Recombinant Aprotinin Produced in Transgenic Corn Seed: Extraction and Purification Studies

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Abstract: Expression in transgenic plants is potentially one of the most economical systems for large-scale production of valuable peptide and protein products. However, the downstream processing of recombinant proteins produced in plants has not been extensively studied. In this work, we studied the extraction and purification of recombinant aprotinin, a protease inhibitor used as a therapeutic compound, produced in transgenic corn seed. Conditions for extraction from transgenic corn meal that maximize aprotinin concentration and its fraction of the total soluble protein in the extract were found: pH 3.0 and 200 m*M* NaCl. Aprotinin, together with a native corn trypsin inhibitor (CTI), was captured using a tryspin–agarose column. These two inhibitors were separated using an agarose-IDA-Cu^{2+} column that proved to efficiently absorb the CTI while the recombinant aprotinin was collected in the flowthrough with purity of at least 79%. The high purity of the recombinant aprotinin was verified by SDS-PAGE and N-terminal sequencing. The overall recombinant aprotinin recovery yield and purification factor were 49% and 280, respectively. Because CTI was also purified, the recovery and purification process studied has the advantage of possible CTI co-production. Finally, the work presented here introduces additional information on the recovery and purification of recombinant proteins produced in plants and corroborates with past research on the potential use of plants as biorreactors. *Biotechnol Bioeng* **80**: 268–276, 2002.
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INTRODUCTION

Aprotinin, also known as bovine pancreatic trypsin inhibitor, is a serine protease inhibitor that was first isolated from bovine tissue by Kunitz and Northrop (1936). It is a single-chain polypeptide of 58 amino acid residues and with a molecular mass of 6.5 kDa. In addition to its application as a protease inhibitor in tissue cultures, aprotinin is an effective but expensive drug used during cardiac surgery to reduce postoperative organ dysfunction and bleeding disorders caused by blood activation (Beath et al., 2000). It reduces contact activation fibrinolysis via kallikrein and plasmin inhibition, respectively (Baufreton et al., 1996). Although it is a bovine protein, aprotinin is well tolerated by humans, probably because of its similarity to human Kunitz-type inhibitors (Vedvick et al., 1991).

In the current commercial production of aprotinin from bovine lung, the main concerns include storage cost and possible contamination of the bovine tissue with bovine spongiform encephalopathy (BSE). Consequently, finding an alternative production system for aprotinin has been of interest for some time. During the last several years the expression of recombinant aprotinin in *Escherichia coli* (Auerswald et al., 1987, 1988), *Pichia pastoris* (Vedvick et al., 1991), *Saccharomyces cerevisiae* (Barthel and Kula, 1993; Norris et al., 1990), and *Hansenula polymorpha* (Zurek et al., 1996) has been reported. More recently, maize transformation and generation of transgenic maize lines producing recombinant aprotinin were reported by Zhong et al. (1999). The main advantages of a plant system over other systems include low production and scale-up costs, the natural storage stability of the recombinant proteins in tubers or seeds, and well-established post-harvest handling and crop processing (Kusnadi et al., 1997). A particular advantage of producing therapeutic proteins in plants is the fact that transgenic plants do not propagate human or animal pathogens (Cramer et al., 2000; Zhong et al., 1999). Furthermore, plant cells tend to produce protein identical to those of the native source, e.g., properly processed and assembled (Hood and Howard, 1999). A potential concern for plant-derived biopharmaceuticals is the difference between the glycosylation patterns of plant and mammalian systems. Fortunately, aprotinin is not glycosylated and its structure–function characterization does not include glycosylation.

Although a significant number of proteins have been expressed and extracted from transgenic plants (Giddings et al., 2000), very few studies have reported conditions for

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recombinant protein isolation and purification from plant biomass (Austin et al., 1994; Kusnadi et al., 1988; Leite et al., 2000; Zhang and Glatz, 1999; Zhong et al., 1999). This knowledge is needed because the purification of identical recombinant proteins may differ significantly from that developed for the native or recombinant host system. Because process development can be the most time-consuming part of a research project (Evangelista and Suttnar, 1997), a fundamental understanding of the factors that affect the quality and quantity of purified protein is necessary to advance the commercialization of the recombinant product from plant systems. The main contribution of the work we present here is along this line: the study of the extraction and purification of recombinant aprotinin produced in transgenic corn seeds.

In purifying a recombinant aprotinin from corn seed, besides the presence of oil and various nonprotein soluble solids in the aqueous extract, the main challenge was the presence of a native corn protease inhibitor (corn trypsin inhibitor, CTI). CTI is a 112-amino acid protein with a calculated molecular mass of 12.5 kDa (Mahoney et al., 1984). The purification of CTI has been carried out by using a combination of affinity (immobilized trypsin or antibody), ion-exchange, and reverse-phase chromatography (Hojima et al., 1980; Lei and Reeck, 1986; Swartz et al., 1977; Wen et al., 1992). Peculiarity of CTI is the presence in its amino acid sequence of three tryptophan residues usually not found in plant protease inhibitors (Mahoney et al., 1984) and one histidine residue. Since the latter has been studied for its ability to interact with IMAC resin, we used immobilized metal affinity chromatography (IMAC, with Cu^{2+} as ligand) for the binding of CTI, providing the final recombinant aprotinin purification, after a previous affinity adsorption step.

