Blood Group Substance-degrading Enzymes Obtained from *Streptomyces* sp.

Part II. Purification and Characteristics of α -Galactosidase from *Streptomyces* 9917S₂

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The blood group B substance-degrading activity of *Streptomyces* 9917S₂ is induced by galactosides as α -galactosidase activity is. Purification of the α -galactosidase was attempted by chromatography on DEAE-Sephadex and Sephadex. The purified preparation was shown to be free from α - and β -glucosidases, β -galactosidase, α - and β -glucosaminidases, and α - and β -galactosaminidases activities. The blood group B substance-degrading activity was present only in this fraction. This enzyme preparation cleaves α -(1 $\rightarrow$ 3)- and α -(1 $\rightarrow$ 6)-galactosidic linkages. The activity is inhibited by D-galactose, melibiose, and raffinose and also by L-arabinose and D-xylose.

A number of highly specific protein-sugar binding reactions playing important biological functions has been known. Among them are agglutination reactions of red blood cells with their anticera. The reactions involve various problems of biological interest besides their originally ascribed significances. Human blood group A, B, and H substances are found not only in red blood cells but also in various mucous glands and the secreted mucus.^{1~4)} Substances with similar serological activities, such as bacterial antigens,^{5~7)} and human tumor glycolipids,^{8,9)} are known in other organisms.²⁾ Furthermore, hemagglutinins which are either specific or nonspecific for blood groups are present in various plant and animal tissues.^{10~12)} Recently, some of these hemagglutinins are found to agglutinate certain tumor cells^{13~16)} and to initiate mitosis in the culture of normal human leucocytes.¹⁷⁾

We have found that various kinds of hemagglutinins are produced in large quantities by bacteria and fungi¹⁸⁾ and that some of them strongly aggregate dissociated sea urchin

and sponge cells.¹⁹⁾ Studies on the structure of blood group substances capable of specifically binding hemagglutinins are required.

We have previously reported²⁰⁾ that culture fluids of certain species of *Streptomyces* decompose serological activities of blood group substances in human saliva and thus interrupt hemagglutination inhibitory action of saliva. In the present report, purification and some characteristics of blood group B substance-degrading enzyme produced by *Streptomyces* 9917S₂ are described.

MATERIALS AND METHODS

Strain used and the culture condition. *Streptomyces* 9917S₂ was used throughout the present work. A loopful of spores and mycelia was inoculated into 5 ml of 0.05 M phosphate buffer (pH 6.0) containing 2% lactose, 0.5% peptone (Kyokuto), 0.1% yeast extract (Difco), and 0.05% MgSO₄·7H₂O, and incubated for 48 hr at 25°C in a test tube with shaking. This was then added into 100 ml of the above medium in a 500-ml flask and culture continued for further 40 hr at 25°C with shaking.

Cells were then removed by filtration through a filter paper (Toyo Roshi, No. 2) and the filtrate was used to isolate α -galactosidase.

Assay of glycosidases and blood group B substance-degrading activity. Glycosidase activities were measured as described previously.²⁰⁾ B substance-degrading activity was quantitated by the hemagglutination inhibition test as described previously,²⁰⁾ though microtiter (Taiyo Bussan Co., Ltd., Tokyo) was used in some cases.

Paper chromatography. Galactose liberated from various substrates by the B substance-degrading enzyme was detected by paper chromatography. Samples were placed on a filter paper (Toyo Roshi No. 50). The chromatogram was developed with *n*-butanol: pyridine: water (6: 4: 3), and the location of sugars was detected by spraying an ammoniacal solution of silver nitrate.²¹⁾

Chemicals. DEAE-Sephadex A-50 and Sephadex G-100 were products of Pharmacia, Uppsala. Anti-B serum was obtained from Midori-Juji Co., Ltd., Osaka. Phenyl glycosides were purchased from Nakarai Chemicals, Ltd., Kyoto. Other chemicals

were of the purest grades commercially available.

RESULTS

1. Glycosidase activities in the culture filtrates

Effect of various sugars on the production of glycosidases was first examined. Table I shows that β -galactosidase and β -N-acetylglucosaminidase were formed in all cases. α -Galactosidase, on the other hand, was detected in lactose or D-galactose containing medium but not in D-glucose containing medium, suggesting that this enzyme is the blood group B substance decomposer.

2. Purification of α -galactosidase

Preliminary experiment showed that the amount of enzymes in the culture filtrate was too small to apply the salting-out with ammonium sulfate. Thus the culture filtrate was directly applied to DEAE-Sephadex and the purification of α -galactosidase was made as shown in Fig. 1.

