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RECOVERY OF CYCLODEXTRIN GLUCANOTRANSFERASE (CGTase) USING IMMOBILIZED METAL CHELATING AFFINITY CHROMATOGRAPHY

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Abstract - Immobilized metal affinity chromatography (IMAC) was chosen as a method of purification for the recovery of CGTase from *E. coli* homogenate. *E. coli* harbouring the Bacillus sp. G1 gene expressed extracellularly secreted CGTase into ampicillin supplied LB broth. Culture was pre-purified using SnakeSkin dialysis tubing (3.5 MWCO) with an enzyme activity of 147.80 U/mL. Three strategies (A, B and C) were employed for the purification of CGTase using column adsorption chromatography with Ni^{2+}-Sepharose resin. Strategy A employed an elution buffer of 50 mM EDTA, pH 7, Strategy B used 0.1 M imidazole, pH 7 and Strategy C employed 45 mM imidazole pH 7 as the elution buffer. Strategy C was found to be most suitable yielding a total CGTase recovery of 87.04% from an initial activity of 147.80 U/mL.
Keywords: Affinity chromatography; Binding capacity; CGTase; New chromatographic adsorbent; Nickel-Sepharose chelating.

INTRODUCTION

Cyclodextrin glucanotransferase (CGTase) is a class of enzymes consisting of three subtypes namely α, β and γ-CGTase. They are monomeric enzymes that are secreted extracellularly and catalyze transglycosylation reactions via their glucosyl residues, which are used as an acceptor in forming cyclodextrins (CD). CD's are widely used in the pharmaceutical, medicine, food, textile, agriculture and the cosmetic industries. They have the unique property of solubilizing hydrophobic material and have the ability to entrap volatile compounds by forming inclusion complexes. Purifying CGTase is often a complicated task due to its heterogeneity, complexity and instability. obtaining it requires downstream processing, which typically consist of a cascade of recovery steps. Current purification strategies include starch adsorption (Higuti *et al*., 2013, Atanasova *et al*., 2011, Kitayska *et al*., 2011, Vassileva *et al*., 2007, Yampayont *et al*., 2006 and Martins and Hatti-Kaul, 2002), α-cyclodextrin bound epoxy-activated Sepharose 6B affinity chromatography (Goh *et al*., 2012, Guru *et al*., 2012, Qi *et al*., 2007, Rahman *et al*., 2006 and Sian *et al*., 2005), ion exchange chromatography (Ibrahim *et al*., 2012, Savergave *et al*., 2008, Alves-Prado *et al*., 2007 and Doukyu *et al*., 2003), hydrophobic interaction chromatography (Shetty *et al*., 2011 and Charoensakdi *et al*., 2007) and aqueous two-phase separation (Rosso *et al*., 2005).

Immobilised metal affinity chromatography is a type of purification method that exploits high-affin-

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ity coordination binding between a group of amino acids (such as histidines, cysteine and tryptophan), with divalent metal ions (such as $Zn^{2+,}$ Cu^{2+}, Ni^{2+}, Co^{2+}, Fe^{3+} and Ga^{3+}) chelated to IMAC ligands pre-immobilised onto the resin (Ueda *et al*., 2003).

The IMAC interaction is based on the interaction of surface accessible side-chains of amino acid (mostly histidine) residues with the immobilized chelated metal ions on the resin (Westra *et al*., 2001). Various factors such as the nature of the chelating groups, metal ion, ligand density on the adsorbent, surface amino acid composition of the protein, molecular size and the surrounding environment (e.g., pH, nature of buffer salts, ionic strength and temperature) affect protein adsorption in IMAC (Vunnum *et al*., 1995, Porath, 1992, Arnold, 1991). Advantages of IMAC include the ability of high protein loading, ligand stability, mild elution condition, low cost and simple regeneration and these have been intensively reviewed by several authors in the past (Yang *et al*., 2011, Prasanna *et al*., 2010, Gaberc-Porekar *et al*., 2001).

