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# Comparison of Cutinase Separation in Different Chromatographic Media

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Cutinase is a hydrolytic enzyme that has both properties of lipase and esterase, thus finding its use in many areas. Previous studies have investigated both upstream and downstream processes for cutinase production from microbial source. However, no study has yet to address the use of membrane chromatography for cutinase purification, which is more favourable in terms of process resolution and product throughput as compared to the conventional gel chromatography. Hydrophobic interaction was chosen as the separation mechanism for cutinase purification in this study. The optimisation of cutinase purification in two different types of chromatographic media; conventional packed-gel and membrane matrix, were pre-determined by the best compromise between the recovery and purity of the purified cutinase. It was found that the optimised condition were of pH 4.0 and 1.0 M ammonium sulfate for the conventional column (50% recovery, 4.8-fold purity) and pH 6.0 with 1.5 M ammonium sulfate for the membrane–matrix column (87% recovery, 30-fold purity). Preferential interaction analysis was used to describe the protein chromatographic behaviour in each chromatographic media. Graph of natural algorithm of protein retention data to the function of salt concentration at pH 4.0 and 6.0 for each column were plotted. It was found that at the optimum pH condition for gel-packed column, a small amount of ammonium sulfate was sufficient to achieve maximum cutinase recovery and purity since the effect of salt at that particular pH were less significant. Consequently, the number of released water molecules were calculated and it was observed that for membrane column, larger number of water was released at pH 6.0 illustrating more protein were bounded to the stationary phase, thus explaining the optimum pH condition of the particular column.

**Keywords:** Purification, Cutinase, Packed-bed, Membrane matrix, Hydrophobic interaction, Preferential interaction analysis

## INTRODUCTION

Cutinase (EC 3.1.1.74) is a cutin-degrading enzyme which originates from phytophatogenic fungi (Carvalho *et al.* 1998). Fungal cutinase is mostly consist of single peptide having molecular weight of 22-26 kDa in range (Kolattukudy *et al.* 1981). Cutinase have double properties of an esterase and lipase, hence it has been applied in many area such as detergent and personal care products, oleochemicals and dairy industries (Dutta *et al.* 2009). Such wide application has led to numerous studies of cutinase production and purification. Crude cutinase from the fermentation process has been purified using many separation techniques such as expended bed adsorption (EBA), affinity chromatography and also hydrophobic interaction chromatography (HIC) (Wang *et al.* 2000, Nilsson *et al.* 2003, Kepka *et al.* 2005, Lienqueo *et al.* 2008). However, cutinase has yet to be purified using hydrophobic interaction membrane chromatography (HIMC) which has favorable advantage in mass transport properties as compared to the conventional packed-bed HIC (Ghosh 2001, Yu *et al.* 2008).

HIC has gained a lot of attention as the choice of chromatographic separation as it is able to produce equally efficient separation at relatively low cost and more benign separation (Ghosh and Wang 2006). However, there are some restriction in solutes transport in the packed-bed chromatography which complicates the scale up of such process (Yu *et al.* 2008). Recent studies have developed an alternative media for chromatographic process which utilises the advantages of membrane technology in overcoming the problem experienced in the conventional packed-bed column (Ghosh and Wong 2006, Huang *et al.* 2009, Pereira *et al.* 2010).

Perkins *et al.* in 1997 has expended the preferential interaction theory (PIT) to describe the salt effect in HIC. This theory used the same approach as solvophobic theory which examines the effect of salts on the properties of the solvent with the advantage of direct physical meaning of the parameters as compared to the lumped factors in the solvophobic theory. Theoretically, during binding of protein to the hydrophobic ligand, water around the protein-ligand complex will redistribute which results in a decrease in hydrophobic exposed area, thus water is released (Xia *et al.* 2004). Therefore, the analysis could be further discussed by analysing the number of released water molecules of the respective solutes and stationary phase applied.

This study will focus on the separation behaviour in different chromatographic media used for the purification of cutinase. A mixture of cutinase and bovine serum albumin (BSA) was prepared to act as the model protein in simulating the purification process. The cutinase purification process was initially determined by optimising the mobile phase pH and the kosmotropic salt concentration condition to achieve the maximum cutinase recovery and purity possible. Ammonium sulfate was chosen as the kosmotropic salt used to enhance the binding of protein to the stationary phase. Isocratic elution experiments were conducted to obtain the protein retention data which were eventually used in

$$\text{Recovery} = \frac{\text{Total activity (U)after HIMC}}{\text{Total activity (U)in feed solution}}\ x\ 100 \quad (1)$$

$$\text{Purification factor} = \frac{\text{Specific activity } (\text{Umg}^{-1})\text{after HIMC}}{\text{Specific activity } (\text{Umg}^{-1})\text{ in feed solution}} \quad (2)$$

determining the preferential interaction parameters. The resulting parameters were then utilised to interpret the chromatographic behaviour of protein in each media.

