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STUDY OF THE OPERATION OF CO-IMMOBILIZED GLUCOSE-6-PHOSPHATE ISOMERASE AND GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN A FLOW INJECTION SYSTEM

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Abstract

Glucose-6-phosphate isomerase and glucose-6-phosphate dehydrogenase were co-immobilized covalently on Akrilex C 100, a polyacrylamide bead polymer possessing carboxylic functional groups, using a water-soluble carbodiimide for activation of the support. The activities of the immobilized isomerase and dehydrogenase were 110.8 U/g solid and 48.4 U/g solid, respectively.

The immobilized enzymes were packed in a reactor (3 ml), the enzyme reactor was inserted into the flow injection system, and the operation was studied with glucose-6-phosphate and fructose-6-phosphate as substrates. A linear relationship was observed between the substrate concentration and the peak in the concentration range 0.2-1 mM for both substrates. The dependence of the peak area on the sample volume and the flow rate was also linear. The immobilized enzymes exhibited good operational stability during operation for more than 5 months. In a coupled system with hexokinase, the applicability of the co-immobilized enzymes for the determination of fructose and glucose in different wines and fruit juices was demonstrated.

Key words: co-immobilized glucose-6-phosphate isomerase and dehydrogenase, flow injection analysis, co-determination of fructose and glucose

Introduction

Enzymatic analysis is highly sensitive and specific and is of increasing importance in practical applications. If the enzymes are immobilized, they can be repeatedly employed for many analyses (LOWE, 1985; HO, 1988). Much attention has recently been devoted to the determination of biologically important substances and food components through the use of biospecific sensors or analysers. Flow methods such as flow injection analysis can readily be automated and they have therefore become important (RUZICKA and HANSEN, 1981; LUNDBACK and OLSSON, 1985).

Enzymes attached covalently to a polyacrylamide support containing carboxylic functional groups have been found to have advantageous properties for both preparative processes and analytical measurements (SZAJÁNI et al., 1980; KOTORMAN et al., 1991; SIMON et al., 1992).

Glucose-6-phosphate dehydrogenase and glucose-6-phosphate isomerase are widely used in clinical chemistry and food analysis for the enzymatic determination of $NADP^+$ and hexose phosphates, and for the determination of enzyme activities (phosphoglucomutase and hexokinase). Glucose-6-phosphate dehydrogenase was previously immobilized for NADPH production. (SIMON et al., 1994). The present paper reports on the co-immobilization of glucose-6-phosphate dehydrogenase with glucose-6-phosphate isomerase. The operation of these enzymes in a flow injection system was studied.

Materials and Methods

Materials

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase, EC 1.1.1.49) was isolated from bakers' yeast according to NOLTMAN et al. (1961). The specific activity of the enzyme was 2.6 U/mg protein. Glucose-6-phosphate isomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9) was isolated from rabbit muscle (Noltman, 1966). The specific activity of the enzyme was 110 U/mg protein. Akrilex C 100, a polyacrylamide bead (100-320 μm) polymer containing carboxylic functional groups (6.4 mequiv./g xerogel) was a commercial product of Reanal (Budapest, Hungary). 1-Cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-4-toluenesulfonate was purhased from Fluka (Buchs, Switzerland). All other chemicals were commercial, reagent grade preparations (Reanal).

Immobilization

The covalent attachment of glucose-6-phosphate dehydrogenase and glucose-6-phosphate isomerase to Akrilex C was carried out according to SZAJÁNI et al. (1980).

Measurement of protein

Protein determination was performed as described by LOWRY et al. (1951). The amount of immobilized protein was determined indirectly from the difference between the amount of protein introduced into the coupling reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

Assay of enzyme activities

The glucose-6-phosphate dehydrogenase activity was measured in 0.1 M triethanolamine buffer (pH 7.6) containing 1.6 mM glucose-6-phosphate, 0.37 mM $NADP^+$, 6.7 mM $MgCl_2$ and 0.3 U/ml glucose-6-phosphate dehydrogenase. The reaction was initiated by the addition of enzyme. The rate of reaction was calculated from the increase in absorbance at 340 nm at 25°C. The activity of the immobilized enzyme was measured in the same reaction mixture, as follows: 100 mg of immobilized enzyme was suspended and swollen in 5 ml reaction mixture. The suspension was continuously stirred with a mechanical stirrer for an appropriate time (usually 1-10 min) at 25°C. The immobilized enzyme was than filtered off quickly and the amount of NADPH was determined at 340 nm. One unit of enzyme activity (U) was defined as the amount of enzyme that catalyses the formation of 1 μmol of NADPH per minute at 25°C.

