Immunoaffinity Purification and Characterization of Cyclodextrin Glycosyltransferase from *Bacillus circulans* A11

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ABSTRACT The purified IgG fraction of anti-CGTase was coupled to CNBr-activated Sepharose 4B and used as immunoaffinity gel to purify CGTase from *Bacillus circulans* A11. The enzyme was successfully purified to approximately 155 folds with a 45% yield and specific activity of 3302 units/mg protein. It was a single polypeptide of 72 KDa. The amino acid composition of this CGTase was found to contain high amounts of Asx, Glx, Gly, Ala, and Thr and low amounts of His, Met, Cys, and Trp. N-Terminal sequence was A P D T S V S N K Q N F S T D V I Y Q I. Chemical modification and substrate protection studies indicate the presence of Trp, His, Tyr, and Asx/Glx residues at the active site of CGTase, while Cys, Lys, and Ser were proved to have no influence on CGTase catalysis. From HPLC analysis of products of enzymatic reaction, this CGTase produced mainly β-CD. The ratio of α : β : γ - CD was 1 : 4.1 : 1.1. When analyzed by non-denaturing PAGE, the enzyme demonstrates the isoform pattern. Four isoforms with the same molecular mass but different in quantity and pI values were observed. All isoforms demonstrate amylolytic and cyclizing activities of CGTase.

KEYWORDS: cyclodextrin glycosyltransferase, anti-CGTase, immunoaffinity purification, characterization, *Bacillus circulans*.

INTRODUCTION

Cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) catalyzes two main reactions: cyclodextrin (CD) production from starch and related α-1, 4-glucans through the intramolecular transglycosylation reaction, and the coupling and disproportionation reactions that transfer the glucosyl residue in starch or CD to acceptor molecules through the intermolecular transglycosylation reaction.[1] CGTase is produced extracellularly by various microorganisms, with those of *Bacillus* species being most extensively characterized.[2-6] The gram-negative species reported to secrete an α-CGTase is *Klebsiella oxytoca* M5a1.[7] The enzyme is divided into three main types, α-, β- and γ-, according to the major type of CD formed.[8] CGTases from different sources show different biochemical characteristics and yield different ratios of CD-product. Cyclodextrins are biochemical products of industrial importance, especially in foods, cosmetics and medicine, due to their ability to form inclusion complexes with appropriate organic or inorganic compounds.[9]

Several reports on CGTase purification were found to be involved the precipitation with organic solvents or ammonium sulphate, the adsorption on starch, chromatography on DEAE-cellulose, and electrophoresis.[3-4,10] Very efficient purification of CGTase from *Bacillus macerans* was achieved on α-CD-derivatized agarose.[11] Affinity chromatography on a β-CD polymer of CGTases from different strains of *Bacillus* sp. INMIA was able to separate 2-4 subforms of the enzyme.[12] Techniques of higher separating performance ie, isoelectric focusing or FPLC on a Mono Q column which could separate different subforms of CGTases with very close pI values were later reported.[13-14]

Purification of the enzyme from *Bacillus circulans* A11 required at least two consecutive columns after starch adsorption or ammonium sulphate precipitation.[15-17] The attempt to use specific antibody-linked chromatographic resin as an immunoaffinity column to improve CGTase purification was raised. Anti-CGTase was previously reported to be used in the detection of the expression of CGTase of *E. coli* transformants and of the parental strains,[7, 18-19] while no studies on the immunoaffinity application have been performed. The aim of the

present work is to develop the use of immunoaffinity chromatography in the purification of CGTase from *Bacillus circulans* A11 and characterization of the purified enzyme.

Materials and Methods

CNBr-activated Sepharose 4B, cyclodextrins, oligosaccharides, N-bromosuccinimide, and other group-specific reagents were obtained from Sigma, St. Louis, USA. Ethanolamine, trichloroethylene, and α-amylase from *Bacillus* sp. were purchased from BDH, Dorset, England. All other chemicals were of reagent grade.

