Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 26 (2003) 287–292
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Stability and activity of cross-linking enzyme crystals of cyclodextrin glucanotransferase isolated from *Bacillus macerans*

Wonsuk Kim [b], Huiwoog Chae [b], Chungung Park [a], Kangmin Lee [a,*]

[a] *Department of Molecular Biology, University of Chonbuk National, Chonju 561-756, South Korea*
[b] *Department of Chemistry, University of Chonbuk National, Chonju 561-756, South Korea*

Received 31 March 2003; received in revised form 28 July 2003; accepted 28 July 2003

Abstract

Recently, a new technique using cross-linking enzyme crystals (CLECs) was introduced in the field of enzyme technology. CLECs are solid crystalline particles which are insoluble in both water and organic solvents. Chemical cross-linking of the enzyme crystals preserves the catalytic activity even in harsh conditions such as at high temperature, at extreme pH, in organic solvents, and in the presence of proteases and radical chemicals. CLECs of cyclodextrin glucanotransferase (CGTase) could be useful biocatalysts because they were stable at elevated temperature, in organic solvents, and in the presence of enzyme inactivation surfactant. They also maintained their activity against protein-digesting enzyme.

Keywords: CLECs; CGTase; Proteases; Surfactant; Organic solvents

1. Introduction

The benefits of exploiting enzymes in the process of organic synthesis and the design of biosensors are well understood. The properties of high selectivity and mild reaction conditions have made the use of enzymes very attractive for the synthesis of biologically active compounds, which are often difficult to obtain by chemical processing. Despite the enormous potential of enzymatic catalysis, only a tiny number of enzymes are used in industry, for instance, in the preparation of natural products, pharmaceuticals, fine chemicals and food ingredients. Although significant improvements in enzyme technology have been achieved, applications in industry are quite limited. The industrial process may cause the loss of enzyme activities within the reaction media. Thus, a variety of methods to stabilize enzyme activity under harsh reaction conditions have been developed [1]. Recently, a new technique, cross-linking enzyme crystals (CLECs), was introduced [2]. CLECs are solid crystalline particles which are insoluble in both water and organic solvents. Chemical cross-linking of enzyme crystals preserves their catalytic activity in harsh conditions such as at elevated temperature, at extreme pH, and in the presence of proteases and radical chemicals [3–6].

Cyclodextrin glucanotransferase [1,4-α-glucan-4-α-D-(1,4-glucano)-transferase, CGTase, EC2.4.1.19] is an extracellular enzyme which catalyzes the formation of cyclodextrin (CD) from starch by intramolecular transglycosylation [7,8]. CGTase-catalyzed CD is a cyclic oligosaccharide comprising six, seven or eight

* Corresponding author. Tel.: +82-63-270-3342; fax: +82-63-270-3345.
E-mail address: kmlee@chonbuk.ac.kr (K. Lee).

1381-1177/$ – see front matter © 2003 Published by Elsevier B.V.
doi:10.1016/j.molcatb.2003.07.007

glucose units. These units respectively are named α-, β-, and γ-CD according to the number of circularly linked glycosidic units such as G6, G7 and G8, and their relative proportions in a reaction medium depend on bacteria form which the enzyme originated [9,10].

In this study, we demonstrate using CGTase that CLECs have many advantages in enzyme technology.

2. Experimental

2.1. Chemicals

Soluble starch, polypeptone, yeast extract, KH_2PO_4 and $MgSO_4 \cdot 7H_2O$, and calcium chloride were purchased from Sigma. Piperazine-1,4-bis[2-ethanesulfonicacid] (PIPES), Tris(hydroxymethyl)aminomethane (Tris), and *N*-2[hydroxyethyl]piperazine-*N*-[2-ethansulfonic acid] (Hepes) were obtained from Merck Co. 2-*N*-Morpholinoethane sulfonic acid monohydrate (MES) was purchased from Gerbu, Biotechnik GmbH.

The column resins DEAE-cellulose, Sephacryl G-200SF, and Superdex G-200FF were from Sigma and Pharmacia Fine Co. Three endo/exogenous proteases, α-chymotrypsin from bovine pancrease, Pronase E from *Streptomyces griseus*, and Proteinase K from *Tritirachium album*, were purchased from Sigma. All solvents were of the highest purity and commercially available. The organic solvents used in this study were of spectrophotometric or HPLC grade. All other chemicals were of analytical grade and purchased from Sigma.

