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Note

A new amyloclastic method for the selective determination of α-amylase using cross-linked amylose as an insoluble substrate

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The amylolytic activity of amylase can be determined by amyloclastic (iodometric)[1], reductometric[2], chromogenic[3], and multienzyme (kinetic)[4] methods. The method presented herein is amyloclastic in nature and is based on the use of artificially cross-linked amylose as a specific substrate for α-amylase only and not for exoamylases (β-amylase and glucoamylase)[5–7]. By acting on the cross-linked (insoluble) amylose, α-amylase liberates soluble polysaccharide chains. These chains are of sufficient size to form inclusion complexes with iodine.

EXPERIMENTAL

Crystalline bacterial α-amylase and potato β-amylase were obtained from Sigma, Chemical Co., amylose was obtained from Koch–Light and Sigma, and the iodine–potassium iodide reagent from Reactivul Bucureşti. Cross-linked amylose (Amylose X-10, Amylose X-15, Amylose X-20, Amylose X-40, and Amylose X-60)* was prepared by treating amylose (100 g) with 5M sodium hydroxide (300 mL) and with 1-chloro-2,3-epoxypropane (epichlorohydrin) (10, 15, 20, 40, and 60 g, respectively), at 0–5°, followed by heating for 1 h at 40–45°, and 2–3 h at 70–75° as described[8,9]. Before being used as substrates, each of the cross-linked amylose preparations were incubated for 48 h at 20° with β-amylase (5 mg). This removed any free amylose chains located outside of the glycerol-interchain bridges[5,6]. The treated amylose was finally dried with acetone[10,11].

In the assay, cross-linked amylose (60 mg) was added to 50mM phosphate buffer, pH 6.9, containing 0.5mM calcium chloride and 50mM sodium chloride (2 mL) at 37°, and allowed to swell for 5 min. The enzyme solution (1 mL), also at 37°, was added, and the mixture incubated for 3 min. The reaction was stopped by ad-

*The letter X indicates that the amylose is cross-linked and the number corresponds to the amount in g of reagent used to cross-link 100 g of amylose.

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ding M hydrochloric acid (1 mL) and 2mM iodine–potassium iodide solution (6.0 mL). The absorbance of the supernatant solution was measured at 570 nm and compared to that of a control sample that contained distilled water instead of the enzyme solution.

The iodometric[1], dinitrosalicylic[2], and the Phadebas[3] methods were used for comparison with the proposed method. The unit of enzyme is the amount of enzyme producing the equivalent of 1 mg of starch hydrolyzed per min. The relationship between increase of absorbance (ΔA/min) and enzyme units was found by analyzing the same α-amylase samples with the iodometric method[1].

RESULTS AND DISCUSSION

A relationship was established between the various degrees of cross-linking and the enzymic hydrolysis (Fig. 1). At all concentrations of enzyme tested, the amylose having the smallest degree of cross-linking produced the highest reaction velocity. Those amyloses having a high degree of cross-linking (Amylose X-40 and Amylose X-60) were found to be very poor substrates for α-amylase. CL-amylose itself (control) is not able to form inclusion compounds with iodine. By use of the most suitable Amylose X-10 as substrate, it was found that 2mM iodine solution gave the most sensitive analysis (Fig. 2). The limit of detection with the proposed method (10 ng of α-amylase) is of the same order as for chromogenic methods and below the detection limits of the iodometric and reductometric methods.

Under the experimental conditions described herein, the α-amylase activity can be calculated as follows:

$$\text{Enzyme activity} = 4.46 \times \Delta\text{A/min/mL [mg of starch hydrolyzed/min/mL]}$$

$$\text{Specific activity} = 4.46 \times \Delta\text{A/min/mg of protein}$$

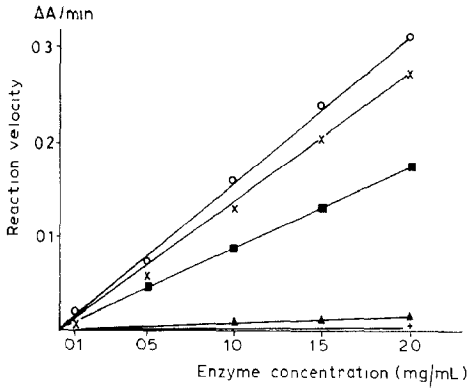

Fig. 1. Influence of the reticulation degree on the hydrolysis rate of cross-linked amylose: (○—○) Amylose X-10, (×—×) Amylose X-15, (■—■) Amylose X-20, (▲—▲) Amylose X-40, and (+) Amylose X-60.