The activities of the soluble and immobilized glucose-6-phosphate isomerase were determined by measuring the rate of transformation of glucose-6-phosphate to fructose-6-phosphate as described by ROE (1934). One unit of enzyme activity was defined as the amount of enzyme which catalyses the formation of 1μmol of fructose-6-phosphate per minute at 30°C.

Analytical system

In the analytical measurements, LKB 2238 Uvicord S II equipment (Broma, Sweden) with a flow-through cell (10 μl) was used to monitor the changes in absorbance at 365 nm. The output signal was displayed on an OH-814/1 recorder (Radelkis, Budapest, Hungary). The carrier stream was pumped with an LKB 2132 microperpex peristaltic pump.

Wine and juice analysis

The samples were diluted 50-700-fold to obtain appropriate glucose/fructose concentrations for measurement. A 2 ml aliquot was taken, containing 2.7 mM ATP, 6.7 mM $MgCl_2$ and 10 U hexokinase was added. After standing for 20 min at room temperature, the samples were diluted 1:1 with 10 mg/ml $NADP^+$ and analysed in a flow injection system.

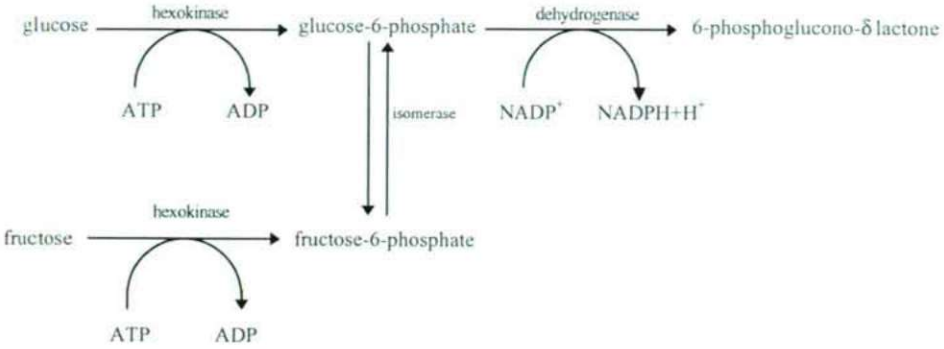
Results

For the covalent immobilization of glucose-6-phosphate dehydrogenase and isomerase enzymes Akrilex C 100 support was used. The enzymes were co-immobilized on the same support under optimized conditions. The catalytic activity of the immobilized glucose phosphate isomerase was 110.8 U/g xerogel, and that of the dehydrogenase was 48.4 U/g xerogel. A 3 ml bed reactor (isomerase activity: 3.5 U, dehydrogenase activity: 2.1 U) was introduced into a flow injection system. The carrier stream was composed of 100 mM triethanolamine buffer, pH 8.0, 5 mM $MgCl_2$ and 2 mM $NADP^+$. The glucose-6-phosphate and fructose-6-phophate concentrations varied in the range 0.2-1 mM. Conditions affecting the operation of this two-enzyme system were studied. The peak shape (A) and the dependence of the peak area on the glucose-6-phosphate concentration (B) are shown in Fig. 1. Up to 1 mM substrate concentration, a linear relationship was observed. Similar results were obtained with fructose-6-phosphate as substrate (Fig. 2). The operation of the two enzymes in co-immobilized form did not need a much longer reaction time than the one-enzyme system. The peak shapes for injection of different volumes of glucose-6-phosphate (A) and fructose-6-phosphate (B) are presented in Fig. 3. The reaction times for measurement in the one- and the two-enzyme systems were the same. The changes in peak shape at different flow rates for the one- (A) and two- enzyme (B) systems are shown in Fig. 4. The best and reproducible results were obtained at the higher flow rates of 80-100 ml/h.