MATERIALS AND METHODS

Materials

The transgenic corn seed containing the recombinant aprotinin used in this work was the same as that used by Zhong et al. (1999). The Sepharose 4 Fast Flow resin (agarose matrix) was purchased from Amersham-Pharmacia (Uppsala, Sweden). The His●Bind IMAC resin (agarose-IDA) was obtained from Novagen, USA. *N*-α-Benzoil-DL-arginine-*p*-nitroanilide (BApNA), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin, bovine trypsin (Type I), imidazole, and aprotinin were purchased from Sigma Chemical Company (St. Louis, MO). CTI was purified from nontransgenic corn seed according to Swartz et al. (1977). The Coomassie Plus Protein Reagent was from Pierce (Rockford, IL). All other chemicals used in this work were of at least reagent grade.

Methods

Transgenic Corn Seed Grinding

Extraction studies were carried out with seeds ground to a particle size smaller than 1.70 mm. For purification studies, seeds were ground to a particle size smaller than 0.85 mm using an Intermediate Model laboratory-scale mill from Thomas-Wiley, USA.

Analytical Assays

Total protein concentration was determined by following the Pierce Coomassie Plus Protein Assay Reagent instructions (Pierce). Trypsin inhibition assay was carried out according to the Sigma Enzymatic Assay of Aprotinin per Kassel (1970). One trypsin unit was defined as the amount of enzyme that hydrolyzes 1.0 μmol of BApNA per minute at pH 7.8 and 25°C. One trypsin inhibition unit was defined as the amount of inhibitor that decreases the activity of two trypsin units by 50%. Aprotinin was quantified by ELISA according to Zhong et al. (1999). All assays were done in triplicate, and the final aprotinin concentration of each sample was expressed as the mean of the three replicates. Electrophoresis was carried out under denaturing conditions (SDS-PAGE) as described by Zhong et al. (1999). The flowthrough fractions from the IMAC column containing recombinant aprotinin were pooled and sent to the Protein Chemistry Laboratory at Texas A&M University (College Station, TX) for N-terminal amino acid sequencing.

Effect of pH and Ionic Strength on Protein Extraction

Twenty-gram batches of corn meal were suspended in either 100 mL of extraction buffer of varying pH or 100 mL of NaCl solution with an increasing ionic strength. The buffers were the following: 200 m*M* glycine (pH 3.0), 200 m*M* sodium acetate (pH 4.0), 200 m*M* NaP_i (pH 6.0 and 8.0), and 200 m*M* sodium carbonate/bicarbonate (pH 10.0). NaCl concentrations were 0, 50, 100, 200, and 300 m*M*. The suspensions were stirred for 60 min with a magnetic bar in a 250-mL beaker and then centrifuged at 9,000*g* for 15 min. The supernatants were collected and stored at 4°C for analysis. All samples were assayed for aprotinin concentration, trypsin inhibition, and total protein concentration. Initial and final pHs and conductivities of all extracts were monitored.

Determination of Adsorption Isotherms

The adsorption isotherms of aprotinin and CTI on trypsin agarose and CTI on agarose-IDA-Cu^{2+} resin were generated at 25°C using 1.5-mL Eppendorf tubes. The agarose-trypsin resin was equilibrated with 50 m*M* NaP_i buffer, pH 7.8. The agarose-IDA resin was first saturated with Cu^{2+}; the unbound metal was washed out with deionized water, and a 100 m*M* imidazole solution in the adsorption buffer (50 m*M* NaP_i buffer, pH 7.0, containing 0.5 *M* NaCl), and in the end, the resin was equilibrated with adsorption buffer. Ten milligrams of drained resins were used per batch. The adsorption isotherms determination was conducted as described by Azzoni and Miranda (1999). Nonlinear regression fit of the

experimental data was done using the Langmuir adsorption model (Langmuir, 1916).

Aprotinin Purification

Two hundred grams of aprotinin corn meal were extracted in a beaker for 30 min with 1.0 L of deionized water at pH 3.0 (pH controlled by addition of 1.0 *M* HCl) and then filtered using a 3-μm pore filter paper. The pH of the filtrate was adjusted to 7.8 using a 1.0 *M* NaOH solution, and the filtrate was stored overnight at 4°C. It was again filtered immediately before being loaded into the affinity column. The trypsin agarose affinity chromatography was carried out using an XK 16 column (16 × 100 mm) with 10 mL of trypsin-agarose resin. TPCK-treated trypsin was immobilized on the Sepharose 4 Fast Flow resin according to the resin supplier's instructions (Amersham-Pharmacia). The column was equilibrated using 50 m*M* NaP_i buffer, pH 7.8, with 0.25 *M* NaCl. After the sample was loaded (775 mL at 2.0 mL/min), the column was washed with 50 m*M* NaP_i buffer, pH 7.8, containing 0.6 *M* NaCl. The elution step was done with 50 m*M* KCl-HCl at pH 2.0 (at 3.0 mL/min). The eluted fractions (4.0 mL) were immediately diluted with 0.2 *M* NaP_i buffer, pH 7.6, containing 2.0 *M* NaCl.

