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Purification of the *Candida utilis* Extracellular Invertase using Affinity Chromatography*1

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ABSTRACT

The extracellular invertase (EC 3.2.1.26) (*Candida utilis*) preparation was obtained from the liquid medium after desalting and freeze drying. This prepared enzyme was used for the comparative purification on 4 activated matrices by liquid column affinity chromatography method. In this method there were used controlled porous glass (CPG) silanized covalently activated by keratin, silanized silica gel and silica gel covalently covered by keratin. It was found that the invertase purification process was better using both CPG matrices (silanized CPG and keratin activated CPG) than these with two silica gel supports. Also the elution coefficient of the invertase from the two CPG columns was about 93 to 94%. Two silica gel supports found to be superior in terms of purification efficiency. The invertase purification process was confirmed by PAGE electrophoresis.

Keywords : affinity chromatography, invertase, *Candida utilis*, invertase purification, keratin, glass beads, silica gel, controlled porous glass, immobilization.

1. INTRODUCTION

Invertases (β-fructosidases EC 3.2.1.26) are the important group of enzymes which are able to hydrolyze not only sucrose but also some other sucrose oligosaccharides. The biosynthesis of invertase by many higher plants and micro-organisms (especially yeasts) is valuable for these organisms *per se* (sucrose metabolism translocation and storage) as well as for biotechnological usage. The invertase enzyme hydrolyzes sucrose into glucose and fructose. This principle of sucrose hydrolysis could be in the industries as sweetener and also as the additive of the bean food during the winter season. Therefore demand for the invertase enzyme in the biotechnological industry is huge. There are many reports on purification of invertase using conventional chromatographical method (Asthir and Dingh, 1997; Chen and

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Black, 1992; Vorster and Botha, 1998; Wallis *et al.*, 1997). However simplication of the process for the purification/isolation of the invertase are needed and still open for further research. The present study is the third step in the purification process, two earlier processes published by us elsewhere (Ginalska *et al.*, 2000), in which we tried to optimize the *Candida utilis* extracellular invertase purification using column liquid affinity chromatography technique. This study attempted to improve the purification process of invertase purification by using four supports in columns.

2. MATERIAL and METHODS

2.1 Organisms and culture conditions

The yeast, *Candida utilis,* was grown on the liquid Lindeberg medium (Bollag and Leonowicz, 1984) for 4 days on rotary shaker (130 rpm/min). After 4 days the medium was centrifuged for 15 min. at 10,000 rpm. The supernatant was desalted using Sephadex G-25 column (29 × 9 cm), lyophilized and used as the crude invertase for further chromatography. The invertase activity of lyophilizates was determined according to the method of Lloyd and Whelan (1969). One unit of invertase preparation referred to the amount of invertase which is able to liberate 1 μm of glucose/min. at 25°C, pH 4.7. The invertase activity in the lyophilized invertase was 13.6 U/mg of crude solid. The specific activity of the invertase crude preparation was established 27.2 U/mg of protein.

2.2 The preparation of affinity supports

The purification of extracellular invertase from the *Candida utilis* was performed using column liquid affinity chromatography. Four different columns were used as follows;

2.2.1 The controlled porous glass (CPG) (dm = 315~500 μm, Cormay, Lublin, Poland) activated by γ-aminopropyltriethoxysilane (APTES) (Lappi *et al.*, 1976; Lobarzewski *et al.*, 1985) (Column I)

2.2.2 CPG - APTES activated further by glutaraldehyde was covalently activated and bound to feather keratin (protein) (Ginalska *et al.*, 2000) (Column II).

2.2.3 Silica gel (dm = 200~250 μm) activated by APTES using method of Lappi *et al.*, (1976) (Column III).

2.2.4 Silica gel-APTES activated further by glutaraldehyde and covalently bound to feather keratin (protein) (Fiedurek *et al.* 1986; Ginalska *et al.*, 2000) (Column IV).

2.3 Conditions of invertase affinity chromatography

The affinity column (8 × 1.6 cm) was equilibrated using 0.001 M acetate buffer of pH 4.7. The lyophilized invertase was used in the purification process in the optimal amounts as follows:

Column filled with 20 mg of the support I, 15 mg of the support II, 15 mg of the support III and 10 mg of the support IV. The lyophilized invertase samples were dissolved in the starting buffer and poured to the four columns (Column I, II, III, IV). After addition of the invertase solution, each column was washed with the same buffer to eluate the unbound proteins (A_{280}) about 10 fractions (3 ml). The elution process of invertase protein from the each of the four columns was performed using 1.0 M acetate buffer of pH 4.7. The elution speed was 2 mL/min. The protein concentration was determined following the method of Schacterle and Pollack (Lowry *et al.*,

1951; Schacterle and Pollack, 1973). The invertase activity of the eluates was determined according to the method of Lloyd and Whelan (1969).