Bacillus sp. A11 was isolated from South-East Asian soil and screened for CGTase by Pongsawasdi and Yagisawa.[15] The strain was later identified as *B. circulans*.

Preparation of antibody against CGTase

Antibody against CGTase was prepared by rabbit immunization using the purified enzyme from *Bacillus circulans* A11.[17] In addition to double diffusion analysis in agar plate, antibody titer was also followed by the indirect antibody ELISA technique, in which optimal assay conditions were developed for the first time for the CGTase enzyme.[17] The antibody was purified by ammonium sulphate precipitation and DEAE-cellulose chromatography at pH 6.5.[20] Antibody titer determined by immuno-diffusion increased from $1 : 2^2$ of the crude antiserum to $1 : 2^8$ of the DEAE-cellulose purified fraction. SDS-PAGE of the purified antibody confirmed the two bands corresponded to heavy and light chains of IgG. The purified antibody was specific towards CGTase and no cross-reaction with Bacillus α-amylase was observed.[17] The antibody was dialyzed against 0.1 M sodium bicarbonate buffer pH 8.3 containing 0.5 M NaCl. This preparation of anti-CGTase was used for coupling to chromatographic resin.

Preparation of immunoaffinity resin

CNBr-activated Sepharose 4B was used as supporter resin. Coupling of the anti-CGTase to CNBr-activated Sepharose followed standard methods: coupling 26.3 mg of purified antibody in 10 ml to 1.5 g (5.3 ml) of swollen Sepharose gel overnight at 4°C, followed by blocking with ethanolamine as previously described.[21] The excess uncoupled ligand that remains after coupling was removed by washing alternately with acetate buffer pH 4.0 and sodium bicarbonate buffer pH 8.3.[20] The gel was packed into a column of 0.8 x 4.5 cm and equilibrated with 50 mM acetate buffer pH 6.0 containing 0.5 M NaCl at the flow rate of 6 ml/hour.

Purification of CGTase using immunoaffinity chromatography

CGTase was partially purified from the culture broth of *B. circulans* A11 by the usual first step of corn starch adsorption.[16-17] The enzyme solution was then concentrated by amicon ultrafiltration and applied on the prepared immunoaffinity column at 4°C. The column was washed with 50 mM acetate buffer pH 6.0 containing 0.5 M NaCl at 2 ml / hour flow rate until A_{280} was negligible. Elution was performed by 3.5 M NaSCN in 50 mM NH_4OH pH 10.5 at room temperature and the flow rate of 6 ml / hour. Fractions of 2 ml were collected. The active CGTase fractions were pooled, concentrated, and dialyzed against 50 mM acetate buffer pH 6.0 containing 10 mM $CaCl_2$ at 4°C.

Determination of CGTase activity

CGTase activity was determined by starch degrading (dextrinizing) activity and CD-trichloroethylene (CD-TCE) complex formation.[22-23] For dextrinizing activity, one unit is the amount of enzyme which produces 10% reduction in the intensity of the blue color (A_{600}) of starch-iodine complex per minute. For CD-TCE complex formation, the activity was expressed in terms of the dilution limit ($1:2^n$), as the highest dilution that produces observable CD-TCE precipitate lining between upper starch solution layer and lower TCE layer.

Protein determination

Protein concentration was determined by the Coomassie blue micro method according to Bradford (1976).[24]

Polyacrylamide gel electrophoresis (PAGE)

Native PAGE was performed in 7.5% polyacrylamide with Tris-glycine buffer (pH 8.3) according to the method of Davis.[25] SDS-PAGE was done in 7.5% polyacrylamide containing 0.1% SDS by the method of Laemmli.[26] In both cases, the gels were stained with Coomassie brilliant blue. For activity staining, 0.02% I_2 in 0.2% KI was used.

