Biochimica et Biophysica Acta, 377 (1975) 410—420

BBA 67408

PURIFICATION AND PROPERTIES OF A β-1,6-GLUCOSIDASE FROM *FLAVOBACTERIUM*

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(Received August 9th, 1974)

Summary

An intracellular β-1,6-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) was produced semiconstitutively by *Flavobacterium* M64. This enzyme was purified 180-fold by fractionation with ammonium sulfate followed by chromatographies on carboxymethylcellulose, hydroxyapatite and Sephadex G-100. The final preparation appeared homogeneous on disc electrophoresis on polyacrylamide gel. The molecular weight of the enzyme was determined to be ca. 59 000 by Sephadex G-100 gel filtration and sodium dodecylsulfate-polyacrylamide gel electrophoresis. The optimum pH of the enzyme was 5.8 and the optimum temperature was 40 °C. The enzyme readily hydrolyzed oligomers with β-1,6-glucosidic linkages, converting them to glucose. The K_m values for gentio-biose, -triose, -tetraose and -pentaose were 2.8, 3.0, 4.2 and 4.6 × 10^{-4} M, respectively. The rates of their hydrolyses decreased with increase in their chain lengths. The enzyme was concluded to be a β-1,6-glucosidase from its substrate specificity, production of glucose, transferring ability and inhibition by glucono-δ-lactone. The enzyme activity was inhibited by Hg^{2+}, Cu^{2+}, Ag^{+}, Fe^{3+}, *p*-chloromercuribenzoate, *N*-ethylmaleimide, glucose and trishydroxyaminomethane (Tris) but not by ethylenediaminetetraacetic acid.

Introduction

Previously, it was reported that *Flavobacterium* M64 can grow with succinoglucan as the sole source of carbon [1] and that it produces extracellular succinoglucan depolymerase which converts succinoglucan to a polymer with a degree of polymerization of 12 [2]. It seemed possible that the organism might produce intracellular glucanase(s) and/or glucosidase(s), which could degrade the depolymerized polymer of succinoglucan produced by the extracellular enzyme. Thus, studies were made on its intracellular β-glucanases and (or)

β-glucosidase(s). At least two β-glucanases and one β-glucosidase were detected in a crude cell extract. This paper describes the purification and properties of the β-glucosidase.

Materials and Methods

Cultures

Flavobacterium sp. M64, isolated from soil, was used as the enzyme source [1]. The synthetic medium used for culture contained (per 100 ml): 1 g of carbohydrate, 0.15 g of $(NH_4)_2HPO_4$, 0.1 g of KH_2PO_4, 0.05 g of $MgSO_4 \cdot 7H_2O$ and 2 ml of mixed salt solution. This salt solution contained (per l) NaCl 10 g, $CaCl_2$ 10 g, $MnCl_2 \cdot 4H_2O$ 10 g, $FeCl_3 \cdot 6H_2O$ 10 g, $ZnCl_2 \cdot 7H_2O$ 70 mg, $CuSO_4 \cdot 5H_2O$ 50 mg, $Na_2MoO_4 \cdot 2H_2O$ 20 mg and H_3BO_3 10 mg. The medium was adjusted to pH 7.2. Conical flasks containing 95 ml of medium were inoculated with 5 ml of a seed culture which had been grown in the same medium. The medium was shaken reciprocally at 30°C for 3 days.

Enzyme assay and chemical determinations

Enzyme activity was assayed at 40°C. The standard reaction mixture contained 50 mM sodium acetate buffer, pH 5.8 and 5 mM or 0.25% substrate in a final volume of 1 ml. The reaction was initiated by adding 10 µl of enzyme solution and 0.2 ml samples were removed at appropriate times. Activity was measured as release of glucose from the substrate, determined either with glucose oxidase or by the method of Somogyi and Nelson [3], using glucose as standard. Reactions were stopped for the former method by heating the sample in a boiling water bath for 5 min and for the latter method by adding 1 ml of alkaline copper reagent. In all cases the initial reaction velocity was measured, and the amount of product formed was proportional to the enzyme concentration. 1 unit of enzyme activity is defined as the amount that catalyzes the hydrolysis of 1 µmole of glucosidic linkage per min under the experimental conditions employed.

