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On the Properties of α-Glucosidase and the Binding of Glucose to the Enzyme

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An α-glucosidase was purified from baker's yeast. The molecular weight was approximately 44 000 daltons. SDS-disc gel electrophoresis suggested that the enzyme consisted of four subunits. The isoelectric point was at pH 5.4. The K_m values for *p*-nitrophenyl α-D-glucopyranoside and maltose were 2.9×10^{-4} and 2.5×10^{-2} M, respectively.

Binding of 2-(*p*-toluidino)naphthalene-6-sulfonate to the α-glucosidase was associated with a strong increase in fluorescence. The dissociation constant of the enzyme-TNS complex was 8×10^{-5} M. The fluorescent probe did not interfere with the binding of glucose to the enzyme although the α-glucosidase was inhibited by high concentrations of TNS. The formation of an enzyme–glucose complex was indicated by an increase of fluorescence and by a shift in the wavelength for maximal emission which suggests that the binding process is associated with a change in conformation. The dissociation constant of the glucose–α-glucosidase complex $K_D = 0.57 \times 10^{-3}$ M, was calculated from the increase in fluorescence as a function of glucose concentration.

α-Glucosidase (EC. 3.2.1.20) from yeast has been known for a long time. The older literature on α-glucosidase was extensively reviewed by Gottschalk.[1] Recently, the specificity of the synthetizing reaction of the enzyme has been investigated[2] and evidence for a covalent intermediate between α-glucosidase and glucose was obtained.[3] Functional groups at the active site of the enzyme consisting of a histidine residue and SH-groups have also been determined.[4] Kinetic studies on β-glucosidase at low temperature have resolved the aglycone-releasing step from the glycosyl-releasing one.[5] The mechanism of action of glucosidases has been discussed by Legler.[6]

In the present paper we describe the use of the potassium salt of 2-(*p*-toluidino)naphthalene-6-sulfonate (TNS) as a fluorescent probe for α-glucosidase. The change in the intensity of the fluorescence upon addition of glucose was used to calculate the dissociation constant of the glucosyl-enzyme complex. Furthermore a method for the purification and some characteristics of the enzyme are reported.

MATERIALS AND METHODS

Enzyme assays. The α-glucosidase activity was determined at 30 °C with 2.2 mM *p*-nitrophenyl α-D-glucopyranoside (PNPG) in 0.05 M sodium-potassium phosphate buffer, pH 6.5, by following the release of *p*-nitrophenol at 400 nm.[7] One unit of enzyme activity is defined as 1 μmol PNPG split per minute. When maltose was used as substrate, the α-glucosidase was inactivated by heating in a boiling water bath for 2 min and glucose was determined by the Glucostat-reagent (Boehringer, Mannheim, GmbH).

Enzyme preparation. The α-glucosidase was prepared from commercial baker's yeast obtained from Oy Alko Ab (Rajamäki, Finland). All operations were carried out at 0–4 °C and the sodium-potassium phosphate buffers used throughout the purification contained 10 % glycerol in addition to 10 mM 2-mercaptoethanol.

The yeast cells (80 g/160 ml 0.05 M buffer, pH 6.5) were broken by sonication and the crude homogenate centrifuged at 45 000 *g* for 30 min. A 10 % streptomycin sulfate solution (40 ml) was added to the centrifugate and the precipitate removed by centrifugation. The protein fraction precipitating between 40 and 60 % saturation of ammonium sulfate was collected and re-dissolved in 20 mM sodium-potassium phosphate buffer, pH 7.5. The solution was applied to a column of Ultrogel AcA 44 (2.5 cm × 100 cm) that was eluted (15 ml h^{-1}) with 20 mM sodium-potassium phosphate