Analysis of cyclodextrins by HPLC

0.5 ml of the enzyme sample was incubated with 2.5 ml of starch substrate (2.0% soluble starch in 0.2 M phosphate buffer, pH 6.0) at 40°C for 24 hours. The precipitate was dissolved in distilled water and

boiled for 5 min. Prior to HPLC injection, the sample was filtered through 0.45 µm membrane filter. The column used was Supelco-NH_2 column (0.46 x 25 cm) and the eluant was 75% acetonitrile in water. The flow rate was 2 ml/min. Cyclodextrin peaks were detected using the RI detector.

Amino acid analysis

The amino acid composition of CGTase was determined on a Beckman System 6360 Amino acid Analyzer after hydrolysis of CGTase with 6 M HCl in evacuated tubes at 110°C for 22 hours. For Trp determination, 6 M HCl was replaced by 4 M methanesulfonic acid. When Cys and Met were to be analyzed, the samples were hydrolyzed with performic acid reagent before the addition of 6 M HCl.

Determination of N-terminal amino acid sequence

Amino acid sequencing was performed by Edman degradation on an Applied Biosystems 476A Protein Sequencer with an on-line phenylthiohydantoin-derivative analyzer.

Effect of group-specific reagents on CGTase activity

Purified CGTase at the final concentration of 20 µg/ml was incubated with 1.0 mM of each modifying reagent in 50 mM acetate buffer, pH 6.0, at 40°C for 30 min. The residual enzyme activity was then determined by the Dextrinizing assay method. Group-specific reagents used were N-ethylmaleimide (NEM), Iodoacetamide (IAM), Trinitrobenzene-sulfonic acid (TNBS), Phenylmethylsulfonylfluoride (PMSF), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Diethylpyrocarbonate (DEP), N-bromosuccinimide (NBS), and N-acetylimidazole (NAI).

Chemical modification and substrate protection experiment

Modification of CGTase with certain group-specific reagents, which affected CGTase activity, was carried out in two steps. The first step was to determine the suitable concentration and incubation time of the reagents used.[27] The experiments were performed by incubating purified CGTase (20 µg/ml) with varying concentrations of the reagents at pH 6.0, 40°C for 30 min, then the residual CGTase activities were determined. The suitable concentration is the minimum concentration of the reagent that leads to maximum inactivation of the enzyme. To determine the suitable incubation time used in the modification, the suitable concentration of each modifying reagent was incubated with CGTase by varying time. The suitable incubation time is the time at which 50 percents of the activity remained.

In the second step, the suitable conditions were used in order to identify amino acids at the active site of CGTase using substrate protection technique. α-, β- and γ- CD were used as protective substrate. Purified CGTase (20 µg/ml) was preincubated with 20 mM of each substrate at pH 6.0, 40°C for 5 min prior to the addition of suitable concentration of each modifying reagent. After suitable incubation time, the residual CGTase activity was then determined and compared with chemical modification in the absence of protected substrate.

Results and Discussion

Immunoaffinity purification of CGTase

Partially purified CGTase after starch adsorption and amicon concentration was loaded onto CNBr-Sepharose 4B linked anti-CGTase column. Figure 1 shows the elution profile from immunoaffinity column using conditions described in Materials and Methods. One major protein peak with no enzyme activity was washed off with the equilibrating buffer, and CGTase was eluted as a single peak between fraction 20 and 24. Purification table (Table 1) shows that a yield of 45% with 155 fold purification was obtained.

Immunoaffinity purification of CGTase has so far never been reported while there were reports on the use of affinity columns, ie α-cyclodextrin Sepharose 6B and epichlorohydrin-reticulated β-CD copolymer