Calibration curves with NADPH standard at different flow rates are shown in Fig. 5. In the concentration range 0.2-1 mM, linear calibration curves were obtained.

During these experiments the operational stability of the enzyme reactor for both enzyme activities was tested (Fig. 6). The enzyme reactor was stable and no measurable decrease in activity could be observed after operation for 5 months. The enzyme reactor was stored at 8 °C in a refrigerator after measurements.

The practical application of this two-enzyme reactor for the determination of glucose and fructose in Hungarian wines and fruit juices was studied. The very low levels of sugar phosphates in wines and juices was under the detection limit of our system, and therefore in a coupled reaction with soluble hexokinase the glucose and fructose amounts were determined. The approach used in this work was based on the following reaction sequences:



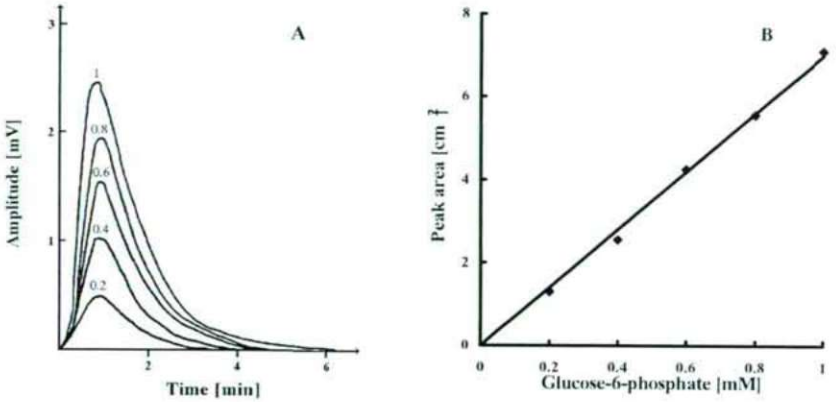


Fig. 1. Peak shapes (A) and change in peak area with glucose-6-phosphate concentration (B). Flow rate: 100 ml/h. Sample volume: 100 µl.

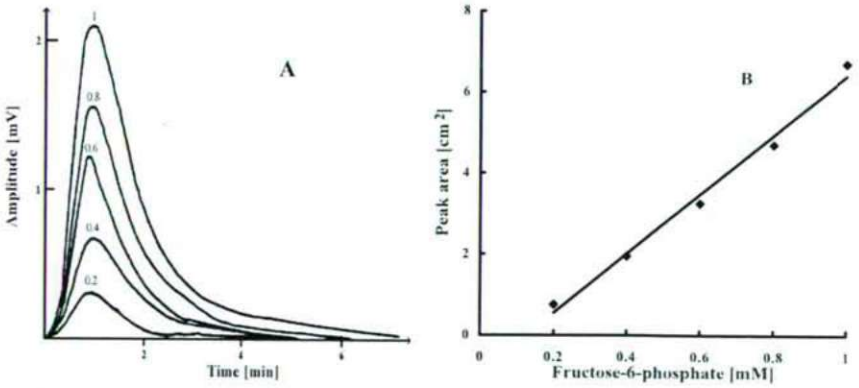


Fig. 2. Peak shapes (A) and change in peak area with fructose-6-phosphate concentration (B). Flow rate: 100 ml/h. Sample volume: 100 µl.

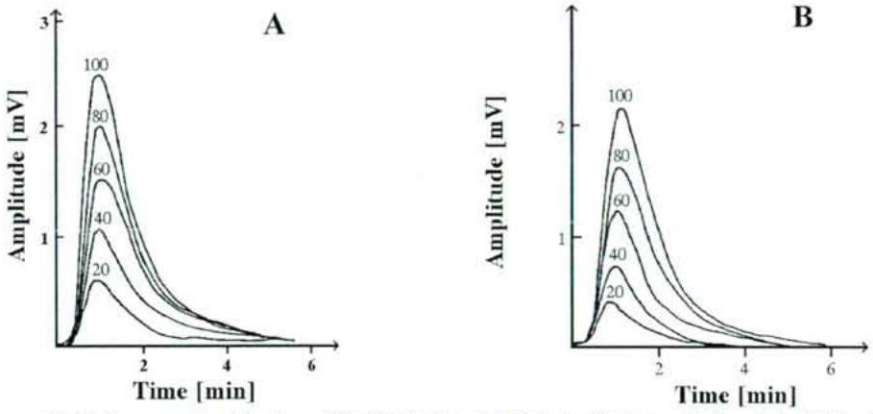


Fig. 3. Responses on injection of 20, 40, 60, 80 and 100 µl. 1 mM glucose-6-phosphate (A), 1 mM fructose-6-phosphate (B). Flow rate: 100 ml/h.