IMAC was performed in an XK 16 column packed with 13 mL of cross-linked agarose-IDA resin. The Cu^{2+} ion was bound to the resin by saturation with copper sulfate, and the unbound metal was removed by washing with deionized water followed by 100 m*M* imidazole in the adsorption buffer (50 m*M* NaP_i buffer, pH 7.0, with 0.5 *M* NaCl). After equilibration, the 34-mL aprotinin pool (fractions #20 to #26 eluted from the trypsin-agarose column) was loaded into the column. After being washed with adsorption buffer, the elution was carried out using an imidazole gradient (0 to 100 m*M* in adsorption buffer, in 10 column volumes). Fractions were of 8.0 mL for the flowthrough and washing and of 9.0 mL for the elution and regeneration steps. The flow rate used during the loading of the sample was 1.0 mL/min. All other steps were carried out at 2.0 mL/min.

RESULTS AND DISCUSSION

Effect of pH and Ionic Strength on Protein Extraction

Extraction is a key step in the recovery of proteins from plant tissue. The appropriate choice of extraction media (i.e., buffer, salts, solvents, protease inhibitors) and conditions (i.e., pH, ionic strength, temperature, time, agitation) is crucial to achieving good extraction yields and reducing the complexity and costs of purification.

Recovery and purification of the recombinant aprotinin from transgenic corn seed that was made available to us for this study presented two major challenges: the relatively low expression level (0.17% of soluble seed protein extracted at pH 3.0) and the presence of CTI. The effect of ionic strength and pH on aprotinin, CTI, and total soluble protein extraction was studied to improve aprotinin extraction and to reduce the presence of CTI and other corn seed proteins in the extract (Fig. 1 and 2). The CTI concentration in the extract was monitored as total trypsin inhibition because CTI contributed to more than 90% of the total inhibition activity in the transgenic corn seed extracts. The ionic strength experiments were performed using different NaCl solutions (0, 50, 100, 200, and 300 m*M*) in deionized water. In these experiments, buffers were not used to eliminate the possible influence of buffer ions on protein extraction. The natural buffering capacity of soluble proteins maintained the pH between 6.2 and 6.4 in all the experiments.

The presence of salt had a strong influence on the extraction of recombinant aprotinin (Fig. 1a). The final concentration of recombinant aprotinin in the extract showed a 12-fold increase (1,200%) when 300 m*M* NaCl solution was used instead of deionized water (from 0.06 to 0.78 μg/mL). Because aprotinin is a highly water-soluble protein, we

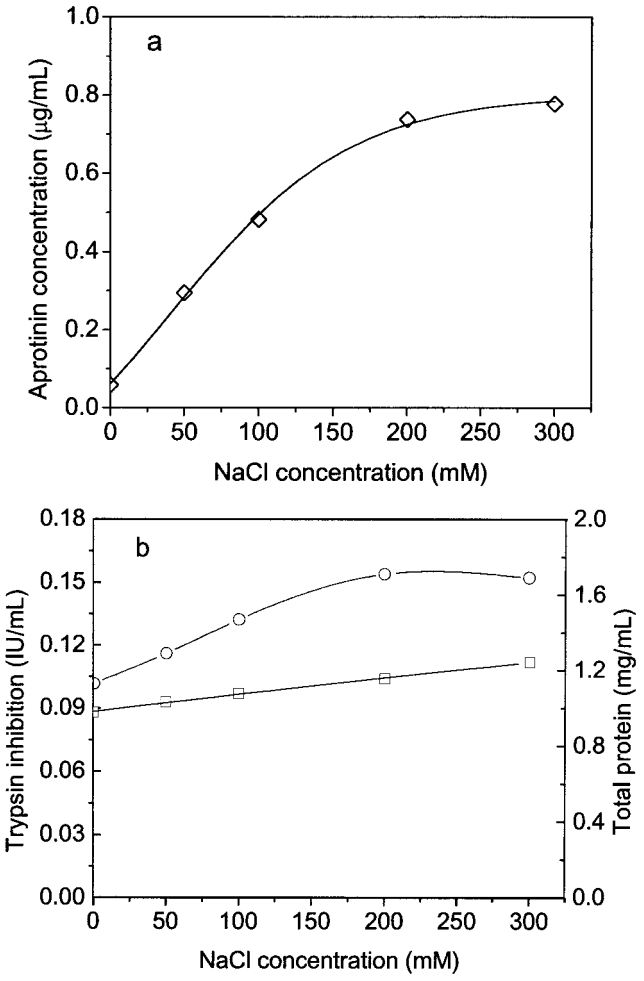

Figure 1. Effect of ionic strength on protein extraction from transgenic corn meal. (a) Recombinant aprotinin concentration; (b) trypsin inhibition (□) and total protein concentration (○). Extractions were carried out in aqueous NaCl solutions. In the experiments, the pH was kept between 6.2 and 6.4, taking advantage of the natural buffering capacity of the corn-soluble molecules. Duplicates did not deviate more than 19%.

