Production and Purification of an Active Bovine Lysozyme in Tobacco (*Nicotiana tabacum*): Utilization of Value-Added Crop Plants Traditionally Grown under Intensive Agriculture

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The goals of this study were to express bovine lysozyme in tobacco and to purify the protein with a scaleable process to >90% homogeneity while retaining antimicrobial characteristics. Results showed that the enzyme was expressed at levels equivalent to 1−1.5% of total fraction 2 protein in each of five different transformant groups. The enzyme was subsequently purified to 93% homogeneity using an easily scaleable process while retaining high activity. It was concluded that tobacco was an excellent choice for expression of the recombinant protein and that the purification process developed in this study demonstrates methodology for isolation of high-value enzymes from tobacco and other crop plants.

Keywords: *Transgenic; lysozyme; recombinant protein; antimicrobial; Nicotiana tabacum*

INTRODUCTION

Lysozymes are a class of antibacterial proteins with molecular masses ranging from 14 kDa (chicken egg white) to 18 kDa (bovine milk) (Durance, 1994). Chicken lysozyme has been suggested for use as a preservative in foods but has had limited use in the United States due to its ineffectiveness at inhibiting Gram-negative bacteria.

Many food pathogens, as well as plant pathogens, are Gram-negative. These cells possess an outer lipid membrane that acts as a barrier to prevent lysozyme from contacting its peptidoglycan substrate. Therefore, researchers have attempted to modify chicken lysozyme to allow the protein to interact with the lipid membrane (Miura and Kumagai, 1989; Ibrahim et al., 1994; Kobayashi et al., 1991). However, these modifications have had limited success and always resulted in a reduction in activity against Gram-positive bacteria. There are lysozymes that naturally act against a broad spectrum of both Gram-positive and Gram-negative bacteria. Lysozyme from the bovine stomach mucosa is one such enzyme (Mirkov and Fitzmaurice, 1995).

Bovine stomach lysozyme (BSL) is more acidic than egg white lysozyme. It has an isoelectric point of 7.65 compared to 10.7 for chicken lysozyme. Its optimum pH range for activity is between 4 and 6, whereas chicken lysozyme's highest activity occurs between pH 5 and 7 (Dobson et al., 1984). In addition, its reported size is slightly larger, at 15 kDa (Denisenko et al., 1985).

BSL is more important industrially than other lysozymes because of its activity against Gram-negative bacteria and fungi. BSL has been shown to exhibit substantial activity against several Gram-negative pathogens at concentrations as low as 25 ppm (Mirkov and Fitzmaurice, 1995). BSL also has a 6-fold higher chitinase activity than chicken lysozyme (Lemos et al., 1993).

Because of the enzyme's broad spectrum of antimicrobial activity, and stability to heat, storage, and proteases, BSL is well-suited for use as a preservative in foods, cosmetics, and agriculture. Therefore, BSL was chosen as a model for expression of high-value animal enzymes in tobacco, a crop traditionally grown under intensive management.

One concern was whether or not the enzyme would be expressed in high enough quantities to be detected in a system of such great complexity. Another concern dealt with the purification process. It had to be simple and scaleable but still retain high antimicrobial characteristics as measured by its lytic action against bacteria cell walls. We proposed that, due to the protocols already in place for tobacco transformation, adequate expression could be obtained. We also believed that the protein could be isolated on the basis of its size and charge characteristics.

By using anti-bovine lysozyme antibodies, we were able to detect relatively low levels of lysozyme in complex mixtures of proteins. Elimination of the proteins dissimilar in size from lysozyme was performed with molecular weight exclusion limit membranes. Cation-exchange chromatography conditions were optimized on the basis of the charge of lysozyme at acidic pH values, thereby isolating the protein of interest. Assays using *Micrococcus lysodeikticus* cells confirmed that the enzyme retained high activity throughout the process.

MATERIALS AND METHODS

Materials. All common laboratory reagents, including protein standards, were purchased from Fisher Scientific (Pittsburgh, PA) or Sigma Scientific (St. Louis, MO). Electrophoresis supplies were purchased from Bio-Rad (Melville, NY) or Pharmacia (Piscataway, NJ).