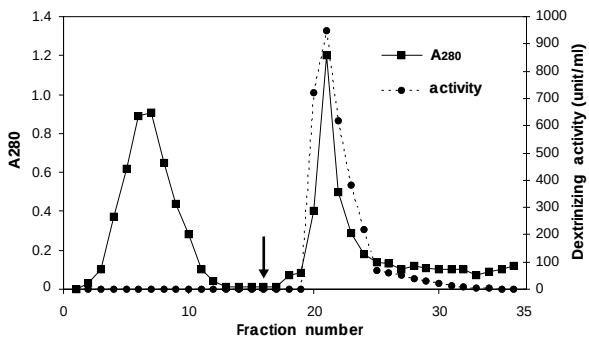

Fig 1. Immunoaffinity purification of CGTase from *B.circulans* A11.
Concentrated CGTase solution from corn starch adsorption was applied onto CNBr-Sepharose 4B linked anti-CGTase column (0.8x4.5 cm).
The column was then washed with equilibrating buffer, 50 mM acetate buffer pH 6.0 containing 0.5 M NaCl. Elution was made by 3.5 M NaSCN in 50 mM NH_4OH, pH 10.5. The arrow indicates where elution starts.

Table 1. Purification of CGTase from *B.circulans* A11.

Step	Volume (ml)	Total Protein (mg)	Activity			Purification fold	Yield (%)
			Total (unit)x10^3	Specific (unit/mg)	CD-TCE (2^n)		
Crude enzyme	1000	620	13.2	21	2^6	1	100
Starch adsorption	7.4	5.1	11.5	2277	2^{10}	107	87
Immunoaffinity column	19	1.8	5.9	3302	2^{11}	155	45

columns for CGTases from *B. macerans* and *B. circulans* E 192, respectively.[11, 28] Cyclodextrin was used as ligand in those reports while anti-CGTase was used in this study. For purification of CGTase from *B. circulans* A11, Techaiyakul reported four purification steps: starch adsorption, ammonium sulphate precipitation, DEAE-cellulose, and Sephadex G-100 columns, with the enzyme yield of only 18% and 59 fold purification.[16] In 1994, Rojtinnakorn improved the yield to 28% by using similar purification steps except changing Sephadex to chromatofocusing column.[17] In this study, purification steps were reduced while purification fold and yield were much improved. In addition to the advantage of immunoaffinity purification, the change in culturing medium for enzyme production, and the addition of ultrafiltration after starch adsorption step (which was found to be able to reduce the amount of small proteins and peptides) should also contribute to the improved purification data.

Purification of CGTase from other strains of *Bacillus*, ie alkalophilic *Bacillus* 38-2 , *B. macerans* ATCC 8514, *B. stearothermophilus* TC 90, *B. megaterium*, and *Bacillus* sp. HA 3-3-2, using various purification techniques, were reported.[2-4, 23, 29] Other genus of which CGTases have been purified were *Klebsiella, Thermoanaerobacter* and *Brevibacterium*.[10,30-31] All strains were found to excrete their CGTases in the extracellular medium.

Characterization of CGTase

The purified CGTase showed one intense protein band with molecular weight of 72 KDa on SDS-PAGE (Fig 2) Elution from Sephadex G-100 proved that this CGTase is a single polypeptide chain.[17] Other CGTases which were also reported to be single polypeptide are those from *B. stearothermophilus, B. fermus/lentus* 290-3, and alkalophilic *Bacillus* 38-2, with the molecular weight of 68 K, 75 K, and 88 KDa, respectively.[4, 29, 32] While CGTases of *B. macerans* IAM 1243 and ATCC 8514 were dimers with identical subunits of 145 K and 139 KDa.[2, 33] The CGTase from *B. circulans* E 192 was also dimeric proteins but with different subunit sizes of 34 K and 49 KDa.[28] Optimum pH and optimum temperature of our purified CGTase were at 6.0 and 40°C which differ from those previously reported.[3-4, 32-33]

When the CGTase of *B. circulans* A11 was characterized by analysis of the products formed using HPLC, one major peak with retention time