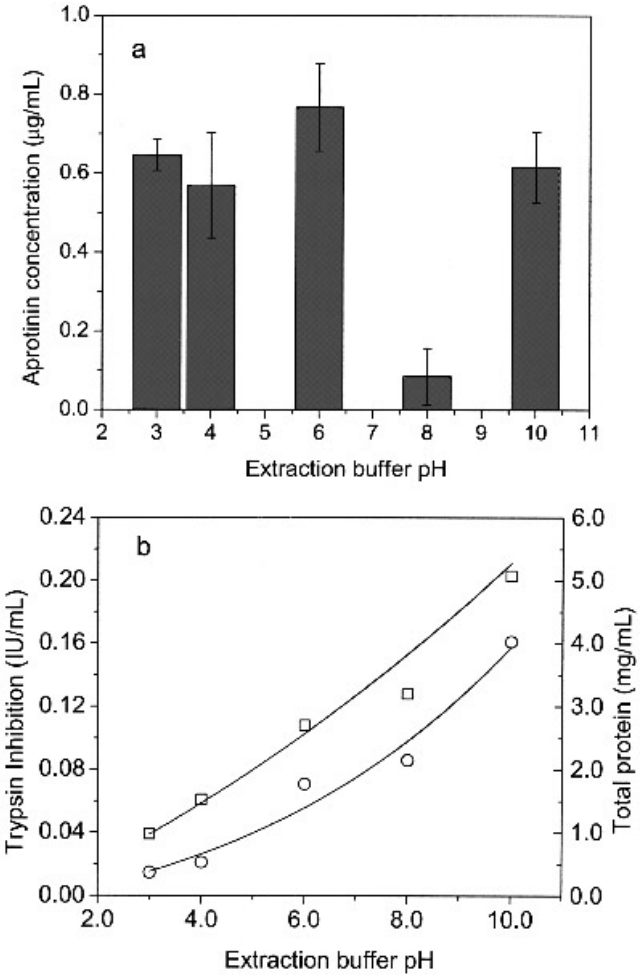

Figure 2. Effect of pH on protein extraction from transgenic corn meal. (a) Recombinant aprotinin concentration; (b) trypsin inhibition (□) and total protein concentration (○). Buffers used were 200 m*M* glycine, pH 3.0; 200 m*M* acetate, pH 4.0; 200 m*M* phosphate, pH 6.0 and 8.0; and 200 m*M* carbonate, pH 10.0. Error bars in panel indicate the standard deviation of five replicates. Duplicates did not deviate more than 18% (panel b).

speculate that the effect of ionic strength on the extraction of aprotinin resulted from disturbing the interactions between the components of the extracellular matrix and aprotinin. The effect of salt on CTI and total protein extraction was not as strong as that for the recombinant aprotinin (Fig. 1b). Increases of 25% and 50% were measured for CTI and total protein concentrations, respectively, as salt concentration increased from 0 to 300 m*M*.

Extraction of aprotinin had the lowest yield at pH 8.0 (Fig. 2a). The extraction was not significantly affected by pH in the range between pH 3.0 and 6.0. The apparent differences between aprotinin extracted at pH 3.0, 4.0, and 6.0 were probably due to the different buffer systems used. However, the lowest efficiency of aprotinin extraction found at pH 8.0 was significant and could not be explained by the difference in buffer since the same buffer (NaP_i) was used for the extractions at pH 6.0 and 8.0. Since the p*I* of the recombinant aprotinin was the same as that of the native aprotinin (Zhong et al., 1999), the phenomenon could be due to the effect of pH on the extracellular microenvironment, where most of the recombinant aprotinin accumulated (Zhong et al., 1999).

The extractability of CTI increased with total protein as the pH was increased (Fig. 2b). The concentration of CTI in the extract increased from 0.039 to 0.203 IU/mL (a 5-fold increase) when the extraction pH increased from 3.0 to 10.0. The concentration of total soluble protein in the extract also increased as the pH of the extraction buffer increased, probably because most of the water-soluble corn proteins have a p*I* around 5.0 (Wilson, 1987).

One of the advantages of extraction at pH 3.0 was the 4.2-fold increase in the mass fraction of aprotinin (from 0.04% to 0.17% of the total soluble protein), compared with that extracted at pH 6.0. Extraction at pH 3.0 also reduced the amount of CTI in the extract. The stability of the recombinant aprotinin at low pH should not be a problem on a commercial scale since the protein is very stable at drastic pH conditions. The inhibitor has a remarkable stability at high temperatures in acidified solutions. Kassel (1970) has demonstrated that bovine aprotinin could be heated for a short time in diluted acid at 100°C and in 2.5% trichloroacetic acid at 80°C without loss of activity.