The results summarized in Table 1 are the mean values of 10 measurements. Determinations in the flow injection system were compared with traditional soluble enzyme measurements. The data agreed within a 5% error and suggest the applicability of this system for practical purposes.

Table 1. Co-determination of glucose and fructose in wines and fruit juices with soluble hexokinase and immobilized glucose-6-phosphate dehydrogenase/ isomerase in a flow injection system. The flow rate was 100 ml/h, with 100 µl of sample. (The results are the mean values of 10 measurements.)

	Glucose and fructose (g / l)	
	with soluble enzymes	with immobilized enzymes
Wine brands		
Zengő	14.26	14.07
3 puttonyos tokaji aszu	196.20	187.28
Édes szamorodni	103.44	99.09
Furmint	13.87	13.87
Fruit juices		
Apple	59.25	58.86
Apricot	75.90	76.49
Grape	114.94	112.96

Discussion

Co-immobilized glucose-6-phosphate dehydrogenase and isomerase were prepared on Akrilex C 100, a polyacrylamide bead support. The immobilized catalytic activity was 110.8 U/g for the isomerase and 48.4 U/g for the dehydrogenase.

The operation of the immobilized enzymes in a flow injection system was studied. A linear response was obtained with glucose-6-phosphate and fructose-6-phosphate in

the concentration range 0.2-1 mM. The shapes of the peaks varied with the flow rate of the carrier stream and the sample volume. At a flow rate of 100 ml/h, using 100 µl of sample, the flow injection system operates with the same reaction time for both substrates and the results are reproducible.

The practical application of the two-enzyme reactor in flow injection analysis was investigated. The comparative co-determination of the glucose and fructose contents in wines and fruit juices offers a simple and novel method for routine analysis with the accuracy of classical soluble enzyme measurements.The enzyme reactor displayed good operational stability: an activity decrease was not observed during 5 months of operation.