Based on the nature of the interactions between metal ions and proteins, the target proteins can be selectively eluted from IMAC resin by an elution buffer with either a suitable pH (via protein protonation) or an appropriate concentration of imidazole (competitive chelator). Some elution protocols also usedstrong chelating compounds, such as EDTA, urea, or guanidine hydrochloride to elute proteins with a strong affinity for IMAC (Sun *et al*., 2005). Most CGTase variants are known to contain approximately 10 histidine residues in their primary structure. In previous studies, (Volkova *et al*., 2000) and (Cristancho *et al*., 2013) described the application of IMAC resin for the purification of CGTase and found this approach to be simple, effective, high capacity, reproducible, stable and cost effective. In order to achieve higher selectivity and efficiency in IMAC separation, it is essential to understand the interaction between adsorbate and adsorbent during binding, washing and elution. Purification of CGTase was performed using Ni^{2+}-Sepharose. Ni^{2+} seemed to be the most suitable in terms of selectivity for distribution of the histidine residues on the protein surface (Dalal *et al*., 2008) and it is important to choose first-row transition metal ions (Cu^{2+}, Ni^{2+}, Co^{2+} and Zn^{2+}) (Clemmitt *et al*., 2000).

Concentration of salt and the pH of the buffer used for loading, washing and elution in the purification of CGTase have to be properly formulated. A well-defined binding/elution condition is crucial to enhance the purification performance and yield, while reducing the overall operation cost. In this study, the efficiency of immobilised metal affinity chromatography was assessed in the recovery of CGTase from *E. coli* homogenate.

MATERIALS AND METHODS

Organism and Culture Conditions of CGTase

The alkalophilic bacteria *Bacillus* sp G1 was originally isolated from soil. CGTase was isolated and inserted into *E. coli* BL21 as per (Goh *et al*., 2008). The recombinant strain was then grown in Luria-Bertani broth supplemented with 50 µg/mL ampicillin (CALBIOCHEM, Massachusetts, USA). Upon harvesting and centrifugation, the supernatant was used as crude enzyme for the subsequent pre-purification steps.

Pre-Concentration of CGTase from *E. coli* Feedstock Via Snakeskin Dialysis Tubing

Twenty ml of *E. coli* feedstock supernatant containing CGTase was dialyzed against 100 mM sodium phosphate buffer, pH 7, in a SnakeSkin (Thermo Scientific, Illinois, USA) dialysis tube having an internal diameter of 3.5 cm. Experiments were performed at 4 °C in an ice bath overnight under gentle mixing. Pre-concentrated CGTase was used for subsequent purification steps.

Preparation of Ni^{2+} Loaded Sepharose Chelating Resin

Sepharose chelating resin (particle size of 45-165 µM) (GE Healthcare, Uppsala, Sweden) slurry was prepared in distilled water at a ratio of 75% settled resin volume to 25% distilled water. Adsorption was performed by mixing 30 mL of an aqueous solution containing 50 mM of nickel chloride (pH 5.5) with 15 ml of IMAC Sepharose resin (GE Healthcare, Uppsala, Sweden) and left to mix on the rotator for 24 hours.

Column Preparation

Prepared resin was packed into the Tricorn 10/50 column and assembled accordingly. The column containing the Ni^{2+} chelated Sepharose resin was washed with 5 CV (19.6 mL) of distilled water to remove excess metal ions. The column was then subsequently washed with 20 mM sodium acetate buffer, pH 5, to elute loosely bound ions that might leak out during a the chromatographic run. For equilibration, 5 CV (19.6 mL) of the binding buffer (20 mM sodium phosphate buffer, pH 7) was

pumped through the bed prior to the loading of the CGTase feedstock.

Static Binding Capacity of CGTase Onto Ni^{2+}-Sepharose IMAC Resin Using an Adsorption Isotherm Model

To each series of tubes, clarified CGTase solutions at concentrations of 0-100% were prepared by dilution in 20 mM sodium phosphate buffer, pH 7. 200 µL of Ni^{2+} loaded IMAC resin were added to each tube at a 50:50 slurry ratio. Each tube was sealed and mixed end over end using a rotator for 2 hours at room temperature, 20 °C. The initial enzyme activity and protein assays were performed. After 2 hours, all the tubes were removed and centrifuged to allow settling. Clarified supernatants were collected and assayed for remaining total protein and enzyme activity.