To conclude, the different extraction behavior of the recombinant aprotinin compared with CTI and other corn seed proteins allows for selection of conditions that would maximize recovery of aprotinin and its fraction of total soluble protein in the extract. The pH and ionic strength of the extraction can be optimized to assure either low or high CTI extractability depending on whether CTI will be purified as a coproduct. A pH of 6.0 and a 300 m*M* NaCl concentration should be used if the designed aprotinin purification process is not affected by the presence of CTI or the latter is considered a valuable coproduct to be co-purified by the same process. If aprotinin is the only target, then extraction at pH 3.0 in 200 m*M* of NaCl concentration is recommended.

Determination of Adsorption Isotherms

Adsorption isotherms were determined to compare the interactions of aprotinin and CTI with trypsin-agarose and agarose-IDA-Cu^{2+} resins and to estimate the equilibrium parameters that would be useful for future process scale-up. The maximum binding capacities (Q_m) and effective dissociation constants (K_d) that describe the equilibrium relationship were calculated by a nonlinear regression fit of the experimental data to the Langmuir adsorption model (Langmuir, 1916).

The adsorption isotherms for aprotinin and CTI on trypsin-agarose were very similar (Fig. 3). The Q_m values calculated for both proteins were virtually the same: 1.55 ± 0.04 mg/mL and 1.55 ± 0.08 mg/mL for aprotinin and CTI adsorption, respectively. This similarity was not expected because the theoretical molar association between these molecules and trypsin should have been 1:1, which translates to 1:3.8 for aprotinin and 1:2.0 for CTI on a mass basis. Since a higher capacity on a mass basis was anticipated

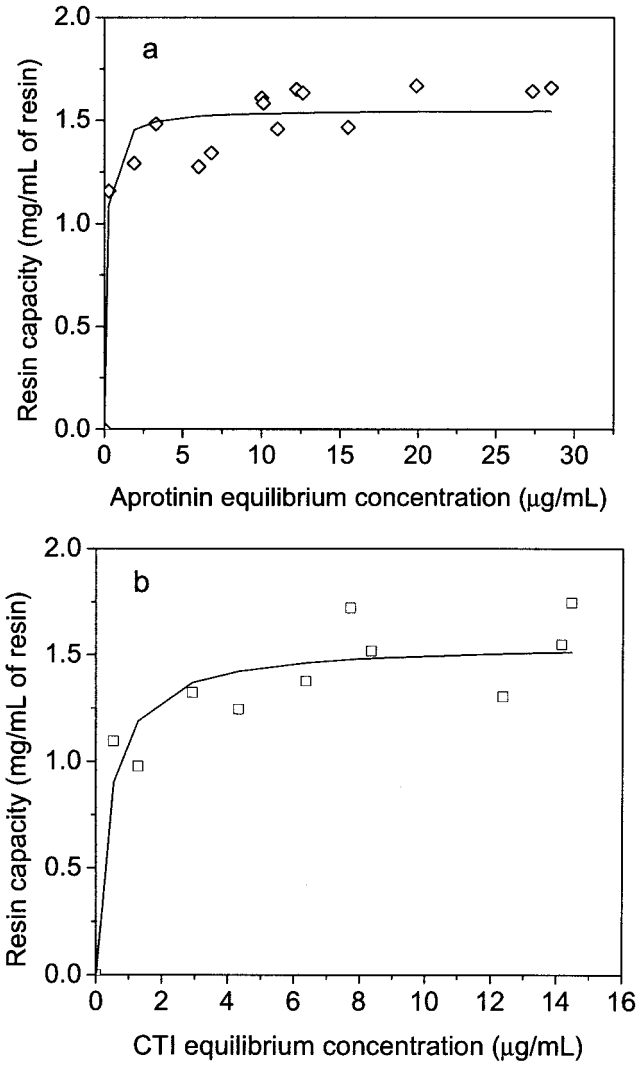

Figure 3. Adsorption isotherms of aprotinin and CTI on agarose-trypsin resin at 25°C. Adsorption was carried out using 50 m*M* NaP_i buffer, pH 7.8, in a batch-stirred tank system. (a) Aprotinin adsorption isotherm: the resin maximum binding capacity (Q_m) and effective dissociation constant (K_d) were 1.55 mg/mL and 0.126 mg/L, respectively. R^2 for the regression was 0.917. (b) CTI adsorption isotherm: Q_m and K_d were 1.55 mg/mL and 0.550 mg/L, respectively, and R^2 was 0.878. The parameters were calculated by nonlinear regression using the Langmuir adsorption model.

