Development of Purification Method of Sesame Sprout (Sesamum indicum) Lipase Using Immobilized Metal Affinity

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ABSTRACT

A high-density matrix was prepared by coating an alumina particle with agarose using an emulsion technique. Imidodiacetic acid was immobilized onto this matrix. Charging this matrix with copper created a useful chromatography matrix for purification of indigenous sesame sprout lipase. Butanediol diglycidyl ether (BDGE) was used as spacer arm. Factors such as, adsorption pH, BDGE concentration in matrix preparation, concentration of NaCl and imidazole were investigated. Based on both the binding capacity of matrix and the eluate adsorbed lipase, the optimum BDGE concentration and the adsorption pH were found about 40 % and 7, respectively. However, an increase in NaCl concentration in adsorption buffer from 0.5 to 1.5 M resulted in 2 times decrease in the ratio between adsorbed lipase and adsorbed total proteins. More interestingly, immobilization of Cu2+ on this matrix was highly effective in the purification lipase, since lipase could be easily eluted from matrix using low concentration of imidazole (10 mM). Enzyme recovery and purification factor were 68% and 9.4, respectively.

Keywords: lipase, immobilized metal ions, sesame, sprout, copper

INTRODUCTION

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) has been produced by mammals, bacteria, fungi, and plants in large amounts (Thirstrup et al., 1993; Kosugi et al., 1995; lima et al., 2003; Yadav et al., 1998; Lamikanra and Watson, 2004, Abigor et al., 2002; Mohamed et al., 2000) and perceived by research scientists as one of the most important classes of industrial enzymes [Arbide and Pitcher, 1989]. Sesame sprout lipase has been produced and characterized its ability as catalyst (Suhendra, 2005). Recently, lipases have been used extensively in the dairy industry, for household...
detergents, in the oleochemical industry and to produce structured triacylglycerols (Falch, 1991; Macrae, 1983). The application of lipases as catalysts in organic synthesis has been paid much more attention due to its several advantages for synthetic chemicals. For instance, the catalytic conditions in nonaqueous media are mild and product yields are usually high. Besides, lipase has stereoselectivity and regioselectivity, just as in aqueous surroundings. Furthermore, lipases are catalysed at hydrophilic–lipophilic interfaces.

However, lipase has to be extracted from original resources and purified before used in the industries. The choice of ligand in chromatographic enzyme purification is often complicated due to the need to compromise between ligand stability and specificity for the objective product. Robust ligands used on the matrices in previous works, such as ion-exchange Streamline matrix (Chang and Chase, 1996a; Frej, 1996; Johansson et al., 1996; Chang and Chase, 1996b), zirconia (Griffith et al., 1997; Mullick et al., 1998), and derivatized glass beads (Thomes et al., 1995), have been used for the protein purification. Although such adsorbents can be used in the purification of a large number of products, their lack of specificity may lead to much adsorption of other components from homogenate. The significant non-specific adsorption of other components to the adsorbent will reduce the available number of ligands for protein binding (Fernadez-Lahore, et al., 2000). In addition, the relatively high ionic strength of some fermentation broth will reduce the performance of ion-exchange ligand (Chang and Chase, 1996; Mullick et al., 1998, Mullick and Flickinger, 1999).

Unfortunately, the use of highly specific ligands, such as antibodies, protein A or lectine is often limited as these ligands cannot withstand the rigorous cleaning in place (CIP) procedures that frequently required in processing homogenate. Immobilized metal affinity chromatography (IMAC) has been proven to be a useful and versatile technique for the isolation and purification of protein (Choe et al., 2002; Michel et al., 2001, Sanchez et al., 2001). Proteins that have exposed histidine residues could be purified using IMAC. As ligands for affinity separation, metal ions complexes have demonstrated important advantages over biological affinity agents such as inhibitors and antibodies. Small and inexpensive metal-complexes are stable under a wide range of conditions, and can be recycled many times without loss of activity. The elution can be performed under relatively mild conditions, and the columns can be cleaned and regenerated easily without reduction in protein-binding capacity. The selectivity can be tailored through the choice of metal ions, solvent conditions, or by modification of the target protein.