When *p*-nitrophenyl-β-glucoside was used as substrate the reaction mixture contained 5 mM *p*-nitrophenyl-β-glucoside, 50 mM acetate buffer and enzyme in a final volume of 1 ml. The reaction was stopped by adding 3 ml of 0.1 M ammonium buffer (pH 10). One enzyme unit was defined as the amount releasing 1 µmole of *p*-nitrophenol per min as measured at 400 nm in a Hitachi 124 spectrophotometer.

Protein was measured by the Folin-Lowry method using bovine serum albumin as standard [4]. The relative amount of protein was expressed as the absorbance at 280 nm per cm of enzyme solution.

Paper chromatography was carried out by the descending method using Toyo filter paper, No. 50 with a solvent system of *n*-butanol/pyridine/water (6 : 4 : 3, by vol.). Sugars were detected on paper chromatograms with silver nitrate reagent.

Disc electrophoresis on polyacrylamide gel was carried out by the method of Davis [5] on 7% gel of pH 4.3 under the following conditions: current, 2 mA/column; time, 150 min; temperature, 30°C; stain, amido black.

The molecular weight of the enzyme was estimated using a column of Sephadex G-100 by the method of Whitaker [6], and using dodecylsulfate-polyacrylamide gel electrophoresis by the method of Weber and Osborn [7].

Materials

Gentiobiose, *p*-nitrophenyl-α(and β)-glucoside, methyl-α(and β)-glucoside, salicin, arbutin, cellobiose, isomaltose and glucono-δ-lactone were purchased from Nakarai Chemical Co. Ltd. Sophorose, kojibiose and nigerose were kindly supplied by Dr Matsuda. Luteose was prepared from lutean [8] which is synthesized by *Penicillium aculeatum* var. *apiculatum* No. 2281. Gentiotriose, gentiotetraose and gentiopentaose were prepared by partial hydrolysis of luteose. Soluble laminaran [9] of *Eisenia bicyclis* and yeast glucan were kindly supplied by Drs Nisizawa and Maeda, and Dr Misaki, respectively. Laminaran was also obtained from the K and K laboratory, U.S.A. Laminaribiose was isolated by partial acid hydrolysis of curdlan. Curdlan [10] and succinoglucan [11] were prepared as reported previously from the culture broths of *Alcaligenes faecalis* var. *myxogenes* 10C3K and 10C3, respectively. Desuccinylated succinoglucan was prepared by treating succinoglucan with dilute alkali at 70 °C. Kefiran [12] and schizophyllan [13] were obtained by courtesy of Drs Kooiman and Kikumoto, respectively. Polytran was a product of Dawe's Laboratories. Inc., Chicago.

Results

Formation of β-1,6-glucosidase

The effects of various carbon sources on the formation of enzymes which attacked luteose, gentiobiose and *p*-nitrophenyl-β-glucoside were examined using cell-free extracts (Table I). The activities for gentiobiose and *p*-nitrophenyl-β-glucoside varied in parallel, depending upon the substrate used. Thus, they seemed to be due to the same enzyme. These activities were highest in cells grown with succinoglucan while the activity for luteose was highest in cells grown with luteose. The two former activities were found to some extent in

TABLE I

FORMATION OF INTRACELLULAR β-1,6-GLUCOSIDASE AND β-1,6-GLUCANASE

Cultures in medium containing (per 100 ml) 1 g of carbon source were incubated for 3 days at 30°C. Then cells were harvested by centrifugation and subjected to ultrasonic oscillation for 30 min at 20 kcycles. The supernatant of the disrupted cells obtained by centrifugation for 30 min at 27 000 g was used for enzyme assay.