## THEORY AND EXPERIMENTAL PROCEDURES

### Calculation for recovery and purity

The performance of the chromatographic process were evaluated by the ability of the process to produce high recovery and purity of cutinase. The recovery and purity of cutinase were calculated using Eq.(1) and Eq.(2)

### Preferential interaction theory of HIC

The PIT model for HIC (Xia *et al.* 2004, Lienqueo *et al.* 2007) were used to describe the correlation between solute's capacity factor and salt concentration. The relationship is expressed as follows:

$$\ln(k') = \alpha + \beta\, m_3 + \gamma \ln(m_3) \quad (3)$$

where $k'$ is the capacity or rentention factor which can be determined using Eq. (4), $\alpha$ is a constant and $m_3$ refers to the molality of the salt.

$$k' = \frac{t_r - t_0}{t_0} \quad (4)$$

The terms $t_r$ and $t_0$ in Eq. (4) are referred to the retention time of the adsorbed protein and the unretained solutes, respectively. The $\beta$ and $\gamma$ are the preferential interaction parameters to calculate the total number of water molecules and salt ions released during binding process by using the following equation (Perkins *et al.* 1997):

$$-(\Delta v_1) = \frac{\beta g m_1}{n} \quad (5)$$

where $m_1$ is molality of water, $n$ is valence of salt ions and $g = (\partial \ln(m_3)/ (\partial \ln(a\pm))$, $a$ is the activity of ions and $-(\Delta v1)$ is the number of water molecules released during binding process. The value of $g$ can be computed using the Debye-Hückel equation.

### Preparation of buffer solutions

All buffers used for the chromatographic process were prepared according to the respective pH range; sodium acetate for pH 4.0 and sodium phosphate for pH 6.0, all fixed to 50 mM of concentration. Two sets of buffers were used for each run, one in presence of ammonium sulfate in range of 0.8-2.2 M; known as the binding buffer, and another without the presence of salting-out salt which act as the elution buffer. All buffers were filtered and degassed prior use.

**Table 1.** Specification of HIC and HIMC column

| | HIC | HIMC |
|---|---|---|
| Media | Sepharose | Stabilised and reinforced cellulose |
| Ligand | Butyl | Phenyl |
| Media size (μm) | 90* | > 3** |
| Column volume (ml) | 1.0 | 3.0 |

Note: *mean bead size ** nominal pore size

**Sample preparation**

The protein samples for the purification experiment, comprising of cutinase (Novozyme 51032) and BSA, were prepared in binding buffer to simulate the separation of protein for the purification process. For isocratic elution experiment, similar approach was used to prepare the samples but only to contain single protein of cutinase. All samples were centrifuged at 10,000 rpm for 20 minutes before injecting samples to the chromatography columns.

**Chromatographic runs**

All the chromatographic experiments were conducted on AKTAprime FPLC system. Flow rate was maintain at 1.0 ml min-1 throughout the process. Two types of column were tested; Butyl FF for HIC column and Sartobind Phebyl nano for HIMC column. The specification of both columns were summarised in Table 1. Purification of cutinase from the protein mixture were performed on both columns by employing gradient elution. The protein isocratic retention data were obtained by performing isocratic elution on each columns. Binding buffer which contained ammonium sulfate were equilibrated for about 5 column volumes (CV) then followed by injection of 100 μl of samples. The obtained retention data were then recorded and process were repeated to obtain duplicate data.

**Protein and cutinase activity assay**

Bradford asssay (Bradford 1976) and cutinase activity assay (Kumar *et al.* 2005) were conducted to determine protein content and cutinase activity in the crude and purified sample. These data were then used to calculate the cutinase recovery and purity.

**Parameter regression**

The isocratic retention data were plotted and Eq. (3) was fitted using Matlab R2008b software to obtain the values of each parameters. Parameter regression was performed to minimised the sum of squared residuals (SSR) between experimental data and the model response.