Dynamic Binding Capacity of CGTase Onto Ni^{2+}–Sepharose IMAC Resin

The Ni^{2+}-Sepharose IMAC resin packed in a Tricorn 10/50 column was equilibrated with 5 CV (19.6 mL) of washing buffer, which was sufficient to achieve the target pH and conductivity. Protein was loaded onto the resin until breakthrough was observed based on absorbance at 280nm (A_{280}). Unbound protein was washed with 5 CV (19.6 mL) of washing buffer (20 mM sodium phosphate buffer, pH 7). All experiments were conducted at ambient temperature (25–27 °C) and a flow rate of 100 cm/h, corresponding to a residence time of 6 min.

Column Purification of CGTase Via Packed Bed Adsorption Approach Using Ni^{2+} –Sepharose IMAC Resin

All buffers were filtered prior to use through 0.2 µm Whatman filter paper with a vacuum pressure pump. A Tricorn 10/50 column (GE Healthcare, Uppsala, Sweden) was loaded with IMAC resin and attached to fast protein liquid chromatography (FPLC) (GE Healthcare, Uppsala, Sweden) equipment. A settled column of Ni^{2+}-Sepharose loaded IMAC resin was equilibrated with 20 mM sodium phosphate buffer, pH 7, at a flow rate of 1.00 mL/min. 20 mL of clarified CGTase was injected once the baseline at A_{280} was observed. Non-bound protein was washed from the resin bed with 25 mL of 20 mM sodium phosphate buffer, pH 7. Elutions were performed via 3 methods. Elution buffers used were: 20 mM sodium phosphate buffer, 50 mM EDTA, pH 7 (gradient elution); 20 mM sodium phosphate buffer, 1 M imidazole, pH 7 (gradient elution); and 20 mM sodium phosphate buffer, 450 mM imidazole, pH 7 (single step elution). Eluents from experiments were collected every one mL and assayed for enzyme activity and protein concentration.

Total Protein Determination

Protein concentration was quantified according to the Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) as a standard (PIERCE, Illinois, USA). For the calibration of the standard curve, 2 mg/mL of BSA was prepared in aliqouts. To 20 µL of the protein sample, 1 mL of Bradford dye reagent was added and was incubated at room temperature for 5 min in a 1 mL cuvette. The absorbance reading was then taken at 595 nm. A standard curve with various BSA (Sigma Aldrich, Misouri, USA) concentrations was generated and then employed to interpolate the protein concentration of unknown samples.

CGTase Enzyme Assay

CGTase activity was determined using the phenolphthalein assay as described by Kaneko *et al*., (1987). The assay was performed by adding 0.1 mL of the sample to CGTase assay reagent containing 1 mL of 0.04 g soluble starch in 0.1 M phosphate buffer, pH 6.0. The mixture was then incubated at 60 °C for 10 min. The reaction was stopped by adding 3.5 mL of 30 mM NaOH (Sigma Aldrich, Misouri, USA) followed by 0.5 mL of 0.02% (w/v) phenolphthalein in 5 mM Na_2CO_3 (Sigma Aldrich, Misouri, USA). This mixture was mixed using a rotator mixer for 15 min (STUART SB2, Fisher Scientific, Pittsburgh, USA). The reduction in colour intensity was measured at 550 nm. Blanks lacking the CGTase were analysed simultaneously with each batch of samples. One unit of enzyme activity was defined as the amount of enzyme that formed 1µmol β-CD (Sigma Aldrich, Misouri, USA) per minute under the defined conditions.

RESULTS AND DISCUSSION

Static Binding Capacity of CGTase from *E. coli* Homogenate on the Ni^{2+} -Sepharose IMAC Resin Using an Adsorption Isotherm Analysis

