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Effect of Ion and Surfactant Choice on the Recovery of a Histidine-Tagged Protein From Tobacco Extract Using Foam Fractionation

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Abstract

Tobacco plants can be used for the production of proteins for pharmaceutical applications. One of the most difficult and expensive tasks associated with this technology is isolating the product of interest from the hundreds of other chemicals found in tobacco. We describe a new recovery strategy in which the protein of interest is "tagged" with a histidine structure, which forms a complex with metal ions and a surfactant that will accumulate in the foamate of a foam fractionation step. His-gus, a histidine-tagged enzyme, was selectively recovered in the presence of two different surfactants and two different metal ions. The foam fractionation with *N*-ε-dodecylamido-*N*-α, *N*-α,-bis(carboxymethyl)-L-lysine surfactant and Ni^{2+} ions resulted in an average His-gus activity recovery value of 88% and an activity enrichment of 2.27. The performance of the recovery strategy without tobacco extract resulted in an average activity recovery value of 63.32% and an average activity enrichment value of 5.16, utilizing lauroyl ethylenediaminetriacetate surfactant and Ni^{2+} ions. It was shown that even though a majority of the native tobacco proteins are removed during the prefoaming step, the presence of tobacco extract does affect the recovery of His-gus.

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Index Entries: Downstream processing; protein recovery; transgenic tobacco; tobacco extract; yeast extract; histidine-tagged protein.

Introduction

Of the systems available for the large-scale production of recombinant proteins, the use of transgenic plants is potentially one of the most economical *(1,2)*. Although there has been much success in the area of transgenic plant development and gene-expression systems *(1)*, several downstream processing issues need to be addressed before these plants can be used to produce plant-made pharmaceuticals commercially *(2,3)*. Typically, proteins are recovered and purified on a commercial scale using the same techniques used in the laboratory (e.g., affinity chromatography). However, these techniques may not be directly scalable owing to the high cost of chemicals, the need to control shear, and the changes in the physical properties of the plant material in laboratory vs industrial equipment *(1)*. For these reasons, there is a well-motivated need to investigate new separation techniques such as foam fractionation, which is potentially cost-effective and easily scalable, for use with transgenic plant ("biomanufacturing") systems.

Foam fractionation, an adsorptive-bubble separation method, has been shown to be a feasible technique for the separation/concentration of a variety of proteins and enzymes *(4)*, including recombinant pharmaceutical proteins *(5)*. Foam fractionation takes advantage of the surface activity (hydrophobic/hydrophilic nature) of the protein of interest. Separation is achieved in the following manner: Gas (e.g., air, N_2, CO_2) is bubbled through a dilute protein solution. The surface-active proteins then adsorb to the gas-liquid interface of the bubbles. The bubbles next rise to the top of the liquid pool and form a protein-rich foam layer. In the foam layer, liquid then drains from between the bubbles back into the liquid pool, further concentrating the foam layer. Finally, foam is collected and collapsed *(6)*, resulting in a protein-rich solution. The performance of a foam fractionation depends on column operating parameters (gas flow rate, feed flow rate, feed solution height, foam layer height, and bubble size) and feed solution conditions (pH, ionic strength, and protein concentration) *(5)*.

Foam fractionation can be utilized in a semibatch *(7–11)* or continuous *(6,12,13)* manner. It involves low capital and operational costs and should be amenable to scale-up *(8,14)*. Several proteins have been investigated for use with foam fractionation *(5,6,8,14,15)*. Foam fractionation conditions have been optimized for the recovery of a target protein from a dilute protein solution containing only the target protein *(6–8,11)* or from solutions containing additional proteins *(9,10,13)*.

