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Selective Recovery of β-Galactosidase With Charged Fusion Tails Using Ion-Exchange Membranes

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We explored the feasibility of attaching charged tails to a target protein, in this case β-galactosidase, for selective recovery. In this process, an ion-exchange membrane was used for selective binding and release of β-galactosidase with the attached purification fusions. Strength of binding and purity of eluate increased with increasing tail length. In addition, activity yield was improved with the implementation of an intermediate partial elution recycle procedure.
INDEX DESCRIPTORS: fusion proteins, purification fusions, protein purification, ion-exchange, β-galactosidase

The ability to genetically manipulate proteins has made it possible to mass produce those of interest. This breakthrough has lead to many technological advancements in science and industry. However, problems arise when genetically engineered proteins need to be separated and purified. The cost associated with separating and purifying proteins to the standard of industrial use is a major production cost. In fact, purification and recovery costs can account for as much as 80 percent of the total manufacturing cost in the large-scale production of recombinant protein products (1). Thus, without new downstream processing techniques, the abundance of genetically engineered proteins may lead to little profitability.

In an effort to keep pace with modern technology, new procedures are being developed for downstream processing in the areas of separation and purification. Ion-exchange is a technique which has been in use for many years. The bind and release process allows proteins with a certain ionic charge to be separated from others having the opposite charge or no charge. Recent developments in biotechnology utilize genetically engineered proteins in ion-exchange procedures, in particular proteins with attached purification fusions. Purification fusions enhance selectivity of ion-exchange operations as well as facilitate difficult separations. In designing fusion "tails", additional charges supplied by charged amino acids are used to provide the fusion with a distinctive charge which readily facilitates purification. The fusion protein is made by combining the DNA of the target protein with DNA coding for the fusion tail. The result is the expression of the fusion tail in the target protein.

For this work, a microporous membrane was selected as the ion-exchange vehicle for application with the purification fusions. Previous research in our laboratory has taken advantage of negatively-charged fusion proteins in examining other ion-exchange separation techniques (2,3). Cationic polyelectrolyte precipitation and aqueous two-phase extraction are two separation methods in which increased selectivity in separation was seen with the purification fusions. The decision to explore ion-exchange membranes for selective separation was based on its low cost, ease of use, and scale-up potential. The membrane provided a uniform matrix that allowed rapid binding kinetics without problems of diffusion or flow problems common with packed bed or chromatographic separations.

The membrane was used in studying a model protein separation system where β-galactosidase was recovered from cell extracts prepared with fusion tails. Prior research (4) has shown success in the use of purification fusions to enhance selectivity using hollow fiber ion-exchange membranes (HFIEM). Over six fold enrichment was observed when the HFIEM was used for ion-exchange. Similar results were expected with the ion-exchange membrane cartridge.

BGCD1	...Gln Lys / Gly *Asp* Pro Met Ala Tyr
BGCD5	...Gln Lys / Gly *Asp* Pro $(Asp)_4$ Ser Tyr
BGCD11	...Gln Lys / Gly *Asp* Pro $(Asp)_{10}$ Ser Tyr

Fig. 1 Amino Acid Sequences for Purification Fusions.

MATERIALS AND METHODS

Fermentation and Extraction

The fusion tails were produced from *Escherichia coli* strain Y1089-1 with coding for β-galactosidase. The fusion tails were attached at the carboxyl terminus of the β-galactosidase and consisted of a series of amino acids with aspartates supplying the negative charge. Three purification fusions of various lengths; BGCD1, BGCD5 and BGCD11 had been prepared previously (5). BGCD1 was used as a control and contained one additional aspartate due to the restriction site. Four more aspartates were fused to BGCD5 and 10 more to BGCD11. Figure 1 shows the specific sequences for each purification fusion tail. From typical pK values for amino acids in proteins, net charges of the fusion tails were estimated to be –22.48, –37.72 and –60.57 for BGCD1, BGCD5 and BGCD11 respectively with the wild type having a net charge of –18.4. A cleavage site exists between the first aspartate in the chain and proline to allow for removal of the tail. Tyrosine allows for ultraviolet detection.