Carbon source	β-1,6-Glucanase activity (luteose) (units/mg protein)	β-1,6-Glucosidase activities	
		gentiobiose (units/mg protein)	*p*-nitrophenyl-β-glucoside (units/mg protein)
Glucose	0.01	0.31	0.013
Gentiobiose	0.02	0.72	0.030
Succinoglucan	0.14	1.0	0.042
Luteose	0.35	0.55	0.023

cells grown on glucose, suggesting that the enzyme responsible is semi-constitutive. However, the latter enzyme with activity for luteose seemed to be inducible.

Purification of β-1,6-glucosidase

Cells were harvested by centrifugation from 12 l of cultures containing 1% succinoglucan from *Flavobacterium* M64 (yield, approx. 40 g as dried matter). The cells were divided into two equal portions. Then the β-1,6-glucosidase was purified according to the following steps.

Step 1. Each portion of the cells was suspended in 240 ml of 0.01 M acetate buffer (pH 5.8) and subjected to sonic oscillation for 30 min at 20 kcycles in a 200 W Kaijo Denki Ultrasonic disintegrator. The sonicate was then centrifuged for 30 min at 27 000 × *g*. The crude extract containing approx. 11.2 g of protein was obtained. The two extracts were combined.

Step 2. The crude extract was brought to 40% saturation of ammonium sulfate. The resulting precipitate was removed by centrifugation. Further solid ammonium sulfate was added to the supernatant to give 80% saturation. The resulting precipitate was collected by centrifugation and dissolved in 50 ml of 0.01 M acetate buffer (ph 5.8). The resulting solution was dialyzed against 1 l of 0.01 M phosphate buffer (pH 6.5) for 24 h.

Step 3. The dialyzed material was applied to a carboxymethylcellulose column (3.5 × 14 cm) equilibrated with 0.01 M phosphate buffer. The column was washed with 500 ml of phosphate buffer and then eluted with 1.6 l of a linear gradient of 0—0.5 M NaCl in phosphate buffer. Fractions containing enzyme activity (200 ml) towards *p*-nitrophenyl-β-glucoside were combined and concentrated to 10 ml at under 30°C and then dialyzed against 0.05 M phosphate buffer (pH 6.5).

Step 4. The dialyzed material was applied to a hydroxyapatite column (5 × 18 cm) equilibrated with 0.1 M phosphate buffer (pH 6.5). The column was washed with 300 ml of the same buffer and then eluted with 1 l of a linear gradient of 0.1—0.4 M phosphate buffer (pH 6.5) (Fig. 1). The activities of the fractions towards gentiobiose, *p*-nitrophenyl-β-glucoside and luteose were meas-

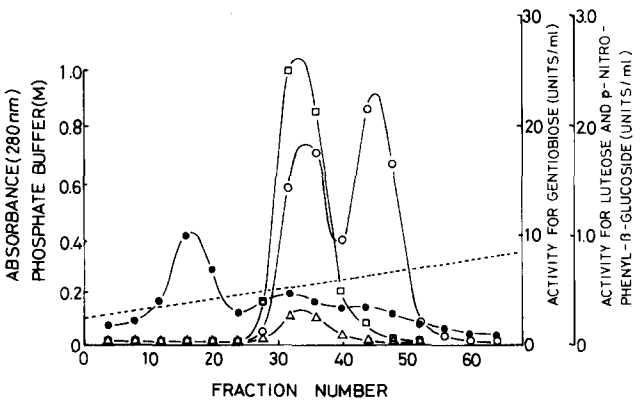


Fig. 1. Hydroxylapatite chromatography of the β-1,6-glucosidase. ●, absorbance at 280 nm; □, activity for gentiobiose; △, activity for *p*-nitrophenyl-β-glucoside; ○, activity for luteose; ------, concentration of phosphate buffer. Fractions of 10 ml were collected. Details are described in the text.

