# ONE-STEP AFFINITY PURIFICATION OF CYCLODEXTRIN GLUCANOTRANSFERASE FROM *Bacillus* SP. 1070

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**Two purification processes of cyclodextrin glucanotransferase (CGTase) form *Bacillus* sp. 1070 on affinity sorbents $\alpha$- and $\beta$-CD-Sepharose 4B have been carried out. Satisfied results have not been obtained using $\alpha$-CD as affinity ligand, whereas the specific enzyme activity isolated on $\beta$-CD-Sepharose 4B increased into 15 times (appr. 95% of purity in SDS Electrophoresis data). It was shown that the extent of starch conversion within crude enzyme did not exceed 70%, while with purified CGTase it was possible to get more than 95%.**

## Introduction

Cyclodextrin glucanotransferase (1,4-$\alpha$-D-glucan: 1, 4-$\alpha$-glucanotransferase EC 2.4.1.19 is a unique enzyme capable to catalyze the cyclization reaction (convertion of starch and related $\alpha$-1,4-glucans into CDs), coupling reaction (opening of CD ring and transfer of linear maltooligosaccharides to acceptors), and disproportionation reaction (transfer of linear maltooligosaccharides to acceptors). Futhermore, the CGTase possesses a weak hydrolyzing activity [1]. CGTases are produced by a variety of bacteria, mainly by *Bacillus*, but also by *Klebsiella, Thermoanaerobacterium, Micrococcus* representatives and other. CGTases are extracellular enzymes and differ in the amount and type of CDs produced.

Cyclodextrins are natural cyclic oligosaccharides with doughnut-shaped structure possessing hydrophilic surface and hydrophobic central cavity (Fig. 1). Due to this unordinary structure CDs are able to form inclusion complexes with different guest organic and inorganic molecules. Recently, these cyclic molecules have been widely utilized in food, agricultural, pharmaceutical, cosmetic and other industries [2, 3].

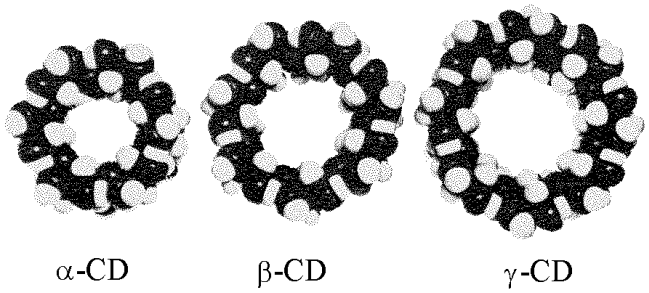


*Fig. 1.* Molecular models of $\alpha$-, $\beta$-, and $\gamma$-CD. Secondary hydroxyl view.

At present in Russia CDs are not industry produced. The problem is in absence of developed highly purified CGTase production technology. But with the aim to get high and specific yield of cyclodextrin homologies ($\alpha$-, $\beta$-, or $\gamma$-CD) it is a very important step. That is why the purpose of this work is the development of simple and effective CGTase purification process immediately from cultural broth.

## Materials and Methods

The strain *Bacillus* sp. 1070 has been isolated from the microorganism collection kindly provided by Moscow State University of Food Production (dep. of Biotechnology, ecology and food sertification) and used as a $\beta$-CGTase producer. The screening has been carried out with the highest cyclic activity of strains, which correlated and located in the range 320-570 U/ml.

**Cyclization activity** of CGTase was measured by slightly modified method [4]. To 1.25 ml of 6% soluble potato starch in 0.05 M Tris-HCl pH 7.2 was added 0.25 ml of enzyme solution. The reaction mixture was incubated 30 min under 56 °C. Reaction was stopped by boiling 5 min and then centrifuged 2 min under 10 000 rotation/min. To 0.5 ml of supernatant 2.5 ml 60 $\mu$M phenolphtalein solution was added in 0.12 M bicarbonate buffer (pH 10.5). Absorption was measured under 550 nm and synthesized $\beta$-CD quantity was detected by calibration curve. One unit of activity was defined as the amount of enzyme able to produce 1 $\mu$mol of CD per minute under the corresponding conditions.

**Dextrinizing activity** of CGTase was measured by slightly modified method [5]. 1 ml 0,2% starch in 0.02 mM Tris-HCl (pH 7.0) was incubated with 50 $\mu$l of enzyme solution during 10 min 56 °C. Reaction was stopped by adding 0.5 ml iodine reagent (0.02% $I_2$, 0.2% KI) and absorption was measured under 700 nm. One unit of activity was defined as the amount of enzyme able to induce 10% decrease of amylose–iodine complex optical density during 1 min under the corresponding conditions.

**Protein concentration** was measured with method described by Bredford on Bovine Serum Albumin as a standard.

**Synthesis of affinity sorbents $\alpha$- and $\beta$-CD-Sepharose 4B** has been carried out with slightly modified method [6]. 12 g pressed on the filter Sepharose 4B was suspended in 12 ml 2 M NaOH, then 3.6 ml of epichlorohydrine was added and stirred for 16 h under $t = 20$ °C. Then it was washed on the filter by distilled water to pH 7.0.