The cells were grown in Luria-Bertani medium and fermented for 8 to 9 hours in an environmental shaker (37°C and 100 rpm). The cells were then harvested by centrifugation (4°C and 15000 g). After 10 minutes of centrifugation, the cells were washed with a neutral Tris-HCl buffer and recentrifuged. At this stage the cells were stored at –70°C until ready for use.

The cell walls were ruptured by sonication (Heat Systems W185 Sonifier, Ultrasonics Inc., Plainview, NY) to extract the protein from inside the cell. Six cycles of 30 second sonification followed by a 30 second cooling period were performed in a cold salt water bath. Cell debris was removed by centrifugation (4°C and 23000 g) for 30 minutes. The filtered supernatants were then adjusted to 10 units/ml by diluting them with a potassium phosphate buffer containing 2-mercaptoethanol and magnesium chloride, pH 5.7 (Sigma Chemical, St. Louis, MO).

Membrane Ion-Exchange Operation

The adjusted cell extracts were passed through a microporous, ion-exchange membrane enclosed in a polypropylene housing (Acti-Disk™ Cartridge, FMC Corp., Rockland, ME). A matrix of quaternary amine came installed on the membrane to supply the fixed positive charge. Prior to loading the cartridge the membrane was equilibrated by washing with 0.089M potassium phosphate buffer (0.1M ionic strength, pH 6). The wash was fed by a peristaltic pump at a flow rate of 2 ml/min for 10 minutes. Twelve milliliters of the cell

extract were prepared and a 2 ml sample of the feed was reserved. The remaining 10 ml were loaded onto the membrane, also at a rate of 2 ml/min. The apparatus assembly is pictured schematically in figure 2.

After all of the feed was loaded onto the membrane, it was washed with 20 ml of the potassium phosphate buffet previously mentioned. The wash cleansed the membrane of any loosely-bound protein.

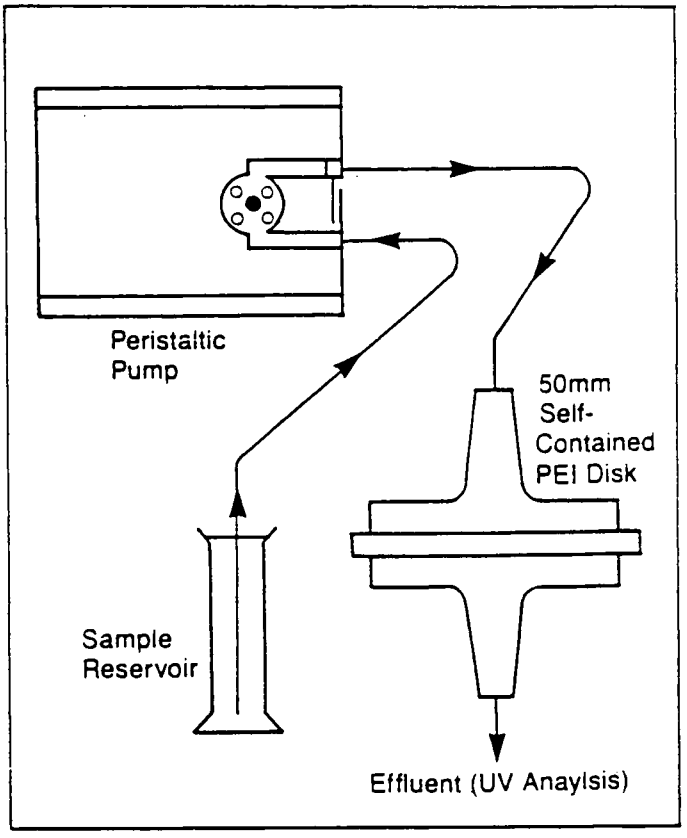

Fig. 2. Apparatus assembly with FMC QUAT. Courtesy of FMC Corporation, Pine Brook, NJ.

Step Gradient Elution

Following the wash, the protein was removed from the membrane by a series of elutions. Buffer solutions were prepared by adding sodium chloride to the potassium phosphate wash buffer with ionic strengths ranging from 0.2 to 1.1 M. A step gradient elution was performed by injecting the buffers through the cartridge in 3 ml aliquots. By the time the 1.1 M solution was injected, nearly all of the protein was eluted. The individual effluents were analyzed for protein and activity content.