Tubes with clarified CGTase weres diluted with 20 mM sodium phosphate buffer, pH 7, in a range of 0-100%. Ni^{2+}-Sepharose resin was added with a 50:50

resin to slurry ratio. Tubes were then subjected to rotational agitation for 2 hours. Figure 1 represents the graph of experimental data for the equilibrium isotherm of CGTase on Ni^{2+} IMAC adsorbent and the least squares fit to the Langmuir equation. The obtained q_m was 666.67 U/mL/ and the K_d was 5.3 x 10^{-1} U/mL (Figure 1). The graph depicts a slow approach towards equilibrium with non-steep initial slopes. Values obtained from this study were seen to be higher in comparison to (Dalal *et al*., 2008) who obtained q_m values of 56.2 U/mL/resin and a K_d values of 21.7 M when adsorption values were tested for green fluorescent protein (GFP) on Ni^{2+}-STREAMLINE. Sharma *et al*. (2001) obtained higher K_d (2 x 10^{-5} M) but lower q_m values (116-131 U/mL) when the adsorption of model proteins, such as lysozyme, ovalbumin and conalbumin on Ni^{2+}-IDA was studied.

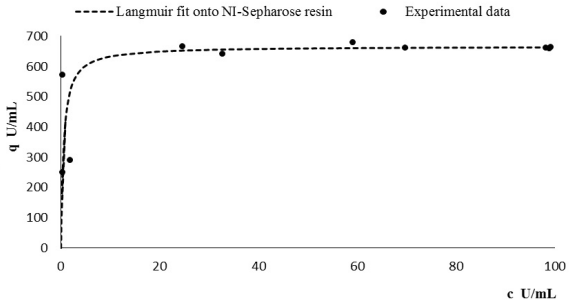

Figure 1: Graph of experimental data of the equilibrium isotherm of CGTase on Ni^{2+} -Sepharose IMAC adsorbent.

During an IMAC adsorption process, various interactions may take place, which includes, in this case, non-specific binding. The lower *qm* obtained could have been due to non-specific hydrophobic interactions that took place between the IMAC-Ni^{2+}-Sepharose and the CGTase (Bornhorst *et al*., 2000). Considering the physical properties of the enzyme CGTase, the isoletric point is 6.5 (carrying a net negative charge at pH > 6.5 and a net positive charge at pH < 6.5). The buffer involved (20 mM PBS) is at the pH 7. The ligand Ni^{2+} is also positively charged. This thus creates a slight negative charge on the surface of the enzyme. A slight ion exchange interaction might have happened, causing the monolayer on the surface of the adsorbent to constantly change, hence explaining a slight deviation from Langmuirian principles, as seen in Figure 1. Foo *et al*. (2010) reported that, for metal binding, a linearized equation such as the Langmuir isotherm, generated problems and faults. These arise from the complex transformation of data, which leads to the violation of the fundamentals underlying the Langmuir isotherm. However, among the many isotherm models, the Langmuir model stands as the most frequently used due to its simplicity (Yang *et al*., 2011), though said to be not suitable in the case of IMAC purification due to factors such as the formation of multiple coordination bonds and low capacities of IMAC resins to bind to protein (Tsai *et al*., 2006, Vunnum *et al*., 1995). Although there are many arguments for the use of a Langmuir adsorption isotherm system in an IMAC operation, this model has been successfully used to describe the binding of protein onto resins (Dalal *et al*., 2008, Tsai *et al*., 2006, Hasar, 2003, Finette *et al*., 1997).

Dynamic Binding Capacity (DBC) of CGTase on Ni^{2+}-Sepharose IMAC Resins Via Column Adsorption Chromatography

The DBC of CGTase on Ni^{2+}-Sepharose was determined at 10% breakthrough. Clarified CGTase was loaded onto a Tricorn 10/50 column pre-packed with 3.92 ml of Ni^{2+} -Sepharose resin. CGTase was loaded at 1 ml/min until a breakthrough (A_{280}) was observed. DBC values were 6.43 mg/ml as seen in Figure 2. This value is coherent with results obtained by Bolanos-Garcia and Davies (2006) when purifying native proteins from *E. coli* using IMAC resin, who mentioned that DBC values of IMAC resins are within the range of 5-10 mg/mL. Results from this study were superior to those obtained by Clemmitt *et al*. (2000), who purified β-galactosidase from *E. coli* homogenate (via an expanded bed adsorption) with DBC values of 0.78 mg/mL. In another study of purification of histidine-tagged nucleocapsid protein of Nipah virus via IMAC (Chong *et al*., 2009), a DBC value of 2.5 mg/ml was obtained using Nickel Sepharose FF, which was also lower compared to this study. Deviations such as these were explained