The most commonly used metal ion ligands in IMAC are the first-row transition metals. Metal ion ligands are usually immobilized on iminodiacetic acid (IDA) that is attached to the matrix through short spacer arm (e.g. epichlorohydrin) or long spacer arm (e.g. butanediol diglycidyl ether). Long spacer arm will adsorb more contaminant proteins than short spacer arm (Armisen et al., 1999). The apparent affinity of protein for immobilized metal ions depends on the metal ion involved in coordination. In the case of IDA chelators, the affinities of retained proteins are in the following order: $\text{Cu}^{2+} > \text{Ni}^{2+} > \text{Zn}^{2+} > \text{Co}^{2+}$ (Sulkowski, 1985; Hemdan et al., 1989; Gaberg-Porekar and Menart, 2001; Chag, 2001). In the case of immobilized $\text{Cu}^{2+}$ on IDA matrix, one exposed histidine residues is enough for weak binding, while $\text{Ni}^{2+}$ will bind a protein with two exposed histidine residues (Sulkowski, 1985; Hemdan et al., 1989).

The adsorption of proteins to derivitised surfaces (e.g. immobilized metal ions) involves multivalent interactions between functional groups on the protein and complementary sites distributed on the surface of metal (Johnson and Arnold, 1995; Todd et al., 1994; Johnson et al., 1996). The fact that adsorption involves multiple interactions has important implications for the design of separation processes and for the interpretation of heterogeneity in biological recognition phenomena. A consequence of multivalent interactions is a significant increase in protein binding affinity, but the recovery of adsorbed protein becomes difficult. Therefore, the elution strategy should be optimized to obtain high recovery of adsorbed protein. On the other hand, multivalent interaction may be reduced by a decrease in ligand density (Wirth et al., 1993; Lesiene et al., 1997), and by immobilization of macromolecules on the adsorbent surface (Mateo et al., 2001) or adsorption of polymers on the adsorbent surface (Kumar et al., 2000; Galaev et al., 1994).

In this paper, the purification of sesame sprout lipase was developed using immobilized metal ions technique. Factors that affect adsorption, such as adsorption pH, concentrations of BDGE, NaCl and imidazole, were investigated. The ability of two-step elution with imidazole for desorption of lipase from immobilized metal ions was also investigated.

**MATERIALS AND METHODS**

**Materials**

Sesame was purchased from local supplier. Activated alumina, sorbitane trioleate (Span 85), imidazole, iminodiacetic acid, BDGE, iso-octane, and agarose powder were purchased from Wako Chemical Industries (Osaka, Japan). EDTA, $\text{Na}_2\text{SO}_4$, $\text{CuSO}_4$, $\text{K}_2\text{HPO}_4$, $\text{KH}_2\text{PO}_4$, NaCl, $\text{Na}_2\text{CO}_3$, and NaOH, were purchased from Merck KGaA (Darmstadt, German). Bovine serum albumin (BSA), oleic acid, olive oil, and folin-ciocalteu were purchased from Sigma Chemical (St Louis, MO, USA).
Crude Enzyme

Crude enzyme was prepared according to a method previously described (Abigor et al., 2002). Sesame sprouts those were prepared as previously described (Suhendra, 2005) were disrupted in 20 mM phosphate buffer at pH 7 containing 0.6 M saccharose and 1 mM EDTA. Homogenate was further centrifuged for 15 min. Supernatant was analyzed for protein and enzyme activity.

Preparation of epoxide agarose-coated alumina matrix. The agarose-coated alumina matrix was prepared according to a method previously described (Hidayat et al., 2003a). Activation of the matrix was performed with BDGE (O’Brien et al., 1996). Eight g of the matrix was washed with distilled water and the matrix was suspended in 45 ml of appropriate BDGE solution containing 0.8 M NaOH. The suspension was mixed for a total reaction time of 18 h at 25°C.

Introduction of chelating groups (IDA matrix). The procedure of coupling IDA into the matrix was as follows: 15 ml of epoxide matrix was suspended in 15 ml of 0.1 M Na₂CO₃ buffer containing 1.35 g of IDA and adjusted to pH 11 with NaOH. The mixture was continuously mixed at 28°C for 16 h. The IDA matrix was then washed with distilled water.

Immobilization of copper ions on matrices. Two g of IDA matrix was charged with 10 ml of 50 mM CuSO₄. Unbound metal ions were removed by washing with water. The matrix was then equilibrated with 20 mM phosphate buffer containing 0.5 M NaCl. It was then equilibrated with 20 mM phosphate buffer containing 0.5 M NaCl.