2.2. CGTase enzyme preparation

CGTase was isolated from *Bacillus macerans* strain IFO 3490 by the modified method of Stavn and Granum [11]. The DEAE-cellulose column (2.6 cm × 20 cm) was prepared and equilibrated overnight with 55 mM NaCl in 10 mM phosphate buffer (pH 7.0). The enzyme was then applied to a column of DEAE-cellulose and a linear gradient was formed by mixing 500 mM NaCl and 175 mM phosphate buffer (pH 7.0). The concentrated enzyme extract was applied to a column (1.6 cm × 95 cm) of Sepharose G-200 SF equilibrated with 10 mM Tris–HCl (pH 7.0) containing 150 mM NaCl and 5 mM $CaCl_2$ as the elution buffer. CGTase purity and activity were determined by SDS-PAGE and colorimetric assay with methyl orange, respectively, as described by Lejeune et al. [12].

2.3. Crystallization of CGTase

Crystallization was performed by the hanging drop vapor diffusion technique [13]. This technique requires 24-well Linbro tissue culture plates with grease to form an airtight seal with the cover slips. Each hanging droplet on a siliconized cover-slip consisted of 4 μl of 20 mg/ml CGTase in 100 mM PIPES buffer (pH 7.0) containing 100 mM NaCl and 5 mM $CaCl_2$ with 4 μl of a precipitating reagent. The reservoir contained 1.0 ml of the same reagent. The cover glass with a hanging drop was placed over the 1 ml reservoir solution in each well of the culture plate.

2.4. Assay of cyclodextrin by HPLC

To determine the concentration of cyclodextrin by HPLC (Waters Associates Inc., Milford, MA, USA), a Waters Bondapak/Carbohydrate column (3.9 mm × 30 cm, Waters Associates Inc.) was used. The operating 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 cyclodextrins were detected using a differential refractometer (Waters Associates Inc.). Non-cyclic compounds of higher molecular weight were precipitated with methanol (1.2 volume) [14] and removed by centrifugation. The elution peaks were calibrated with pure α-, β-,and γ-CD.

2.5. Assay of soluble CGTase

The enzymatic reactions of CGTase in solution were analyzed as follows. The composition of the reaction mixture was 3% soluble starch, 10 mM MES buffer containing 5 mM $CaCl_2$ (pH 7.0), and 1.5 mg/ml purified enzyme protein. After a reaction for 30 min at 50 °C, the amount of cyclodextrins produced in the mixture was determined by HPLC.

2.6. Assay of CGTase-CLEC

The reaction mixture comprised 3% soluble starch, 10 mM MES buffer containing 5 mM $CaCl_2$ (pH 7.0)

and 1.0–1.5 mg of CGTase-CLEC, which were cross-linked by 1% glutaldehyde for 1 h. After 30 min at room temperature, the amount of cyclodextrin produced in the mixture was determined by HPLC. To determine the enzyme's stability, we incubated CLECs in a 1% surfactant solution for 30 min, and added 3% soluble starch to the reaction mixture. The effects of surfactants on the soluble enzyme were studied.

2.7. Cross-linking of CGTase crystals

CGTase crystals were collected in a 1 ml vial. A 25% glutaraldehyde solution (Fluka) was added to the enzyme crystals for 1 h. After the cross-linking reaction with glutaldehyde, CLECs were well washed with 100 mM PIPES buffer (pH 7.0) containing 100 mM NaCl and 5 mM $CaCl_2$. The buffer was then removed by suction. The cross-linked enzyme crystals were stored at 4 °C. They were then washed with the same buffer solution three to four times prior to use. The separation of soluble CGTase from CLECs was performed using an Amicon Centricon. The cut-off size of the Amicon Centricon was 100 K.

2.8. Effect of temperature on CLECs

The effect of temperature on CLECs was analyzed by increasing the temperature from 40 to 80 °C. As controls, the same experiments with soluble CGTase were performed under the same conditions.

2.9. Stability and activity of CGTase-CLECs in organic solvents

The effects of acetonitrile, 2-butanol, isopropanol, formamide and DMSO on the activity of CGTase in CLECs were examined. The enzyme activity of CGTase was measured in the presence of increasing volumes of organic solvent from 0 to 40% (v/v). As controls, the same experiments with soluble enzyme were performed.

2.10. Stability of CGTase-CLEC in the presence of proteases

Three proteases, α-chymotrypsin from bovine pancrease, Pronase E from *S. griseus*, and Proteinase K from *T. album*, were used. The concentration of proteases and CGTase was 0.1 and 0.5 mg/ml for the degradation test, respectively. The reactions were performed in an Amicon Centricon of 10 K for the soluble form, and 100 K for the CLEC form. After the digestion reaction for 0, 6, 12, and 24 h, the filtered solution was assayed using a modified Lowry method [14].

