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Use of Ion Exchange Membranes for Selective Recovery of *Aspergillus awamori* Glucoamylase and Phage T4 Lysozyme

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Our research examined whether enhanced separation and purification of mutant enzymes could be obtained on ion-exchange membranes. Solutions of three mutants of *Aspergillus awamori* glucoamylase were passed through an anionic exchange membrane, as well as one mutant of T4 lysozyme through a cationic exchange membrane. The mutant enzymes were modified by adding "charged fusions", polypeptides of either aspartic acid residues to increase the overall negative charge of the enzyme or arginine residues to increase the overall positive charge. The effect of the mutations on the purification of glucoamylase from a "modified" fermentation broth were examined at two different elution pHs, 4.5 and 6.0.

The use of the charged fusions provided significantly improved purification capabilities over control versions. Both the small scale glucoamylase runs and the scaled up experiments had overall purification factors of around two, with a peak purification factor of near 7 for GACD10. Elution of glucoamylase at pH 4.5 did not lead to an increase in separability as compared to that obtained at pH 6.0.

Initial trials using a purified lysozyme mutant showed significant binding capabilities. Further experiments with this protein need to be done to determine scale up potential.

INDEX DESCRIPTORS: fusion proteins, purification fusions, protein purification, ion exchange membrane

The biochemical processing industry has in recent years made great strides in techniques for producing genetically engineered proteins; however, methods for separating and purifying these proteins cheaply have not shown similar growth. Many purification techniques that have proved successful for small quantities in the research laboratory are simply too difficult and costly to scale up.

One approach to this problem that has been taken in our lab with favorable results is the purification of genetically altered proteins using ion exchange membranes. An ion exchange membrane has a matrix of charged groups on it which attract oppositely charged proteins as they are passed through the membrane. Figure 1 is a schematic of an anionic exchange membrane, which in this case has positively charged quaternary amine groups which bind to negatively charged proteins. An advantage to using an ion exchange membrane is that binding and elution cycles can be run much more quickly than conventional ion exchange chromatography. In addition, the selectivity of this technique may be enhanced by using mutant enzymes containing additional charged amino acids in the form of "fusion fusions"; these extra amino acids create a region of high charge density on the protein that increases the protein's ability to bind to ion exchange groups. This method has worked well for purification of the enzyme β-galactosidase using both hollow fiber membranes (Heng et al., 1992) and ion-exchange membranes (Thiem and Heng, 1993). Our work used two different enzymes, glucoamylase from *Aspergillus awamori* and T4 lysozyme, to continue investigation of ion-exchange membranes as a method for separation and purification of charged fusion proteins.

**MATERIALS AND METHODS**

Microporous Membranes

The membrane used for small scale experiments with glucoamylase was a 25 mm O.D. QUAT Acti-Disk™ Separation and Purification Cartridge (FMC BioProducts, Rockland, ME). The large scale glucoamylase and lysozyme experiments utilized the 50 mm O.D. QUAT and CM Acti-Disk™ cartridges, respectively. The QUAT cartridge has positively charged quaternary amine groups while the CM cartridge has negatively charged carboxymethyl groups.

Fermentation and Preparation of Glucoamylase

The gene for glucoamylase (a gift from Cetus corporation, Emeryville, CA) was genetically altered to produce mutants with additional charged residues in the form of a “fusion tail” on the carboxyl

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**Table 1. Amino Acid Sequences for Purification Fusions**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GACD0</td>
<td>...Gly Ser Met Ala Tyr</td>
</tr>
<tr>
<td>GACD5</td>
<td>...Gly Ser Met Ala (Asp) Tyr</td>
</tr>
<tr>
<td>GACD10</td>
<td>...Gly Ser Met Ala (Asp)10 Tyr</td>
</tr>
</tbody>
</table>
Fermentation and Preparation of Lysozyme

The gene for the triple mutant lysozyme was a gift from Brian Matthews (University of Oregon). The triple mutant has glutamic acids substituted for lysines at amino acid residues 16, 135 and 147 (Dau-Pin et al., 1991). A purification fusion consisting of the residues Arg-Val-(Arg-Val)-Arg was added to this mutant to create the U5V mutant, which was used in these experiments (Bakir, U., Iowa State University, personal communication, 1993).

The microorganism used for production of lysozyme is *Escherichia coli*. Cells were grown in Luria Bertani media, supplemented with 100 mg/l ampicillin, at 32°C in an Environ-Shaker 3597 (Lab-Line, Melrose Park, IL). Growth proceeded for about four hours, at which point IPTG (isopropyl-β-D-thiogalactopyranoside) was added to induce lysozyme production. After 90 minutes, the cells were harvested by centrifugation. The cell pellets were then resuspended in 20 mM sodium phosphate buffer, pH 6.5, and sonicated to disrupt the cells, with the cell debris removed by centrifugation. The supernatant was dialyzed into 20 mM Tris, pH 7.25, then purified on an CM-Sephadex ion exchange column (Sigma Chemical, St. Louis, MO) using a linear 0-300 mM sodium chloride gradient. Fractions were tested for activity, with the purified lysozyme fraction dialyzed into 20 mM Tris solution, pH 7.25, and stored at 4°C for use with the ion exchange membrane experiments.