Transformation of Tobacco. Burley tobacco cv. KY14 was transformed with the chimeric gene encoding bovine

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lysozyme c2 (Digan et al., 1989). The gene was under the control of the CaMV 35S promoter and the Nos terminator. The plasmid pSPI-LYS-1, carrying both the lysozyme gene and the selectable marker NPT II, was transferred into *Agrobacterium* strain LBA 4404, which was subsequently used in transformation of tobacco. Cocultivation, selection, and regeneration of transgenic plants were carried out using standard protocols. Selection was accomplished using kanamycin sulfate at a concentration of 300 mg/mL, with transfers to fresh media at 2-week intervals. Regenerated plants were rooted on standard rooting medium, amended with 100 mg/mL of kanamycin, and transferred to soil.

Plants used as the source material from which lysozyme was extracted were first-generation self-pollinated progeny (R1) of the primary transformants. R1 seeds were germinated on water agar containing a low concentration of kanamycin (100 mg/mL) to eliminate segregants which did not carry the transgene. All lines used as a source material in the study had been tested by Western blot analysis for production of bovine lysozyme (data not shown).

Extraction of Plant Proteins. Young, expanded plants were harvested. The leaf proteins were separated into two fractions (fractions 1 and 2) according to the procedure of Wildman and Kwanyuen (1982). Continuous centrifugation was performed at 6812*g* for 20 min to remove any remaining particulate matter. The exiting solution was cooled at 4 °C for a minimum of 8 h, after which time centrifugation was again performed. F-1 was the material retained in the bowl of the centrifuge, while F-2 was the exiting, amber-colored solution.

Expression Level Analysis. F-2 was fractionated and concentrated with Centricon concentrators purchased from Amicon (Beverly, MA). Two milliliters of F-2 from each of the five transgenic lines was taken. These initial samples were analyzed for individual proteins using SDS−PAGE and for total protein concentration using the bicinchoninic acid assay (BCA). The remaining 1.95 mL was placed into Centricon 100 concentrators and centrifuged in an IEC clinical centrifuge at 5125*g* for 2 h at 4 °C.

The filtrate volumes from the Centricon 100 concentrations were measured using a pipet and then placed into Centricon 3 concentrators and returned to the centrifuge for an additional 2 h. The concentrated retentate volumes were measured. Total protein concentration was determined with the BCA method using 5-μL portions of each concentrated retentate.

Analysis for individual proteins was performed on 40-μL aliquots of the fractionated samples using SDS−PAGE (15−16.5% acrylamide) according to the procedure of Laemmli (1970). The gels were analyzed with a densitometer (Molecular Dynamics, Sunnyvale, CA) to determine the percentage of each protein and subsequently the expression levels.

Protein Concentration. The enhanced protocol for the BCA (Pierce Chemical Co., Rockford, IL) was used to determine the protein concentration of numerous samples throughout the purification procedure. The protein concentrations in the samples were calculated on the basis of a linear fit to the standard curve.

For studies with chicken lysozyme, the protein concentration was determined from the absorbance at 280 nm. Concentrations were calculated from a linear fit to data obtained from measurements of standard solutions of the protein.

Western Blotting. Western blotting (immunoelectrophoresis) on selected gels was conducted according to the method described in Maniatis et al. (1989). The method utilized polyclonal serum raised in rabbits against bovine lysozyme c2 (Digan et al., 1989). The nitrocellulose filter was incubated with the serum, followed by incubation with an anti-rabbit IgG−horseradish peroxidase conjugate.

Cation-Exchange Resin Binding Comparison. For each experimental comparison of the binding capacity of various cation-exchange resins, 3 mL of the resin was packed into a 15-mL-capacity Econo column (Bio-Rad) and equilibrated with 0.05 M sodium phosphate, pH 7.5. A solution of 0.05 M sodium phosphate, pH 7.5, which contained chicken lysozyme at a concentration of 1 mg/mL, was passed through the column at a flow rate of 1 mL/min. Absorbency (280 nm) readings were taken on 3-mL fractions until the measurement exceeded 0.300, at which time elution with 0.25 M sodium phosphate, pH 8.0, ensued. With the Bio Rex 70 resin, the A_{280} surpassed 0.300 very quickly (6 mL); therefore, an additional 37 mL was added to obtain a reasonable chromatographic curve.

