Isolation and Characterization of a Keratan Sulfate-degrading Endo-β-galactosidase from *Flavobacterium keratolyticus*

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An endo-β-galactosidase has been isolated in a highly purified form from a new organism, *Flavobacterium keratolyticus*. The purification procedure includes ammonium sulfate precipitation of the enzyme from the culture medium, Sephadex G-100 filtration, chromatography at the pH of the enzyme on a column which contains equal portions of CM-Sephadex C-50 and DEAE-Sephadex A-50, followed by Matrex gel blue A and DEAE-Sephadex A-50 chromatography. The final preparation shows one major but diffuse protein band on polyacrylamide gel electrophoresis. The molecular weight of this enzyme is about 30,000, and the optimal activity occurs at pH 6.0 using keratan sulfate as the substrate. Hg^{2+}, Ag^+, Cu^2+, and p-chloromercuribenzoate are potent inhibitors for the enzyme. This enzyme is stabilized but not activated by Ca^{2+}.

This enzyme hydrolyzes endo-β-galactosyl linkages in keratan sulfate, and glycoconjugates with N-acetyllactosamine repeating units and milk oligosaccharides. The specificity of this enzyme is similar to the one isolated from *Escherichia freundii* (Nakagawa, H., Yamada, T., Chien, J., Gardas, A., Kitamikado, M., Li, S.-C., and Li, Y.-T. (1980) *J. Biol. Chem.* 255, 5955-5959). The only difference is that the enzyme isolated from *F. keratolyticus* hydrolyzes milk oligosaccharides faster than the one isolated from *E. freundii*. In contrast to *E. freundii* and other organisms that produce endo-β-galactosidase, *F. keratolyticus* produces endo-β-galactosidase without induction by keratan sulfate. Thus this organism is the best source so far for the preparation of the endo-β-galactosidase capable of cleaving sugar chains with N-acetyllactosamine repeating units.

The acidic glycosaminoglycan, keratan sulfate, is widely distributed in animal connective tissues. The major parts of the chain consist of disaccharide repeating units containing N-acetylglucosamine and galactose linked by alternating β1 → 3 and β1 → 4 linkages (1). This type of repeating unit has been also found to occur in the sugar chains of a wide variety of glycosphingolipids and glycoproteins (2-8). Among several keratan sulfate-degrading enzymes, endo-β-galactosidase isolated from *Escherichia freundii* (9-11) and *Pseudomonas* sp. (12) has been found to be useful for the structural analysis of glycoconjugates. The main disadvantage of preparing endo-β-galactosidase from these organisms is the absolute requirement of keratan sulfate which is not readily available in large quantities to induce the enzyme. Recently, Kitamikado and Li (13) found that *Flavobacterium keratolyticus* isolated from soil was unable to produce a keratan sulfate-degrading endo-β-galactosidase without induction by keratan sulfate. This report describes the isolation and characterization of an endo-β-galactosidase from the culture fluid of this organism. A brief account of this work has been presented (14).

**EXPERIMENTAL PROCEDURES AND RESULTS**

This work represents the continuation of our efforts in searching for a better source for the preparation of endo-β-galactosidase useful for the structural analysis of glycoconjugates. Although endo-β-galactosidase has been isolated from *E. freundii* (9-11), *Pseudomonas* sp. (12), and *Cocobacillus* sp. (26), in all cases, the production of endo-β-galactosidase has to be induced. Endo-β-galactosidase from *Cocobacillus* was induced by gastric mucin while that from *E. freundii* and *Pseudomonas* sp. was induced by keratan sulfate. Among these three sources, endo-β-galactosidase isolated from *E. freundii* (9-11) and *Pseudomonas* sp. (12) have been used for the structural analysis of glycoconjugates. Recently, we have devised a simple scheme to isolate this enzyme from the culture medium of a new strain of *E. freundii* which releases high levels of endo-β-galactosidase with relatively low levels of exoglycosidases (11). The main disadvantage of preparing endo-β-galactosidase from *E. freundii* is the absolute requirement of keratan sulfate which is not readily available in large quantities. We therefore undertook the search for organisms capable of producing endo-β-galactosidase without keratan sulfate. *F. keratolyticus* was found to release endo-β-galactosidase to the culture medium in the absence of keratan sulfate (13). Furthermore, this organism releases very low levels of exoglycosidases and proteases to the culture medium. Therefore, this organism is the organism of choice for producing endo-β-galactosidase. We have purified endo-β-galactosidase.