The overall objective of this research is to develop a separation strategy that results in the efficient and cost-effective recovery of biopharmaceuticals, which have been engineered to be compatible with a foam fractionation separation step, possibly produced in tobacco plants. The over-

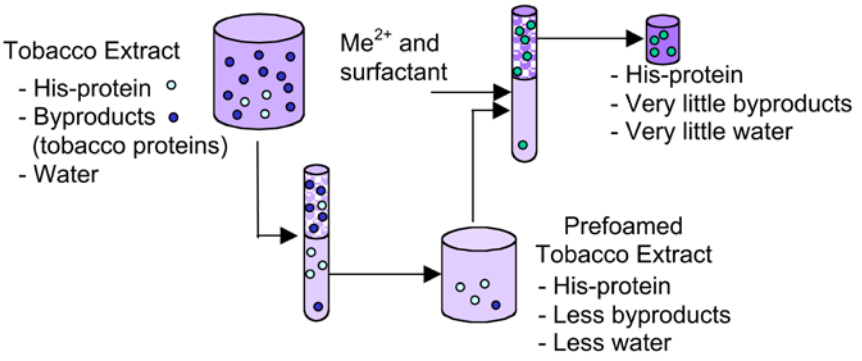

Fig. 1. Schematic of foam fractionation recovery strategy.

all procedure includes several steps, illustrated in Fig. 1. In the first step, the His-protein is extracted from tobacco leaves (tobacco extract [TE]) along with other proteins native to tobacco. The TE is diluted and foam fractionated to remove as much of the native proteins (having high surface activities) as possible while removing a minimum amount of His-protein (having a low surface activity). The retentate from this step, containing the bulk of the His-protein, referred to as prefoamed TE (pTE), is combined with the surfactant and metal ions before the second foam fractionation step. In the presence of metal ions and a surfactant possessing a chelating group, the histidine tag and the surfactant bind to the metal ions, forming a complex where the surfactant adsorbs to the bubble surface during foam fractionation (bubble/surfactant/metal ion/histidine-tagged protein). In this way, the protein does not need to be surface active to be selectively recovered by foam fractionation. In the second foaming, His-protein would be complexed with the surfactant and recovered in the foamate with a concentration higher than that of the initial solution. After separation, the histidine tag is cleaved from the protein of interest *(16,17)*.

This methodology is attractive because the optimum foaming conditions would be the same for any protein of interest; it allows for separation by foam fractionation when the protein of interest is not easily foamed; and it allows for selective separation of the protein of interest, leaving behind surface-active byproducts.

In the present work, the performance of the strategy with two different surfactants was investigated in the presence and absence of tobacco proteins. The first surfactant, *N*-ε-dodecylamido-*N*-α,*N*-α,-bis(carboxymethyl)-L-lysine (DCL), was synthesized (unpublished data) and previously tested with the proposed strategy *(18)*. The second surfactant, lauroyl ethylenediaminetriacetate (LED), was a commercially available multipurpose surfactant from Hampshire Chemical (Dow, Midland, MI). The DCL is compatible with both Co^{2+} and Ni^{2+} ions, whereas the LED is only compatible with Ni^{2+} ions. The structures of DCL and LED are shown in Figs. 2 and 3, respectively.

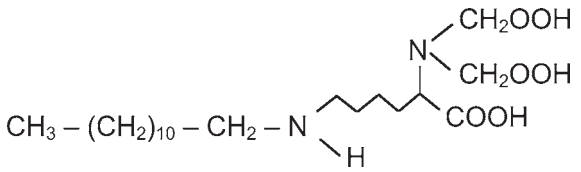

Fig. 2. Structure of DCL.

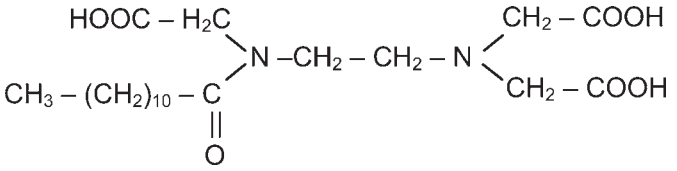

Fig. 3. Structure of LED.

Materials and Methods

Production of His-Protein

Histidine-tagged β-glucuronidase, His-gus, was synthesized using a gus gene transformed into yeast cells where the protein could be expressed *(18)*. The cloning was performed by the procedure described in ref. *19*. After cloning, the vector was transformed into INVSc1 yeast (Invitrogen) by using LiAc TRAFO method *(20)*. The yeast was grown for 12 h and harvested at 5000 rpm for 5 min. The yeast was induced to produce the targeted protein by suspending the harvested pellet with S-URA + 2% Raffinose medium *(21)* to obtain an optical density (OD) of ~0.7 at 600 nm. After 12 h of incubation at 30°C, the cells were diluted with S-URA + 2% galactose medium to obtain an OD of ~0.7 at 600 nm and incubated for another 6 h at 30°C. The induced yeast was harvested at 5000 rpm for 5 min at 4°C. The cells were then mixed with Yeast Protein Extraction Reagent (Y-PER; Pierce, Rockford, IL). After a 20-min incubation in Y-PER, debris was removed by spinning it at 13,000 rpm for 10 min. The collected supernatant, the yeast extract containing His-gus (YE), was stored at –20°C and used without further purification.