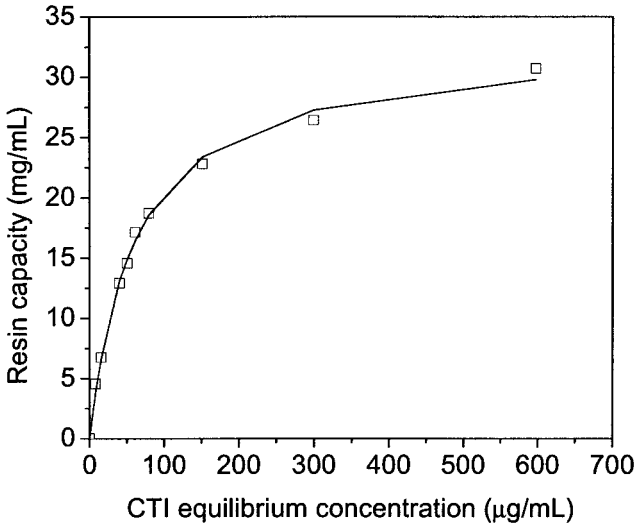

Figure 4. Adsorption isotherm of CTI on immobilized copper resin (agarose-IDA) at 25°C. Adsorption was carried out in 50 m*M* NaP_i buffer, pH 7.0, containing 0.5 *M* NaCl, using a batch-stirred tank system. The resin maximum binding capacity (Q_m) and effective dissociation constant (K_d) were 32.8 mg/mL and 60.7 mg/L, respectively. The parameters were calculated by nonlinear regression using the Langmuir adsorption model. R^2 for the regression was 0.996.

for CTI, a steric hindrance may explain the lower-than-expected adsorption capacity value. The estimated K_d values were 0.126 ± 0.051 mg/L and 0.550 ± 0.248 mg/L for aprotinin and CTI absorption, respectively. In spite of some scattering and our inability to collect experimental data points on the steepest parts of the isotherms, the values for the dissociation constant suggest that aprotinin and CTI can be efficiently captured in a trypsin-agarose column, even considering the very low concentration of these proteins in the extract. The binding capacity of the tryspin-agarose column was rather low, and an increase in this parameter would be required for large-scale applications.

The adsorption isotherm for CTI on immobilized copper resin is shown in Fig. 4. The choice for the use of copper ion was based on the fact that CTI has only one histidine residue and copper is known to promote the strongest interaction in such cases (Sulkowski, 1989). The Langmuir model gave a very good prediction of the experimental adsorption data, with maximum binding capacity and effective dissociation constant of 32.8 ± 0.7 mg/mL and 60.7 ± 0.35 mg/L, respectively. The K_d value is sufficiently small to predict efficient CTI binding and separation from the recombinant aprotinin during an IMAC step. The binding capacity was sufficiently large to suggest that a small column volume could be used for CTI and aprotinin separation.

An adsorption isotherm for aprotinin binding to immobilized copper resin was not generated because aprotinin does not interact with metal affinity resins (Tamagawa et al., 1999).

Recombinant Aprotinin Purification

Recovery and purification of recombinant aprotinin from corn seed extract was carried out by a process outlined in Fig. 5. To maximize the aprotinin concentration and minimize the amount of CTI and other corn proteins in the extract, the corn meal was extracted with water at pH 3.0 and no salt. The use of affinity adsorption as the first purification step was designed to take advantage of the low dissociation constant of the aprotinin-immobilized tryspin interaction (K_d of 0.126 ± 0.051 mg/L) and therefore to achieve high efficiency in the capture and purification of the recombinant product from the dilute extract. Because the affinity column was expected to bind CTI equally well,

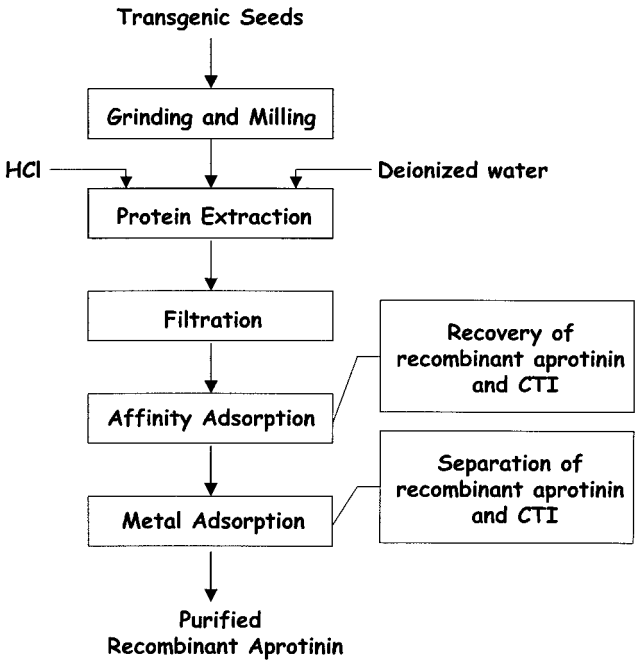

Figure 5. Block diagram of the process studied for the recovery and purification of recombinant aprotinin.

IMAC was used as the second step to separate the two proteins and to purify aprotinin to a higher degree.