Cartridge Storage

The cartridge was sterilized by washing with a 25 ml sample each of deionized water and 70% methanol. The disk was dried by passing air through a syringe and stored at room temperature for reuse.

Assays

Protein content and activity assays were performed immediately after the step gradient elution. A dye-binding assay was performed to determine protein content and concentration. Coomassie Brilliant Blue G-250 stain (Bio-Rad, Richmond, CA) was used with a bovine serum albumin standard. Enzyme activity was monitored by a timed assay where o-nitrophenyl-β-D-galactoside (ONPG), was hydrolyzed by the β-galactosidase. One unit of activity is defined as the amount necessary to hydrolyze one micromole of ONPG to o-nitrophenol and galactose per minute at pH 7.3 and 37°C.

Recycle With Intermediate Partial Elution

In later runs a recycle was implemented to increase the activity yield. The cartridge was prepared, loaded and washed as was done previously. At this point the membrane was eluted with 3 ml of the potassium phosphate salt solution via a syringe. The ionic strength of the elutant depended on the number of aspartates in the fusion tail. BGCD1 was eluted with 0.2M solution, BGCD5 with 0.3M and 0.4M for BGCD11. The increasing concentration reflected the increase in binding strength with tail length as discovered in our initial runs. With the membrane eluted, the feed was recycled and the cartridge was washed again with 20 ml of potassium phosphate buffer. The protein was then eluted through the step gradient procedure as before.

The purpose behind the partial elution was to remove any undesired proteins and create additional space on the membrane surface for the successful binding of more β-galactosidase. In addition, an accompanying reduction of undesired protein components in the final product would be favorable.

RESULTS AND DISCUSSION

The selectivity in this ion-exchange operation can occur either during the binding or release of the protein. Selectivity enhancement provided by the fusion tails was examined in both cases.

Selective binding took place as a result of the intermediate partial elution. In this process the intermediate elution caused all loosely-bound proteins to be eluted from the membrane. In addition, unwanted, neutral and positively charged ions were rinsed through. Hence, the membrane was cleaned and cleared in preparation for the reapplication of the feed during the recycle.

The activity recovered in the effluent increased significantly with the recycle procedure. For example, an initial run of BGCD1 resulted in an activity recovery of 39 U/ml. In contrast, when the BGCD1 feed was recycled, the activity recovered rose to 55 U/ml. Figure 3

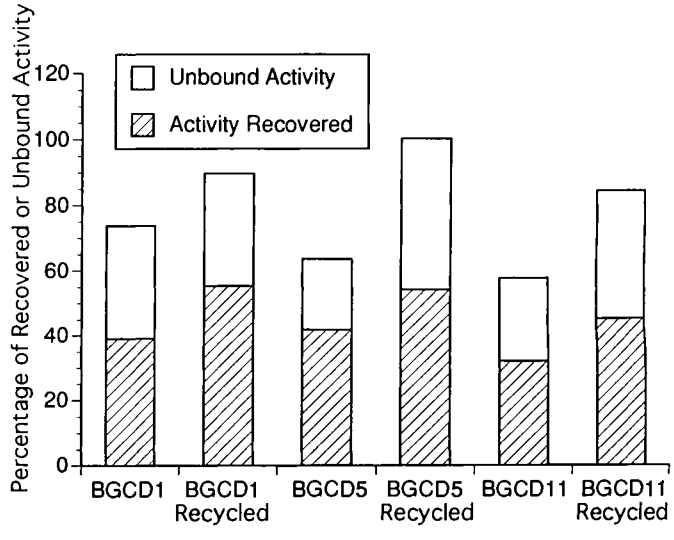

Fig. 3. Activity recovery with intermediate elution and recycle procedure.

shows the relationship between the increased activity and the intermediate elution procedure. The first column of each set shows the amount of activity recovered without the recycle procedure and the second column corresponds to the amount of recovered activity with the feed recycled. Some of the activity was never recovered and may have been lost due to inactivation or remained bound to the membrane during elution. Inactivation occurred when the enzyme was allowed to sit at room temperature for lengthy periods of time. In a measure to reduce the procedure time, the two, 20 ml buffer washes were perfotmed using a syringe rather than the peristaltic pump. This step reduced the length of the recycle procedure and the assay results showed less missing activity.