buffer, pH 7.5. Fractions containing α-glucosidase activity were pooled and adsorbed to a column of DEAE-cellulose (Whatman DE 32, 2.5 cm × 45 cm) previously equilibrated with 20 mM sodium-potassium phosphate buffer, pH 7.5. Elution (50 ml h^{-1}) was performed using a linear gradient formed from 1000 ml of 20 mM and 1000 ml of 0.3 M sodium-potassium phosphate buffer, pH 7.5. The collected fractions were assayed for α-glucosidase activity and two active peaks were distinguished (peak I and peak II). Both peaks were concentrated in a Diaflo pressure filtration unit using filter PM 30 and the buffer concentration was finally reduced by dilution to about 20 mM. Only peak II containing the main α-glucosidase activity was purified further. It was adsorbed to a column of a mixture of 80 % hydroxylapatite, 15 % DEAE-cellulose and 5 % Sephadex G-25 (1.5 cm × 30 cm) equilibrated with 20 mM sodium-potassium phosphate buffer, pH 7.5. Elution (25 ml h^{-1}) was performed with a linear gradient formed from 200 ml of 20 mM and 200 ml of 0.3 M sodium-potassium phosphate buffer, pH 7.5. The enzymatically active fractions were pooled and concentrated by pressure filtration in a Diaflo unit, using filter PM-30. The concentration of the buffer was diluted to about 20 mM. The concentrated enzyme solution was adsorbed to a column of hydroxylapatite containing 10 % cellulose (Whatman CF 11, 1 cm × 15 cm). α-Glucosidase was eluted (15 ml h^{-1}) with a linear gradient formed from 250 ml of 20 mM and 250 ml of 0.3 M sodium-potassium phosphate buffer, pH 7.5. Fractions containing enzyme activity were pooled. The solution was concentrated by pressure filtration and the buffer concentration diluted to about 20 mM. The enzyme obtained was stored at −20 °C in 10 % glycerol.

Determination of protein. Protein was determined by the method of Lowry *et al.*[8] The elution profiles of protein in column chromatography was followed by measuring the absorbance of each fraction at 280 nm.

Disc gel electrophoresis. Disc gel electrophoresis[9] of 75 μg of the enzyme was carried out in polyacrylamide gels which contained 7.5 % polyacrylamide. The gels were stained for protein in 0.05 % Coomassie Brilliant Blue and for enzyme activity in a 2.2 mM solution of *p*-nitrophenyl α-D-glucopyranoside (PNPG) in a 0.05 M sodium-potassium phosphate buffer, pH 6.5. The enzyme was analysed by sodium dodecylsulfate disc gel electrophoresis according to Weber and Osborn[10] in order to determine the molecular weight of possible subunits.

Determination of molecular weight. The molecular weight of the isolated enzyme was determined by thin-layer gel filtration using Sephadex G-200 "superfine".

Determination of isoelectric point. The isoelectric point was determined by the LKB 8100 Electrofocusing column using Ampholine pH 4 – 6.

Fluorescence experiments. A Perkin-Elmer 512 fluorescence spectrophotometer thermostated at 20 °C and equipped with quartz cuvettes was used. When the binding of TNS to the α-glucosidase was studied the excitation wavelength was 340 nm, and emission was measured at 440 nm. All measurements were performed at pH 6.5 in 0.05 M sodium-potassium phosphate buffer. Concentrations of other components are stated separately in the figure texts. The ligand was added from a micro syringe to 2.0 ml of buffer already pipetted into the quartz cuvette.

RESULTS AND DISCUSSION

Enzyme properties. A summary of the purification procedure is given in Table 1. Two enzymatically active peaks were separated by

Table 1. An outline of the purification procedure. The α-glucosidase activity and protein were determined as described in Materials and Methods.

Step	Volume/ ml	Units [a]	Protein/ mg	Units/ mg protein	Degree of purification	Recovery/ %
Crude extract	218	3382	33787	0.1	1	100
Streptomycin	200	2633	1980	1.3	13	78
$(NH_4)_2SO_4$ precipitate	21	3491	1334	2.6	26	103
Ultrogel	120	2873	924	3.1	31	85
DEAE-cellulose						
peak I	17	102	15	6.8		
peak II	22	1814	174	10.4	104	54
Mixed column	23	1072	25	42.8	428	32
Hydroxylapatite	11	691	7.2	95.9	959	20
Concentrate	1.4	559	5.1	109.6	1096	17

[a] One unit of enzyme activity is defined as 1 μmol PNPG split per minute.