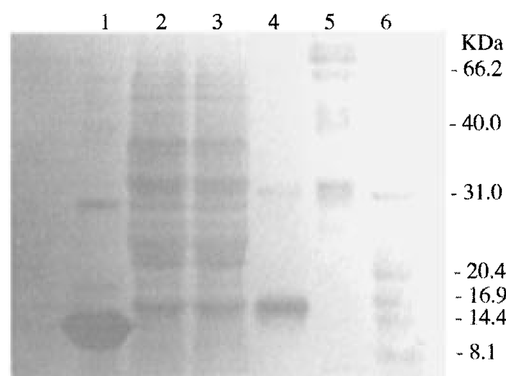

Figure 1. SDS−PAGE (15% acrylamide) representing the migration pattern of proteins present in fraction 2 extracts. Lanes 5 and 6 contain molecular weight markers. Lanes 1 and 4 contain chicken and bovine lysozyme standards, respectively. Lanes 2 and 3 contain duplicate bulked fraction 2 extracts.

Bovine Lysozyme Binding. Recombinant bovine lysozyme was obtained from Sigma and used as a standard to identify its elution pattern on the Hyper D-S column. A 15-mL-capacity Econo column was packed with 2 mL of Hyper D-S resin and equilibrated with 0.01 M acetate, pH 5.85. Addition of 1.5 mg of recombinant lysozyme in 5 mL of equilibration buffer was performed at a flow rate of 1.5 mL/min. The column was then washed with 100 mL of equilibration buffer and eluted with 0.1 M acetate, pH 7.20. Fractions (5 mL) were collected, and the absorbance was measured at 280 nm.

Membrane Fractionation. Membrane fractionation of F-2 extracts was carried out with a 30-kDa exclusion limit spiral wound membrane ultrafiltration cartridge using an Amicon RA2000 (Amicon) ultrafiltration system. Sample solutions were ultrafiltered, using 2-L batches at a flow rate of 250 mL/min and a pressure of 25 psi. A total of 5.5 L of filtrate was collected from 6 L of starting solution. Subsequent ultrafiltration with a 1-kDa exclusion limit Pellicon (Amicon) cassette system was carried out at 20 psi, resulting in a concentration of the retentate to 600 mL and simultaneous dialysis with 12 volumes of 0.01 M acetate, pH 6.0.

Ion-Exchange Chromatography. Purification of F-2 samples by chromatography was performed with the following conditions. Ultrafiltered sample was passed through an Econo column containing either 5 or 30 mL of Hyper D-S (Bio Sepra) resin equilibrated with 0.01 M acetate, pH 6.0. Equilibration buffer was used to wash the column until no significant absorbance at 280 nm was measured (approximately 100 mL buffer for the smaller column and 250 mL for the larger column). Elution was performed with 0.1 M acetate, pH 8.0, and 5- or 10-mL fractions were collected for the small or large columns, respectively.

Activity Assays. Purified lysozyme fractions were tested for their ability to lyse *M. lysodeikticus* cells using the standard protocol from Sigma. Some purified enzyme fractions were also freeze-dried using dry ice and hexane prior to being assayed for activity.

RESULTS

Expression. Analysis of the proteins in the fraction 2 (F-2) extracts from two transgenic tobacco groups by SDS−PAGE (Figure 1) showed that a band which appeared to be lysozyme migrated to a position above the chicken standard and similar to that of the bovine standard. A Western blot (Figure 2) of the same samples confirmed that these bands indeed contained the recombinant bovine lysozyme. A typical SDS−

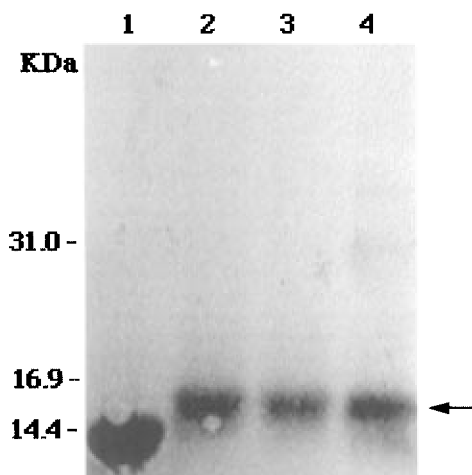

Figure 2. Western blot on nitrocellulose depicting bovine lysozyme antiserum recognition of the proteins in lanes 1−4 of Figure 1. The arrow points to the location of the lysozyme.