Recovery of Recombinant Aprotinin and CTI in the Trypsin-Agarose Column

Protein and aprotinin concentrations and trypsin inhibition profiles for the affinity chromatography are shown in Fig. 6. No significant recombinant aprotinin or trypsin inhibition was found in the fractions collected in the flowthrough and washing steps, indicating that the recombinant aprotinin and the CTI were efficiently captured in the column. The column was not saturated: it was loaded 30% of its equilibrium capacity. A protein peak containing the recombinant aprotinin and trypsin inhibition activity was collected in the eluted fractions at low pH. By comparing the total protein, aprotinin concentration, and trypsin inhibition curves, a delay in aprotinin elution was detected. At the beginning of the elution step, the mixture of incoming elution buffer (pH 2.0) and washing buffer (pH 7.8) may have resulted in a pH gradient that caused a slight separation of aprotinin and CTI. The recombinant aprotinin corresponded to approximately 9.5% of the eluted protein with a purification factor of 33 (Table I). The rest of the protein was CTI. Recoveries of trypsin inhibition and aprotinin were 82% and 71%, respectively. A substantial loss of aprotinin occurred during the elution because the eluate pH was not sufficiently low to effectively dissociate the inhibitor from the immobolized trypsin (peak tail was not included in the pooled fractions). Significant improvement of the aprotinin recovery may be obtained by optimization of the elution step (i.e., pH and buffer).

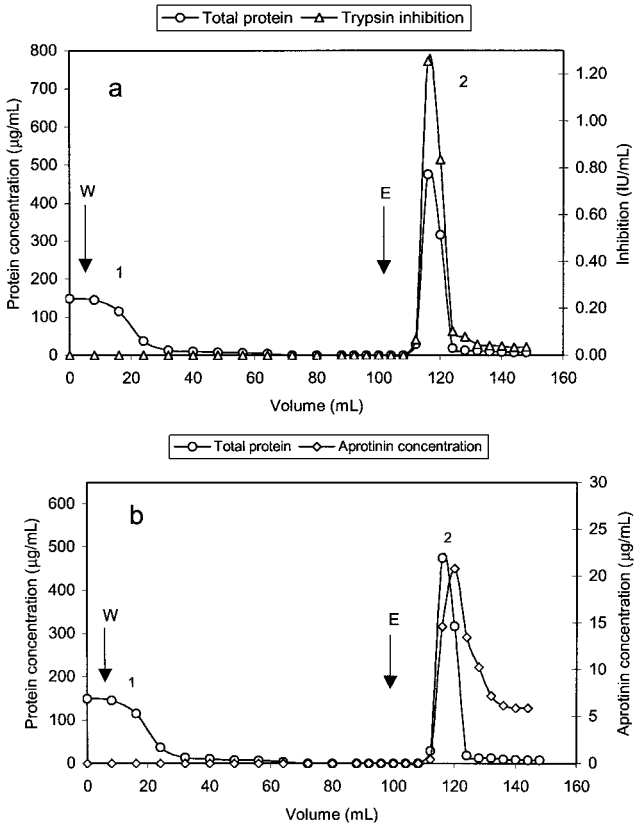

Figure 6. Chromatographic profiles the recovery of recombinant aprotinin and CTI from transgenic corn seed extract using a trypsin-agarose column. (a) Total protein and inhibition profiles. (b) Total protein and aprotinin concentration profiles. A 775-mL sample of extract was loaded into the column and washed with 50 m*M* NaP_i buffer, pH 7.8, containing 0.6 *M* NaCl. The bound proteins were eluted with 50 m*M* KCl-HCl at pH 2.0. W and E indicate the beginning of the washing and elution steps, respectively.

Separation of Recombinant Aprotinin and CTI by IMAC

The recombinant aprotinin and CTI rich fractions from the affinity column elution were pooled and loaded into the IMAC column (Fig. 7), except for fractions eluted between 110 and 116 mL (Fig. 6b). These first two fractions of the trypsin agarose column elution peak were not pooled because their aprotinin concentration was low compared to that of CTI. As in the previous chromatographic step, an excess of resin was used to avoid saturation and capture all corn protein impurities (we designed to use 1% of maximum equilibrium capacity). Trypsin inhibition activity was detected in the flowthrough as well as in the eluted peak (Fig. 7a). The ELISA assay indicated that aprotinin was present mainly in the flowthrough fractions; less then 1% of the aprotinin was detected in the eluted fractions (Fig. 7b). The recoveries of total tryspin inhibition and recombinant aprotinin for this adsorption step were 97% and 81%, respectively, resulting in an overall purification factor and aprotinin recovery (trypsin-agarose and IMAC steps) of 280 and 49% respectively (Table I). Table I shows a purity of

Table I. Purification of recombinant aprotinin (rAprotinin) from transgenic corn seed extract.[a]

Step	Volume (mL)	Protein (mg)	Inhibition (IU)	rAprotinin (mg)	rAprotinin yield (%)	rAprotinin purity (%)	Purification factor
Extraction	775	225	17	0.64	100	0.28	1
Affinity							
Flowthrough	837	119	0	0	0	—	—
Eluate	48	4.8	14	0.46	71	9.6	33
IMAC							
Load	34[c]	2.2	4.3	0.39	61	18	62
Flowthrough	48	0.39	1.5	0.31	49	79[b]	280
Eluate	39	1.7	2.7	0.002	0.30	—	—

[a]Extraction condition: 1:5 solid to liquid ratio (w/v) using de-ionized water at pH 3.0 for 30 min and then filtered through 3.0-μm filter paper.
[b]Direct analysis of the purity, e.g., using inhibition activity, gave values >95% (data not shown).
[c]The first two fractions from affinity elution containing mainly CTI were discarded.

