Biotechnol. Bioprocess Eng. **1998**, 3, 78-81

Organic Solvent and pH Induced Alteration of Product Specificity of CGTase

Kyo-Sun Park[2], Hyun-Mi Oh[1], Hui-Woog Choe[2], Chung-Ung Park[1], and Kang-Min Lee[1]*

[1]Department of Molecular Biology, Chonbuk National University, Chonju, Korea
[2]Department of Chemistry, Chonbuk National University, Chonju, Korea

Cyclodextrin glucanotransferase [CGTase, E.C.2.4.1.19] is an extracellular enzyme, which catalyzes the formation of α-, β-, γ- CDs from starch. Their proportions of formations depend on enzyme sources and reaction conditions. To understand what determines the product specificity of CGTases, we examined the alteration of product specificity of CGTase from *Bacillus macerans* by organic solvents and pH. At acidic pH range less than pH 6 where the enzyme was unstable, the ratio of α-/β-CD production was increased 4 times more than that at neutral pH range. As we increased the concentration of 2-butanol, α-/β-CD ratio was proportionally increased but /ratio remained constant. The α-/β-CD ratio of products was increased in the reaction media which yielded low products.

Key Words: Cyclodextrin glucanotransferase, Product specificity, Organic solvents, Cyclodextrin

INTRODUCTION

Many bacteria and fungi excrete enzymes that degrade starch so as to facilitate the uptake of carbohydrates into the cell. A small group of bacteria produced enzymes that are able to convert starch into cyclic compounds called cyclodextrins (CDs) via an intramolecular transglycosylation reaction. Cyclodextrin glucanotransferase [CGTase, E.C.2.4.1.19] is an extracellular enzyme that catalyzes the formation of cyclodextrins from starch [1, 2]. These enzyme-catalyzed compounds are cyclic oligosaccharides consisting of six, seven or eight glucose units (α-, β- or γ-CD). They also catalyze the intermolecular transglycosylation reaction in the presence of acceptors. Furthermore, they display a weak hydrolyzing activity.

The proportions of α-, β- and γ-CD are varied with enzyme sources and reaction conditions. The CGTase from *Bacillus macerans* produced α-, β- and γ-CD in the proportion of 2.7:1:1[3]. CGTase from *B. macerans* had advantages on the production of α-CD. On the other hand, the *B. megaterium* and *B. circulans* enzyme produced α-, β- and γ-CD in the proportion of 1:6.3:1.3 and 1:6.4:1.4 respectively in the early stage of reaction and the enzyme from *B. ohbensis* produced negligible amount of α-CD [4, 5]. External additives such as organic solvents and surfactants could influence the activtity of CGTases and the proportion of produced CDs [6, 7]. Surfactants which have straight carbon chains as hydrophobic moiety promoted the formation of α-CD but surfactants which have more bulky hydrophobic moiety activated that of β-CD.

To know what determines the differences in product specificity of CGTases, we were obligated to in vestigate the effects of pH, metal ions, organic solvents and other additives on specificity relaxation of CGTase. In this work, we examined the alteration of product specificity of CGTase from *Bacillus macerans* by organic solvents and pH.

*Corresponding author
Tel· 0652-270-3342 Fax: 0652-270-3345
e-mail: kmlee@moak.chonbuk.ac.kr

MATERIALS AND METHODS

Reagents and Equipments

Soluble starch, polypeptone, yeast extract, KH_2PO_4 and $MgSO_4 \cdot 7H_2O$, calcium chloride, cyclodextrins, corn starch, 2-(N-morpholinoethane sulfonic acid monohydrate (MES), Tris (hydroxymethyl) aminomethane (Tris) were purchased from Sigma (St. Louis, USA). Column materials such as DEAE-cellulose, Sephacryl G-200 SF, Superdex G-200 FF were purchased from Pharmacia Fine Co (Upsala, Sweden). All organic solvents we used in the experiments were spectrophotometric or HPLC grade. High Performance Liquid Chromatography (Waters Associates Inc., Milford, USA) equipped with a differential refractometer R401 and μ-Bondapak/Carbohydrate column was used. UV/Vis spectrophotometer (Cary-3, Varian) was used for determinating CGTase activity.