Chromatography. The chromatographic column containing the IDA matrix (2 ml) was first washed with distilled water, and then loaded with 6 ml of 50 mM CuSO₄ solution. The column matrix was then washed with distilled water to remove loosely bound metal ions. The column was equilibrated with 20 mM phosphate buffer containing 0.5 M NaCl at pH 7. Homogenate was applied into the column, and washed with buffer. The adsorbed proteins were eluted using appropriate elution buffer.

PROTEIN assay. Total protein concentration was determined by the Lowry method. The samples to be assayed were diluted with buffer to obtain protein concentrations in the ranges of 0 to 0.8 mg/ml. The sample was added to the assay reagent according to the standard protocol. The absorbance of the sample was determined at 540 nm. BSA was used to obtain the standard curve.

Lipase assay. Lipase activity was determined by adding 100 µL of sample to 2 ml appropriate solution containing of olive oil and iso-octane. The mixture was incubated at 37°C for 20 min. The formation of oleic acid was determined according to a method previously described (Marseno et al., 1998). One unit of enzyme activity is defined as the amount of released oleic acid (µmol) from olive oil per min at 37°C.

RESULTS AND DISCUSSIONS

Effect of BDGE on Protein Adsorption

Binding capacity of immobilized metal affinity matrices depend strongly on ligand density of matrices that may be prepared by different concentration of spacer arm materials, such as BDGE, epichlorohydrin and glycidoxypropyltrimethoxysilane (Armisen et al., 1999; Wirth et al., 1993; Liesiene et al., 1997; Hidayat et al., 2003a). Fig. 1A shows the effect of BDGE concentration on binding of proteins and lipase on immobilized copper ions. The capacity of matrix increased with an increase in BDGE concentration until equilibrium was reached. Maximum binding capacity of immobilized copper ions for total proteins and lipase were

Figure 1. Effect of BDGE concentration on the adsorption of proteins (A) and eluted enzyme activity (B).
reached at BDGE concentration of 50% and 40%, respectively. The adsorption of proteins increased 19% with an increase in BDGE concentration from 20 – 50%. The adsorbed lipase increased 30% with an increase in BDGE concentration from 20 – 40%. These findings are in good agreement another works (Armsen et al., 1999; Liesiene et al., 1997).

The retention of lipase depended on addition of BDGE concentration at matrix preparation (Fig. 1B). At low BDGE concentration (20 – 30%), lipase could easily be eluted from the matrix using low imidazole concentration (2 mM). At higher BDGE concentration (> 40%), the eluted lipase decreased about 2 times. Finally, the rest of adsorbed lipase was effectively eluted with 50 mM imidazole. A decrease in eluted lipase with an increase in BDGE concentration may be related with an increase in ligand density of matrix (Sanchez et al., 2001; Wirth et al., 1993; Liesiene et al., 1997; Hidayat et al., 2003a). As a consequence of this condition, adsorption of proteins to matrix surface involved multivalent binding interactions (Johnson and Arnold, 1995; Todd et al., 1994; Johnson et al., 1996). Therefore, binding affinity of proteins to the matrix increased. As a result, the recovery of adsorbed proteins became difficult (Hidayat et al., 2003b; Hidayat et al., 2004). Thus, based on both the adsorbed and eluted lipase, BDGE concentration of 40% was chosen for further lipase purification.

Effect of NaCl Concentration

Concentration of NaCl in adsorption buffer affects protein adsorption in the immobilized metal affinity system. Fig. 2 shows the effect of NaCl concentration on the adsorption of lipase on the immobilized copper ions. An increase in NaCl concentration up to 1.5 M resulted in an increase in 2 times adsorbed total proteins (contaminant proteins and lipase). As a result, the adsorbed specific activity decreased with an increase in NaCl concentration in buffer. An increase in adsorbed contaminant proteins may be caused by character of interactions between proteins and immobilized metal ions.

Porath (1990) suggested that the molecular interaction in metal-affinity adsorption may be classified as follows: i) ionic bond formation due to electrostatic forces; ii) coordinative bonds with electrons in overlapping orbitals; iii) hydrophobic interaction. Since NaCl in solution will dissociate as Na\(^+\) (cation) and Cl\(^-\) (anion), they affect the outer sphere ion cloud of proteins and ligands, thereby suppressing ionic adsorption. Beside, a high NaCl concentration alters the partitioning of the protein in the interfacial region between the matrix and immobilized metal ions. When immobilized metal ions and nucleophile (e.g., histidine) in a protein locate close each other, electron orbital overlapping occur (Porath, 1990; Porath et al., 1975; Arnold, 1991).