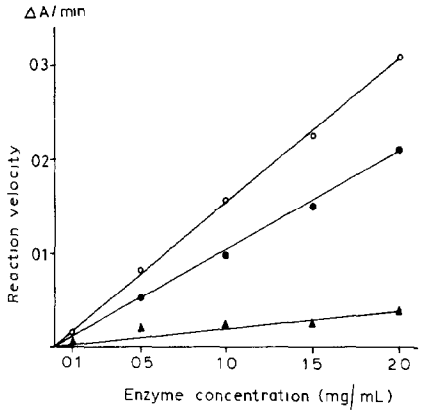

Fig. 2. Influence of the iodine concentration on the sensitivity of the method: the following concentrations were used: (○—○) 2mM, (●—●) 0.5mM, and (▲—▲) 50μM.

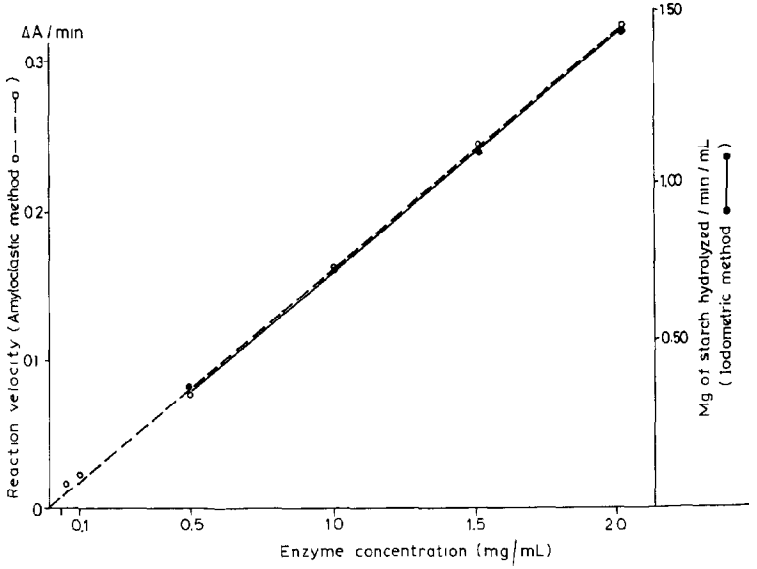

Fig. 3. Influence of the concentration of α-amylase on the rate of hydrolysis, and correlation of the amyloclastic method (○—○) with the iodometric method[1] (●—●).

The constant 4.46 was found by comparing the increase of absorbance (ΔA/min) determined by the new amyloclastic method and the number of enzyme units obtained, for the same α-amylase samples, with the iodometric method[1] (Fig. 3).

The decrease in reaction velocity (measured as ΔA/min) as a function of the increasing degree of cross-linking (Fig. 1) may be explained in two ways: (a) the cross-linking of Amylose X-10 allows access of the enzyme to the interior of the CL-amylose, whereas this access is hindered for amyloses having a higher degree of cross-linking. (b) The soluble polysaccharide chains released from amylose of low degree of cross-linking may be sufficiently large to form ideal inclusion complexes with iodine, whereas this is not the case for amyloses having a high degree of cross-linking.

The proposed method has much in common with the chromogenic ("Phadebas") method. Both use insoluble substrates [β-limit amyloses (starch)] that are not susceptible to attack by β-amylase or β-D-glucosidase, and in both cases the enzymic reaction is followed by the release of soluble polysaccharide fragments. In the proposed method, these fragments are determined by the iodine reaction. Unlike the classical iodometric methods, the reaction is followed by an increase in iodine color. The polysaccharide fragments released are sufficiently large and in suitable conformation to allow the formation of iodine-inclusion complexes. For the CL-Amylose X-10 used, the release of the soluble polysaccharide chains was linear for the concentration of enzyme tested (Fig. 3). This novel form of iodometric analysis, where the color yield increases as a result of the action of α-amylase, may have an application to the selective analysis of α-amylase. Under certain circumstances, the sensitivity of the method (10 ng) may make it the method of choice. Some preliminary clinical analyses with this new amyloclastic method were performed. The α-amylase level in human normal serum determined by the chromogenic method using a Phadebas substrate was 295 $\pm$3 U/L, and 295 $\pm$10 U/L as determined by the proposed amyloclastic method using amylose X-10 as substrate.

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