Tobacco Extract

TE was obtained from tobacco leaves based on the method described in ref. *22*. Tobacco plants (*Nicotiana tobaccum cv*. SamsunNN) were grown at room temperature (26°C) in a growth chamber at the Kentucky Tobacco Research and Development Center. Fully expanded young leaves (~15 g) were placed in a cold mortar, liquid nitrogen was poured over the leaves, and the leaves were crushed into a fine powder. The resulting powder was suspended in 20 mL of extraction buffer and mixed using a high-speed Omni Mixer (Warrenton, VA) at 17,105 rq!8for 2 min in 10-s intervals.

The extraction buffer contained 25 m*M* Tris, 50 m*M* NaCl, 2 m*M* β-mercaptoethanol, 1 m*M* phenylmethylsulfonyl fluoride, and 2 μg/mL of leupeptin; all reagents were obtained from Sigma (St. Louis, MO) and used as received. After mixing, the solution was placed in a 25-mL centrifuge tube and spun for 10 min at 10,000 rpm using an RC5C Sorvall centrifuge with an SA-600 rotor (Albertville, MN). The supernatant was transferred to a clean centrifuge tube and spun again at the same speed. The resulting supernatant, the TE, was stored at –20°C for later use.

Foam Fractionation Performance

The performance of a foam fractionation system is quantified using the enrichment and recovery of the separation. The *enrichment* (E) is defined as the concentration of protein in the foamate divided by the protein concentration of the initial solution (as the protein concentration in the foamate increases, the enrichment increases). The *recovery* (R) refers to the ratio of the amount of protein in the foamate to the amount in the initial solution (as the mass of protein in the foamate increases, the recovery increases):

$$\text{enrichment (E)} = \frac{[\text{Protein}]_{\text{foamate}}}{[\text{Protein}]_{\text{initial}}} \tag{1}$$

$$\text{recovery (R)} = \frac{[\text{Protein}]_{\text{foamate}}\,\text{Volume}_{\text{foamate}}}{[\text{Protein}]_{\text{initial}}\,\text{Volume}_{\text{initial}}} \tag{2}$$

In the present work, the His-gus activity per volume was used as a representation of the His-gus concentration. The total protein (the amount of His-gus plus tobacco proteins) was measured in an effort to determine whether changes in surfactant or metal ion resulted in a change in the total protein recovered.

Prefoaming Conditions

TE was subjected to a prefoaming step, prior to the addition of surfactant and metal ions, in an attempt to remove as many of the surface-active native tobacco proteins as possible. The foam fractionation setup is shown in Fig. 4. Previously, the optimum pH for this prefoaming step was determined without the addition of His-gus *(18)*. Since His-gus is not surface active, it was assumed that it would not be selectively recovered during the prefoaming step. In our work, His-gus was added prior to the prefoaming step and the same three pH values—3.0, 7.5, and 10.0—were tested. Owing to the limited quantity of His-gus, these prefoaming experiments were performed in a smaller 5-mL column (8-mm id, 150-mm length, custom made by Ace Glass, Vineland, NJ) equipped with a porous glass filter disk (pore sizes of 70–100 μm). Bubbles were produced as nitrogen gas passed through the porous glass filter near the bottom of the column. In the smaller

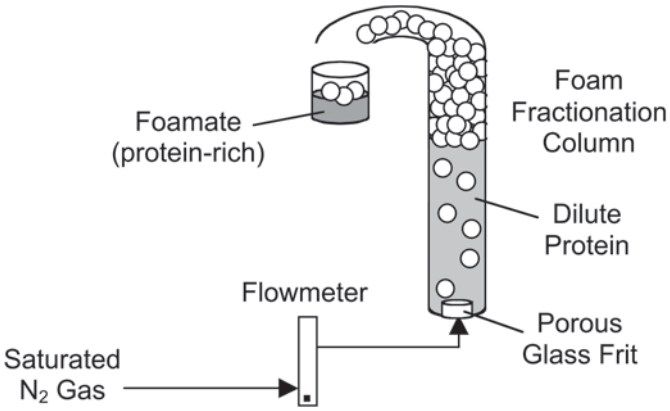

Fig. 4. Schematic of semibatch foam fractionation setup.