2.11. Effects of surfactants on stability of CGTase-CLEC

The effects of the surfactants effects of sodium dodecy sulfate (SDS), haxadecyltrimethyl ammonium bromide (CTAB), Tween 80, TritonX-100 and Lubrol PX on the stability of CGTase-CLECs were studied. The experiments were conducted in a 1% surfactant, 10 mM MES buffer containing 5 mM $CaCl_2$ (pH 7.0).

2.12. Effects of metal ions on activity of CGTase-CLEC

The effects of metal ions such as $CaCl_2$, $MgCl_2$, $HgCl_2$, $ZnCl_2$ and $FeCl_2$ on the activity of CGTase-CLECs were studied. The concentration of metal was 5 mM in 10 mM MES buffer containing 5 mM $CaCl_2$ (pH 7.0).

3. Results and discussion

The insolubilization of CLECs was attributed to cross-linking, followed by crystallization. The cross-linking of CGTase was regulated by glutaraldehyde, one of the most popular cross-linking agents. The insolubilization of crystals was examined at 0.5, 1 and 2% glutaldehyde for 1 h at 4. The best compromise between stability and activity of the CLECs was achieved with 1% glutaldehyde. Therefore we used 1% glutaldehyde for cross-linking of CGTase and a 1 h cross-linking of crystals. The stabilization of CLECs by cross-linking with glutaraldehyde may stem from the formation of a mixture of oligomers of different lengths and structures. However, the mechanism remains unresolved. The optimization of cross-linking seems to depend on a particular protein and its crystal form although little information is currently available. It may be due to the enzyme purity. CLECs are not needed for further purification.

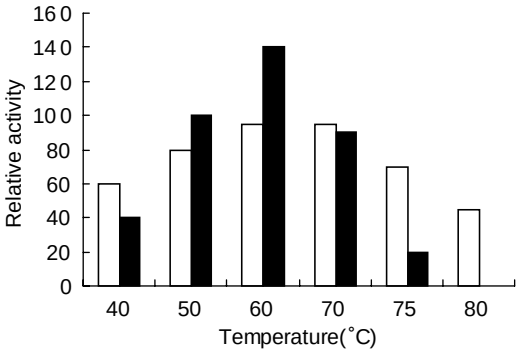

Fig. 1. The activity of soluble CGTase and CGTase-CLECs as a function of temperature from 40 to 80 °C. The reaction mixtures of soluble CGTase were composed of 3% soluble starch, 10 mM MES buffer containing 5 mM $CaCl_2$ and 1.5 mg/ml purified CGTase. For CLECs, reactions were under the same conditions with 1.0 mg/ml CGTase-CLECs. Soluble CGTase (■) and CGTase-CLECs (□).

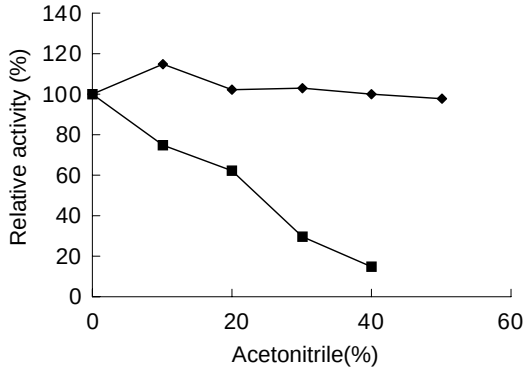

Fig. 2. The activity of soluble CGTase and CGTase-CLECs in acetonitrile solution from 0 to 50% (v/v). The reaction mixture of soluble CGTase was composed of 3% soluble starch, 10 mM MES buffer containing 5 mM $CaCl_2$ and 1.5 mg/ml purified CGTase. For CLECs, reactions were run under the same conditions with 1.0 mg/ml CGTase-CLECs. Soluble CGTase (■) and CGTase-CLECs (◆).

The effect of temperature on the stability of CGTase-CLECs and soluble CGTase was studied. The soluble CGTase was more sensitive to high temperature than the crystalline form. At 70 °C, more than 85% of the activity was lost in the case of the soluble enzyme while 70% of the activity remained in the case of CLECs. The soluble CGTase exhibited no activity at 80 °C whereas CGTase-CLECs retained 48% of its original activity at the same temperature (Fig. 1). In general, CLECs were found to be very stable at elevated temperatures [15].

