EFFECTS OF CARBODIIMIDES COUPLING AGENTS ON THE PROPERTIES OF IMMOBILIZED GLUCOAMYLASES

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Abstract

Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3. 2. 1. 3) produced by *Aspergillus niger* was immobilized on polyacrylamide beads possessing carboxylic functional groups activated by water-soluble carbodiimides bearing different substituents. The most favourable carbodiimide was N-*t*-butyl-N'-dimethyl-aminopropyl carbodiimide methyl iodide. The structure of the carbodiimide was found to influence the pH and temperature dependence of the catalytic activity, and the substrate specificity of the immobilized enzymes.

Key words: glucoamylase, carbodiimides, immobilized

Introduction

Carbodiimides are excellent reagents for the activation of carboxylic groups of supports under mild conditions (WELIKY and WEETALL, 1965; MOSBACH, 1970; MOSBACH and MATTIASSON, 1970). For successful immobilization, the amino acid side-chains essential for the catalytic activity should mostly remain intact during the immobilization. Therefore the reactive sites of the activated support should react with the amino acid side-chains on the enzyme surface, rather than with those of the active site. In the case of an activated support bearing bulky reactive groups of the 0-acyl-isourea type, access to the active site hole is sterically hindered

and, consequently, the undesirable reactions become inhibited. On the basis of the structural characteristics of an enzyme to be immobilized, the most adequate carbodiimide can be selected for activation of the support (SZAJÁNI et al., 1991). In the present work, the effects of disubstituted carbodiimides have been studied using glucoamylase, an enzyme of great practical importance in the starch processing.

Materials and methods

Materials: Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3) with a specific activity of 900–1500 units/g protein was produced by *Aspergillus niger*. One unit is defined as the amount of enzyme that catalysed the liberation of 1 g of D-glucose from soluble starch per hour at pH 4. 0 at 60°C. The enzyme was shown to be homogeneous by polyacrylamide gel electrophoresis. Akrilex C-100, a polyacrylamide bead polymer containing carboxylic functional groups (6. 4 meq/g solid), was a commercial product of Reanal Factory of Laboratory Chemicals (Budapest, Hungary). Carbodiimides were synthetized according to JÁSZAY et al. (1987). Soluble starch was purchased from E. Merck GmbH (Darmstadt, Germany). All other chemicals were reagent grade commercial preparations of Reanal.

Immobilization: Akrilex C-100 xerogel (1 g) was suspended and swollen in 50 ml of 0.1 M potassium phosphate buffer (pH 7.5). A water-soluble carbodiimide, in a stoichiometric quantity relative to the carboxylic functional groups located on the support, dissolved in 25 ml of cold (0°C) buffer, was added, with continuous stirring and cooling in an ice bath. After 10 min, 25 ml of enzyme solution containing 0.5g of protein was added, and the pH was adjusted to 7.5. The mixture was incubated at 0–4°C for 48 h, with two 6-h periods of agitation. The gel was filtered by suction and successively washed three times with 100 ml of buffer, three times with 100 ml of buffer containing 1.0 M sodium chloride and three times with 100 ml of buffer to remove unbound proteins, and finally, with a large volume of distilled water to remove the buffer ions. The products were lyophilized.

Protein measurements: Protein was determined according to the method of LOWRY et al. (1951) as modified by SCHACTERLE and POLLACK (1973). The amount of immobilized protein was calculated from the difference between the amount of protein introduced into the reaction mixture and the protein present in the filtrate and washing solution after immobilization (SZAJÁNI et al., 1980).

Assay of glucoamylase activity: In the activity testing of the soluble enzyme, the reaction mixture (5.1 ml) contained 40 mg/ml soluble starch (pH 4.0) and 5–12 μg/ml enzyme. After 30–90 min at 60°C , the reaction was terminated by alkali treatment. The control containing only substrate was treated in an identical manner. In the case of immobilized glucoamylases, 1.5–2.0 mg of immobilized enzyme (dry) suspended in 5.0 ml 40 mg/ml soluble starch at the optimum pH for the catalytic activity was stirred for 45–120 min at 60°C. The enzyme was then filtered off quickly (a few seconds) and the concentration of liberated D-glucose was determined by iodometric titration (ERDEY, 1956.).