5-mL column, an initial solution of TE (4.5% [v/v]) and yeast extract containing His-gus (0.5% [v/v]) in 50 m*M* Tris buffer was subjected to foam fractionation with a superficial gas velocity of 0.2 mm/s. Once the correct pH was confirmed, larger quantities of pTE were prepared without His-gus, using a larger glass column (25-mm id, 510-mm effective height, Model # 5904-28; Ace Glass). In the larger column, initial solutions of 5 mL of TE and 100 mL of 50 m*M* Tris buffer were subjected to foam fractionation with a superficial gas velocity of 1.8 mm/s. For all foaming experiments, the foaming was allowed to proceed as long as the foam layer was sufficient to be collected. The volume of the resulting foamate and retentate was measured, the protein concentrations were determined using the Bradford method *(23)*, and the enrichment and recovery for each test were calculated. Bovine serum albumin (BSA) was used as a standard for the Bradford's calibration curve. BSA and Bradford reagent were purchased from Sigma.

Foam Fractionation Recovery of His-Protein

The recovery of His-gus from prefoamed tobacco extract was tested in the 3-mL column. The column was charged with a 3-mL initial solution containing pTE (98.35% [v/v]), yeast extract containing His-gus (1.0% [v/v]), surfactant (0.18 mg/mL of DCL or 0.0065% LED solution), and metal ions (0.1 mg/mL of cobalt chloride or nickel chloride) in 50 m*M* Tris buffer, adjusted to a moderate pH of 7.5. The DCL was synthesized (unpublished data), the cobalt chloride and nickel chloride were purchased from Sigma, and the LED was generously provided by Hampshire Chemical (Dow Chemical, Midland, MI). For each experiment, the volumes of the foamate and the retentate were recorded, and the His-gus activities in the initial, foamate, and retentate were determined (described in the next section). To examine the recovery of His-gus regardless of the decrease in activity owing to denaturation, adjusted enrichment and recovery values were

calculated, where the total activity after foaming was used as the denominator in Eqs. 1 and 2 *(18)*.

Enzyme Activity Assay

To measure the activity of His-gus, 225 µL of 1 mg/mL β-4-MU-glucuronide substrate was incubated with 25 µL of the sample (crude yeast extract, initial foaming solution, retentate, or foamate) at 37°C *(24)*. During incubation, 20 µL of the mixed solution was added to 2 mL of stop buffer (0.20 *M* sodium carbonate) in a glass cuvet at 0, 15, 30, and 60 min from start of incubation. The fluorescence intensity of the mixture was measured using a TKO 100 Mini Fluorometer (Hoefer, San Francisco, CA). The activity was then calculated based on the following equation in terms of international units (U, µmol of substrate consumed per time) per microliter of the sample being tested:

$$\frac{\text{U}}{\mu\text{L sample}} = \left(\frac{\text{fluorescence}}{\text{time}}\right) \frac{\text{standard curve slope}}{\mu\text{L sample}} \tag{3}$$

Total Protein Assay

The total protein for the initial foaming solution, foamate, and retentate from each experiment was measured using a trichloroacetic acid (TCA) treatment and Quanti-Pro BCA assay. The TCA treatment was required owing to the levels of surfactant and metal ion used. Starting with 250 µL of the sample, the volume was adjusted to 1 mL using deionized water. Then 100 µL of 0.15% (w/v) sodium deoxycholate was added and mixed, and the sample was incubated at room temperature for 10 min. Next, 100 µL of TCA was added, mixed, and allowed to incubate at room temperature for 5 min. The protein was spun down using a Centra CL2 Micro Centrifuge (International Equipment, Needham Heights, MA) for 20 min at 7500 rpm. The supernatant was decanted for each sample, and the pellet was dissolved in 40 µL of 5% (w/v) sodium dodecyl sulfate in 0.1 *N* NaOH. The volumes were then adjusted to 1 mL for the Quanti-Pro BCA assay using deionized water. After a 1-h incubation, the absorbancies were measured using a Varian UV-VIS (Cary 300, Cary, NC) at a wavelength of 562 nm. Calibration curves were generated using a standard solution of BSA. The Quanti-Pro BCA assay kit and other reagents were purchased from Sigma.