DEAE-cellulose chromatography. In a preliminary report on the purification of α-glucosidase from yeast we described two active peaks which catalysed the hydrolysis of maltose, α-methylglucoside and PNPG.[11] Khan and Eaton [12] also found two enzymes that catalysed the hydrolysis of PNPG but one was specific for maltose and the other for α-methylglucoside. Besides, Van Wijk [13] described both an inducible and a constitutive α-glucosidase synthesis in yeast. In the present study only peak II which contained the main α-glucosidase activity was purified further and characterized. The protein pattern of the polyacrylamide disc gel electrophoresis of the isolated enzyme showed one distinct sharp band and three very faint ones. The yellow band of *p*-nitrophenol in gels stained for enzyme activity coincided with the site of the sharp protein band. On the basis of visual inspection of the intensity of the protein bands the enzyme was estimated at least 95 % pure. The K_m for maltose and PNPG was determined to 2.5×10^{-2} and 2.9×10^{-4} M, respectively. This is in agreement with values previously reported.[14-15] The isoelectric point of the enzyme was at pH 5.4. The molecular weight was approximately 44 000 daltons as determined by thin layer gel filtration. In gel-chromatography on Ultrogel AcA 44 or Sephadex G-150 the α-glucosidase was, however, eluted close to the void volume of the column. This indicates that the molecular weight of the enzyme in the crude extract was considerably higher than 44 000 daltons or that the enzyme in the extract was associated with some other material. A similar phenomenon was observed when α-glucosidase was purified from *Bacillus subtilis*.[16] By chromatography on a Sephadex G-100 column Khan and Eaton [12] obtained the molecular weight of 68 500 daltons.

In sodium dodecylsulfate disc gel electrophoresis the isolated enzyme showed one strong and one faint band of molecular weights of 10 500 and 20 000 daltons, respectively. The isolated α-glucosidase thus seemed to consist of four subunits. The polypeptide with a molecular weight of about 20 000 daltons might represent a dimer.

Binding of glucose to the enzyme. When TNS dissolved in water was added to the cuvette containing buffer a weak fluorescence was observed with a maximum emission at 500 nm.

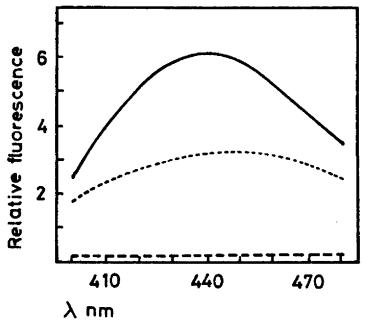

Fig. 1. Emission spectra for TNS in buffer (- - -), for α-glucosidase – TNS complex (. . .) and for α-glucosidase – TNS – glucose complex (———) in 0.05 M sodium-potassium phosphate buffer pH 6.5 at 20 °C. Excitation wavelength was 340 nm. The concentrations were 0.015 μM TNS, 0.2 μM enzyme and 1.3 mM glucose.

When α-glucosidase was added the fluorescence increased strongly with a maximum emission at 450 nm (Fig. 1). Addition of glucose to the solution containing enzyme and TNS caused a 100 % increase in fluorescence accompanied by a shift of the emission maximum to a shorter wavelength (440 nm). The shift of the wavelength for maximum emission suggests that binding of glucose to the enzyme is associated with a conformational change of the enzyme-TNS complex.

The reaction between α-glucosidase and TNS was reversible and followed a typical saturation

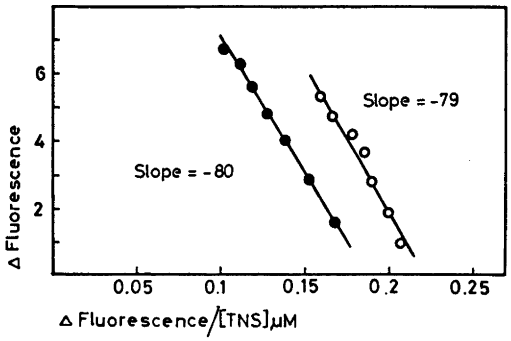

Fig. 2. Determination of the dissociation constant of the complex between TNS and α-glucosidase according to Benesi and Hildebrand [17] in the absence (●) and presence (○) of 1.0 mM glucose. Wavelengths were 340 nm for excitation and 440 nm for emission. The concentration of α-glucosidase was 0.2 μM.