Feed Preparation

The preparations of each of the three mutants of glucoamylase were diluted 1:1 with 50 mM sodium acetate and then adjusted to pH 6.0. The protein concentration for the set of experiments at elution pH 4.5 was 0.1 mg/ml, while the protein concentration for the large scale operation was 0.1 mg/ml.

Membrane Loading and Eluting of Lysozyme

For those samples eluted at pH 4.5, the membrane was equilibrated with about 20 ml of 50 mM sodium acetate buffer, pH 6.0. Five milliliters of the feed solution were passed through the membrane six times using a syringe. The membrane was then washed with six 5 ml passes of equilibration buffer to remove any loosely bound protein. The removal of the bound protein was done by step gradient elution using 2 ml each of 50 mM sodium acetate solutions, pH 4.5 with NaCl concentrations varying from 0.1 M - 0.5 M in 50 mM graduations. One and a half milliliter samples of the feed, effluent, buffer wash, and each of the salt steps were set aside for protein and activity assays. Samples were stored at 4°C until assaying.

For the samples eluted at pH 6.0, the membrane was again equilibrated with 50 mM sodium acetate buffer, pH 6.0, though the solution was passed through the membrane using a Tris pump (ISCO, Lincoln, NE) at a flow rate of 5 ml/min. for ten minutes. The feed was then passed through the membrane at 1 ml/min. and recycled twice. After loading, the membrane was washed with 20 ml of equilibration buffer to remove loosely bound protein. The protein was removed with a step elution from 0.1 to 0.5 M NaCl in 50 mM sodium acetate at pH 6.0, with a step size of 100 mM NaCl. A solution of 50 mM sodium acetate with 1 M NaCl was also used to ensure complete protein removal. Elution buffers were passed through the membrane in 3 ml aliquots. Syringes were attached to either end of the cartridge and each salt solution was passed through four times. Samples of the feed solution and all effluents were saved for protein and activity assays.

Membrane Loading and Eluting of Lysozyme

DISCUSSION OF RESULTS

The results of the small scale glucoamylase runs with elution pH 6.0 are shown in Figures 2 and 3 and Table 2. Figure 2 shows the transmittance at 540 nm as a function of time (Parry et al., 1965). The activity was calculated from the slope of this line.

The protein assay used for both enzymes was the Pierce BCA Protein Assay (Pierce, Rockford, IL), both standard and enhanced protocols, with BSA as a standard.

The purity of a sample is indicated by its specific activity, which is defined by the amount of activity per unit mass of protein in the sample. The specific activity is calculated by dividing the activity in U/ml by the protein concentration in mg/ml, both determined by the assays described earlier. A purification factor was also calculated for each sample, which is a comparison of a sample's purity compared to that of the initial feed. It is a dimensionless quantity calculated by dividing the specific activity of a sample by the specific activity of the feed, and indicates the relative number of times the protein was purified.

Small Scale Glucoamylase Experiments

The results of the small scale glucoamylase runs with elution pH 6.0 are shown in Figures 2 and 3 and Table 2. Figure 2 shows the percentage of protein and activity recovered at each salt elution step, as well as the specific activity. The percentage of protein recovered is defined here as the amount of protein in a given fraction divided by the total amount of protein recovered from the membrane. Note that the protein elution peak for each mutant is at the 0.2 M elution step. Also note here that the activity peak shifts from between 0.2-0.3 M for GACD0 to 0.3 M for GACD5 to between 0.3-0.4 M for GACD10.

The specific activities of glucoamylase generally increase with increasing tail length. Additionally, the elution steps at which the maximum activity peaks depend on the particular mutant and the elution buffer used.
SELECTED RECOVERY OF GLUCOAMYLASE AND T4 LYSOZYME

Fig. 2. Activity (●) and protein (■) profiles for GACD0 (a), GACD5 (b) and GACD10 (c). Specific activity (Δ) is calculated as the ratio of glucoamylase activity (U) to protein content (mg). Loading conditions were 25 mM sodium acetate, pH 6.0. Elution conditions were 50 mM sodium acetate, pH 6.0 with NaCl step gradient.

Fig. 3. Purification profiles of step gradient elution of GACD0, GACD5, and GACD10.

Specific activity occurs also increases with tail length, from 0.3 M for GACD0 to 0.5 M for GACD10, with GACD5 in the middle at 0.3-0.4 M. All of these trends point to enhanced binding of the proteins with purification fusions to the ion exchange membranes relative to the control version. The purification factors for each of the salt elution steps are shown in Figure 3. This number generally increased both with the increasing ionic strength of the salt elution steps and with increasing tail length. Finally, Table 2 gives results for representative experiments.

Shifting the elution pH from 6.0 to 4.5 resulted in a marked decrease in the ionic strength required to elute the proteins and also a decrease in the specific activities in the fractions. This result is shown in Figure 4 where the activity elution peak is at the 0.15 M elution step.

Table 2. Results for mutant Glycoamylase trials at elution pH = 6.0.

