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General Properties of Beta-Galactosidase of *Xanthomonas campestris*

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Partially purified β-galactosidase of *Xanthomonas campestris* required 32 to 37°C and pH 5.5 to 5.8 for optimum activity. The enzyme had low affinity for lactose hydrolysis (K_m = 22 mM) and was inhibited by thiol group reagents, ethylenediaminetetraacetic acid, galactose, and D-galactal.

Xanthomonas campestris is the source of xanthan gum, an extracellular polysaccharide which is widely used as a viscosity-building agent in foods and has shown promise in other types of industrial applications (6, 15). The successful production of xanthan gum in inexpensive industrial waste fluids, such as cheese whey, would be of considerable importance. However, xanthan gum is not readily produced in lactose-based media (16), although many species in the genus *Xanthomonas*, including *X. campestris*, are known to synthesize β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) (4). This paper describes the partial purification and general properties of *X. campestris* β-galactosidase.

The strain of *X. campestris* used in this study (B-1459) was obtained from the United States Department of Agriculture, Northern Regional Research Center, Peoria, Ill. The organism was grown in lactose-mineral salts medium containing 10 g of lactose, 5 g of KH_2PO_4, 2 g of NH_4Cl, 1 g of NaCl, and 0.25 g of $MgSO_4 \cdot 7H_2O$ in 1 liter of distilled water, with adjustment to pH 7.0 before autoclaving. During storage, the culture was maintained in the same medium with 1.5% agar (Difco).

Enzyme extraction and purification. Lactose-mineral salts medium (500 ml per 2-liter Erlenmeyer flask) was inoculated with 0.5% (vol/vol) of a 72-h liquid culture of *X. campestris* and incubated for 5 days at 30°C on a shaker (200 rpm). All steps in the enzyme purification scheme were carried out at 4°C. Cells from 10 liters of medium were centrifuged at 15,000 × *g* for 15 min and resuspended in 100 ml of 100 mM tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.4) containing 50 mM $MgCl_2$, 0.1 ml of Triton X-100, and 100 mg of egg white lysozyme. After stirring overnight and centrifuging at 26,000 × *g* for 25 min, deoxyribonuclease was added (1 mg/ml) to the supernatant and incubation was continued for 3 h. Saturated ammonium sulfate solution (pH 7.0) was added to 30% saturation. The precipitate was discarded, and salt saturation was increased to 50%. After 3 h of stirring, the precipitate was centrifuged, resuspended in 50 mM sodium acetate buffer (pH 5.5) containing 10% glycerol, and dialyzed against 16 liters of the same buffer for 16 h. The protein solution was applied to a column (3 by 45 cm) of Sephadex G-100 (Pharmacia). Elution was with the buffer described above. Active fractions were combined and concentrated by ultrafiltration under nitrogen pressure (24 kg/cm^2) with an Amicon model 52 ultrafiltration cell with a UM-10 membrane. The concentrated enzyme solution was adjusted to pH 8.0 with 20 mM tris(hydroxymethyl)-aminomethane and applied to a column (1 by 16 cm) of diethylaminoethylcellulose (Whatman DE 52) equilibrated with 20 mM tris(hydroxymethyl)aminomethane hydrochloride buffer (pH 8.0). The column was eluted with a linear gradient of NaCl ranging from 50 to 250 mM in a total volume of 250 ml. Fractions (5 ml/tube) positive for β-galactosidase were combined, concentrated by ultrafiltration as described above, and dialyzed against 50 mM sodium acetate buffer (pH 5.5). This preparation was used for studying the properties of β-galactosidase.

Enzyme assays. During enzyme purification, β-galactosidase reaction mixtures contained 5 μM of *o*-nitrophenyl-β-D-galactoside (ONPG), 50 μM of sodium acetate (pH 5.5), and enzyme in a total volume of 2.0 ml. Incubation was at 32°C for 30 min. Reactions were terminated by the addition of 4 ml of ice-cold Na_2CO_3. Absorbance was read at 420 nm in a spectrophotometer, and the amount of *o*-nitrophenol released was determined from a standard curve. In experiments where substrate saturation was required, reactions were carried out in a 2-ml total volume containing 5 × K_m concentration of ONPG. One

unit of enzyme activity was defined as the release of 1 nmol of *o*-nitrophenol per min under standard assay conditions. Specific activity was defined as units per milligram of protein. Protein was measured according to Lowry et al. (11), with bovine serum albumin as the standard. With lactose as the substrate, reaction time was increased to 2 h. Reactions were terminated by heating in boiling water for 3 min. The amount of glucose released was measured by a glucose oxidase assay method (Sigma Chemical Co., St. Louis, Mo.).

