Enzymatic Determination of Anomers of Glucose Released by Glucosidases

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The determination of the anomers of glucose is of special interest in connection with the reaction mechanism of glucosidases. The present method can be used for glucosidases which are inhibited by tris (2-amino-2-hydroxymethylpropane-1,3-diol) or mercuric chloride. The method is based on the ability of glucose oxidase to catalyse the oxidation of β-glucose, but not of α-glucose, to gluconic acid, the α-glucose being determined after the oxidation of the β-anomer.[1]

A mutarotated solution of maltose contains 39 % of α-maltose and 61 % of β-maltose. If an α-glucosidase releases the non-reducing glucose unit from maltose as α-glucose, 70 % of the glucose will be α-glucose, neglecting the mutarotation of the glucose liberated. If on the other hand the α-glucosidase releases the glucose unit as β-glucose, 20 % of the glucose will be the α-anomer. It is assumed that a glucosidase will release the non-reducing glucose unit either as α- or as β-glucose. As mutarotation cannot be completely avoided the results found will be lower than 70 % or higher than 20 %, respectively. A glucose solution brought to the mutarotational equilibrium contains 37.4 % of α-glucose. If the glucosidase releases the glucose from maltose as the α-anomer, the α-glucose content in a hydrolysed maltose solution will be found to be between 70 % and 37.4 % of the total amount of glucose in the reaction mixture. If the glucose is released as the β-anomer, the α-glucose content will be between 20 % and 37.4 % of the total amount of glucose.

For the hydrolysis of other glucosides similar calculations can be made. If the glucosidase hydrolyses methyl glucoside, this substrate is even better for the purpose. Phenyl glucoside cannot be used since phenol interferes with the glucose oxidase reagent.

A malt α-glucosidase[2] was incubated with 2 mM mutarotated maltose for 5 min and the total amount of glucose (α + β) was found to be 17.2 μg/0.5 ml. The amount of α-glucose released was 9.3 μg/0.5 ml or 54 % of the total amount of glucose, which shows that malt α-glucosidase releases the glucose unit from maltose as α-glucose. A similar result was found with maltotriose as substrate.

The reaction time for the glucosidase should be as short as possible (≤ 5 min) to reduce the mutarotation of glucose and for the same reason the pH should be about 4.5. If the glucosidase contains too much mutarotase, it is often possible to inhibit the mutarotase by xylitol.[1]

Procedure: Reagent I: Dahlqvist's tris/glucose oxidase/peroxidase reagent.[3,2] Reagent II: Tris/glucose oxidase reagent: 50 mg glucose oxidase (Sigma, type II) dissolved in 20 ml 0.5 M tris buffer pH 7.0.

At zero time 200 μl suitably diluted α-glucosidase solution is added to 300 μl 3.33 mM maltose in 0.02 M acetate buffer, pH 4.6 at 37°C. (The amount of enzyme is adjusted to release 15-20 μg glucose in 0.5 ml of the mixture in 5 min). After a reaction time of 5 min 500 μl of reagent II is added with careful mixing, and the reaction mixture is incubated for 15 min at 0°C. The reaction mixture is now placed for 1 min in a boiling water bath to inactivate the glucose oxidase and to expel H_2O_2. The remaining glucose (α-glucose) in the reaction mixture is then determined as by Dahlqvist by addition of 3 ml reagent I, and suitable glucose standards are run simultaneously. The total amount of glucose (α- + β-glucose) released by the α-glucosidase is determined by running a similar reaction, in which 500 μl boiled reagent II is added instead of reagent II.

The malt α-glucosidase is totally inhibited by the tris in reagent II. If the glucosidase is not inhibited by this reagent, it is often possible to inhibit the glucosidase by addition of 2 mg mercuric chloride at the same time as reagent II. Glucose oxidase is not inhibited by mercuric chloride in this concentration.