In addition, Porath (1992) suggested that addition of NaCl resulted in an increase in entropy due to the disorganization of water molecules surrounding the immobilized spacer arm and the polymer matrix. The increase in entropy promotes protein binding. Therefore, the adsorbed lipase increased with an increase in NaCl concentration. Since sesame sprout has high concentration of lipid (48-58%), then it is suggested that most of proteins have hydrophobic character. Thus, it is suggested that an increase in protein adsorption may be caused by altering the hydrophobic interactions and electrons in overlapping orbitals. These interactions are probably the dominant forces in adsorption of lipase at high NaCl concentrations.

![Figure 2. Effect of NaCl concentration on the adsorbed proteins and lipase.](image-url)

Effect of pH Buffer

The molecular interaction between proteins and metal ions in metal-affinity adsorption is caused by the interaction of certain amino acid residues of protein with immobilized metal ions and the involvement of non-specific binding including electrostatic interactions of a positively charged amino acid residues and a negatively charged of matrix surface and/or hydrophobic interactions. However, histidine is the most responsible amino acid residues of protein for the interaction with immobilized metal ions (Sulkowski, 1985; Hemdan et al., 1989; Bal et al., 1998). The interaction is probably due to the formation of coordination complex of imidazole nitrogen of histidine residues only in an unprotonated state with immobilized metals. This was indicated by the very strong elution power of imidazole and imidazole derivatives (Porath, 1990). Therefore, pH has an effect on binding capacity of immobilized metal ions.

Fig. 3 shows the effect of pH on the adsorption of proteins on immobilized metal ions. An increase in binding capacity was significant observed for increasing pH in the range of 5 – 7. This may be due to an increase in...
unprotonated imidazole nitrogen of histidine residues, since pK\textsubscript{a} of surface histidine residues is generally between 6 and 7. The unprotonated nitrogen of histidine residues enhanced the interaction between proteins and immobilized metal ions. As consequence, binding capacity increased. Further increase in pH resulted in a decrease in binding capacity due to electrostatic repulsion, since some amino acids have negatively charged at high pH.

Figure 3. Effect of pH buffer on the adsorption of proteins and lipase.

Purification of Lipase

Imidazole, which is usually used as an eluent in the metal-affinity chromatography system, was investigated. It is suggested that imidazole, as electron donor, competes with the functional groups of protein for the immobilized metal ions. The concentration of imidazole for proteins desorption depends on the binding affinity of the target protein. Proteins that have a low binding affinity can be eluted at low imidazole concentrations.

In this paper, two-step elution was investigated. Fig. 4 shows chromatogram elution of lipase from immobilized metal ions. Most of the adsorbed lipase was eluted with 10 mM imidazole containing 0.5 M NaCl during first-step elution. It is suggested that lipase has a low binding affinity toward immobilized copper ions, so that it could be easily desorbed from matrix at low concentration of imidazole. The recovery of lipase and the purification factor of the first-step elution were 68 % and 9.4, respectively. The specific activity of the eluted enzyme was 5.3 U/mg protein. The most strongly adsorbed lipase was eluted out at second-step elution using a solution of higher imidazole concentration (50 mM).

Figure 4. Two-step elution on purification of sesame sprouts lipase using immobilized copper ions technique.

CONCLUSIONS

Maximum binding capacity of immobilized copper ions was reached at BDGE concentration of 50% and 40% for protein and lipase, respectively. An increase in adsorbed proteins (19%) and lipase (30%) occurred when BDGE concentration increased from 20 – 50% and 20 – 40%, respectively. An increase in NaCl concentration up to 1.5 M resulted in an increase in the adsorbed contaminant proteins. As a result, the adsorbed specific activity decreased with an increase in NaCl concentration in buffer. Based on both the binding capacity of matrix and the eluted adsorbed lipase, the optimum BDGE concentration and the adsorption pH were 40 % and 7, respectively. More interestingly, immobilization of Cu\textsuperscript{2+} on this matrix was highly effective in the purification lipase, since lipase could be easily eluted from matrix using low concentration of imidazole (10 mM). Enzyme recovery and purification factor were 68% and 9.4, respectively. The specific activity of the eluted enzyme was 5.3 U/mg protein.

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