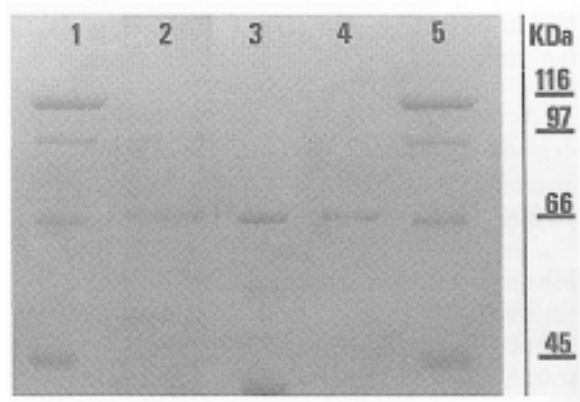

Fig 2. SDS-PAGE of CGTase from different steps of purification Lane 1 and 5,molecular weight protein markers(5 µg); lane 2, crude enzyme (20 µg); lane 3, starch adsorbed enzyme(20 µg); lane 4, immunoaffinity-purified enzyme (5 µg).

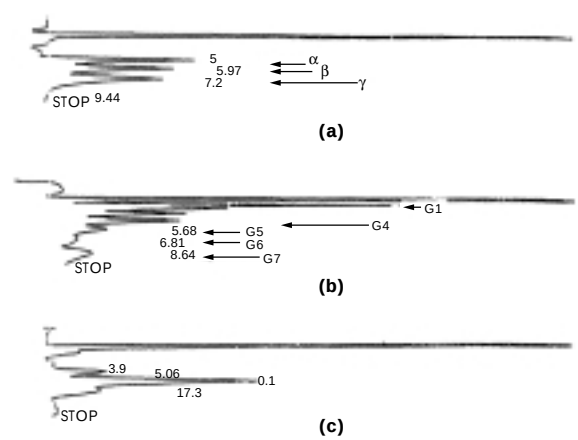

Fig 3. HPLC chromatograms of (a) standard α-, β-, and γ-CD; (b) standard saccharides (G1-G7); (c) reaction products formed from CGTase of *B.circulans* A11. The column used was Supelco-NH_2 (0.46 x 25 cm) column. Elution was made by 75% acetonitrile in water, with the flow rate of 2 ml/min.

corresponded to that of standard β-CD was obtained (Fig 3). The ratio of α : β : γ-CD was 1.0 : 4.1 : 1.1. The result indicates that this CGTase produced mainly β-CD in the experimental conditions used. Different ratios of CD products were reported for CGTases from other bacteria, ie 2.7 : 1.0 : 1.0 for α-CGTase of *B. macerans*,[2] and 1.0 : 11.0 : 1.5 for β-CGTase of alkalophilic *Bacillus* sp. 38-2, [10] while the CGTase of *B. fermus/lentus* 290-3 was known to produce γ-CGTase in the initial phase of enzyme production.[32] The enzyme source is hence one important parameter in specifying the product ratio.

The amino acid composition of *B. circulans* A11 CGTase was shown in Table 2. The result demonstrates that this CGTase was rich in Asx, Glx, Gly, Ala, and Thr while contained low amounts of His, Met, Cys, and Trp, respectively. When compared to other CGTases of closely-related species and strains such as *B. circulans* E192 and *B. macerans*,[14,34] the CGTase from *B. circulans* A11 had very similar amino acid composition when expressed in the term of mole %. However, some differences were observed in the contents of Glx, Gly, Thr, Arg, and Cys. *B. alkalophilus* 38-2 enzyme also showed very similar composition.[18] Significant differences were observed between CGTases of *B. circulans* A11 and *Bacillus* sp. INMIA 1919, especially in the content of Ser, Gly, His, Arg, Ala, and Cys.[12]

Table 2. Amino acid composition of CGTase from *B. circulans* A11 as compared to CGTases from other strains.