2.4 Polyacrylamide gel electrophoresis (PAGE)

Non-reducing polyacrylamide gel electrophoresis was performed in 7.5% gel using discontinuous buffer system (Laemmli, 1970). Protein bands were detected using Bio-Rad silver staining kit. Invertase activity was detected *in situ* by the method of Gabriel and Wang (1969). Electrophoresis was performed in 150 V for 5 hours.

3. RESULTS and DISCUSSION

The elution profiles of proteins present in the crude invertase preparation were as shown in Fig. 1. The crude invertase preparations added

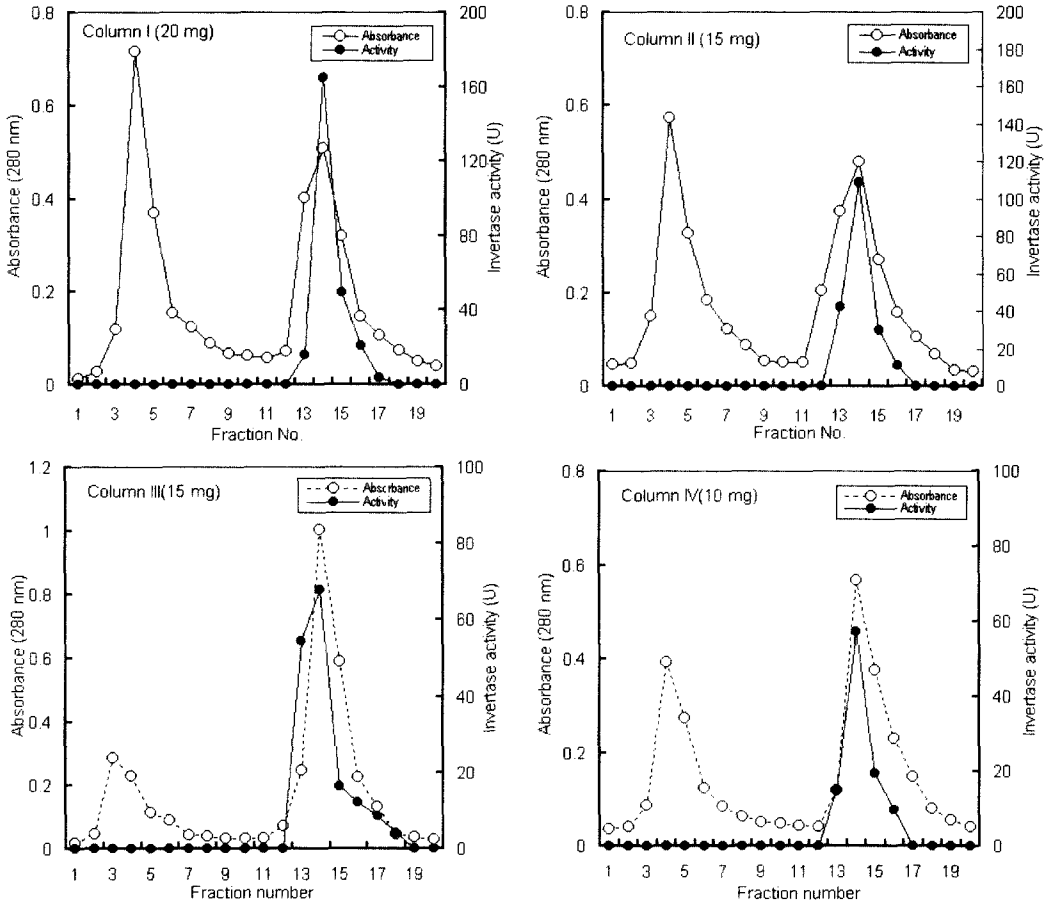

Fig. 1. Chromatographic purification of extracellular invertase from *Candida utilis* using affinity technique. The four matrices were used 8 × 1.6 cm with the elution speed 20 ml/h. Fraction vol. 3 ml. Column I: CPG-APTES; Column II: CPG-APTES-glutaraldehyde-keratin; Column III: siliica gel-APTE; Column IV: silica gel-APTES- glutaraldehyde-keratin.

to each of four columns were optimized experimentally. Fig. 1 shows the chromatographic elution profile of invertase after addition of Column I: 20 mg, Column II: 5 mg, Column III: 15 mg, and Column IV: 10 mg. Initially 1 mM acetate buffer eluted only ballast proteins without any invertase activity. This acetate buffer allowed eluting the proteins containing invertase activity.