The CGTase of CLECs was stronger than the soluble enzyme in 15% organic solvent. In water immiscible organic solvents, more than 50% of activity was lost in the case of the soluble form, but most activity was retained by CLEC (Table 1). CLEC was slightly more resistant to 2-butanol and acetonitrile than DMSO. In 15% solution of 2-butanol, over 100% of the activity was retained in CGTase-CLECs while 28% of the activity remained in the case of soluble CGTase. The crystallization and cross-linking of alcohol dehydrogenase from horse liver (HLADH) was investigated by Lee [4]. The HLADH of CLECs had more than 60% activity in a reaction medium containing 65% organic solvents while a soluble form of HLADH was totally inactivated under the same conditions. The effect of acetonitrile on activity of CGTase-CLECs and soluble CGTase by increasing the concentration to 50% was studied. The soluble form of CGTase in acetonitrile was more sensitive to acetonitrile than the crystalline form (Fig. 2). In the medium of 40% acetonitrile, most activity of soluble CGTase was lost while 150% of the activity was retained for the CLECs.

Enzyme stability was tested in protease solutions. Two types of CGTase were used. Activities of soluble enzymes and CLECs were analyzed at the same level for control experiments. The stability tests for CGTase using proteases were performed for 0, 6, 12 and 24 h. Different proteases such as α-chymotrypsin from bovine pancreas, Pronase E from *S. griseus*, and Proteinase K from *T. album* solutions were exploited.

Table 1
The activity of soluble CGTase and CGTase-CLECs against 15% (v/v) organic solvents

Solvent	CGTase-CLEC	Soluble CGTase
None	100	100
2-Butanol	128	28
Acetonitrile	101	60
DMSO	93	54
Isopropanol	87	48
Formamide	85	40

The reaction mixture of soluble CGTase was composed of 3% soluble starch, 10 mM MES buffer containing 5 mM $CaCl_2$ and 1.5 mg/ml purified CGTase. For CLECs reactions were run under the same conditions with 1.0 mg/ml CGTase-CLECs.

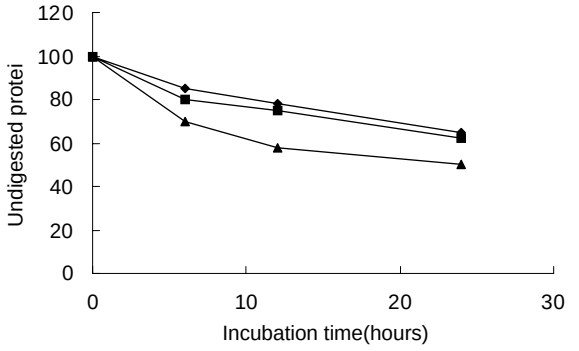

Fig. 3. The stability of soluble CGTase against various proteases. The concentrations of proteases and CGTase were 0.1 and 0.5 mg/ml for the degradation test. α-Chymotrypsin from bovine pancreas (◆), Pronase E from *Streptomyces griseus*, (■) Proteinase K from *Tritirachium album* (▲).

As shown in Fig. 3, 40% of proteins were cleaved to small fragments (<10 K) which exhibited no enzymatic activity while less proteins were not digested in CLECs even when filtered with a 100 K membrane filter. The remarkable feature observed in CLECs may result from a crystal packing re-enforced by covalent cross-linking. The proteolysis resistance of CLECs is attributed to the size of the solvent channels defined by the crystal lattice. When an enzyme molecule is in a crystal lattice, which is surrounded in three dimensions by other protein molecules, it is much more difficult to unravel the tertiary structure than when it is dissolved in solution. In a crystal lattice, where the concentration of protein is close to the space limit, a large number of protein–protein interactions occur.

This may significantly enhance the protein stability to heat and other denaturants by preventing unfolding, aggregation or dissociation. When proteins were transferred from a solution to a crystalline environment, an increase in both electrostatical polarity and hydrophobic interactions occurred among the protein molecules [16]. This may significantly enhance protein stability in response to temperature, solvents and other denaturants [17].

The effects of surfactants on the stability of CGTase-CLECs and soluble CGTase were studied. Cationic surfactant CTAB, anionic surfactant SDS and nonionic surfactants TritonX-100, Tween 80 and Lubrol PX were chosen for this experiment. While nonionic surfactants known as “mild surfactants” such as TritonX-100, Tween 80, and Lubrol PX exhibited a small effect on CGTase activity, ionic surfactants such as SDS and CTAB exhibited a remarkable effect (Table 2). In the presence of 1% of SDS which generally is known as a detergent inactivating protein, [18] only 7% of the activity of soluble CGTase remained, but CLECs exhibited very strong activity. CLECs were very active in $FeCl_2$ whereas the soluble CGTase was not active (Table 3). These findings are not fully understood. CLEC biotechnology seems to be different from traditional enzymology.