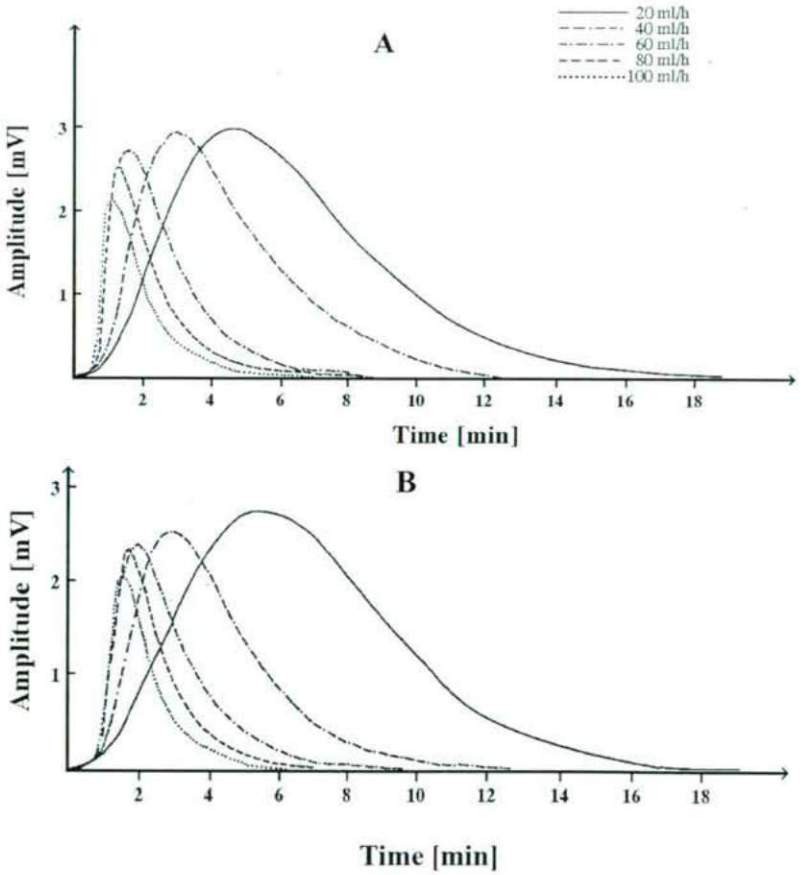


Fig. 4. Responses with flow rates of 20, 40, 60, 80 and 100 ml/h. (A) with glucose-6-phosphate, (B) with fructose-6-phosphate as standards.

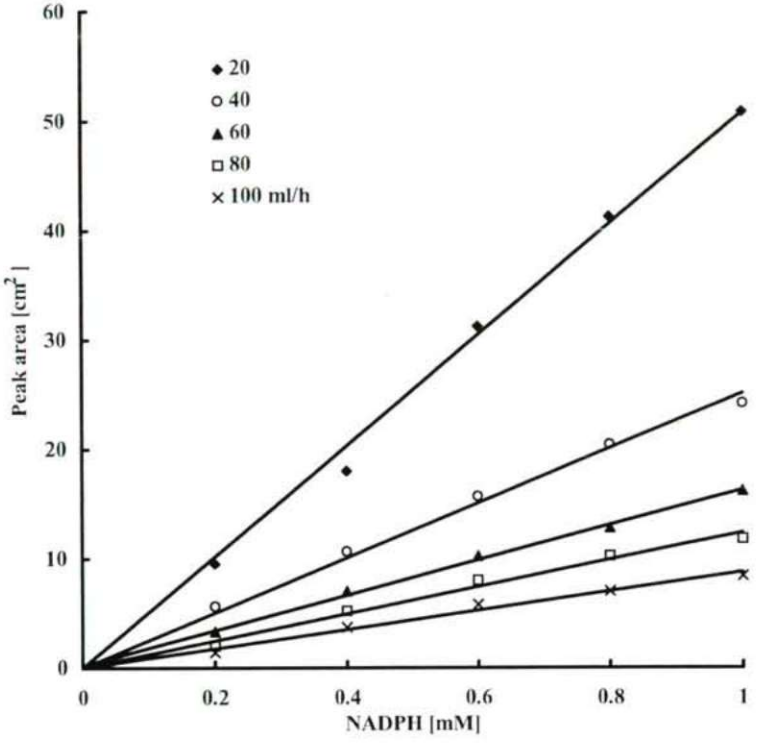


Fig. 5. Peak area as a function of NADPH concentration at different flow rates. Sample volume: 100 µl.

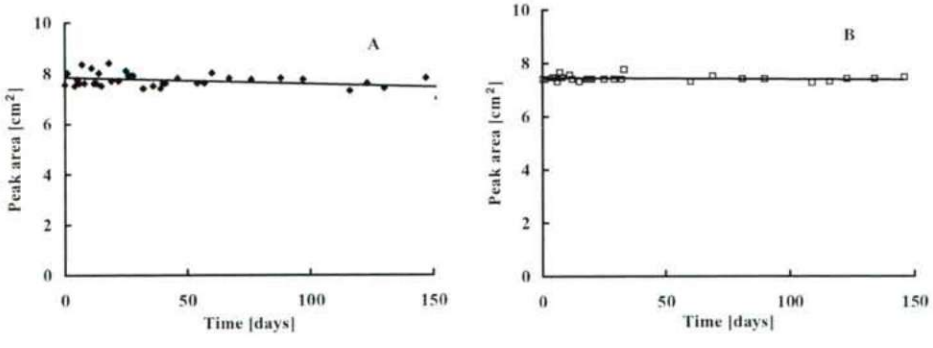


Fig. 6. Operational stability of co-immobilized glucose-6-phosphate dehydrogenase and isomerase bioreactor. Glucose-6-phosphate dehydrogenase activity (A) and isomerase activity (B) with 1 mM glucose-6-phosphate (fructose-6-phosphate) standard solution.

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