The Column I and Column II produced the second peaks and the invertase activity level was higher than its protein adsorption. It indicates that the specific activity of the purified invertase will be higher (Fig. 1). Compared to the purified invertase activity (in the second peak) in the elution profile from Column I and II with results from Column III and IV, it is easily to say that the last results of invertase purification were worse than the above (Fig. 1). The results shown in Table 1 agree with the above straight observation from the columns elution profiles of invertase (Fig. 1)

The invertase elution coefficient (Table 1) was highest (14.6 times) in Column II, but also slightly less after Column I. The elution efficiency of the invertase activity was also better after Column I and Column II (Table 1) than after Columns III and IV (Table 1). Based on the results shown in Fig 1 and Table 1, CPG supports used for Column I and II were better than the silica gel (Column III and IV).

The invertase purification using affinity chromatography on the four columns used was controlled by using PAGE electrophoresis (Fig. 2). After the electrophoresis, protein and invertase activity bands were coloured. The invertase activity was separated to two bands before the chromatography. After the chromatography, the invertase activity was also separated to two bands, but had higher activity of the second band (Fig. 2). The crude invertase preparation contained 15 protein bands. After the chromatography, the crude protein separated by electrophoretically separated protein contained only 6

Table 1. Results of the purification of the invertase (*Candida utilis*) on the affinity columns used four matrices.

Support	Optimal amount of crude invertase preparation used for chromatography (mg)	Invertase activity added to the column (U)	Invertase activity eluted from the column (U)	Invertase elution yield (%)	Invertase activity put on the column (U × mg^{-1} protein)	Invertase activity eluted from the column (U × mg^{-1} protein)	Invertase purification coefficient
Silanized CPG	20	272	255	93.9	27	302	11.1
Silanized CPG activated covalently by keratin	15	204	193	94.4	27	397	14.6
Silanized silica gel	15	204	164	80.4	27	217	8.0
Silanized silica gel activated covalently by keratin	10	136	101	74.4	27	275	10.1

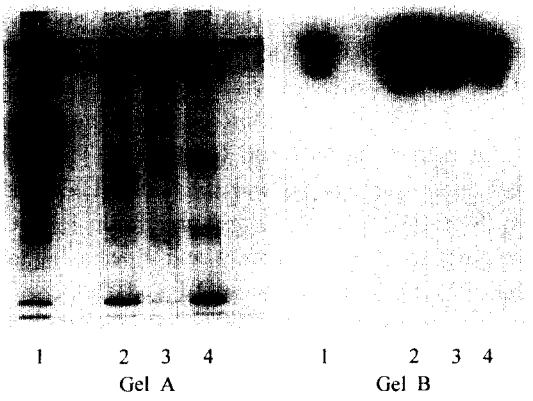

Fig. 2. The electrophoretically compared results of crude and purified (on four supports) invertases. Gel A was silver stained for protein bands (each lane contained 10 μg of total protein), gel B was stained for invertase activity (each lane contained 100 μg of total protein). 1. crude invertase; 2. purified invertase on column I (CPG-APTES); 3. purified invertase on Column II (CPG-APTES-glutaraldehyde-keratin); 4. purified invertase on Column III (Silica gel-APTES).

bands (Fig. 2). It means that the affinity chromatography procedure used in this experiment is able to remove the most balast proteins from the crude invertase together with the activation process of the enzymatic activity (Fig. 1 and Fig. 2). Reassuming these results it could be concluded that the comparative use of some number of differently activated affinity supports allows choosing the better purification condition as result obtained in our earlier reports (Ginalska *et al.* 2000).

4. CONCLUSIONS

This study attempted to improve the purification process of invertase purification by using four supports in columns such as CPG-APTES, CPG-APTES activated further by glutaraldehyde, Silica gel activated by APTES and Silica gel-APTES activated further by glutaraldehyde and covalently bounds to feather keratin. The extracellular invertase of *Candida utilis* was used for the comparative purification on 4 activated matrices by liquid column affinity chromatography method. It was found out that the invertase purification process was better using both CPG matrices (silanized CPG and keratin activated CPG) than these with two silica gel supports. Two silica gel supports found to be superior in the experiment. After the chromatography, the crude protein separated by electrophoretically separated protein contained only 6 bands. The affinity chromatography used in this experiment is able to remove the most balast proteins from the crude invertase. The elution coefficient of the invertase from the two CPG columns was about 93~94%.

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