances hardly induce keratanase production in *E. freundii.*

Strain no, F-179 was assigned to the genus *Flavobacterium,* but its morphological and biochemical characteristics were different from any of the 12 species of the genus *Flavobacterium* described in the Bergey's manual of determinative bacteriology (Buchanan and Gibbons, 1974). Moreover, the strain was totally different from *Flavobacterium heparinum* ATCC 13125, since clear differences were found such as the size and color of cells and motility. Therefore, we tentatively designate the strain *Flavobacterium keratolyticus* based on its high productivity of keratanase.

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