<table>
<thead>
<tr>
<th>Feed Protein (mg)</th>
<th>GACD0</th>
<th>GACD5</th>
<th>GACD10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed Activity (U)</td>
<td>31.6</td>
<td>52.2</td>
<td>50.0</td>
</tr>
<tr>
<td>Feed Specific Activity (U/mg)</td>
<td>1.6</td>
<td>4.9</td>
<td>2.8</td>
</tr>
<tr>
<td>% Feed Activity Captured</td>
<td>39.7</td>
<td>93.1</td>
<td>76.1</td>
</tr>
<tr>
<td>% Captured Activity Recovered</td>
<td>106</td>
<td>85.5</td>
<td>87.7</td>
</tr>
<tr>
<td>Overall Purification Factor</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Peak Specific Activity (Ionic Strength)</td>
<td>7.4 (0.3M)</td>
<td>19.0 (0.3M)</td>
<td>19.9 (0.5 M)</td>
</tr>
<tr>
<td>Peak Purification Factor (Ionic Strength)</td>
<td>4.5 (0.3M)</td>
<td>3.9 (0.3M)</td>
<td>7.2 (0.5 M)</td>
</tr>
</tbody>
</table>
Purification trials with the 0.5 M elution step were all saved. The results of a number of glucoamylase are given in Table 3) and thus smaller interaction with large 4. In all cases, the purification factor is near two with the resulting specific activity being equal to that of the purified GA'.CD5 instead of the 0.3 M step as shown in Figure 2. This is due to a decrease in the net charge on the protein (estimated net charges for glucoamylase are given in Table 3) and thus smaller interaction with the ion exchange groups. Since more of the target protein was released at lower ionic strengths as are the undesired proteins, the resultant purity was lower than seen at pH 6.0.

Table 3. Estimated Net Charge on Glucoamylase at pH 4.0-4.5, 6.0. Charge estimations determined using Henderson-Hasselbalch equation with amino acid pK's from Stryer (1988).

<table>
<thead>
<tr>
<th>pH</th>
<th>GA'CD0</th>
<th>GA'CD5</th>
<th>GA'CD10</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>12.0</td>
<td>10.6</td>
<td>9.2</td>
</tr>
<tr>
<td>4.1</td>
<td>9.4</td>
<td>7.7</td>
<td>6.0</td>
</tr>
<tr>
<td>4.2</td>
<td>6.6</td>
<td>4.6</td>
<td>2.7</td>
</tr>
<tr>
<td>4.3</td>
<td>3.6</td>
<td>1.4</td>
<td>-0.9</td>
</tr>
<tr>
<td>4.4</td>
<td>0.5</td>
<td>-2.0</td>
<td>-4.5</td>
</tr>
<tr>
<td>4.5</td>
<td>-2.5</td>
<td>-5.3</td>
<td>-8.1</td>
</tr>
<tr>
<td>6.0</td>
<td>-25.7</td>
<td>-30.6</td>
<td>-35.4</td>
</tr>
</tbody>
</table>

Large Scale Glucoamylase Experiments

A large scale glucoamylase procedure has been examined for the GA'CD5 protein. The feed effluent, the 0.1 M elution step, and the 0.5 M elution step were all saved. The results of a number of purification trials with the GA'CD5 mutant protein are shown in Table 4. In all cases, the purification factor is near two with the resulting specific activity being equal to that of affinity chromatography purified GA'CD5 (Forney, C. unpublished results). Further experiments are under way to recycle the feed effluent and the 0.1 M elution step by reloading those fractions on the ion exchange membranes.

Table 4. Large scale Glucoamylase Results for GA'CD5.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Feed Specific Activity (U/mg)</th>
<th>0.5 M Elution Specific Activity (U/mg)</th>
<th>Purification Factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.6</td>
<td>15.9</td>
<td>1.85</td>
</tr>
<tr>
<td>2</td>
<td>6.2</td>
<td>14.4</td>
<td>2.32</td>
</tr>
<tr>
<td>3</td>
<td>6.7</td>
<td>11.4</td>
<td>1.70</td>
</tr>
<tr>
<td>4</td>
<td>9.1</td>
<td>12.8</td>
<td>1.41</td>
</tr>
</tbody>
</table>

Lysozyme Experiments

Figure 5 shows the specific activities obtained at each step in the elution of lysozyme from a cation exchange membrane; the peak at 0.3 M indicates that significant binding is being obtained by this method. More experiments are needed to verify this behavior over an expanded pH range.

CONCLUSIONS

In the small scale application of ion exchange membranes for glucoamylase, the results show that excellent purification can be achieved. Specific activities are similar or superior to those obtained using affinity chromatographic techniques. We have also shown that the purification fusions enhance binding of the target protein to the membrane relative to the control versions.

The large scale application of the ion exchange membranes seems to work very well, and the technique can be used efficiently for separation and purification of large quantities of glucoamylase with a significant decrease in processing time over affinity chromatography. More experimentation is needed to determine whether using the longer tailed mutants enhances separation on a large scale and the effect of charge on separability of lysozyme.

ACKNOWLEDGEMENTS

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REFERENCES