1 Portions of this paper (including "Experimental Procedures," "Results," Figs. 1 to 4, and Table I) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 80M-2316, cite author(s), and include a check or money order for $5.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
over 16,000-fold with 11% recovery from the culture medium of Flavobacterium Endo-β-galactosidase. The final preparation was completely free from exo-glycosidases, proteases, and endo-β-N-acetylgalactosaminidase (see under "Enzyme Assay" and "General Properties of Endo-β-Galactosidase" in the miniprint supplement).

It gave one major band on disc gel electrophoresis. Among various steps used, Sephadex C-100 filtration was found to be the most effective, resulting in a 23-fold increase in specific activity with 84% recovery. The increase in specific activity could be due to the removal of small peptides and inhibitors by this step. A column composed of CM-Sephadex C-50 and DEAE-Sephadex A-50 was used to remove the contaminating proteins. Since the PI of endo-β-galactosidase was found to be about pH 6.0, the enzyme was not retained by this column at this pH. Using this simple procedure, the specific activity of the enzyme increased more than three times. Another effective step was Mätrix gel blue A chromatography. By this step, endo-β-galactosidase activity was separated into one major and one minor peak. The specificity of the enzyme from the major peak toward keratan sulfate, milk oligosaccharides, and glycosphingolipids was found to be similar to that of the enzyme from the major peak. For practical purposes, the enzyme preparation obtained from Mätrix gel blue A column is quite suitable for the structural analysis, since the enzyme is free from exoglycosidases and proteases.

From the results presented above, the specificity of endo-β-galactosidase isolated from Flavobacterium Endo-β-galactosidase toward different glycoconjugates is very close so that isolated from E. freundii. Since F. keratolyticus produces endo-β-galactosidase without induction by keratan sulfate, this organism is the best source so far for the preparation of the endo-β-galactosidase capable of cleaving sugar chains with N-acetyllactosamine repeating units.

REFERENCES

18. Li, Y.-T., and Li, S.-C. (1972) Methods Enzymol. 28, 702-713
**Flavobacterium Endo-b-galactosidase**

**Isolation and Characterization of a Protein**

**Endo-b-galactosidase from Flavobacterium sp.**

**Materials:**

- Lipopectin B-2000 (from Sigma Chemical Co.)
- BSA (bovine serum albumin)
- DEAE-Sephadex A-50 (Pharmacia LKB Biotechnology, Inc.)

**Methods:**

- Lipopectin B-2000 was used as the substrate for the enzyme assay.
- The enzyme was assayed using the method of Ferrel et al. (15, 16).
- The reaction was stopped by the addition of 50 mM sodium citrate buffer, pH 5.0.
- The enzyme activity was determined by measuring the decrease in absorbance at 415 nm.

**Results:**

- The enzyme was found to be active over a pH range of 4.5 to 8.0 and a temperature range of 25 to 45°C.
- The enzyme was stable at pH 6.0 and 37°C for 24 hours.

**Discussion:**

- The enzyme is a novel endo-b-galactosidase from Flavobacterium sp. that has potential applications in the food industry.
- Further studies are needed to characterize the enzyme's properties and potential uses.

**References:**


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Flavobacterium Endo-β-galactosidase


Fig. 2. Polyacrylamide disc gel electrophoresis of endo-β-galactosidase preparations at pH 6.4. 1, preparation obtained after chromatography on CM-Sephadex G-50 and DEAE-Sephadex A-50 combined column (50 μg); 2, the entire fraction obtained after chromatography on CM-Sephadex C-25 (50 μg); 3, the major fraction obtained after chromatography on Nutres Blue A (50 μg). The gels were stained with Comassie blue. The endo-β-galactosidase activity was found to coincide with the protein band shown as A.

Table 1

<table>
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<th>Procedure</th>
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