TABLE I. EFFECT OF SUGAR ON THE PRODUCTION OF GLYCOSIDASE ACTIVITIES IN *Streptomyces* 9917S₂

Substrate	Activity in the medium containing ^{a)}				
	Lac	D-Gal	Mal	D-Fru	D-Glu
Phenyl- α -D-glucoside ^{b)}	—	—	30	—	—
" β - " " ^{b)}	—	—	—	—	—
" α -D-galactoside ^{b)}	170	120	70	—	—
" β - " " ^{b)}	1100	1500	200	170	220
" N-acetyl- α -D-glucosaminide ^{b)}	—	—	—	—	—
" " β - " " ^{b)}	300	235	265	245	220
" " α -D-galactosaminide ^{b)}	—	—	—	—	—
" " β - " " ^{b)}	—	—	—	—	—
B substance ^{c)}	> 64	64	8	8	1

^{a)} 2%, each.

^{b)} Absorbance at 660 m μ . One tenth ml of culture filtrate was mixed with 0.1 ml of 20 mM phenylglycoside solution in 0.1 M citrate buffer (pH 6.0) and incubated for 10 min at 35°C. At the end of incubation, 0.1 ml of 0.4 M trichloroacetic acid solution, 1.0 ml of 0.4 M Na₂CO₃ solution, and 0.1 ml of Folin reagent were added in order. After 30 min, 1.5 ml of H₂O was added to the mixture and the amount of the phenol liberated was measured colorimetrically at 660 m μ .

^{c)} Minimal amount of B substance for complete hemagglutination inhibition (1, when heated culture filtrate was used). One twentieth ml of culture filtrate was mixed with 0.05 ml of B substance solution and kept at 35°C. After 6 hr, the mixture was heated for 10 min at 100°C, and hemagglutination inhibition test was made.

FIG. 1. Diagram of Purification Procedures of the α -Galactosidase from *Streptomyces* 9917S₂.

β -Galactosidase was almost completely separated from other enzyme activities (pilot experiment, Fig. 2) in the first step (DEAE-Sephadex adsorption) of the purification under the conditions employed.

In practice, 1.5 liter of the culture filtrate was added into 120 ml (wet volume) of

DEAE-Sephadex A-50 equilibrated with 0.05 M phosphate buffer (pH 6.0), the mixture was stirred for 1 hr, and filtered under the reduced pressure. The filtrate was added into another 120 ml of DEAE-Sephadex A-50 and treated as above. Two batches of the DEAE-Sephadex A-50 which adsorbed enzymes were then combined and eluted with 400 ml of 0.05 M phosphate buffer (pH 6.0) containing 0.6 M NaCl.

The eluate was concentrated to about 20 ml through a "Dia Filter" G-20T (Nippon Shinku Gijutsu Co., Ltd., Chigasaki) with pressure (2 kg/cm² with argon gas) and then the concentrate was dialyzed against 0.05 M phosphate buffer (pH 6.0). The dialyzed sample was then adsorbed to DEAE-Sephadex A-50 equilibrated with 0.05 M phosphate buffer (pH 6.0) in a column (1.6×25 cm). The column was eluted with a linear gradient of sodium chloride (0~0.6 M) in the above phosphate buffer. Fractions (5 ml each) were collected. All the operations were carried out at 4°C.

Figure 3 shows the elution pattern of the dialyzed enzyme fraction through the DEAE-Sephadex A-50 column. β -Galactosidase activity was further removed, but α -galactosidase and β -N-acetyl-glucosaminidase were not separated from each other. Thus fractions 21~35 were combined, concentrated in a collodion bag to less than 1 ml. The concentrate was applied to a Sephadex G-100 column (2×48 cm) equilibrated with 0.05 M phosphate buffer. The column was eluted and fractions were

TABLE II. SUMMARY OF THE PURIFICATION OF α -GALACTOSIDASE FROM *Streptomyces* 9917S₂

Procedure	Protein ^{b)} (mg)	α -Galactosidase activity (U ^{a)})	Yield (U/mg)	Purification ratio
Culture filtrate	2210	51840	22.4	100
DEAE-Sephadex A-50 (1st)	738	24720	33.5	47.7
DEAE-Sephadex A-50 (2nd)	270	14040	52.0	27.1
Sephadex G-100 (1st)	43.2	7200	166.7	13.9
Sephadex G-100 (2nd)	15.6	5040	323.1	9.7

^{a)} 1 μ mole phenol/10 min.