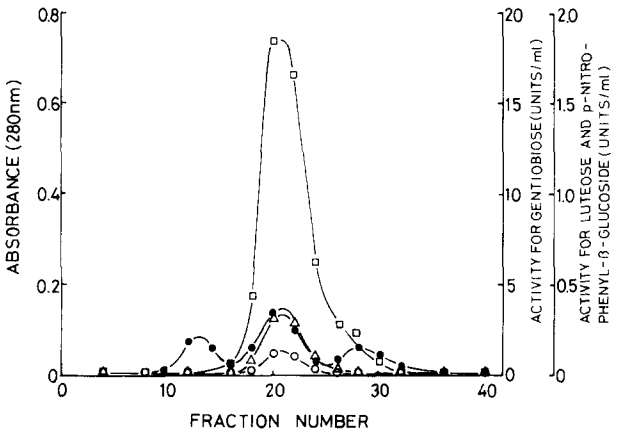


Fig. 2. Sephadex G-100 chromatography of the β-1,6-glucosidase ●, absorbance at 280 nm; □, activity for gentiobiose; △, activity for *p*-nitrophenyl β-glucoside; ○, activity for luteose. Fractions of 5 ml were collected. Details are described in the text.

ured. The activities towards the two former compounds appeared in the same fractions (Peak I) while activity towards luteose appeared in the same fractions and also in another peak (Peak II). Fractions 28—40 (Peak I), were combined and concentrated to 3 ml at under 30°C and then dialyzed against 0.05 M phosphate buffer (pH 6.5). The enzyme in Peak II seemed to be β-1,6-glucanase but it was not examined further.

Step 5. The dialyzed enzyme solution was applied to a Sephadex G-100 column (3 × 50 cm) equilibrated with 0.05 M phosphate buffer (pH 6.5). The column was eluted with the same buffer (Fig. 2). The three enzyme activities and protein appeared in the same fractions, while a small amount of protein was eluted in another position. The fractions in the major peak (Nos 18—24) were combined and concentrated to 5 ml, and then dialyzed against the same buffer. The results of a typical purification are shown in Table II.

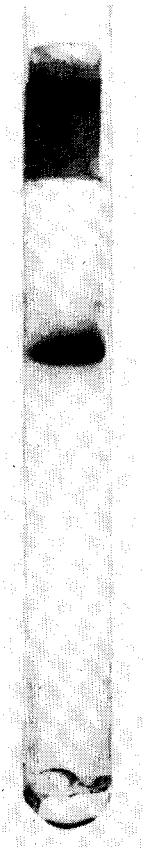
Disc gel electrophoresis of the purified enzyme at pH 4.3 revealed a single protein component (Fig. 3). On extraction of the slices of an unstained gel, the enzyme activity was found to coincide with the protein band. The overall yield

TABLE II

PURIFICATION OF β-1,6-GLUCOSIDASE

Enzyme units are expressed as μmoles of gentiobiose hydrolyzed per min.

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
1. Crude filtrate	11200	4140	0.37	100
2. $(NH_4)_2SO_4$, 40—80%	2190	2780	1.27	68
3. Carboxymethylcellulose	71.8	1910	26.6	47
4. Hydroxyapatite	8.4	486	57.9	12
5. Sephadex G-100	1.9	124	65.4	3.1



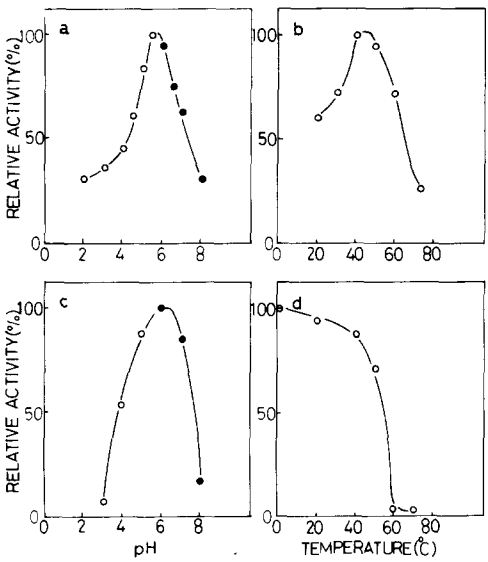


Fig. 3. Polyacrylamide gel electrophoresis of purified β-1,6-glucosidase. Fifty micrograms of protein were applied to the gel and run as described in the text.