Amino acid	Content (mole%)				
	B. circulans A11	*B. circulans* E192[14, 28]	*B. alkalophilus* 38-2[18]	*B. macerans*[34]	*B.* sp. INMIA 1919[12]
Asx	15.42	16.62	16.06	14.70	16.0
Glx	8.63	5.17	7.01	5.82	8.2
Ser	6.25	6.28	5.68	6.84	10.1
Gly	12.35	9.36	9.34	11.50	5.6
His	1.29	1.68	1.75	1.31	4.5
Thr	8.31	10.89	8.47	9.75	11.3
Arg	2.82	1.12	3.36	3.06	6.0
Ala	10.24	10.34	8.32	8.30	5.0
Pro	5.49	3.49	4.23	3.49	4.1
Tyr	2.94	3.91	4.96	4.95	6.0
Val	6.95	6.28	7.01	7.13	6.2
Met	1.11	1.26	2.04	1.75	1.5
Cys	0.29	0.00	0.29	0.00	0.0
Ile	4.28	5.17	6.28	5.24	3.7
Leu	5.54	5.59	5.26	5.83	3.2
Phe	4.31	5.59	4.68	4.66	5.7
Trp	0.16	2.65	1.90	1.89	ND
Lys	3.56	4.60	3.36	3.78	2.4

Asx = Aspartic acid plus asparagine
Glx = Glutamic acid plus glutamine
ND = Not Determined

The N-terminal amino acid sequence of the purified CGTase was also determined. The sequence of twenty residues at the N-terminus was A P D T S V S N K Q N F S T D V I Y Q I which was identical to CGTases of alkalophilic *Bacillus* 38-2,[18] *Bacillus* sp. 1011,[35] and *Bacillus* sp. 17-1,[36] while 70-75% sequence identity was found with CGTases of *B. circulans* 8, E192, and *B. macerans*.[14,34,37] When compared with the N-terminus of CGTases from *B. ohbensis*,[38] and *B. stearothermophilus*,[39] only 50% sequence identity was observed. No homology was found with N-terminal sequence of *K. oxytoca* M5a1 CGTase.[7] From the amino acid composition and the N-terminal sequence comparison, CGTase from *B. circulans* A11 was more similar to other β-CGTases[14,18,36] than those CGTases which were known to produce mainly α-CD.[7,12,38-39]

The amino acid residues required for enzyme catalysis were investigated by chemical modification with group-specific reagents. The results in Table 3 shows that CGTase activity was totally inhibited by DEP and NBS and partially inhibited by NAI and EDC, when 1 mM of the reagents were incubated with 20 μg/ml CGTase at pH 6.0, 40°C for 30 min. NAI and EDC, at 30 mM and 5 mM were equally effective to 1 mM DEP or NBS. This finding indicates the involvement of His, Trp, Tyr, and carboxylic amino acids in catalysis. The inhibitory activity of DEP, NBS, and NAI on His, Trp, and Tyr was confirmed by monitoring the formation of N-carbethoxyhistidine, N-acetyltryptophan, and O-acetyltyrosine at 246, 280 and 278 nm, respectively.[27] Modifying reagents which had no effect on enzyme activity were NEM / IAM, TNBS, and PMSF. These are known to modify Cys, Lys, and Ser, respectively.[27]

Table 3. Effect of various group-specific reagents on CGTase activity

Group-specific reagents (1 mM)	Residual activity* (%)
None	100
N-ethylmaleimide	96.1
Iodoacetamide	99.3
Trinitrobenzenesulfonic acid	100
Phenylmethylsulfonyl fluoride	100
N-bromosuccinimide	0
Diethylpyrocarbonate	0
N-acetylimidazole	85.0
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide	67.3

* These data are average values from two separate experiments.

It was found that these reagents, even at the concentration of 100 mM, showed no significant inhibition of the enzyme CGTase. From these chemical modification studies, it can be concluded that His, Trp, Tyr, and Asx/Glx had significant contribution to CGTase catalysis while Cys, Lys, and Ser had no obvious involvement.