Isolation and Purification of CGTase from *Bacillus macerans*

CGTase was isolated from *B. macerans* strain IFO

3490 according to the modified method of Stavn et al.[8]. Cells were harvested after 2 days of cultivation and were removed by centrifugation at 4℃ for 30 min at 10,000 g. The CGTase enzymes were precipitated between 35 to 65% saturation with ammonium sulfate. The enzymes were then subjected to chromatography on the columns of DEAE-cellulose, Sepharose G-200SF and Superdex G-200FF.

Determination of Cyclodextrins by HPLC

Concentration of cyclodextrins was determined by high performance liquid chromatography with μ-Bondapak/Carbohydrate column(Waters, 3.9 mm × 30 cm). The conditions were as follows: the eluent was a mixture of 65 % acetonitrile and 35 % water and the flow rate was 0.6 mL/min. The eluate was detected using a differential refractometer (Waters). To prepare the sample, the complexing agent was removed by boiling and non-cyclic compounds of higher molecular weight were precipitated with methanol and were removed by centrifugation, together with other insoluble material [9].

Effect of pH on the Activity of CGTase and the Product Specificity

The activity of CGTase in different pH (pH 3.0-5.5, citrate buffer; pH 5.5-pH 7.0, MES buffer; pH 7.0-pH 9.0, Tris/HCl buffer; pH 9.0-pH 13.0, glycine buffer) was determined. To initiate the enzyme reaction we added 0.1 mL of buffer solution containing 3 % soluble starch and 5 mM calcium chloride to 0.9 mL of various buffer solution containing 5 mM calcium chloride and 0.15 mg CGTase. The mixtures were reacted for 30 min at 50℃. The amount of cyclodextrins produced by CGTase was determined by HPLC.

Effect of pH on the Stability of CGTase

To examine the pH effects on the stability of CGTase, the enzyme reaction solutions were used same buffers as above. 0.9 mL of various buffer solution containing 5mM calcium chloride and 0.15 mg CGTase was incubated for 30 min at 50℃ without substrates and 0.1 mL of 3 % soluble starch solution was added to the 0.9 mL enzyme solutions for initiating. The reaction mixture was incubated for 30 min at 50℃. The amount of produced cyclodextrins was determined.

Effect of Organic Solvents on the Activity of CGTase

The enzyme activity of CGTase in different concentrations of various organic solvents were determined. The activities of enzyme reaction were measured in the presence of 5-40% organic solvents such as glycerol, formamide, ethylene glycol, dimethyl sulfoxide, N-methyl formamide, N,N-dimethyl formamide, acetonitrile, ethanol, isopropanol, 2-butanol and t-butanol. Reaction mixture containing 3% soluble starch, 10 mM calcium chloride and organic solvents was reacted with enzyme (0.15 mg) at 50℃ for 30 min.

Effect of Organic Solvents on the Stability of CGTase

To examine the effect of organic solvents on stability of CGTase, CGTase was incubated in 0.9 mL of buffer solution containing 5 mM calcium chloride, 0.15 mg CGTase and different concentration of various organic solvents for 30 min at 50℃. After this step 0.1 mL of 3% soluble starch solution was added to the 0.9 mL enzyme solutions and then the enzyme reaction was carried out for 30 min at 50℃. The amounts of produced cyclodextrins in the mixtures were determined by HPLC.

RESULTS AND DISCUSSIONS

The pH dependency on the activity and the stability of the enzyme was investigated in an aqueous buffer by soluble starch through the cyclodextrin formation activity. As shown in Fig. 1, CGTase from *B. macerans* had the maximum activity at pH 6.0. CGTase had similar enzyme activity in wide pH range from 5.0 to 9.0. However, at pH values below 4.5 and above 9.0, the enzyme activity remarked decreased. In our experimental conditions CGTase was found to be quite stable at pH 6.0~8.0, nevertheless it was rapidly inactivated at pH values below 5.5. The product specificities of CGTase were also influenced by the pH of reaction medium. At more acidic range (pH 4.5-5.0) the proportion of α-CD formation by CGTase from *B. macerans* was increased, but that of β-CD formation was increased at neutral pH range. α-CD was more produced in any pH range where the enzyme was not stable. One possible mechanism which explains an increase of α-CD production at low pH is the change of the net charge on the protein. As the functional groups of the enzymes, both in the catalytic center and the surface have their inherent pK_a values, the degrees of ionization of those fuctional groups could be changed with the variation of external pH. A change in pH caused the ionization of a catalytically essential group that might be possible to change specificity in enzyme reactions.