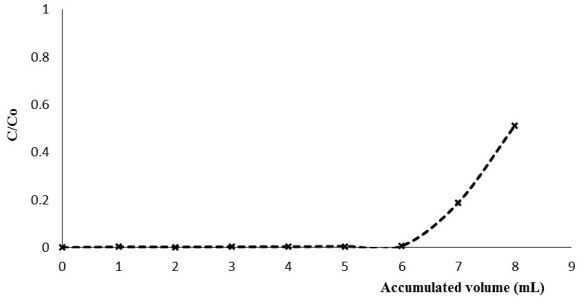

Figure 2: Breakthrough curve of CGTase on the Ni^{2+}-Sepharose IMAC resin.

by Sharma *et al*. (2001) as being due to the suitability or deviation of the interaction of the protein with the resin, which depends heavily on the nature of the protein. One disadvantage associated with the IMAC is its vulnerability to potential binding interference by metal chelating species that sometimes are present in cell cultures. The consequences include a pronounced reduction in protein binding efficiency and leakage of immobilized ions from the packed column during a chromatographic run (Zhang *et al*., 2011).

Purification of CGTase from *E. coli* Homogenate Using Ni^{2+}-Sepharose IMAC Resin Loaded in a Packed Bed Adsorption Column Chromatography

Purification of CGTase from *E. coli* homogenate using Ni^{2+}-Sepharose IMAC resin was performed using three elution strategies which are:

Strategy A: Elution buffer: 20 mM sodium phosphate buffer, 50 mM EDTA, pH 7 (gradient elution);

Strategy B: Elution buffer: 20 mM sodium phosphate buffer, 0.1 M imidazole, pH 7 (gradient elution);

Strategy C: Elution buffer: 20 mM sodium phosphate buffer, 45 mM imidazole, pH 7 (single step elution).

Elution of Bound CGTase on Ni^{2+}-Sepharose IMAC Resin Via Strategy A

Twenty ml of clarified *E. coli* homogenate containing the enzyme CGTase was loaded onto a packed column of Ni^{2+}-Sepharose resin. The column was then equilibrated and washed with 25 ml of 20 mM PBS, pH 7, with a fixed flow rate of 1 mL/min throughout. Once unbound protein was removed from the column, bound proteins were eluted via 20mM PBS, 50 mM EDTA, pH 7. This was performed via a 0-50 mM gradient elution with 20 mM PBS, pH 7, and 20 mM PBS, 50 mM EDTA, pH 7.

From the chromatogram (Figure 3), 20 mL of feedstock containing the CGTase enzyme were loaded onto the Tricorn 10/50 column containing the Ni^{2+} -Sepharose IMAC resin. About 20% enzyme loss was observed during the flowthrough step (Table 1). Protein concentrations (mg/mL) followed closely the enzyme activity (U/mL). A further 13% enzyme loss was observed during the washing step using the buffer of 20 mM PBS, pH 7. The elution buffer**,** 20 mM PBS, 50 mM EDTA, pH 7, was applied in a gradient manner from 0-50 mM EDTA. This was achieved using two buffers, 20 mM PBS, pH 7, and 20 mM PBS-50 mM EDTA, pH 7. The elution buffer were applied gradiently for 40 mL and resulted in an overall enzyme yield of 45%. The elution step gave a 1.01-fold purification relative to the initial feedstock.