In the case of CLECs, structural rigidity makes them resistant to the protease and organic solvents. We, here, ensured the stability of CGTase-CLECs in the presence of proteases, at high temperature and in organic solvents emphasizing that it has great potential as a biosensor. CLEC technology may contribute to the acceleration of practical chemical synthesis and other areas.

Table 2
Effect of surfactants on stability of CGTase-CLECs

Surfactant	CGTase-CLEC	Soluble CGTase
None	100	100
SDS	220	7
CTAB	88	10
TritonX-100	118	96
Lubrol PX	80	98
Tween 80	110	96

The effect on CLEC was examined with 1% surfactant, 3% soluble starch, 10 mM MES buffer containing 5 mM $CaCl_2$ and 1.5 mg/ml purified CGTase. For CLECs, reactions were run under the same conditions with 1.0 mg/ml CGTase-CLECs.

Table 3
Effect of metal ions on activity of CGTase-CLEC

Metal	CGTase-CLEC	Soluble CGTase
No metal	100	100
$CaCl_2$	120	102
$MgCl_2$	85	78
$HgCl_2$	32	60
$ZnCl_2$	70	20
$FeCl_2$	210	16

The effect on CLEC was examined with 5 mM metal, 3% soluble starch, 10 mM MES buffer containing 5 mM $CaCl_2$ and 1.5 mg/ml purified CGTase. For CLECs, reactions were run under the same conditions with 1.0 mg/ml CGTase-CLECs.

4. Conclusion

The rigidity of structure produced by chemical cross-linking makes it possible to resist heat, protease and organic solvents. We, here, ensured the stability of CGTase-CLEC at high temperature, in organic solvents and in the presence of proteases emphasizing that it has great potential in CLEC biotechnology. CLEC enzymology seems to differ from traditional solution enzymology. CLEC technology may contribute to an acceleration of practical chemical synthesis, therapeutics, biosensor and other areas.

Acknowledgements

This work was supported by Chonbuk National University Research Funds.

References

[1] H.G. Davies, R.H. Green, D.R. Kelly, S.M. Roberts, CRC Crit. Rec. Biotechnol. 10 (1990) 129.
[2] J.S. Dordick, Enzyme Microb. Technol. 11 (1989) 194.
[3] N.S. Clair, Y.F. Wang, A.L. Margolin, Angew Chem. Int. Ed. 39 (2000) 380.
[4] K.M. Lee, M. Blaghen, J.P. Samama, J.F. Biellmann, Bioorg. Chem. 14 (1986) 202.
[5] L.Z. Vilenchik, J.P. Griffith, N. St. Clair, M.A. Navia, A.L. Margolin, J. Am. Chem. Soc. 120 (1998) 4290.
[6] R.A. Persichetti, N.S. Clair, J.P. Griffith, M.A. Navia, A.L. Morgolin, J. Am. Chem. Soc. 17 (1995) 2732.
[7] J.A. Depinto, L.L. Campbell, Biochemistry 7 (1968) 114.
[8] H. Bender, Carbohydr. Res. 206 (1990) 257.
[9] T. Kaneko, T. Hamamoto, K. Horikoshi, J. Gen. Microbiol. 34 (1988) 97.
[10] G. Schmid, Trends Biotechnol. 7 (1989) 224.
[11] A. Stavn, P.E. Granum, Carbohydr. Res. 75 (1979) 243.
[12] A. Lejeune, K. Sakaguchi, T. Imanaka, Anal. Biochem. 181 (1989) 6.
[13] J. Jancarik, S.H. Kim, J. Appl. Crystallogr. 24 (1991) 409.
[14] H. Bender, R. Siebert, A. Stadler-Szoke, Carbohydr. Res. 110 (1982) 245.
[15] J.J. Lalonde, Chem. Tech. 27 (1997) 38.
[16] S.A. Islam, D.L. Weaver, Proteins 8 (1990) 1.
[17] C. Otero, A. Ballesteros, J.M. Guisan, Appl. Biochem. Biotechnol. 19 (1988) 163.
[18] J.P. Samama, K.M. Lee, J.F. Biellmann, Eur. J. Biochem. 163 (1987) 609.