Fig. 4. Effect of pH (a,c) and temperature (b,d) on activity and stability of β-1,6-glucosidase, respectively. Gentiobiose was used as substrate. To examine the pH stability, enzyme solutions of various pH values were incubated for 1 h at 40°C. Then each solution was adjusted to pH 5.8 and the residual activity was measured by the standard method. To examine temperature stability, the enzyme in 0.1 M acetate buffer solution (pH 5.8) was incubated for 5 min at the various temperatures indicated, and then the remaining activity was assayed. ○, 0.1 M acetate buffer; ●, 0.1 M phosphate buffer.

was 3.1% and the specific activity of the purified enzyme with gentiobiose was 65.4 units per mg. In this way the enzyme was purified 180-fold with a yield of about 3.8 mg of enzyme from 12 l of culture.

Properties of β-1,6-glucosidase

The molecular weight of the enzyme was estimated by comparing its elution volume from a column of Sephadex G-100 with those of a series of proteins of known molecular weight (Mann Res. Lab.). The molecular weight of the enzyme determined by interpolation is 59 000. This value coincides with that obtained by dodecylsulfate-polyacrylamide gel electrophoresis. The optimum pH of the enzyme was approximately 5.8 in acetate buffer at 40°C (Fig. 4a). The enzyme was stable at pH 5.0 to 7.0 at 40°C for 1 h (Fig. 4c). Enzyme activities in 5 min were determined at pH 5.8 at various temperatures. The

TABLE III

SUBSTRATE SPECIFICITY OF β-1,6-GLUCOSIDASE

Enzyme activity with various substrates was assayed as described in the Methods.

Substrate 5 mM	Specific activity (μmoles/mg/min)
Gentiobiose	65.4
Gentiotriose	40.9
Gentiotetraose	35.7
Gentiopentaose	29.6
Luteose*	0.9
Laminaran* (Eisenia)	1.6
Laminaran*	0.4
Laminaribiose	0.5
Sophorose	0
Cellobiose	0
p-Nitrophenyl-β-glucoside	2.7
Methyl-β-glucoside	0.2
Salicin	0
Arbutin	0

* 0.25% substrate was used.

optimum temperature was about 40°C (Fig. 4b). Next the enzyme solution in 0.05 M acetate buffer (pH 5.8) was incubated at various temperatures for 5 min and the remaining activity was assayed at 40°C. The results shown in Fig. 4d indicate that the enzyme was stable below 40°C and that about 70% of the initial activity remained after heating at 50°C for 5 min.

To characterize the enzyme, its rates of hydrolysis of various oligo- and polysaccharides were measured and the results are shown in Table III. The results indicate that this enzyme is very specific for oligosaccharides composed of β-1,6-glucosidic linkages and that activity decreases with increase in the number of glucose residues. Among the polysaccharides tested, luteose which is composed largely of β-1,6-glucosidic linkages, was hydrolyzed, although the activity was low. Laminaran from *Eisenia*, which is known to have many β-1,6-glucosidic linkages, was also hydrolyzed slightly. *p*-Nitrophenyl-β-glucoside and methyl-β-glucoside were slightly hydrolyzed but the α-forms of these compounds were not. Sophorose and cellobiose, which are dimers with β-1,2- and β-1,4-glucosidic linkages, respectively, were not attacked. Aryl glucosides, such as salicin and arbutin, were not hydrolyzed. From these results, this enzyme was considered to be a β-1,6-glucosidase although it had very weak activity with laminaribiose. Yeast glucan, shizophyllan, succinoglucan, desuccinylated succinoglucan and polytran, which have some β-1,6-glucosidic linkages, were not attacked by this enzyme.