Substrate protection experiment was then performed to help identify which of the four residues among His, Trp, Tyr, and Asx/Glx were the active site residues of CGTase. Using the suitable conditions (modifier concentration, incubation time) found for each modifier (DEP, NBS, NAI, and EDC), modifications of CGTase in the presence or absence of substrate were compared. Specificity of chemical modification was known to increase when mild treatment of suitable conditions was used.[27] Protective substrates in the experiment were α-, β-, and γ-CD. The concentration used was 20 mM as preliminary data of K_m values of the three substrates were in the range of 1-2 mM.[40] Table 4 shows the result of chemical modification of NBS in the presence or the absence of protective substrate. Modification by 0.05 mM NBS led to 54.6% loss of CGTase activity. When CGTase was protected by substrate before NBS modification, the loss of CGTase activity was significantly reduced. Trp modification was totally protected by γ-CD and almost totally protected by α- and β-CD. Substrate protection prior to modification of His, Tyr, and Asx/Glx by DEP, NAI, and EDC were also performed. The result showed that the loss of CGTase activities were partially or totally reduced in the presence of protective substrate. These results support the involvement of Trp, His, Tyr, and Asx/Glx residues in the active site of CGTase. When compared to other studies, these residues have been demonstrated as essential residues for other CGTases as well. Trp was protected against NBS by glucose and the maltosaccharides G_2-G_4, which indicates Trp to be located at the substrate binding site of CGTase from *B. stearothermophilus*.[41] Bender (1991) reported that histidine residues of CGTase from *B. circulans* 8 and *K. oxytoca* M5a1 were modified by DEP and proposed to be involved in the cyclizing reaction of CGTase.[42] His and Asx/Glx were present at the active site of CGTase from *B. circulans* var alkalophilus (ATCC 21783) since their inactivation was prevented by α- and β-CD, while Tyr was essential for *B. circulans* E 192.[43-44]

Table 4. Effect of substrate on the inactivation of CGTase activity by N-bromosuccinimide

Compound added	Relative activity * (%)
1. None	100
2. 20 mM α-CD	100
20 mM β-CD	97.9
20 mM γ-CD	97.5
3. 20 mM α-CD, then 0.05 mM NBS	89.9
20 mM β-CD, then 0.05 mM NBS	91.4
20 mM γ-CD, then 0.05 mM NBS	97.4
4. 0.05 mM NBS	54.6

* These data are average values from two separate experiments.

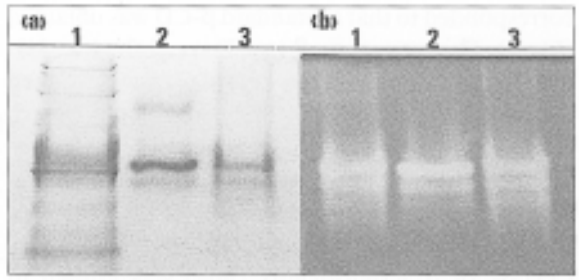

Fig 4. Native PAGE of CGTase from different steps of purification. (a) Coomassie blue stain; Lane 1, crude enzyme (20 μg); lane 2, starch adsorbed enzyme (20 μg); lane 3, immunoaffinity-purified enzyme (5 μg); (b) Amylolytic activity stain; Lane 1-3, as in (a) 0.2 units of dextrinizing activity was loaded to each well.

CGTase isoforms

When the purified CGTase which showed a single band on SDS-PAGE, was analyzed by native PAGE and stained for protein and amylolytic activity, 3-4 bands with different intensity were observed (Fig 4). All these bands also showed cyclization activity of CGTase. Western blot analysis confirmed this heterogeneity (data not shown). pI values were preliminarily estimated from chromatofocusing to be in the range of 4.4-4.9.[17] Only three studies on isoforms of CGTase have been reported so far. Six isoforms with pI range of 4.75-4.99 were reported in alkalophilic *Bacillus* ATCC 21783[13] while only two, pI 6.7 and 6.9 were detected in *B. circulans* E 192.[14] *Bacillus* INMIA-T6, -T42, -A7/1 contained 2, 4 and 2 isoforms, respectively.[12] In some of those studies, amino acid compositions and CD product ratios among isoforms were shown to be different. However, not much more information is known due to difficulty in isoform separation and the small amounts obtained. For further study, we intend to isolate isoforms of our CGTase and look into their properties for more understanding of this enzyme.

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