During preliminary work on enzyme purification, significant losses in activity were sustained. However, the incorporation of 10% (vol/vol) glycerol in all stages of enzyme purification stabilized the β-galactosidase. The enzyme was separated from approximately 97% of the total protein in the cell-free extract, resulting in a 19-fold increase in β-galactosidase activity with a total recovery of 59% of the enzyme units present in the crude extract (Table 1). Analysis of the purified enzyme by discontinuous polyacrylamide gel electrophoresis at pH 9.3 by the method of Davis (2) showed a major band and three minor bands when the gels were stained with coomassie brilliant blue.

The temperature optimum of the enzyme was 32 to 37°C, as determined in 50 mM acetate buffer (pH 5.5), with a rapid decline occurring above 37°C. The pH optimum of the β-galactosidase was between 5.5 and 5.8, as determined in sodium acetate–acetic acid (pH 4.0 to 5.8), Na_2HPO_4–NaH_2PO_4 (pH 5.8 to 8.0), and Na_2HPO_4–citric acid (pH 3.0 to 8.0) buffer systems. The specific activity was highest when assayed in acetate buffer. The relatively low optimum pH of the β-galactosidase was of interest since the optimum pH requirement for xanthan gum synthesis in a glucose medium is 7.0 (15), where β-galactosidase activity was only 45% of the maximum. The temperature optimum of the enzyme was also higher than the range (28 to 30°C) known to be optimum for growth (13). This implied that initial growth and subsequent xanthan gum synthesis by *X. campestris* would be slow in lactose media adjusted to neutral pH and 28 to 30°C.

Among metals tested, Mg^{2+}, Ca^{2+}, Fe^{2+} at 5 and 25 mM, and K^+ at 50 mM did not affect enzyme activity, whereas Zn^{2+} at 2 and 10 mM caused 20 and 46% loss of β-galactosidase activity, respectively. Ethylenediaminetetraacetic acid (5 mM) caused 30% loss of enzyme activity, indicating the involvement of a metal ion in enzyme function which remained unidentified. The effectiveness of the thiol group reagents iodoacetamide at 0.5 mM and *p*-hydroxymercuribenzoate at 5 mM in causing 100 and 20% enzyme inhibition, respectively, pointed to the possible involvement of free sulfhydryl group(s) in enzyme activity, similar to some other bacterial β-galactosidase (5, 12).

The activation energy for the β-galactosidase, 11.9 kcal/mol, was calculated from an Arrhenius plot after measuring the reaction velocity of ONPG hydrolysis at 15, 25, and 35°C.

The apparent affinity constants (K_m) of the *X. campestris* β-galactosidase, determined from Lineweaver-Burk plots (10) of substrate saturation data, were 10 mM for ONPG and 22 mM for lactose. The K_m for ONPG was about 6-fold higher than that reported for *Lactobacillus thermophilus* (14) and *Clostridium perfringens* (9) and 77-fold higher than that found for *Escherichia coli* β-galactosidase (9). Only the β-galactosidase of *Bacillus subtilis* is known to have a higher K_m (42 mM) for ONPG (1). A higher K_m for ONPG in contrast with that for lactose was also reported for the β-galactosidase of *B. megaterium* (8) and *L. thermophilus* (14).

Galactose and D-galactal were competitive inhibitors of the *X. campestris* β-galactosidase, similarly to other microbial β-galactosidases (7–9). The inhibition constants (K_i) determined from the Dixon plots (3) of inhibitor saturation data were 5.3 and 0.19 mM for galactose and D-galactal, respectively, with ONPG as the substrate, whereas the K_i for D-galactal was 0.39 mM with lactose as the substrate. The relatively low K_i for galactose indicated that lactose hydrolysis by β-galactosidase may be under negative feedback control by one of the products of the reaction during the growth of *X. campestris*

TABLE 1. *Purification of β-galactosidase from X. compestris B-1459*[a]

Fraction	Total protein (mg)	Total U[b]	Sp act[c]	Recovery (%)	Purification (-fold)
I. Crude cell-free extract	880	18,500	21	100	
II. Ammonium sulfate precipitate	215	14,700	68	79	3
III. Sephadex G-100 chromatography	48	12,500	260	67	12
IV. Diethylaminoethyl-cellulose chromatography	27	11,000	407	59	19

[a] Purification steps are described in the text.
[b] Nanomoles of *o*-nitrophenol released per minute.
[c] U, Units of enzyme activity per milligram of protein.

in a lactose medium. In addition, the low level of substrate affinity shown by the β-galactosidase may be yet another contributing factor to the inability of *X. campestris* to synthesize large amounts of extracellular polysaccharide in lactose-based media.

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