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To 10 g epoxyactivated Sepharose 1 g $\alpha$- or $\beta$-CD in 25 ml of 0.1 M NaOH was added and stirred for 24 h under $t = 45$ °C.

Afterwards these sorbents were washed by $H_2O$ to pH 7.0, treated 0.05 M $H_2SO_4$ according to method [3] to the opening of non reacted epoxy groups and than washed again by $H_2O$.

Synthesized affinity sorbents contained 90 $\mu$mol $\alpha$- and 53 $\mu$mol $\beta$-CD/ml of gel. The amount of jointed CDs was determined as difference between those in the reaction and non reacted.

**Affinnity Chromatography.** On the column ($1 \times 1.5$ cm) with $\beta$-CD-Sepharose 4B previously washed with 0.1 M Na–acetate buffer pH 7.0 50 ml of crude enzyme was applied with the flow rate 25 ml/h. Adsorbed CGTase was eluted by 0.1 M Na–acetate buffer pH 7.0 with the addition of 0.25 M NaCl and $\beta$-CD 5 $\mu$mol. The chromatography on $\alpha$-CD-Sepharose 4B was similar.

**Immobilized Metal Ion Affinity Chromatography.** On the column ($1 \times 1.3$ cm) with Cu(II)–IDA–Agarose previously washed with 0.1 M Na–acetate buffer pH 7.0 and 0.25 M NaCl the fraction (10.2 ml) after affinity chromatography was applied with the flow rate 15 ml/h. The enzyme was desorbed by 25 mM of Imidazole addition to washed buffer.

**Electrophoresis** in the presence of SDS in 12% polyacrylamide gel was carried out by Laemmli method [7] using Mini Protein II (Bio-Rad). The molecular weight proteins used were from 14.2 to 66.0 kDa (Sigma).

**Isoelectrical focusing** was realized on the PAG plates with the pH range 3.5–9.5 and 4.0–6.5 according to Pharmacia Biotech (Sweden) recommendations.

**Starch hydrolysis** was carried out on 6% soluble potato starch with 1% of isooctane addition under the constant stirring during 7 days and $t = 25$ °C. Every 12 h certain amounts of reaction mixture were picked out and $\beta$-CD formed was measured according method [4]. The inclusion complex of $\beta$-CD with isooctane was destructed by boiling for 1 h.

**HPLC analysis** of starch hydrolysis products was undergo on Gilson chromatograph on the column Separon $SGN \times NH_2$, 5 $\mu$M, ($3 \times 150$ mm) in $CH_3CN$:$H_2O$ eluent—70:30. The injection volume 100 $\mu$l, the flow rate 1 ml/min.

## Results and Discussion

The purpose of this work is the development of simple and effective CGTase purification process immediately from cultural broth. Microorganisms for screening were grow on identical composition medium. The strain producer of CGTase was selected according the highest cyclization provided activity (for 1 ml of cultural broth). The crude enzyme was shown previously to convert 6% of starch to CDs mixture $\alpha$:$\beta$:$\gamma$—12:65:23. These data allowed consider CGTase from *Bacillus* sp. 1070 as $\beta$-specific enzyme. It is possible from literary data to provide affinity chromatography on $\beta$-CD- [6, 8] or $\alpha$-CD-Sepharose [9]. Thus it was decided to compare two purification processes using such kinds of sorbents. During chromatography on $\beta$-CD-Sepharose 4B the specific $\beta$-CGTase activity increased into 3 times, whereas on $\alpha$-CD-Sepharose 4B enzyme purity was shown to be less for 1.5 times. Probably those results are explained by lower sorbent capacity on protein.

Usually the gel-filtration is used for CD removal from affinity complex with CGTase [6, 8]. But the pretreatment of applying matter, for instance, concentration and careful calculation, makes this step hard. Hence it was proposed to carry out on-line IMAC on Cu(II)–IDA Agarose since the protein affinity for metal ion immobilized on chromatographic matrix is based on coordination bonds formation between metal ion and imidazole groups of accessible histidines [12]. And from literary data most of CGTases are known to contain approximately 10 histidine residues in their primary structure. The specific activity of $\beta$-CGTase has fold into 15 times in comparison to initial matter (Table 1).

Most of the bacterial strains are known to produce other amylolitic enzymes besides CGTases [14]. Thus the measurement of cyclyzing and dextrinizing activity was conducted during purification process. And these two activities was shown to correlate after every step of purification (Table 1). This datum allowed to refer them to the same enzyme.