The K_m and V values for the homogeneous series from gentiobiose to gentiopentaose and luteose were determined (Table IV). The kinetic data show that their rates of hydrolysis decreased with increasing chain length.

Action of β-1,6-glucosidase on luteose

Luteose (2.5 mg) was incubated with the purified enzyme in 50 mM buffer in a total volume of 1 ml at 40°C and at intervals the product was

TABLE IV

KINETIC PARAMETERS OF ENZYMATIC HYDROLYSIS OF GENTIODEXTRINS AND LUTEOSE

Substrate	K_m ($M \times 10^{-4}$)	V (μmoles/mg/min)
Gentiobiose	2.8	101
Gentiotriose	3.0	52.4
Gentiotetraose	4.2	36.2
Gentiopentaose	4.6	32.1
Luteose	2.6*	20.0

* Expressed as mg/ml.

examined by paper chromatography (Fig. 5). Only one spot was detected which had the same R_F value as D-glucose. This gives further evidence that this enzyme is a β-1,6-glucosidase.

Transglucosylation

Gentiobiose (50 mg) was incubated with the enzyme (0.16 units) in a

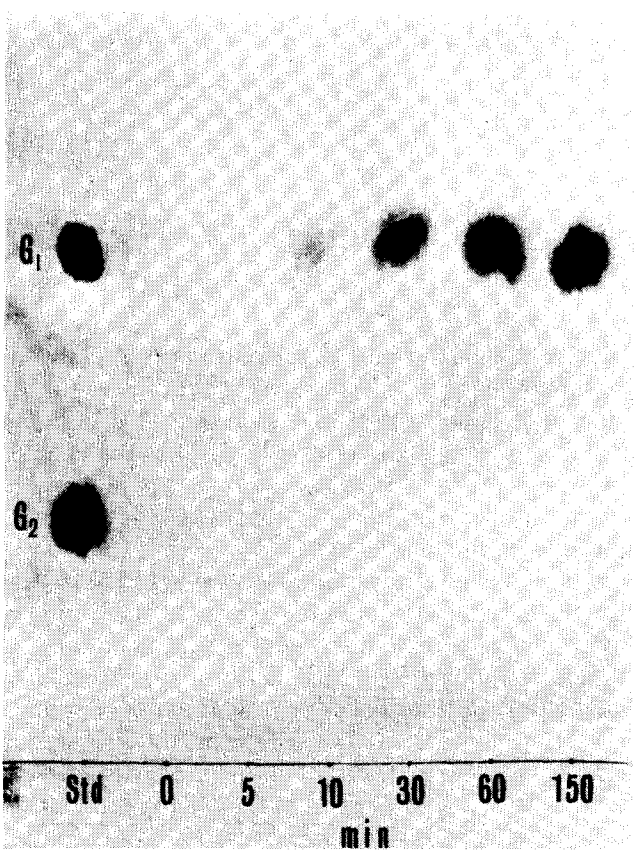


Fig. 5. Paper chromatogram of hydrolysis product from luteose. G_1, glucose; G_2, gentiobiose.

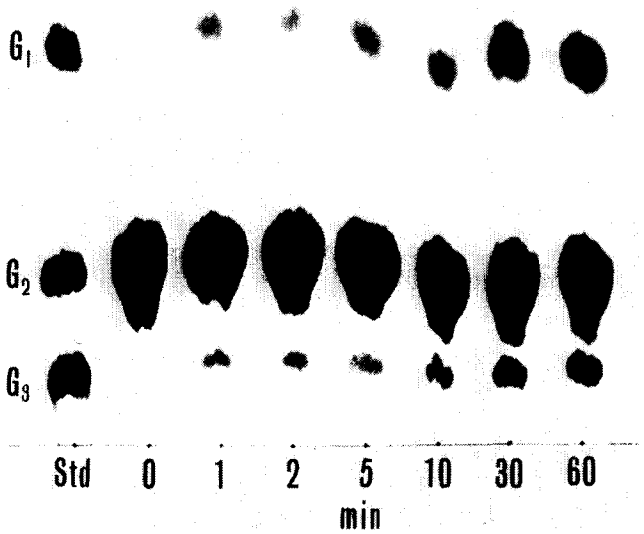


Fig. 6. Paper chromatogram of transglucosylation product from gentiobiose. G_1, glucose; G_2, gentiobiose; G_3, gentiotriose.