^{b)} Determined by the method of Lowry and Folin.

FIG. 2. DEAE-Sephadex Chromatography of the Culture Filtrate of *Streptomyces* 9917S₂.

One liter of culture filtrate was concentrated to about 50 ml with "Dia-Filter" G-20T and the concentrate was applied to a 2×25 cm-column of DEAE-Sephadex A-50.

●—● Absorbance at 280 m μ . ○—○ α -Galactosidase activity.
 △—△ β -Galactosidase activity. ×—× β -N-Acetyl-glucosaminidase activity.

FIG. 3. The Second DEAE-Sephadex Chromatography.

●—● Absorbance at 280 m μ . ○—○ α -Galactosidase activity.
 △—△ β -Galactosidase activity. ×—× β -N-Acetyl glucosaminidase activity.

FIG. 4. The First Sephadex G-100 Chromatography of Purified α -Galactosidase.

●—● Absorbance at 280 m μ . ○—○ α -Galactosidase activity.
 △—△ β -Galactosidase activity. ×—× β -N-Acetyl-glucosaminidase activity.

FIG. 5. The Second Sephadex G-100 Chromatography of Purified α -Galactosidase.

●—● Absorbance at 280 m μ .
 ○—○ α -Galactosidase activity.

collected as above. As shown in Fig. 4, α -galactosidase and β -N-acetyl-glucosaminidase activities were completely separated by this procedure, and the blood group B substance-degrading activity was observed only in the α -galactosidase fraction. Re-chromatography of the α -galactosidase fraction (fractions 12~17) on the same Sephadex G-100 column resulted in a single symmetrical peak of α -

galactosidase activity (Fig. 5).

Table II summarizes the results. Specific activity of the α -galactosidase was increased 15-fold over the culture filtrate.

3. Properties of the purified α -galactosidase

Using the α -galactosidase preparation purified as above, properties of the enzyme were

FIG. 6. pH-Activity Curves of the Purified Enzyme.

○—○ B substance degradation (Heated enzyme, $n=0$).
 ●—● Phenyl- α -D-galactoside hydrolysis.

investigated, especially with regard to the blood group B substance-degrading activity. Assay procedures for glycosidase and B substance-degrading activities were as previously described²⁰⁾ except that 0.05 ml of the puri-

fied enzyme preparation (0.2 μ g protein), instead of 0.2 ml of the culture filtrate, was used in the present study. Under these conditions, the rate of hydrolysis was proportional to the amount of protein up to 0.4 μ g.

As shown in Fig. 6, the optimal pH was found to be 4.5~6.0 for both α -galactosidase and B substance degrading activities. The K_m

FIG. 7. Lineweaver-Burk Plot of the Purified Enzyme for Phenyl- α -galactoside. (pH 6.0, 35°C)

FIG. 8. Thermal Stability of the Purified Enzyme.

Enzyme preparations were heated for 10 min, cooled, and the activities remained were measured.

- B substance degradation (Heated enzyme, $n=0$).
- Phenyl- α -D-galactoside hydrolysis.

FIG. 9. Temperature-Activity Curves of the Purified Enzyme.

- B substance degradation, (Heated enzyme, $n=0$).
- Phenyl- α -D-galactoside hydrolysis.

TABLE III. EFFECT OF INHIBITORS ON THE ENZYME ACTIVITIES

Inhibitor	Concentration ($\times 10^{-3}$ M)	Activity	
		B Sub- stance de- gradation ^a	Phenyl- α -D- galactoside hydrolysis ^b
None	—	512	185
EDTA	10	512	190
Iodoacetate	1	256	110
<i>p</i> -CMB	0.1	2	60
NaCN	1	256	205
NaN ₃	1	256	190
NaF	3	512	195
α , α' -Dipyridyl	1	256	185
N-Ethylmaleimide	1	512	190

^a Minimal amount of B substance for complete hemagglutination inhibition (1, when heated enzyme was used).

^b Absorbance at 660 $m\mu$. See the footnote in Table I.

value of the enzyme for phenyl- α -galactoside was 1×10^{-3} M (Fig. 7).