Some of the physical and chemical properties was identified for purified CGTase. SDS electrophoresis gave us one protein track (purity degree was no less than 95%, Fig. 2), whereas isoelectric focusing—two isoelectric points (pI 5.1 and 5.3). These two protein tracks are in the slightly acid

Table 1

**Purification of CGTase FROM Bacillus species 1070**

| Purification steps | $V$ (ml) | Protein (mg) | Sp. activity Ac (U/mg) | Sp. activity Ad (U/mg) | Purity Ac | Recovery Ac % | Purity Ad | Recovery Ad % |
|---|---|---|---|---|---|---|---|---|
| Crude enzyme | 50 | 3 | 4560 | 2708 | 0 | 100 | 0 | 100 |
| Affinity chromatography on $\beta$-CD-Sepharose 4B | 10.2 | 1.3 | 13542 | 7210 | 3.0 | 130 | 2.7 | 116 |
| Immobilized Metal Ion Affinity Chromatography on Cu(II)–IDA Agarose | 10.5 | 0.15 | 68673 | 39780 | 15.0 | 75 | 14.7 | 73 |

Ac is cyclyzing activity, Ad is dextrinizing activity.

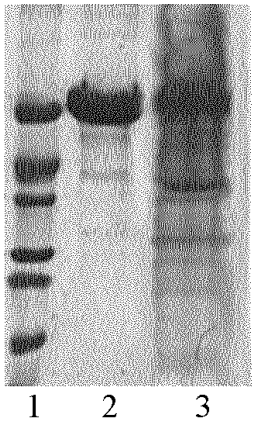


*Fig. 2.* SDS Electrophoresis: 1—Protein molecular weight markers—14.2 kDa, 20.1 kDa, 24.0 kDa, 29.0 kDa, 36.0 kDa, 45.0 kDa, and 66.0 kDa (Sigma); 2—Purified CGTase; 3—Crude enzyme.

region as for most of the bacterial CGTases [8, 13] and their belonging to CGTase was shown by the extraction form gel and determination of cyclyzing (Ac) and dextrinizing (Ad) activities. Obtained data allowed to consider these proteins as isoenzyme, but not subunits.

Also we detected pH optimum (7.2), pH stability (5.0–9.0), temperature optimum (60 °C) for $\beta$-CGTase. The enzyme was not thermostable (it retain just 20% of activity under 60°C during 1h of incubation). This enzyme was shown to be metal independent since it retains more than 75% of activity with EDTA, but strong inhibition of its activity with $Zn^{+2}$, $Ni^{+2}$, $Cu^{+2}$, and $Fe^{+3}$ metal ions confirms the presence of histidine and tyrosine residues in the active center. In the presence of $Hg^{+2}$ ions $\beta$-CGTase retains approximately 70% of its activity, that probably proves the sulfur-containing amino acids absence in the active center of the enzyme. $Mg^{+2}$, $Ca^{+2}$, and $Mn^{+2}$ do not lower the CGTase activity.

During starch hydrolysis with crude enzyme and purified $\beta$-CGTase the equilibrium in reaction mixture was found to be attained at the same time. However in the first case conversion extent did not exceed 70% while $\beta$-CGTase usage shows more than 95% (Fig. 3). And the target product content ($\beta$-CD) in the hydrolyzate with purified enzyme was mach more higher than with crude (Table 2). It should be noted that substrate conversion extent more than 90% with CGTase only (without any other amylolitic

Table 2

**Conversion of Starch**

| Hydrolysing agent | Content of cyclodextrins (%) | | | Recovery (%) |
|---|---|---|---|---|
| | $\alpha$-CD | $\beta$-CD | $\gamma$-CD | |
| Crude enzyme | 6.0 | 57.0 | 6.3 | 69.3 |
| Purified CGTase | 6.0 | 90.2 | — | 96.2 |

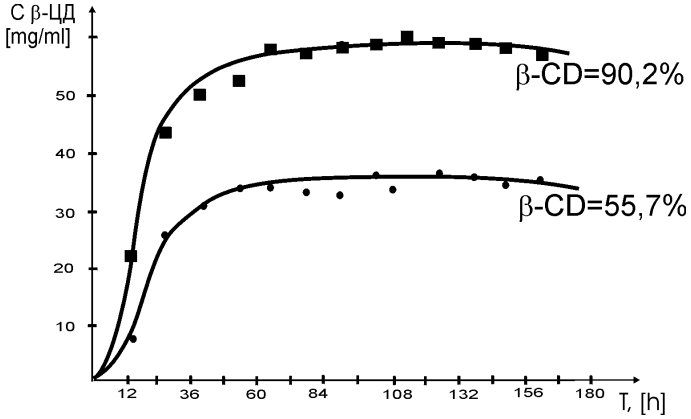


*Fig. 3.* Kinetic of $\beta$-CD accumulation during 6% starch hydrolysis. ■—Purified CGTase; ●—Crude enzyme.

enzymes—amylases, pollulanases, and isoamylases) have not been described previously [14, 15]. Thus on our opinion $\beta$-CGTase purified with the proposed chromatographic scheme is of benefit in comparison to crude enzyme in starch hydrolysis process.

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