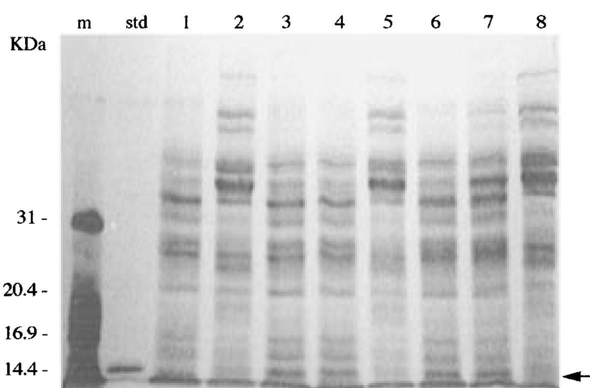

Figure 3. Typical SDS−PAGE (16.5% acrylamide) analysis of concentrated fraction 2 extracts of transgenic tobacco groups 1283 (lanes 2−4), 1285 (lanes 5 and 6), and 1289 (lanes 7 and 8). Lanes 3, 4, 6, and 7 were concentrated, while lanes 2, 5, and 8 were original extracts. A nontransgenic control was run in lane 1. A bovine standard was run in the lane marked std. The arrow represents the position on the gel to which the recombinant lysozyme migrated. Bands were analyzed by densitometry to obtain the results found in Table 1.

Table 1. Expression of Bovine Lysozyme in Transgenic Tobacco Plants

	% of fraction 2		% of total fresh wt	
transgenic line	mean ($n = 4$)	± SD	mean ($n = 4$)	± SD
1283	1.04	0.2	0.056	0.011
1285	1.81	0.209	0.097	0.012
1289	1.27	0.271	0.068	0.015
1291	0.93	0.169	0.05	0.009
1293	1.38	0.235	0.074	0.012

PAGE analysis of concentrated F-2 extracts, which was used to determine lysozyme levels in the transgenic plants, is shown in Figure 3. By performing densitometry on the concentrated F-2 extracts and calculating the protein concentration of the samples from BCA analysis, expression levels in the tobacco were calculated. The percentages of the total F-2 protein represented by lysozyme are shown in Table 1. Transformants of group 1285 had the highest level of expression at 1.8% of F-2 protein and 0.1% on a fresh weight basis.

Resin Comparison. Comparison of three cation-exchange resins led to the choice of Hyper D-S (Bio Sepra) for further studies. It not only bound the most lysozyme but also gave the sharpest elution peak, as shown in Figure 4. The Hyper D-S resin (3 mL) bound approximately 43 mg of the 50 mg (86%) of lysozyme that was added, while Duolite C464 (3 mL) bound 12 mg of the 20 mg (60%) that was added. Bio-Rex-70 resin (3 mL) bound little lysozyme.

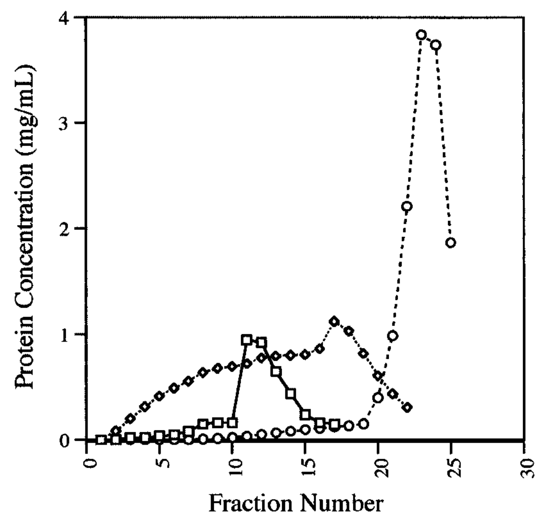

Figure 4. Comparison of the abilities of various cation-exchange resins to bind standard chicken lysozyme. Buffer change for Duolite C464, Bio Rex 70, and Hyper D-S occurred at fractions 9, 16, and 18, respectively. Fractions were collected every 3 mL.

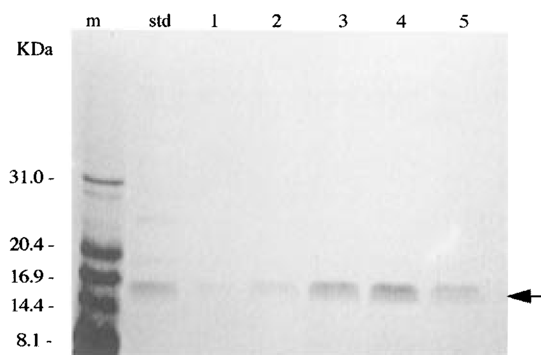

Figure 5. SDS−PAGE (16.5% acrylamide) analysis of the proteins present after purification of fraction 2. Lanes 1−5 represent separate, lysozyme-containing fractions eluted from the cation-exchange column, each being between 90 and 95% homogeneous. Lane m contained markers, and lane std contained a bovine standard. The arrow shows the position of the purified protein.