total volume of 1 ml containing 50 mM buffer and the resulting products were examined by paper chromatography (Fig. 6). A spot with the R_F value of gentiotriose was clearly detected with gentiobiose and glucose. This enzyme catalyzed transglucosylation from gentiobiose to gentiotriose. This is a characteristic of glucosidase.

Effects of ions and other compounds on β-1,6-glucosidase

The effects of various ions and other compounds on the activity of the enzyme were investigated by preincubating the enzyme with them for 6 min at 40 °C and then assaying the residual activity for *p*-nitrophenyl-β-glucoside. Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Na^{+}, Ni^{2+} and Zn^{2+} had little or no effect at a concentration of $2 \cdot 10^{-3}$ M. Ag^{+}, Cu^{2+}, Fe^{3+} and Hg^{2+} caused 80—100% inhibition at the same concentration. The enzyme was not inhibited by EDTA but was affected by *N*-ethylmaleimide ($5 \cdot 10^{-4}$ M) and *p*-chloromercuribenzoate (10^{-4} M). Thus, a thio or disulfide group seems to be essential for enzymatic activity. The inhibition by *p*-chloromercuribenzoate was overcome by the addition of cysteine. Glucono-δ-lactone, which is a specific inhibitor of many glu-

cosidases [14], was also inhibitory. Glucose and Tris were somewhat inhibitory.

Discussion

The wide distribution of β-1,6-glucanase in fungi such as various strains of *Penicillium*, *Aspergillus*, *Trichoderma* and *Fusarium* was demonstrated by Reese et al. [15]. Some strains of *Actinomycetes* [16], *Rhizopus* [17] and *Gibberella* [18] have also been found to produce the enzyme well. These enzymes form oligosaccharides, such as gentiobiose or gentiotriose, from β-1,6-glucan, although the enzyme in some strains also forms a slight amount of glucose. None of these enzymes attacks gentiobiose. The intracellular β-glucanase from yeast [19] releases glucose from pustulan as well as laminaran, gentiobiose and *p*-nitrophenyl-β-glucoside. This enzyme does not split cellobiose, methyl-β-glucoside or salicin.

Our enzyme is very different from the enzymes mentioned above. Walker and Builder [21] named the enzyme from *Streptococcus mitis* α-1,6-glucosidase. However, later the name was changed to α-1,6-glucan glucohydrolase (dextran-glucosidase) [22] since the enzyme showed equal affinities for α-1,6-glucans, irrespective of their chain lengths. The simplest test to differentiate between a glucosidase and an exoglucanase was to determine the relative activities of the enzyme on a dimer and on the corresponding tetramer or polymer [15]. An exo-glucanase would be more active on the tetramer and a glucosidase on the dimer. Our enzyme split a series of β-1,6-gluco-oligosaccharides. The rate of the reaction with equimolar concentrations of these compounds gradually decreased with increase of chain length from gentiobiose to gentiopentaose. Gentiobiose was a much better substrate than luteose, weight for weight. From its substrate specificity, ability to cause transglucosylation and its inhibition by glucono-δ-lactone [20], our enzyme was concluded to be a β-1,6-glucosidase, yielding glucose from the non-reducing end of β-1,6-glucose oligosaccharides and β-1,6-glucans. No similar enzyme has yet been demonstrated in nature. It has been suggested that *Phytophthora palmivora* contains β-1,3-glucosidase, but the enzyme was not purified [23].

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