## RESULTS AND DISCUSSION

**Purification of cutinase**

Purification of cutinase were performed on various buffer pH and ammonium sulfate concentration. The eluted protein were collected in fractions and checked for its protein concentration and cutinase activity. The recovery and purity of the purified cutinase were quantified. It was observed that the best compromise of

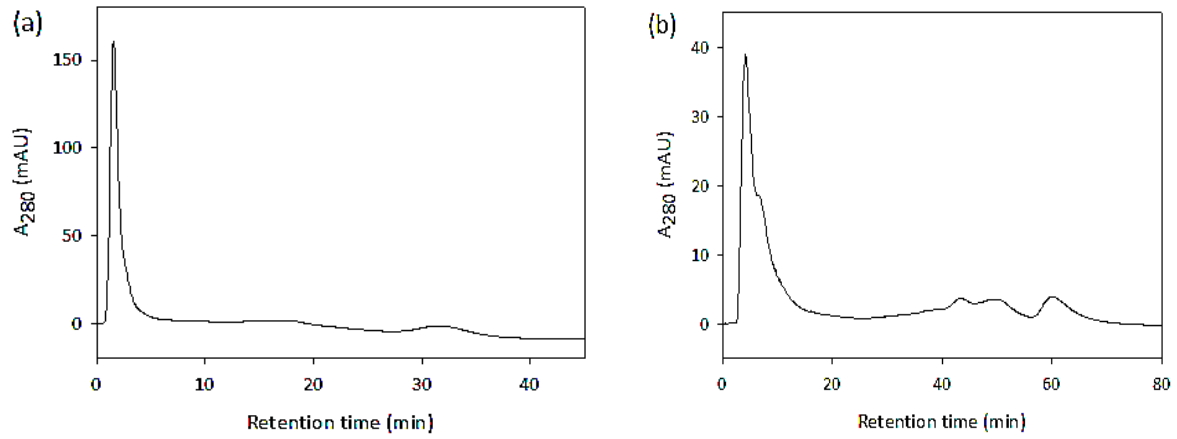


**Fig. 1:** Chromatograms of the optimised condition for (a) HIC and (b) HIMC columns

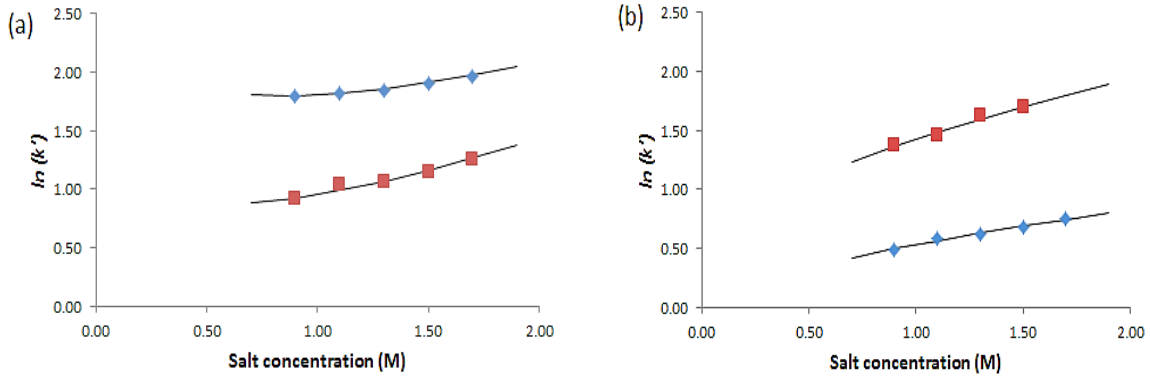


**Fig. 2:** Plots of natural algorithm of protein retention data against ammonium sulfate concentration at pH 4.0 and 6.0 for (a) HIC and (b) HIMC columns. (◆), pH 4.0; (■), pH 6.0.

recovery and purity obtained for HIC was at pH 4.0 and 1.0 M ammonium sulfate. Meanwhile for HIMC column, pH 6.0 and 1.5 M salt concentration was found to be the optimum condition for the maximum output of cutinase recovery and purity. Table 2 shows the result summary of the optimum condition for cutinase purification for each type of column.