Results and Discussion

Prefoaming Experiments

In the prefoaming step, the objective was to maximize the amount of protein in the foamate (maximize R) while minimizing the volume of the foamate (maximize E), considering a portion of the His-protein would be carried into the foamate in the interstitial liquid. Various pH values—

Table 1
Summary of Results (% ± SE)
for Prefoaming of Tobacco Extract at Various pH Values

pH	Protein removed (%)	Loss of His-gus (%)[a]	Activity retention (%)[b]	Volume removed (%)
3.0	31.7 ± 2.3	5.4 ± 0.9	86.3 ± 9.5	8.6 ± 0.8
7.5	20.9 ± 2.7	7.8 ± 0.1	75.4 ± 12.3	8.5 ± 0.7
10.0	47.0 ± 2.8	12.6 ± 1.6	80.2 ± 9.1	24.5 ± 1.6

[a]Based on the activity measured in the foamate.
[b]Based on the activity measured in the retentate.

3.0, 7.5, and 10.0—were tested. In addition, combinations of foam fractionating steps with varying pH values were tested. For example, the pH of the initial solution was adjusted to 3.0 and foam fractionated; the resulting retentate was adjusted to pH 7.5 and foam fractionated; and, finally, the resulting retentate was adjusted to pH 10.0 and foam fractionated again. In this manner, the proteins that favored removal at a different pH value would be removed. However, so many of the proteins were removed at the first pH (no matter which was chosen) that subsequent foaming was not possible. The prefoaming results in terms of percentage of protein removed, percentage loss of activity retention, and percentage of the volume removed are summarized in Table 1. A pH of 3.0 resulted in the highest activity retention, accompanied by high protein removal and low volume-removed percentages. Hence, a pH of 3.0 was selected as appropriate for the prefoaming step.

Instead of using His-gus produced in tobacco leaves, His-gus was added to the TE for the prefoaming experiments or to previously pTE with surfactant and metal ions for the second foaming step. The aforementioned results show that His-gus alone (without the surfactant) is not selectively removed in the foam fractionations step and that the resulting His-gus concentration in the foamate of the prefoaming step is equal to or less than that of the initial solution; that is, the His-gus in the foamate was carried in the liquid and not on the bubble surface. With this assumption, the His-protein does not need to be added to the TE but can be added to the pTE as long as the percentage of His-protein that would have been removed in the prefoaming step is taken into consideration. From this, one can appreciate the importance of removing as much of the protein as possible with as little volume as possible in the prefoaming step.

Adjuvant Compatibility

As already discussed, ultimately the activity recovery and enrichment will determine the usefulness of this separation strategy. The target protein may be denatured by the foaming process or the mere presence of the various strategy adjuvants. The results in Table 2 show that there is no statistical difference between the His-gus activity in extraction buffer and

Table 2
His-gus Activities with Various Adjuvants
Associated With Recovery Strategy

Buffer added to His-gus	Average activity (U/μL of sample ± SE)	*p* Value[a]
Extraction buffer	218 ± 16	
Tris buffer	154 ± 32	0.20
pTE, Tris buffer	220 ± 38	0.97
TE, Tris buffer	203 ± 14	0.57
DCL, Tris buffer	287 ± 17	0.06
LED, Tris buffer	287 ± 32	0.17
$CoCl_2$, Tris buffer	239 ± 5	0.33
$NiCl_2$, Tris buffer	163 ± 21	0.14

[a]Compared to the His-gus activity in extraction buffer.

the activity of His-gus with the various adjuvants. It appears that the surfactants may increase His-gus activity (154 vs 287) while the addition of the metal ions may decrease the His-gus activity (287 vs 238 and 162). However, none of these changes rise to the level of statistical significance. Since the extraction buffer is specifically designed to maintain the His-gus activity, the activity in extraction buffer is assumed to be ideal.