Thin-layer chromatography: For the thin-layer chromatography of saccharified starches, HPTLC aluminium sheets (20x20 cm) with a silica gel 60F layer were purchased from E. Merck. The sheets were developed in a Chrompress 10 pressurized ultramicro chamber (Labor MIM, Budapest, Hungary). The concentration of the saccharified starches and the standard was 0.1% (w/v) in water. Each spot represents about 3 μg of carbohydrate. The chromatoplates were developed in an acetonitril-water 1:2 (mol/mol) solvent system. The development required 20–25 min at room temperature. The membrane pressure was 1.2 Mpa. After drying at 80°C for 5 min, the carbohydrates were identified with a staining reagent composed of 50 ml of solution A+50 ml of solution B Solution A contained 2 g of diphenylamine and 2 ml of aniline in 50 ml of acetone, and solution B 10 ml of phosphoric acid (85%) diluted to 50 ml with deionized water.

Results and discussion

As regards the activity on a dry weight basis, the application of N-*t*-butyl-N'-dimethylaminopropyl carbodiimide methyl iodide (BDAPI) as coupling agent proved to be most favourable, but the highest activities on a protein basis were experienced for BDAPI and N-ethyl-N'-dimethylaminopropylcarbodiimide methyl chloride (EDAPC).

Table 1. Effects of carbodiimides as coupling agents on catalytic activity of immobilized glucoamylases

Carbodiimide	activity on a dry weight basis (unit/g)	activity on a protein basis (%)
BDAPI	41. 6	10. 0
CMC	17. 7	5. 0
EDAPC	13. 5	13. 0

[a] The activity of the soluble enzyme was taken as 100 %.

pH dependence of catalytic activity: The pH dependence of the initial rate of hydrolysis of soluble starch by immobilized glucoamylases was studied at the same ionic strength in the pH range from 2.5 to 5.5 (Fig. l). The curves for the immobilized glucoamylases produced with EDAPC and BDAPI as coupling agents were practically identical, with a maximum value at pH 4.5. The immobilized glucoamylase prepared with N-cyclohexyl-N'-morpholinoethyl carbodiimide metho-*p*-toluenesulfonate displayed a somewhat different function, with a maximum at pH 4.0, which is the same as that for the soluble enzyme (SZAJÁNI et. al., 1985).

Dependence of catalytic activity on temperature: The temperature dependence of the activity of the immobilized glucoamylases was studied in the temperature range 40 to 70 oC at the optimum pH for the catalytic activity. Initial velocities were derived from the amount of D-glucose liberated from soluble starch during a 60-min incubation at selected temperatures (Table 2.).

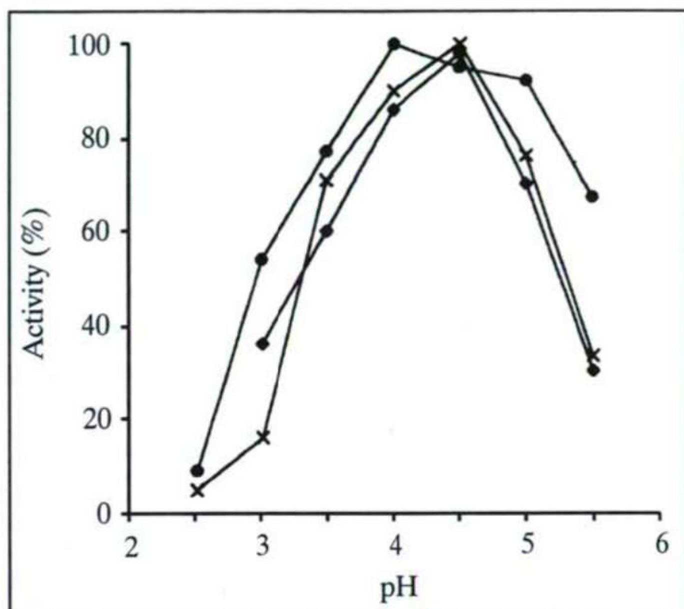

Figure 1 Effect of pH on catalytic activity of immobilized glucoamylases prepared with different disubstituted carbodiimides as coupling agents. Experiments were performed at 60°C. ◆, BDAPI; ●, CMC; x, EDAPC. The maximum activities were taken as 100%.