Bovine Lysozyme Binding. Results of column chromatography using the Hyper D-S resin to bind a bovine lysozyme standard (data not shown) confirmed that the resin would bind and release BSL. Subsequent benchtop trials using a 100-mL Econo (Bio-Rad) column (30 mL of resin) equilibrated with 0.01 M acetate, pH 6.0, and eluted with 0.1 M acetate, pH 8.0, yielded a substantially purified lysozyme fraction, as shown in Figure 5. On the basis of densitometric analysis, the purified protein was 93% homogeneous.

After several successful trials indicating substantial purificaton by ion exchange, another Western blot was performed on purified fractions and on crude extracts to verify that the purified protein was indeed bovine lysozyme. Tests on fractions collected from small column purification, large column purification, and F-2 extracts verified the presence of lysozyme in those fractions (not shown). These results confirmed that the correct protein had been purified consistently.

Activity assays using *M. lysodeikticus* cells were performed with purified BSL fractions. A typical tur-

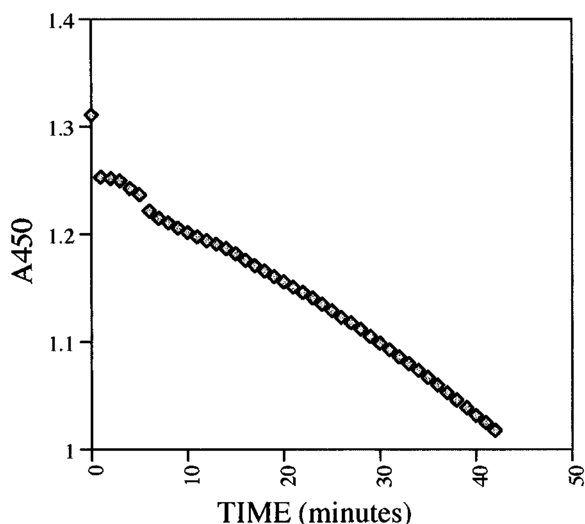

Figure 6. Typical turbidity reduction curve depicting the lytic action of active, recombinant lysozyme from purified fractions. The assay was based on lysis of Gram-positive *M. lysodeikticus* cells.

bidity reduction curve is shown in Figure 6. Activity levels between 7 150 and 29 000 units/mg of lysozyme were obtained from isolates. Units of activity represent a reduction in absorbance per unit of time. Activity was also maintained after the samples were freeze-dried. Assays suggested that the resolubilized enzyme had an activity between 6 000 and 16 500 units/mg of lysozyme. These values are lower than those determined for a chicken standard from Sigma (35 000 units/mg) but are higher than those for a Sigma bovine standard (4 000 units/mg).

DISCUSSION

The finding that the transgenic plants were expressing bovine lysozyme at levels above 1% of total F-2 protein was very encouraging. This level of efficiency suggests that extraction of lysozyme from transgenic plants can be made cost-effective. The level of expression observed was typical of reported levels of successful recombinant protein expression in plants (Destefano-Beltran et al., 1993).

It is important to note that the population of transgenic plants from which lysozyme was extracted was a heterogeneous R1 progeny array from each of the primary transformants tested. These arrays were not intended to be representative of populations that would be used for commercial production. For the purpose of this series of experiments, these genetic stocks constituted an appropriate, though less than optimal, source set. The primary consideration was that the plants produced detectable (and isolatable) quantities of the target protein. This provided a working stock sufficient for proof of principle.

For large-scale commercial production, extraction of lysozyme (or other target proteins) would require further optimization of several key steps in the prepurification process. First, transformation vectors would need to be optimized to enhance expression of the product peptide in the harvested tissue (i.e., leaves or fruits). Second, ideal physiological conditions for the production of the product would have to be determined empirically. Third, the most appropriate genotypes of recipient tissue would have to be chosen on an empirical basis. This requires that the plant genotype be sufficiently amenable to transformation to permit the generation of large numbers of transgenic plants from among which optimal expressers might be selected. The genotype must also be selected on the bais of empirical measurement of transgene expression within the specific genotype. While this phenomenon has been cataloged to a limited extent, it would be essential to improve upon this aspect of genotype choice. In fact, there are strong proponents for the development of one or more pure-line varieties bred exclusively for use in systems for the production and extraction of transgenic products. Finally, current extraction protocols have been developed for Maryland-type tobacco since this tobacco yielded the highest levels of F-1 protein. While selection of lines for production of transgenic product would ideally conform to this requirement, preliminary data suggest that higher levels of recombinant product are typically obtained from burley tobacco genotypes. This disparity could require some adjustment in the overall extraction protocols.