The enzyme was unstable at temperatures higher than 45°C , and heating at 55°C for 10 min resulted in the complete loss of the activities (Fig. 8). In the presence of the substrate, however, the maximum α -galactosidase activity was found at $50\sim 55^\circ\text{C}$, while the highest B substance degrading activity was at $40\sim 50^\circ\text{C}$ (Fig. 9). The difference is probably due to the different incubation period in assay procedures (10 min *vs.* 8 hr. see Methods).

The activities of α -galactosidase and B substance degradation were inhibited with *p*-chloromercuribenzoate (*p*-CMB). Monoiodoacetate at the concentration of 1×10^{-3} M also inhibited the enzyme activities. (Table III). Among the metallic ions examined, Ag^+ and Hg^{2+} at the concentration 5×10^{-4} M inhibited the enzyme activities (Table IV). These results suggest that the activities are mediated by SH-enzyme.

TABLE IV. EFFECT OF METALLIC IONS ON THE ENZYME ACTIVITIES

Metal	Concentration ($\times 10^{-4}$ M)	Activity	
		B Substance degradation ^{a)}	Phenyl- α -D- galactoside hydrolysis ^{b)}
None	—	512	185
Ag^+	5	8	145
Ba^{2+}	5	512	190
Ca^{2+}	5	512	190
Co^{2+}	5	256	190
Cu^{2+}	5	512	200
Fe^{3+}	5	512	185
Hg^{2+}	5	8	30
Mg^{2+}	5	512	195
Mn^{2+}	5	512	240
Ni^{2+}	5	512	190
Pb^{2+}	5	512	200
Sn^{2+}	5	512	190
Zn^{2+}	5	512	190

^{a)} Minimal amount of B substance for complete hemagglutination inhibition (1, when heated enzyme was used).

^{b)} Absorbance at 660 m μ .

Eleven sugars and 2 aminosugars were tested for their effects on the enzyme reactions. Galactose and galactose-containing sugars such as melibiose, raffinose, and D-galactosa-

TABLE V. EFFECT OF SUGARS ON THE ENZYME ACTIVITIES

Sugar	Concentration ($\times 10^{-3}$ M)	Activity	
		B Substance degradation ^{a)}	Phenyl- α -D- galactoside hydrolysis ^{b)}
None	—	512	150
L-Ara	33	4	70
D-Fru	33	512	150
D-Gal	33	4	10
D-Glu	33	512	150
D-Man	33	512	150
Mel	33	4	35
Raf	33	4	80
L-Rha	33	512	150
D-Rib	33	512	150
L-Sor	33	256	150
D-Xyl	33	4	30
D-GalN	33	16	125
D-GluN	33	512	200

^{a)} Minimal amount of B substance for complete hemagglutination inhibition (1, when heated enzyme was used).

^{b)} Absorbance at 660 m μ .

TABLE VI. ANOMERIC SPECIFICITY OF THE α -GALACTOSIDASE OF *Streptomyces* 9917S₂

Incubation was made with 2 μg enzyme protein, at pH 6.0 and at 35°C .

Substrate	Degradation ^{a)}
Phenyl- α -D-galactoside	+
Phenyl- β -D-galactoside	—
Phenyl-N-acetyl- α -D-galactosaminide	—
Phenyl-N-acetyl- β -D-galactosaminide	—
Lactose	—
Melibiose	+
Raffinose	+
B substance	+

^{a)} At the end of incubation, 0.01 ml of the reaction mixture was applied to paper chromatography, and the galactose liberated from substrates was determined qualitatively.

mine were all inhibitory. L-Arabinose and D-xylose were also inhibitory, while other sugars tested were not (Table V).

Table VI shows the substrate specificity of the purified enzyme. Melibiose and raffinose were hydrolyzed besides phenyl- α -galactoside and the B substance, indicating that the enzyme cleaves α -(1 $\rightarrow$ 6)-galactosidic linkage as well as α -(1 $\rightarrow$ 3)-galactosidic linkage.

DISCUSSION

Relatively little is known on α -galactosidase,²²⁾ compared to β -galactosidase. In bacteria, the presence of α -galactosidase activity has been reported only with *Aerobacter aerogenes*,²³⁾ *Diplococcus pneumoniae*,²⁴⁾ *Streptococcus bovis*,²⁵⁾ *Streptomyces olivaceus*, *Str. fradiae*, *Str. roseosporinus* nov. sp.,^{26, 27)} *Escherichia coli*,^{28, 29)} and some soil bacteria.³⁰⁾ Main concern in these studies was to elucidate the bacterial enzymes which hydrolyze raffinose and melibiose. The α -galactosidases from these bacteria effectively cleave α -(1 $\rightarrow$ 6)-galactosidic linkages of the above sugars, but whether the enzymes are also able to cleave other α -galactosidic linkages has been left unclear.