The pK_a values of the charged polar side chain

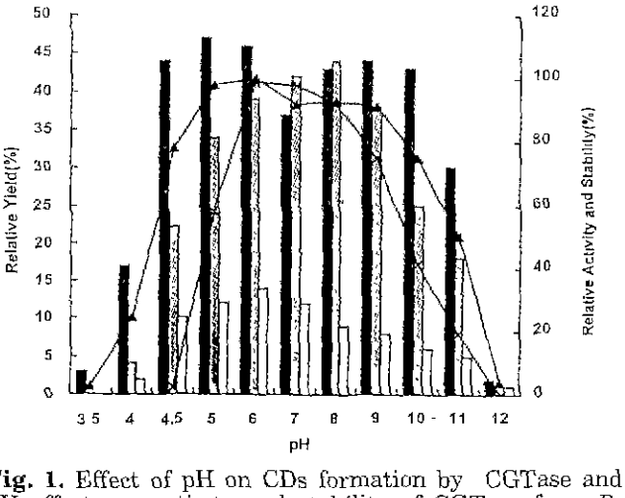

Fig. 1. Effect of pH on CDs formation by CGTase and pH effects on activity and stability of CGTase from *B. macerans* : the formation of cyclodextrins α-( ), β-( ), γ-( ), activity of CGTase(▲) and stability of CGTase(✕).

(Asp, Glu, His) were affected by neighboring chemical groups on the molecule, principally by electron withdrawing or electron donating inductive effects. CGTase from *B. macerans* have very high percentage of Asp and Glu (21-22%) these residues were concerned with catalytic reaction. Glu-257, Asp-229 and Asp-328 have been proposed to be the catalytic residues in CGTase from *B. circulans* [10]. And also three histidine residues located in conserved region active center of various amylolitic enzymes. Around the catalytic site in *B. macerans* CGTase were Glu-258, Asp-229, Asp-329, Asp-196, Asp-372, His-233, His-328, His-399, Glu-194 and Glu-258. The pI's of Asp and Glu were near pH3. Therefore, these molecules have a net negative charge below pH 4.0. The deamination of asparagine and glutamine residues, which introduced negative charges into the hydrophobic interior of the protein induced to inactivate in the condition of high acidity. Histidine residues were also largely responsible for the unfolding of proteins at acid pH.

There are numerous advantages when conducting enzymatic conversions in organic solvents from the biotechnological standpoint. One of the most profound revelations arisen in nonaqueous enzymology is that the specificity of an enzyme strongly depends on the solvent [10,11]. All the types of enzyme specificity which is particularly important for synthetic applications proved to be controlled by solvent systems. The use of organic solvent in the reaction not only ensured the stability and the production specificity, but also resulted in the high yield of the products. The questions concerning the dependence of the enzymatic activity, stability and product specificity of reactions catalyzed by the solvent is interesting in view of biotechnology. The enzymatic reactions of CGTase in organic solvents acquired some remarkable and new properties. They revealed the increase of activity, stability and product specificity of the enzyme. As shown in the Fig. 2, the maximum formation of total CDs were dramatically increased in the presence of 5% of 2-butanol (200% yields), 5% t-butanol (180% yields), and 10% DMSO (176% yields). CDs formation by CGTase was increased even in the reaction media containing 40% organic solvent such as DMSO, ethyleneglycol, glycerol and DMF. CDs productions by CGTase in 2-butanol were much greater than aqueous Tris/HCl buffer (pH 7.0) as shown in Fig. 3. Addition of small amounts of 2-butanol resulted in a considerable change of the product specificity. For instance, α/β and α/γ ratio of products were 1.1 and 3.9 in a buffer solution, however the addition of 5% 2-butanol as cosolvent to buffer solution increased the α/β and α/γ ratio of products 3.3 and 12 respectively. As we increased the concentration of 2-butanol, the α/β ratio was proportionally increased up to 8.0 while the α/γ ratio remained constant. The α/β ratio of products was increased in reaction media which yielded low products and unstable reaction conditions (Fig. 3). In DMSO solution CGTase is more stable and quite active as shown Fig.4. The activities of the enzyme remained constant even in 30% DMSO solution. As we added DMSO, the α/β ratio remained constant but the α/γ ratio increased continuously.