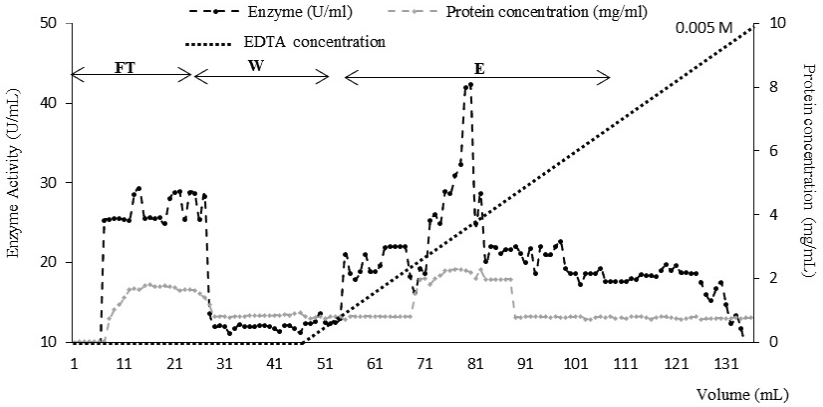

Figure 3: Chromatogram of CGTase separation via Ni^{2+}-Sepharose IMAC resin at 1 mL/min. Buffers used: equilibration buffer: 20 mM sodium phosphate, pH 7, washing buffer: 20 mM sodium phosphate buffer, pH7, gradient elution 20 mM sodium phosphate, 50mM EDTA, pH 7.

Table 1: Purification table for CGTase using Ni^{2+}-Sepharose IMAC resin. (Strategy A).

Purification stage	Volume (ml)	Total Activity (U)	Total Protein (mg)	Specific activity (U/mg)	Fold	Yield (%)
Clarified homogenate	20	2956	77.6	38.09	1.	100
Flowthrough	20	573	21.4	26.78	0.7	19
Washing	25	370	17.75	20.85	0.55	13
Elution	50	1325.6	34.4	38.53	1.01	45

With every IMAC column, some leaching of metal ions occurs, depending on the type of chelating compound involved and the sort of elution. To assess this phenomenon, the elution samples collected during the elution peak were subjected to nickel ion leaching analysis using an inductively coupled plasma-mass spectrometer (ICP-MS). For the effluent using 50 mM EDTA buffer, very high Ni^{2+}, 158 mg/L, was co-eluted in the elution step. Metal leaching may generate charged groups which act as a cation exchanger and bind to the positively charged groups on the surface of the proteins (Block *et al*., 2009) which accounts for low CGTase yields. Ni^{2+} compounds are also established human carcinogens (Kozlowski *et al*., 2000) and thus must be removed from the final product. Although the role of Ni^{2+} in carcinogenesis is not clear, some molecular models suggest interaction with histones in the cell nucleus, leading to DNA damage. Application of a strong chelating agent, such as EDTA, resulted in co-elution of the bound proteins. Elution with EDTA was also found to cause a co-elution of a small amount of enzyme before and after the main peak (Figure 3). This could also be due to the distribution of histidine residues on the surface of CGTase. Hemdan *et al*. (1989) mention that locations of histidines residues is critical for the exploitation of IMAC chromatography. These histidine residues could be on the surface or interior, localized, accessible or non-accessible for coordination, distant or vicinal. Sometimes intramolecular interaction such as hydrogen bonding may also occur, which results in a non-attachment of the histidine to the Ni^{2+}-Sepharose resin. Thus, the EDTA was too strong as a chelating compound and eluted most of the Ni^{2+} ion from Ni^{2+} -Sepharose IMAC resin. A second competitive agent, imidazole, was therefore chosen to replace EDTA in the elution buffer.

Elution of Bound CGTase on Ni^{2+}-Sepharose IMAC Resin Via Strategy B

Twenty mL of clarified *E. coli* homogenate containing the enzyme CGTase was loaded onto a packed column containing Ni^{2+}-Sepharose resin. The column was then equilibrated and washed with 25 ml of 20 mM PBS, pH 7, with a flow rate of 1 mL/min. Once unbound protein was removed from the column, bound proteins were eluted via 20 mM PBS, pH 7, 0.1 M imidazole. This was performed via a 0-100 mM gradient elution with 20 mM PBS, pH 7, and 20 mM PBS, 0.1 M imidazole, pH 7.

From the chromatogram in Figure 4, about 30% enzyme loss was observed in the flowthrough step (Table 2). In the washing step, a 13% enzyme loss was noted. This was the same as the results obtained when using the same washing buffer (20 mM PBS, pH 7) in the earlier purification step (Strategy A). The elution buffer used was 20 mM PBS, pH 7, 0.1 M imidazole. This was applied in a gradient manner using two buffers, i.e., 20 mM PBS, pH 7, and 20 mM PBS, 0.1 M imidazole, pH 7. Yields of 57% enzyme were obtained, which were higher than that obtained using Strategy A (0.05 M EDTA as chelating agent) with a 45% elution yield. However, a similar chromatogram pattern (Figure 3 and Figure 4) with the formation of "shoulder" peaks (elution before and after the main peak) was observed, which resulted in a large amount of buffer loss. An apparent peak was observed at 45 mM imidazole concentration (Figure 4). It indicated that most of the elution occurred at 45 mM imidazole, which is more cost effective. Hence to reduce cost, buffer loss and the harmful effects of this chelating agent, subsequent elutions were done at 45 mM of imidazole in a single step manner.