**Table 2.** Summary of cutinase purification result

| | HIC | HIMC |
|---|---|---|
| **Recovery (%)** | 50 | 87 |
| **Purity (fold)** | 4.8 | 30 |

Figure 1 shows the chromatogram of the optimised condition for HIC and HIMC column. Cutinase was eluted at 32 and 60 minutes at retention time, respectively. It can also be observed that the peak in HIC is rather broad as compared to the sharp peak produced by the HIMC column.

**Protein retention factor data**

PIT was employed to describe the protein behaviour in different chromatographic condition. Isocratic elution experiment were conducted to obtain the capacity factors data for each type of columns at various pH and ammonium sulfate concentration. The protein retention factors data (*ln k'*) were plotted against salt concentration in range

of 0.9 – 1.7 M. Generally, the capacity factors would increase with increasing of kosmotropic salt concentration (Xia *et al.* 2004). A high value of ln k′ would indicate that the hydrophobic interaction was strong due to the increasing time needed to desorp the protein from the adsorber.

Figure 2 shows the plots of natural algorithm of the retention factors versus the ammonium sulfate molal concentration at pH 4.0 and 6.0 for both columns. From this plot, it was observed that the optimum pH of each column has higher *ln k′* values compared to the other pH. It can also be seen that at pH 4.0 of HIC column, the effect of salt concentration to *ln k′* were less obvious than in pH 6.0. Therefore, low salt concentration were sufficient to obtain maximum recovery and purity of cutinase.

**Determination of preferential interaction parameters**

The plots as in Figure 2 was fitted with Eq. (3) to obtain the values of the preferential interaction parameters. These values could later be used to evaluate the effects of salt, pH and stationary phase (Lienqueo *et al.* 2007) of the particular purification process. The values are tabulated in Table 3.

**Table 3.** Values of the preferential interaction parameters

| Column | HIC | | HIMC | |
|---|---|---|---|---|
| **pH** | **4.0** | **6.0** | **4.0** | **6.0** |
| ***α*** | 1.094 | 0.098 | 0.346 | 1.079 |
| ***β*** | 0.711 | 0.853 | 0.185 | 0.345 |
| ***γ*** | -0.628 | -0.535 | 0.157 | 0.253 |

From Table 3, both columns for each pH recorded positive values of $\beta$, which shows that the protein retention increases with increasing salt concentration (Chen *et al.* 2008). The significance of the $\beta$ parameter will be described in the later section.

**Water release values**

The total number of released water andsalt ions molecules were calculated based on Eq. (5) using the $\beta$ values determined in section Protein retention factor data. A high number of water molecules released during binding process demonstrate that the total wetted area has decreased due to the binding of hydrophobic patches on protein surface to the hydrophobic ligands (Perkins *et al.* 1997). Thus stronger bind should show higher number of water release value. Table 4 summarised the number of released water molecules at pH 4.0 and 6.0 of the HIC and HIMC column. Sartobind Phenyl nano column has relatively low number of released molecules as compared to the Butyl FF. A study done by To and Lenhoff (2007) found that the number released values are statistically independent of protein retention strength and also on the adsorbent and protein properties. It is also not sensitive enough to allow prediction of adsorption selectivity. Nonetheless, the result shows that number of water released increased as increasing pH for both columns. For HIMC column, protein binding is higher at pH 6.0, which explains the better purification result at that particular pH. For HIC column, although the $-(\Delta v1)$ value is slightly higher at pH 6.0, but since the *ln (k')* range is higher at pH 4.0; which indicates better separation, therefore pH 4.0 is the best chromatographic condition.

**Table 4.** Water released values at pH 4.0 and 6.0 of HIC and HIMC columns

| Column | HIC | | HIMC | |
|---|---|---|---|---|
| **pH** | **4.0** | **6.0** | **4.0** | **6.0** |
| $-(\Delta v_1)$ | 21 | 25 | 5 | 10 |

## CONCLUSION

It was observed that for each chromatographic media, different optimum condition were recorded in achieving maximised recovery and purity of cutinase. The recovery and purity obtained by HIC column is much lower from the HIMC column. Such behaviour is observed by using the PIT to evaluate the effect of pH, salt concentration and the stationary phase. The plots of natural algorithm of the protein retention data versus the ammonium sulfate concentration all increases with increasing salt concentration. At pH 4.0 for HIC column, the effect of salt concentration was less obvious, thus only small amount of ammonium sulfate in necessary to promote separation. It can also be observed that higher protein binding had occurred at pH 6.0 which correlates with the optimum pH condition for the HIMC column.

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