Effect of Choice of Surfactant and Ion

The resulting recovery and enrichment values in terms of total protein and His-gus activity for foam fractionation with two different surfactants (DCL and LED) with Ni^{2+} ions and two different metal ions (Ni^{2+} and Co^{2+}) with DCL are shown in Fig. 5. It is possible that the surfactants may result in an apparent increase in His-gus activity, but it is likely that the activity enrichment is higher than the protein enrichment owing to the fact that some of the non-His-gus protein remains in the retentate following foaming. Thus, the His-gus was being enriched while the other proteins were not. For the recovery and enrichment values based on total protein, there was no statistical difference for all treatments with $\alpha = 0.1$. With $\alpha = 0.05$, the only statistical difference found was between the LED/Ni^{2+} and DCL/Co^{2+} treatments at the higher flow rate ($p = 0.04$). For the recovery and enrichment values based on the activity of His-gus, the DCL/Ni^{2+} treatment performed better than either of the other two treatments, with recoveries of 66.7 and 88.0% and enrichments of 2.44 and 2.27 for superficial gas velocities of 2.8 and 4.1 mm/s, respectively. As expected, the higher superficial gas velocity corresponds to the higher recovery and lower enrichment. The recovery and enrichment of the total proteins remain statistically the same for all treatments, whereas the recovery and enrichment for His-gus specifically is better for the DCL/Ni^{2+} treatment. These results indicate that the choice of surfactant and the choice of metal ion can significantly affect the performance of the His-tagged strategy, increasing both the

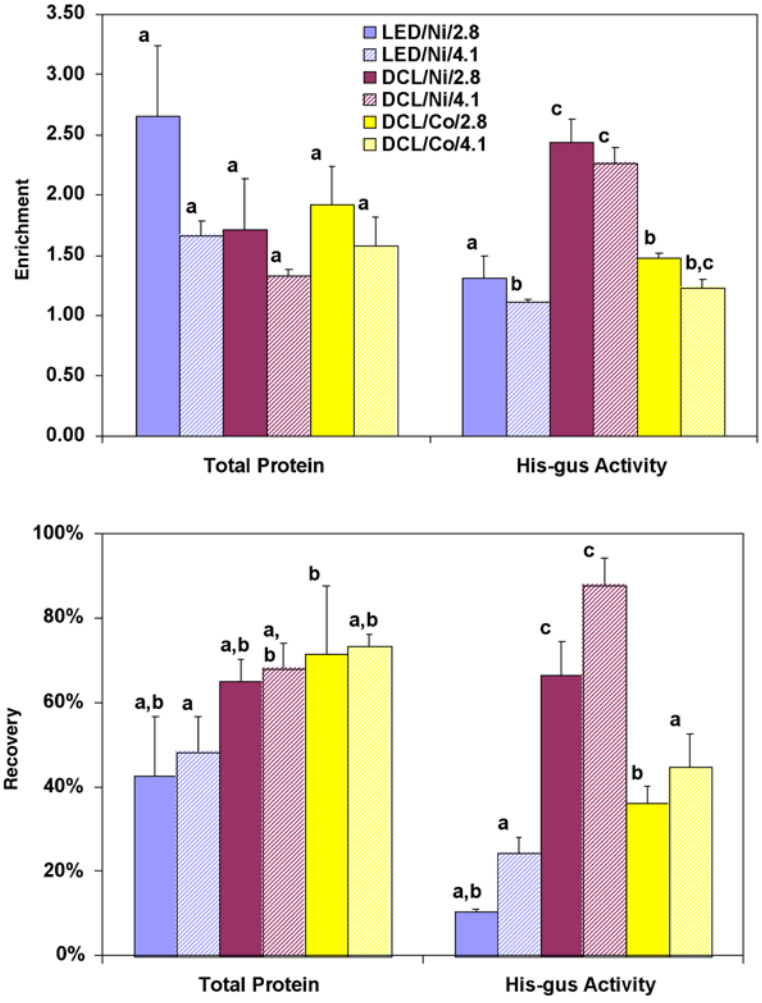

Fig. 5. Recovery (%) and enrichment in terms of total protein and His-gus activity for foam fractionation with two different surfactants (DCL and LED) with Ni^{2+} ions and two different metal ions (Ni^{2+} and Co^{2+}) with DCL at two different superficial gas velocities (2.8 and 4.1 mm/s). Error bars show the SE for each average (n = 3). Treatments with the same letter are not statistically different.

enrichment and the recovery. Further studies will focus on determining what surfactant qualities are most desirable, such that a surfactant and metal ion combination can ultimately be selected that results in recoveries above 80% with enrichment values above 8 or 9.