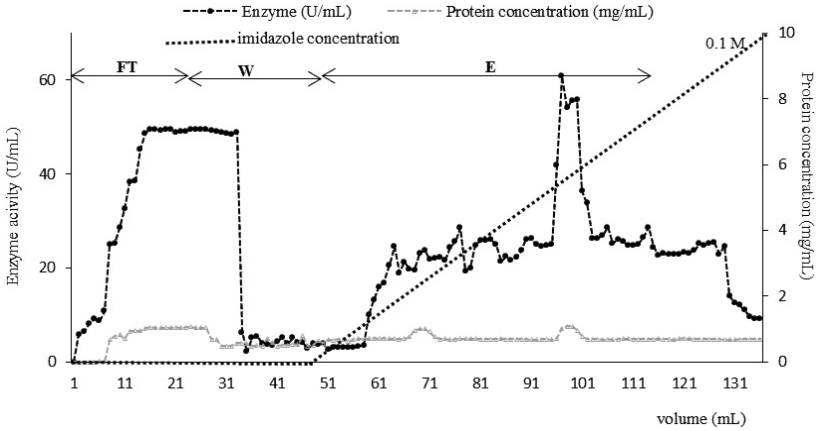

Figure 4: Chromatogram of CGTase separation via Ni^{2+} IMAC resin at 1 mL/min. Buffers used: equilibration buffer: 20 mM sodium phosphate, pH 7, washing buffer: 20 mM sodium phosphate buffer, pH 7, gradient elution 20 mM sodium phosphate, 0.1 M Imidazole, pH 7.

Table 2: Purification table for CGTase using Ni^2 -Sepharose IMAC resin (Strategy B).

Purification stage	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold	Yield (%)
Clarified homogenate	20	2956	77.6	38.09	1	100
Flowthrough	20	885.33	19.4	45.64	1.2	30
Washing	25	371.25	14.33	25.91	0.68	13
Elution	50	1684.25	51.5	32.7	0.86	57

Elution of Bound CGTase on Ni^{2+}-Sepharose IMAC Resin Via Strategy C

Twenty mL of clarified *E. coli* homogenate containing the enzyme CGTase was loaded onto a packed column containing Ni^{2+}-Sepharose resin. The column was then equilibrated and washed with 25 ml of 20 mM PBS, pH 7, with a flow rate of 1 mL/min. Once unbound protein was removed from the column, bound proteins were eluted via 20 mM PBS, 0.045 M imidazole, pH 7. This was performed via a single step elution.

The chromatogram in Figure (5) presented a good IMAC separation. A single step elution was done using 45 mM imidazole throughout. Purification of CGTase performed with 45 mM imidazole as its elution buffer showed a 5-fold purification during the elution step with yields of 49% (Table 3). The loss of enzyme was 13%, which indicated a good separation by Ni^{2+} -Sepharose IMAC resin. The remaining enzyme in the Ni^{2+} -Sepharose IMAC column was regenerated by washing the column with 50 mM EDTA.