Waterhouse and Johonson have reported the effect of bulk solvent environment on the secondary structure of several peptides and equivocal peptide sequences that could be predicted to be α-helical from amino acid preparence in alcohol solvents

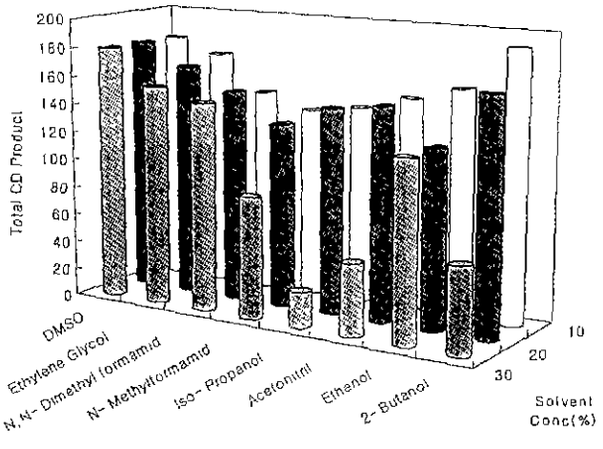

Fig. 2. The formation of cyclodextrins by *B. macerans* CGTase in the presence of various organic solvents [%,(v/v)].

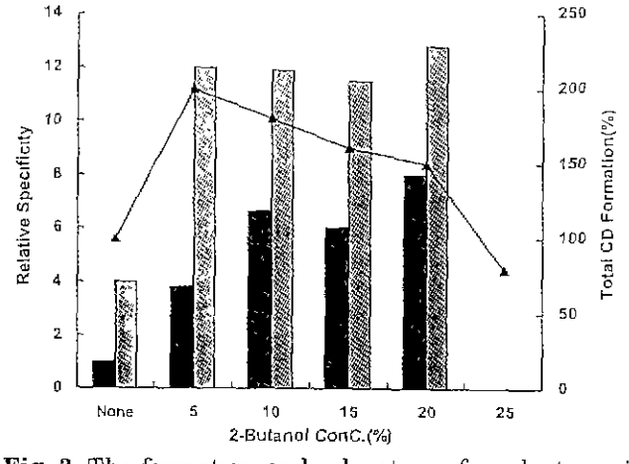

Fig. 3. The formation and relaxation of product specificity of cyclodextrins by *B. macerans* CGTase in the presence of 2-butanol. α/β-CD( ), α/γ-CD( ) and total CD products(-▲-).

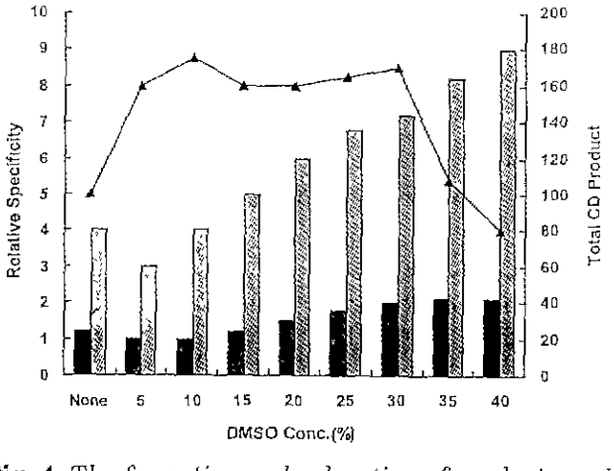

Fig. 4. The formation and relaxation of product specificity of cyclodextrins by *B. macerans* CGTase in the presence of DMSO. α/β-CD( ), α/γ-CD( ) and total CD products(-▲-).