With the implementation of the intermediate partial elution recycle procedure, an increase in product yield was seen when compared to the single pass procedure. More active β-galactosidase was recovered in the elutions than without the recycle. However, no increase in specific activity was seen for the recycled runs.

Selective release was enhanced through the use of the step gradient elution. Each fusion showed a peak at the ionic strength (I_{max}) required for eluting the fraction with the maximum specific activity.

The I_{max} values remained quite reproducible throughout the battery of runs. An expected correlation between the fusion tail length and the I_{max} peak was soon proven; with the inctease of aspartates in the tail the ionic strength required to release the protein increased. This can be attributed to the additional charge the aspartates provide to the fusion tails.

Although protein was eluted throughout the gradient steps, at I_{max} the majority of the protein was washed off. Specific activity and purification factor peaks coincided with the I_{max} peaks; however, the maximum total protein concentration for all three fusions was eluted at 0.25 M. For BGCD1 and the wild type enzyme, the peak came at 0.3 M. BGCD5 showed a broader range spanning from 0.4 M to 0.5 M with an I_{max} at 0.45 M. Similarly, BGCD11 had a broad peak, its maximum was at 0.55 M. An I_{max} value of 0.3 M for commercial β-galactosidase shows that the purification fusion tails exhibit stronger binding. More information about the elution steps can be derived from figures 4, 5 and 6. The figures show the step gradient elution profiles for BGCD1, BGCD5 and BGCD11 respectively. Protein concentration peaks can be seen at 0.25 M for all three tails. As seen in figure 4, the fraction with the maximum specific activity had a value of 350 U/mg. BGCD5 peaked at 400 U/ml and BGCD1 at 700 U/ml as shown in figures 5 and 6.

To understand the level of enrichment each effluent sample had obtained, the purification facror was calculated. The purification factot α is defined as the total specific activity divided by the specific activity of the feed. The purification factor for BGCD1 ranged from 2.5 to 5 throughout the tests with an average of 4.0. As with specific activity, α increased with tail length. BGCD5 and an α of 4.5 and BGCD11 an average α of 7.0. Figure 7 shows a step gradient elution profile overlay with each fusion tail being represented. The different specific activity peaks have a direct correlation with the purification factor. Just as BGCD11 has the highest specific activity of the three tails, it too has the highest α.

Throughout the testing, BGCD11 showed superior binding capabilities far exceeding those seen for BGCD1 and BGCD5. Its high specific activity brought upon by its longer tail led to a high purification factor. The difference in specific activity and purification factor between BGCD5 and BGCD11 was substantially greater than that between BGCD1 and BGCD5. It was hypothesized that more charges on the fusion tail yield stronger binding traits. Because BGCD11 bound to the membrane much stronger than either BGCD1 or BGCD5, 11 aspartates in the tail could approach an optimal number for selective binding. Continued research with even longer fusion tails would prove or disprove this hypothesis.

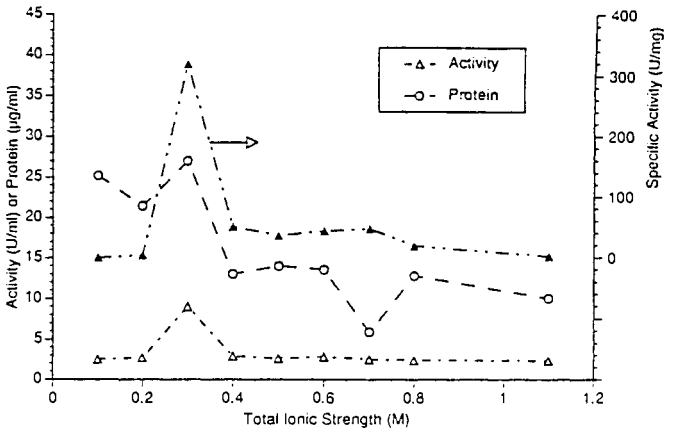

Fig. 4. Step gradient elution profile for BGCD1 cell extracts treated using FMC QUAT.