In terms of purification, Hyper D-S resin was chosen for use in purifying lysozyme on the basis of its higher capacity (from Figure 4), its ability to be reused indefinitely, its scaleability, and its ability to be rapidly sterilized (Horvath et al., 1994). Unfortunately, chicken lysozyme had to be used for the resin trials because of the high cost of purified native bovine lysozyme.

It was therefore necessary to purchase a small amount of bovine lysozyme to verify that conditions could be found such that the Hyper D-S resin would bind this lysozyme efficiently. It was predicted that binding would occur because sample addition could still be performed at a pH lower than the reported isoelectric point of bovine lysozyme (7.65) (Dobson et al., 1984). Because of this enzyme's less basic characteristics, it was also necessary to change chromatographic conditions from those used with chicken lysozyme. Instead of equilibrating the resin with 0.1 M potassium phosphate, pH 7.5, the resin was equilibrated with 0.1 M acetate, pH 5.8. These conditions were similar to those used by Dobson et al. (1984) in purifying lysozyme from bovine stomach mucosa using steps that included ion-exchange chromatography with carboxymethylcellulose. Results showed that Hyper D-S resin bound the bovine lysozyme in a similar fashion as it had the chicken lysozyme and that this resin could be used to purify lysozyme from the F-2 extract. Because of BSL's lower net positive charge, a lower ionic strength buffer had to be used during application to the column to allow electrostatic interactions between the resin and the lysozyme to become more favorable. Therefore, the equilibrating buffer used was 0.01 M acetate, pH 6.0. Lysozyme was selectively eluted by raising the pH to 8.0.

The conditions described in this study for the purification of lysozyme facilitated its isolation from a very complex and concentrated protein mixture. The complexity of F-2 is illustrated in Figure 3. Membrane fractionation of F-2 proteins eliminated higher molecular weight proteins that would have otherwise contaminated the ion-exchange column.

The isolation procedure developed in this study yielded lysozyme of 93% purity. Furthermore, the isolation process required only two readily scaleable steps. The levels of activity obtained from purified fractions were quite high. Substantial lytic activity was consistently present in purified fractions when tested on *M. lysodeikticus* cells. This assay showed that the activity from which the enzyme derives its antimicrobial

properties remained intact. Even after freeze-drying, lysozyme isolated according to these protocols retained most of its lytic activity.

It is likely that this process would retain its efficiency if used on a larger scale. For example, processing with a larger column resulted in greater specificity of elution during chromatographic runs. In addition, greater precolumn concentrations of the protein of interest are known to increase the binding efficiency of columns. Therefore, larger volumes processed by ultrafiltration should lead to increased efficiency of the process.

While the goal of the work described here was to achieve maximum enzyme purity and to retain the greatest possible activity, there are many industrial applications that require neither the purity nor the high levels of specific activity obtained here. For example, dilute solutions of lysozyme have been used to preserve sausage. A similar approach might be used to spray fruits, vegetables, and meats to retard bacterial contamination. Partially purified product could also be used in cosmetics and an array of other products.

Many transgenic proteins are likely to be industrially valuable and purified in a very efficient manner such as by precipitation or by bioselective adsorption to their specific substrates. In the case of lysozyme, this has been done using chitin (Weaver and Kroger, 1977). Isolation methods could be developed on a case by case basis, taking into account purity and processing requirements and subsequently adjusting the appropriate chromatographic parameters (i.e. resin particle size, pore size, and column design) (Lawlis and Heinsohn, 1993).

At the heart of all of these possibilities lies a crop that until recently was thought to have an uncertain future. However, due to its ease of manipulation and ability to be intensely cultivated to produce phenomenal biomass, value-added, nontraditional tobacco products are on the horizon, giving rise to a healthy future for tobacco.

ABBREVIATIONS USED

BCA, bicinchoninic acid assay; R1, self-pollinated, first-generation progeny; BSL, bovine stomach lysozyme; F-1, fraction 1, the 4 °C precipitate from leaf extract; F-2, fraction 2, the 4 °C soluble fraction from leaf extract.

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