and β-strand in nonmicellar sodium dodecyl sulfate by circular dicroism spectroscopy [12]. They found that solvent was a very important factor in determining the secondary structure of an amino acid sequence in vitro and could override the propensity for a secondary structure of an amino acid sequence. In the presence of more hydrophilic organic solvents such as ethyleneglycol and DMSO, the stability of CGTase slightly increased, but the residual activity was significantly decreased in the presence of 30% organic solvents or more. CGTase was stabilized by some organic solvents such as ethyleneglycol, DMSO, DMF at low concentrations, although they could inactivate enzymes at higher concentrations (Fig. 4). They were able to form multiple hydrogen bonds and to act like the solvent water. Polyhydric compounds such as glycols and alcohols as well as neutral salt were known to have both stabilizing and destabilizing effect. Increasing the number of attachment sites caused the increase of rigidity of protein and thermal stabilization. DMSO is a dipolar, aprotic solvent with a dielectric constant of 46.45. It might compete with protein hydrogen bond acceptors for hydrogen bond donors. This was similar to acetonitrile, but DMSO is a much better hydrogen bond acceptor and is one of the few protein-dissolving solvents. DMSO has been widely used as an alternative solvent to water due to its high solubility of peptides and proteins. The main driving force of the enzyme-substrate binding in the case of CGTase appears to be hydrophilic interactions between the side chain of the amino acid and substrate in the binding pocket of the enzyme. The soluble starch had also different solubility in various organic solvents. Solvent molecules could bind within the enzyme active site and could block the normal binding mode of the substrate and energetics of substrate solvation was the dominant means by which the solvent influenced the substrate specificity.

Acknowledgement This study is supported by academic research fund from Korean Ministry of Education. The authors also thank anomymous reviewer for crucial comments.

REFERENCES

[1] Depinto, J. A. and L. L. Campbell (1968) Purification and properties of the cyclodextrinase of *Bacillus macerans*. *Biochemistry* 7: 114-120.

[2] Bender, H. (1986) Production, characterization, and application of cyclodextrins. In *Advances in Biotechnological Processes*, Mizrahi, A. edit. Vol. 6, 31-71, Alan R. Liss, New York.

[3] Kitahata, S. and S. Okada (1975) Transfer action of cyclodextrin glycosyltransferase on starch. *Agr. Biol. Chem.* 39: 11, 2185-2191.

[4] Okada, S. and S. Kitahata (1973) A study of cyclodextrin forrming enzymes. *Proceeding of amylase Symposium*, Osaka 18, 21-27

[5] Yagi, Y., M. Sato, and T. Ishikura (1986) Comparative studies of CGTase from *Bacillus ohbensis*, *Bacillus macerans* and *Bacillus circulans*, and production of cyclodextrins using those CGTases. *J. Jpn. Soc. Starch Sci.* 33: 144-151.

[6] Kobayashi, S., K. Kainuma, and D. French (1983) Effect of surfactants on cyclization of *Bacillus macerans* Cyclodextrin glucanotransferase, *J. Jpn. Soc. Starch Sci.* 30: 62.

[7] Lee Y.D. and H.S. Kim (1992) Effect of organic solvents on enzymatic production of cyclodextrins from unliquefied corn starch in an attrition bioreactor. *Biotechnol. Bioeng.* 39: 977.

[8] Stavn, A. and P. E. Granum (1979) Purification and physicochemical properties of an extracellular cycloamylose cyclodextrin glucanotransferase from *Bacillus macerans*. *Carbohydrate. Res.* 75: 243-250.

[9] Bender, H. (1983) An improved method for the preparation of cyclooctaamylose, Using starches and the CGTase of *Klebsilla pneumoniae* M5al., *Carbohydrate Res.* 124: 225-233.

[10] Lawson, C. L., R. van Montfort, B. Strokopytov, H. J. Rozeboom, K. H. Kalk, G. E. de Vries, D. Penninga, L. Dijkuizen, and B. W. Dijkstra (1994) Nucleotide sequence and X-ray structure of cyclodextrin glycosyltranferase from *Bacillus circulans* strain 251 in a maltose-dependent crystal form. *J. Mol. Biol.* 236: 590-600.

[11] Carrea G. (1984) Biocatalysis in water-organic solvent two phase systems. *Trends Biotechnol.* 2: 102

[12] Waterhuse, D. V. and W. C. Johnson (1994) Importance of environment in determining secondary structure in proteins, *Biochemistry* 33: 2121-2128.