In the past, only two studies of the purification of CGTase using metal affinity chromatography were performed. In the first study by Berna *et al*. (1996), the enzyme CGTase was purified in a single step metal affinity chromatography using two metals Cu (II), Zn (II) and a tandem combination of Cu (II) and Zn (II). 25 mM imidazole was used as the eluting agent coupled with 50 mM EDTA. When using the tandem combination of Cu (II) and Zn (II) there were no deleterious effects on enzyme activity. IMAC was demonstrated to be a viable technique that can outperform biospecific affinity chromatography (such as β-CD). This is because metal affinity chromatography gives similar purity and activity recovery with minimal or no additional steps required, which is an additional advantage when it comes to industrial applications. In the second study by Volkova *et al*. (2000), CGTase was purified using Cu (II)-IDA-Agarose and desorbed with the addition of 25 mM of imidazole to the washing buffer in a single step elution manner. The specific activity of the CGTase increased 15-fold in comparison to the initial value (273.6 U/mL). Results showed a 73% activity recovery, which is lower that than obtained from this study (87%). The author suggested the use of IMAC because most of the CGTases are said to contain approximately 10 histidine residues in their primary structure. The affinity of CGTase to Cu (II)-IDA-Agarose is based on the coordination bond formation between the metal ion and the imidazole groups of the accessible histidine groups in the primary structure

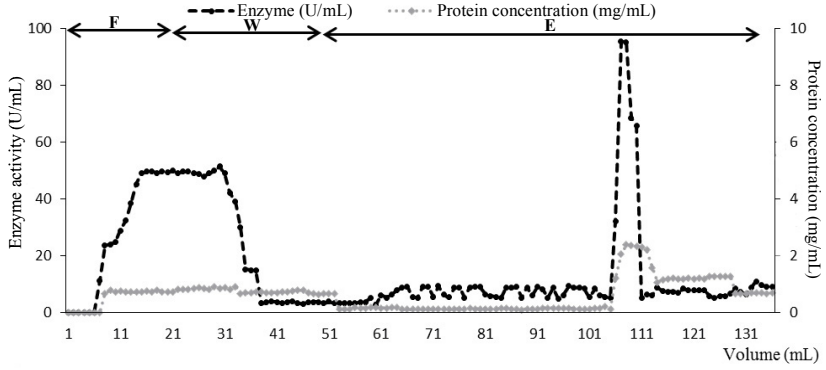

Figure 5: Chromatogram of CGTase separation via Ni^{2+} IMAC resin at 1 mL/min. Buffers used: equilibration buffer: 20 mM sodium phosphate, pH 7, washing buffer: 20 mM sodium phosphate buffer, pH 7, single step elution 20 mM sodium phosphate, 45 mM Imidazole, pH 7.

Table 3: Purification table for CGTase using Ni^{2+}-Sepharose IMAC resin (Strategy C).

Purification stage	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold	Yield (%)
Clarified homogenate	20	2956	77.6	38.09	1.	100
Flowthrough	20	870.6	15.4	56.53	1.48	30
Washing	25	411.14	19	21.64	0.57	14
Elution	80	1291.2	7.5	172.16	4.52	45

Total enzyme recovery: 87.04%

(Volkova *et al*., 2000). The enzyme is also said to be metal-independent since activity was retained with EDTA, but inhibition with $Zn^{2+,}$ Ni^{2+}, Cu^{2+} and Fe^{3+} indicated the presence of histidine residues. In another study by Clemmitt *et al*. (2000), single step elutions were studied using various imidazole concentrations for the purification of green fluorescent protein (GFP) via an IMAC expanded bed adsorption. Imidazole with single step elution was found to yield a better separation and higher enzyme yields.

The ICP results showed only 0.4512 mg/L nickel ion was co-eluted with the CGTase. This amount of nickel is considered insignificant (< 0.5 ppm) and proved that 20 mM sodium phosphate with 45 mM of imidazole, pH 7, was a suitable elution buffer.

CONCLUSION

The equilibrium binding capacity of CGTase toward Ni^{2+} IMAC resin was high, 666.67 U/mL, and the dissociation constant is 5.3×10^{-1} U/mL This indicated a strong binding capacity and affinity of the Ni^{2+} IMAC resin towards the enzyme. The dynamic binding capacity was 6.43 mg/mL of resin. The separation using 45 mM imidazole in the elution buffer gave 4.5-fold purification during the elution step and an 87% overall recovery of CGTase. Moreover, the nickel ion concentration in the eluted sample was only 0.4512 mg/L, which is approved for usage in the cosmetic and textile industry. All in all, IMAC separation has proven to be reliable, efficient and inexpensive in our preparatory scale test. It played an important role in reducing metal leaching into the final product, achieving higher purification and avoiding the denaturing of the enzyme during the process.

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