The amount of α-glucose at the mutarotational equilibrium in the reaction mixtures can be determined by addition of 400 μl 0.5 M tris buffer, pH 7.0, instead of reagent II. After boiling for 2 min, 100 μl of a solution with 5 times as much glucose oxidase as reagent II is added and the procedure is continued as above. Several determinations showed 37 % of α-glucose in agreement with the calculation.

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The Peroxidase-Catalyzed Oxidation of Thyroxine

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The oxidation of thyroxine (T_4) and related *o*-iodophenols by H_2O_2 is catalyzed by horseradish peroxidase,[1-4] myeloperoxidase,[1,5] and peroxidase-like activity in the livers of various animals.[6] When T_4 is oxidized, I^- and diiodotyrosine have consistently been identified among the products [1,4], but little else is known about this reaction. A further study was therefore undertaken.

Experimental. A commercial (Worthington) crystalline horseradish peroxidase was used. Reactions were carried out at 25° in 0.05 M Tris (pH 9) buffer, except where otherwise stated. The choice of the relatively high pH was due to the extreme insolubility of T_4 at neutral pH. Isosbestic points were observed with the reacting system 10^{-4} M T_4, 1.33×10^{-4} M H_2O_2, and 2×10^{-8} M peroxidase in the sample cell and buffer as the only solute in the reference cell. Consecutive spectra were recorded in the range 260−390 mμ at 2 min intervals for a period of 14 min. In kinetic and titration experiments the oxidation of T_4 and related compounds was followed spectrophotometrically at the wavelengths given in Table 1. The effect of the hydrogen ion concentration on the rate of T_4 oxidation was studied by making rate measurements at the pH values 9.00, 9.49, 10.00, and 10.45 in 0.05 M glycine buffer with 10^{-4} M T_4, 10^{-4} M H_2O_2 and suitable peroxidase concentrations in the reacting solution. When the reaction products were to be analyzed, the oxidation system used contained 10^{-4} M T_4, 10^{-4} M H_2O_2, and 5×10^{-8} M peroxidase. In some cases labeled [3′,5′-^{131}I] thyroxine was used. The reaction was stopped after 10 min by adding catalase to a final concentration of 50 μg/ml. Labeled I^- was separated from other products and remaining T_4 by paper electrophoresis in 0.05 M Tris (pH 9) buffer. Labeled T_4 and diiodotyrosine were separated from other products by electrophoresis in 4 M acetic acid. Formation of non-labeled diiodotyrosine from labeled T_4 was confirmed with previously described [7] chromatography methods. Labeled products on paper strips were quantitated with scanning equipment. A chemical method [8] was also used for the determination of I^- in the reaction mixture. In a spectrophotometric titration experiment graded doses of H_2O_2 were added to a system containing 10^{-4} M T_4 and 10^{-7} M peroxidase.

Table 1. Spectrophotometrically determined rate constants (k) for the oxidation of T_4 and related compounds. The rate constant is defined by eqn. (1). Measurements were performed at the wavelengths (λ) given. The proportionalities between absorbance change and consumption of H_2O_2 ($\Delta A/\Delta[H_2O_2]$) were established by measuring the absorbance changes caused by the addition of graded doses of H_2O_2 to systems containing oxidizable substrate and peroxidase. The proportionalities were used in the calculation of rate constants.

Oxidizable substrate	λ (mμ)	$\Delta A/\Delta[H_2O_2]$ (M^{-1})	k ($M^{-1} \times min^{-1}$)
L-Thyronine	310	−3900	9×10^7
L-Thyroxine (3,5,3′,5′-tetraiodo-L-thyronine)	327	3700	9×10^6
3,5-Diiodo-L-thyronine	320	−3900	4×10^6
3,5,3′-Triiodo-L-thyronine	350	−3100	3×10^6
L-Tyrosine	300	−2400	1×10^5
3,5-Diiodo-L-tyrosine	313	2400	9×10^4
3-Iodo-L-tyrosine	330	−2200	8×10^4