Table 2. Dependence of catalytic activity of immobilized glucoamylases on temperature.

Temperature (oC)	Carbodiimide		
	BDAPI	CMC	EDAPC
	Activity (%) [a]		
40	54.1	40.9	56.8
50	70.9	77.4	86.6
60	100.0	100.0	100.0
70	27.0	36.4	31.8

[a]The activity measured at 60°C was taken as 100%.

The data indicated that the temperature dependence of the catalytic activity of the immobilized glucoamylases produced by using various disubstituted carbodiimides differed somewhat.

Starch splitting: The catalytic activity of the immobilized glucoamylases was studied with a thinned starch substrate (1.2 g/ml) containing glucose, maltose, maltotriose, maltotetraose and short – chain dextrins. After incubation for 60 h at 60°C, the reaction was stopped by filtration (a few seconds) and the samples were analysed by means of thin-layer chromatography (Fig. 2). Surprisingly, significant differences could be detected reflecting different rates of hydrolysis in the starch splitting patterns. The immobilized glucoamylases produced with EDAPC and BDAPI as coupling agents showed similar splitting patterns, which differed from that of the immobilized glucoamylase produced with CMC, this resembling the splitting pattern of the soluble enzyme. It is presumed that the differences are caused by the different spatial localization of the enzyme molecules in the support matrix as a special effect of the carbodiimide structure resulted in different steric hidrances. Some changes in the mode of action of the immobilized glucoamylases can not be excluded.

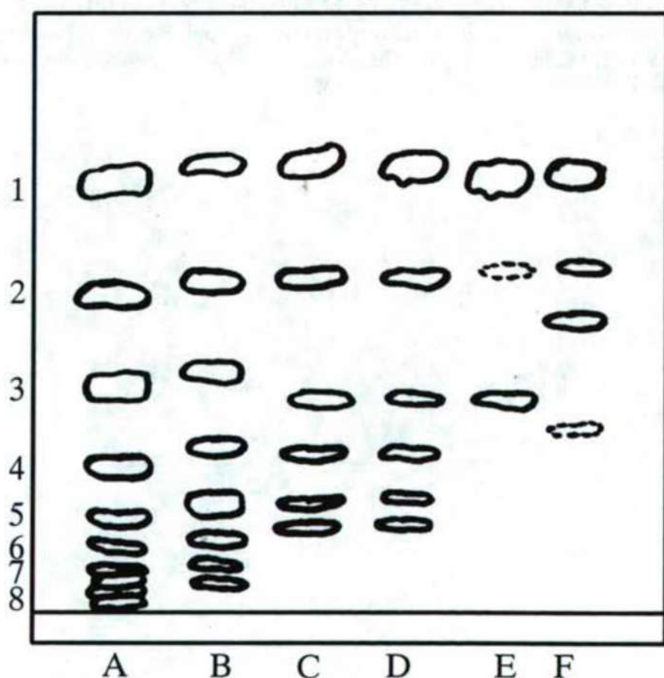

Figure 1 Thin-layer chromatography of starch splitting products. A, Standard; B, thinned starch. Thinned starch processed by glucoamylase immobilized with C, EDAPC; D, BDAPI; E, CMC; F, thinned starch processed by soluble glucoamylase. 1: glucose; 2: maltose; 3: maltotriose; 4: maltotetraose; 5: pentose; 6: hexose; 7: heptose; 8: octose.

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