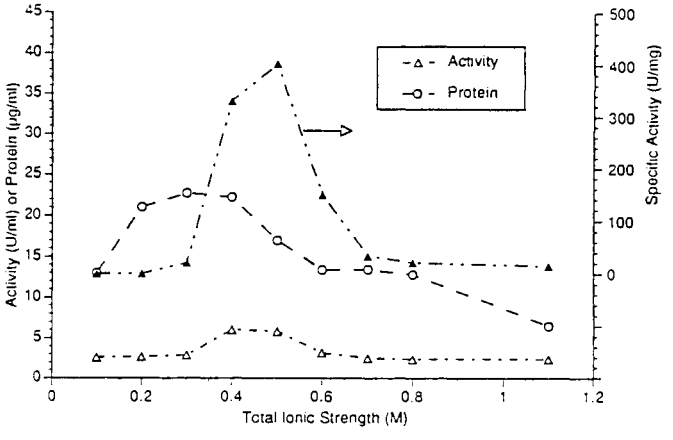

Fig. 5. Step gradient elution profile for BGCD5 cell extracts treated using FMC QUAT.

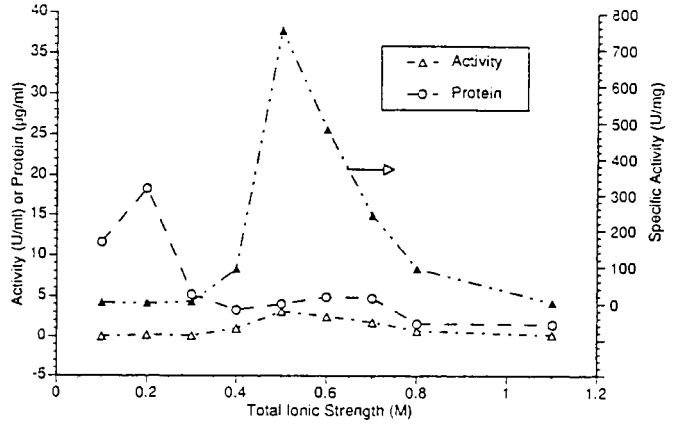

Fig. 6. Step gradient elution profile for BGCD11 cell extracts treated using FMC QUAT.

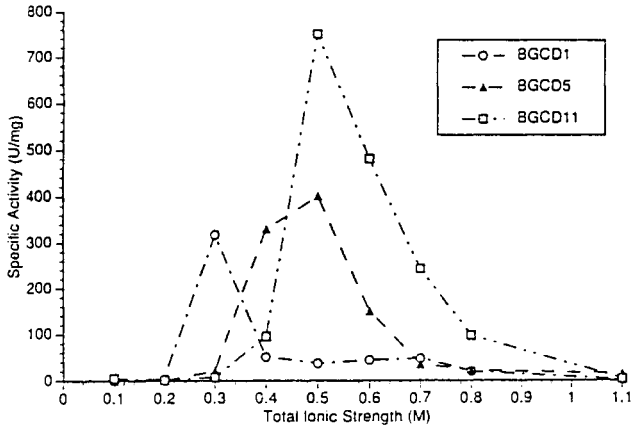

Fig. 7. Specific activity peaks increase with longer tail length. The enzyme binds more strongly to the ion-exchange membrane with an increasing number of aspartates in the tail.

CONCLUSIONS

In this work it was demonstrated that purification fusion tails can be used to enhance separation and recovery on ion-exchange membranes. Selective enhancement increased with fusion tail length. BGCD11 bound to the membrane most strongly followed by BGCD5 and BGCD1. The bind and release process brought sevenfold enrichment of BGCD11 from cell extracts through step gradient elution. The product was recovered in an active state with a specific activity level comparable to commercial β-galactosidase. The intermediate partial elution procedure increased activity recovery by nearly 20%, however, no further enrichment was witnessed.

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