Recovery From TE vs Yeast Extract

More than 2200 compounds have been identified in tobacco plant leaves *(25)*. The performance of the His-tagged strategy with and without TE was tested in an attempt to determine whether the mere composition of the TE will affect the performance of the His-tagged strategy. The resulting

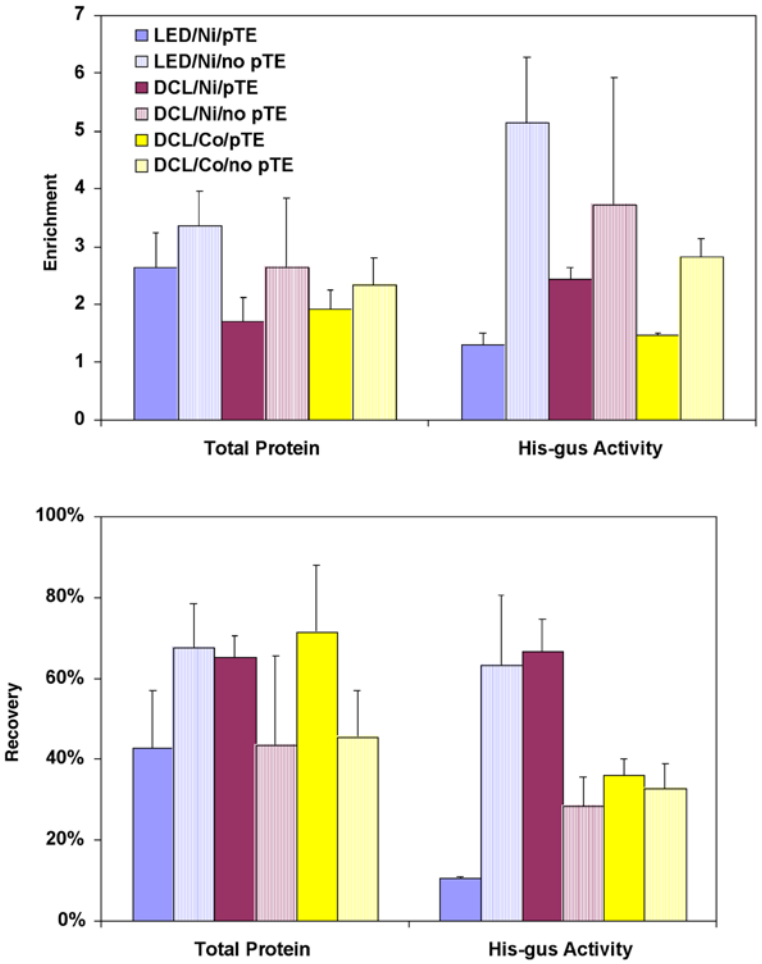

Fig. 6. Recovery (%) and enrichment in terms of total protein and His-gus activity for foam fractionation with two different surfactants (DCL and LED) with Ni^{2+} ions and two different metal ions (Ni^{2+} and Co^{2+}) with DCL at superficial gas velocity of 2.8 mm/s with (pTE) and without (no pTE) prefoamed tobacco in initial solution. Error bars show the SE for each average (n = 3).

recovery (%) and enrichment values in terms of total protein and His-gus activity for the foam fractionation with two different surfactants (DCL and LED) with Ni^{2+} ions and two different metal ions (Ni^{2+} and Co^{2+}) with DCL with and without pTE are shown in Fig. 6. For the recovery and enrichment values based on total protein, there were no statistical differences for all treatments with $\alpha = 0.05$. For the recovery and enrichment values based on the activity of His-gus, there was no statistical difference between the foam fractionations with and without pTE with the DCL/Ni^{2+} treatment combination. However, in the LED/Ni^{2+} treatment, the recovery and enrichment without pTE (63.3% and 5.16) were statistically higher compared with the values with pTE (10.6% and 3.37). In the DCL/Co^{2+} treatment, the recover-

ies were not statistically different (36.2 and 32.9%), but the enrichment value without pTE was statistically higher (2.83 vs 1.48). These results indicate that the various proteins in the pTE will affect the performance of the histidine-tagged strategy, sometimes improving and sometimes hindering recovery and enrichment.