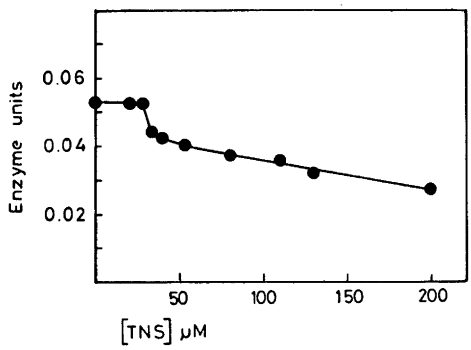

Fig. 3. The α-glucosidase activity as a function of TNS concentration. The activity was measured as described in Materials and Methods.

function when the concentration of TNS was varied. The data obtained were plotted according to the method of Benesi and Hildebrand [17] (Fig. 2). The dissociation constant (8×10^{-6} M) was calculated from the slope of the line according to the method of least squares. The stability of the enzyme-TNS complex was also tested in the presence of glucose (Fig. 2). The dissociation constant in the presence of glucose was 7.9×10^{-6} M. This indicates that the binding of glucose does not affect the stability of the enzyme-TNS complex and that TNS does not bind to the active site of the enzyme. However, when the α-glucosidase activity was measured by following the rate of hydrolysis of PNPG in the presence of varying amounts of TNS, concentrations above 27 μM were inhibitory (Fig. 3). TNS concentrations above 27 μm obviously affected the α-glucosidase activity though no change in the dissociation constant for the enzyme-TNS complex was observed when glucose was added.

In order to determine the dissociation constant of the α-glucosidase – glucose complex a solution containing enzyme and 0.02 mM TNS was titrated with glucose (Fig. 4). Although glucose has to be considered as a substrate for the α-glucosidase saturation was reached at a low concentration of glucose which indicates that the synthetizing reaction does not influence the estimation. ΔG of the synthesis is unfavorable and very high concentrations of glucose were required.[2] The dissociation constant for the α-glucosidase – glucose complex was evaluated from the increase in fluorescence using the method of Benesi and Hildebrand [17] and was found to be 0.57×10^{-3} M (Fig. 4). Evidently, glucose is bound to the enzyme at a much lower concentration than that required to reverse the reaction. This is consistent with the finding that glucose is a competitive inhibitor of α-glucosidase [1] with K_i values from 1.2×10^{-3} to 0.4×10^{-3} M.[14–15] Laid and Axelrod [2] showed that the competitive inhibition by glucose is independent of its anomeric form although only the α-anomer is utilized for the synthetic reaction. Fink and Good [5] presented the following scheme for the β-glucosidase reaction

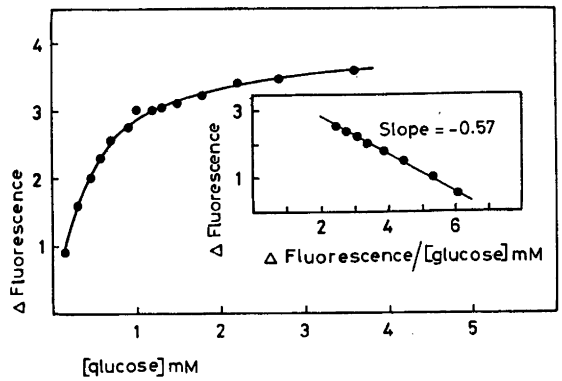

Fig. 4. Increase in fluorescence of α-glucosidase – TNS complex upon binding of glucose as a function of concentration. The data in the inset were plotted according to Benesi and Hildebrand [17] for evaluation of the dissociation constant. The concentrations were 0.015 mM TNS and 0.2 μM enzyme. Wavelengths 340 nm for excitation and 440 nm for emission.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} \underset{+\ P_1}{EG} \xrightarrow{k_3} E + P_2$$

where ES = Michaelis complex, EG = glucosyl–enzyme, P_1 = *p*-nitrophenol and P_2 = glucose, and in which $k_3 \ll k_2$. The results indicate that the mechanism could also apply to α-glucosidase. Obviously the binding of glucose to form a glucosyl–α-glucosidase intermediate occurs with relatively high affinity as compared to the binding of aglycone the concentration of which has to be high compared to that of glucose before the reaction is reversed.

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