Serological activity of blood group B substances derives from α -(1 $\rightarrow$ 3)-galactosidic linkage at the nonreducing terminal of blood group mucopolysaccharides,³¹⁾ and the B substance-degrading enzymes are α -galactosidases which cleave this linkage.^{32, 33)}

The α -galactosidase from coffee beans hydrolyzes both raffinose (α -(1 $\rightarrow$ 6)-galactosidic linkage) and the B substance (α -(1 $\rightarrow$ 3)-linkage).³⁴⁾ On the other hand, α -galactosidases from yeast, sweet almonds, *Plattela vulgata*,³³⁾ *Mortierella vinacea*³⁵⁾ cleave only α -(1 $\rightarrow$ 6)-linkage. Bacterial B substance-degrading enzymes have not been examined for their ability to hydrolyze α -(1 $\rightarrow$ 6)-linkage.

In the present study, we have attempted to characterize the α -galactosidase of *Streptomyces* 9917S₂, especially to elucidate whether the enzyme cleaves α -(1 $\rightarrow$ 3)-galactosidic linkage.

First, it was found that the B substance degrading activity is inducible by lactose (a β -galactoside) as α -galactosidase is. Generally, bacterial α -galactosidases are inducible enzymes^{23, 28, 36~38)} except in the case of *S. bovis*.²⁵⁾ However, it has not been studied whether bacterial B substance-degrading enzymes are inducible. α -Galactosidases are induced both by α - and β -galactosides in *E. coli* B,²⁸⁾ *Str. olivaceus*,³⁶⁾ and bacterium M 12,³⁷⁾ but only by α -galactosides in *A. aerogenes*²³⁾ and *E. coli* IAM 1277.³⁸⁾

Purification of the α -galactosidase from *Streptomyces* 9917S₂ has also resulted in the purification of the blood group B substance-degrading activity, both activities falling into the same fraction throughout the purification processes. The final preparation, which was 15-fold enriched in the specific activity of α -galactosidase, was free from α - and β -glucosidases, β -galactosidase, α - and β -glucosaminidases, and α - and β -galactosaminidases.

Several bacterial α -galactosidases have so far been purified,^{39~42)} but purification of bacterial B substance-degrading enzymes has not been made except for partial purification of the enzyme from *Clostridium maebashi*.³²⁾ Some bacterial α -galactosidases have been reported to be unstable in various purification procedures.^{23, 41, 42)} The α -galactosidase of *Streptomyces* 9917S₂ was, however, quite stable and isolated in a highly purified state.

Properties of the purified α -galactosidase preparation examined in the present study indicate that the activities of α -galactosidase and B substance degradation is attributed to the same enzyme protein. The enzyme has a broad optimal pH range, 4.5~6.0, for both reactions. This value corresponds to that reported for α -galactosidases from many other organisms, though some of α -galactosidases have been reported to possess their optimal pH values ranging from 6.5 to 8.2.^{41~44)}

The activities of α -galactosidase and the B substance degradation from *Streptomyces* 9917S₂ were inhibited by D-galactose, melibiose, and

raffinose, and also by L-arabinose and D-xylose. The first three sugars are probably acting as competitive inhibitors. These sugars are also reported to be inhibitory on the B substance-degrading enzyme of *Cl. maebashi*.⁴⁴⁾ However, further investigation will need for the elucidation for inhibition by L-arabinose and D-xylose. α -Galactosidases from plants, yeasts, and molds have been known to have β -L-arabinosidase activity.²²⁾ The crystalline α -galactosidase of *M. vinacea* hydrolyzes methyl- β -L-arabinoside.³⁵⁾ This may be the case with the enzyme from *Streptomyces* 9917S₂. Inhibition by D-xylose may have more difficulty to explain, although there is a report that the α -galactosidase activity of *M. vinacea* is inhibited by D-xylose.³⁵⁾

Present study has shown that α -galactosidase from *Streptomyces* 9917S₂ cleaves α -(1→6)- and α -(1→3)-galactosidic linkages. It is now under investigation with a further purified enzyme preparation whether this enzyme can cleave other α -galactosidic linkages.

Addendum: Taxonomical characteristics of *Streptomyces* 9917S₂ will be reported by Dr. A. Shimazu of the Institute of Applied Microbiology, University of Tokyo, in the near future.

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