Conclusion

A novel His-tagged/surfactant strategy was investigated with two different surfactants and two different metal ions. The results indicate that DCL performs better than the commercially available LED and that the use of Ni^{2+} ions with DCL performs better than using DCL with Co^{2+} ions. These results suggest that the choice of surfactant and metal ion will have a significant effect on the overall performance of the strategy. In addition, the presence of native tobacco proteins significantly affects the efficiency of the strategy. Once the optimum surfactant and metal ion combination is determined, operating conditions can be optimized.

Acknowledgments

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References

1 Kusnadi, A. R., Nikolov, Z. L., and Howard, J. A. (1997), *Biotechnol. Bioeng.* **56,** 473–484.
2 Boothe, J. G., Saponja, J. A., and Parmenter, D. L. (1997), *Drug Dev. Res.* **42,** 172–181.
3 Cramer, C. L., Boothe, J. G., and Oishi, K. K. (1999), *Curr. Top. Microbiol.* **240,** 95–119.
4 Uraizee, F. and Narsimhan, G. (1996), *Biotechnol. Bioeng.* **51,** 384–398.
5 Lockwood, C. E., Bummer, P. M., and Jay, M. (1997), *Pharm. Res.* **14,** 1511–1515.
6 Brown, A. and Varley, K. (1999), *Biotechnol. Bioeng.* **62,** 278–280.
7 Ko, S., Loha, V., Prokop, A., and Tanner, R. D. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 547–558.
8 Chai, J., Loha, V., and Prokop, A. (1998), *J. Agric. Food Chem.* **46,** 2868–2873.
9 Lockwood, C. E., Jay, M. E., and Bummer, P. M. (2000), *J. Pharm. Sci.* **89,** 693–704.
10 Loha, V., Prokop, A., Du, L., and Tanner, R. D. (1999), *Appl. Biochem. Biotechnol.* **77–79,** 701–712.
11 Varley, J. and Ball, S. K. (1994), *Separat. Biotechnol.* **158,** 525–531.
12 Chen, S., Timmons, M. B., Bisogni, J. J., and Aneshansley, D. (1994), *Aquacult. Eng.* **13,** 163–183.
13 Brown, A. K., Kaul, A., and Varley, J. (1999), *Biotechnol. Bioeng.* **62,** 291–300.
14 Uraizee, F. and Narsimhan, G. (1990), *Enzyme Microb. Technol.* **12,** 315, 316.
15. DeSouza, A. H., Tanner, R. D., and Effler, W. T. (1991), *Appl. Biochem. Biotechnol.* **28/29,** 655–666.
16 Walker, P. A., Leong, L. E. C., Ng, P. W. P., Tan, S. H., Waller, S., Murphy, D., and Porter, A. G. (1994), *Bio-Technology* **12,** 601–605.

17 Cordingley, M. G., Callahan, P. L., Sardana, V. V., Garsky, V. M., and Colonno, R. J. (1990), *J. Biol. Chem.* **265,** 9062–9065.
18 Crofcheck, C., Loiselle, M., Weekley, J., Maiti, I., Pattanaik, S., Bummer, P. M., and Jay, M. (2003), *Biotechnol. Prog.* **19,** 680–682.
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989), *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.
20. Agatep, R., Kirkpatrick, R. D., Parchaliuk, D. L., Woods, R. A., and Gietz, R. D. (1998), Technical Tips Online (http://tto.trends.com).
21. Guthrie, C. and Fink, G. R. (1991), *Guide to Yeast Genetics and Molecular Biology*, Academic, New York.
22 Lige, B., Ma, S., Zhao, D., and van Huystee, R. B. (1998), *Plant Sci.* **136,** 159–168.
23 Bradford, M. M. (1976), *Anal. Biochem.* **72,** 248–254.
24. Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987), *J. Cell. Biochem.* 57–57.
25. Schmeltz, I. and Hoffmann, D. (1977), *Chem. Rev.* **77,** 295–311.