79% for the purified aprotinin based on the mass ratio of aprotinin to total protein. However, total protein was determined in terms of equivalent mass of a reference protein, bovine serum albumin. The results from more direct analysis of aprotinin purity, e.g., based on inhibition activity, suggested that the purity may be higher than this, at greater than 95% (data not shown).

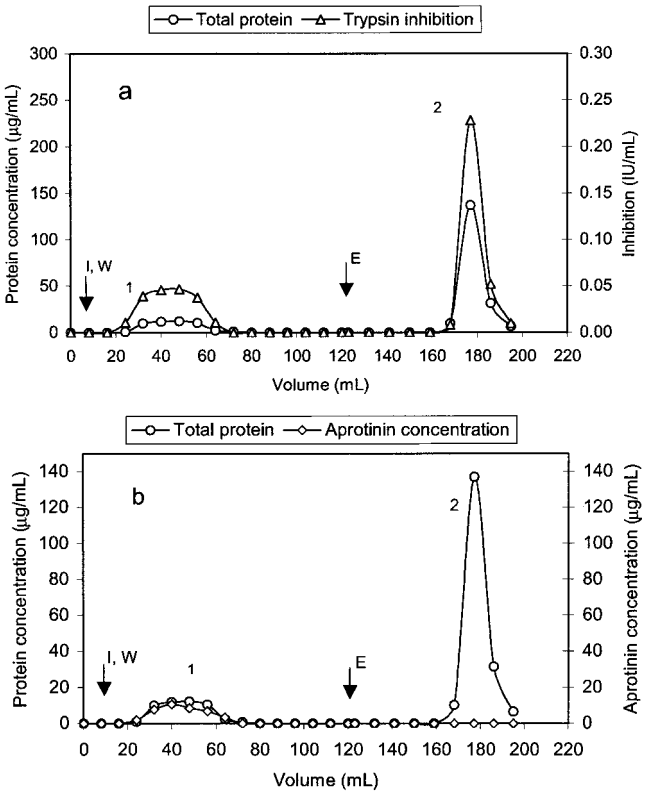

Figure 7. Profiles for the immobilized metal affinity chromatography. (a) Total protein and trypsin inhibition profiles. (b) Total protein and aprotinin concentration profiles. A 34-mL pool of fractions #20 to #26 recovered from the affinity column elution (Fig. 6) was loaded into the column. It was washed with 50 m*M* NaP_i buffer, pH 7.0, containing 0.5 *M* NaCl, and the elution step was carried out using an imidazol gradient in the same buffer. I, W, and E indicate the beginning of the injection, washing, and elution steps, respectively.

The flowthrough fractions containing the recombinant aprotinin (24 to 64 mL fractions) were pooled and a sample was sent for N-terminal amino acid sequencing. The amino acid sequence for the recombinant aprotinin was compared to that for the bovine aprotinin, showing a match between the recombinant and bovine molecules (Table II). The letter X for the fifth amino acid on the recombinant aprotinin sample indicates that a major amino acid was not detected in this cycle. Because the sample was neither reduced nor alkylated, this amino acid is probably a cysteine, which was the amino acid expected to be found at this position in the sequence.

The fractions collected during the purification process were also analyzed by SDS-PAGE (Fig. 8), confirming the purification of the recombinant aprotinin (lane 4). Also, the gel indicates that the fractions collected during elution from the IMAC column contained purified CTI in their single- and two-chain forms (Lei and Reeck, 1986) (lane 5). Under reducing conditions for SDS-PAGE, the two-chain CTI (14 kDa) can be seen on the gel as two polypeptides of approximately 8.0 and 5.0 kDa each. The two-chain CTI results from the cleavage of CTI by the immobilized trypsin in the affinity adsorption step (Lei and Reeck, 1986). A weak band corresponding to a protein with a molecular mass of approximately 24 kDa (not seen in Fig. 8) can also be seen on the original gel in lane 5. This band probably corresponds to trypsin released from the trypsin-agarose column during the low pH elution step (Lei and Reeck, 1986). Because trypsin is also adsorbed on metal affinity resins (Tamagawa et al., 1999) the use of IMAC after the trypsin-agarose column provides a way to overcome the problem of trypsin leakage during purification of the recombinant aprotinin.

Table II. N-terminal sequence for bovine (from native source) and transgenic aprotinin.

Protein	N-terminal sequence
Bovine aprotinin	RPDFCLEPPY[a]
Transgenic aprotinin	RPDFXLEPPY[b]

[a]According to Kassel (1970).
[b]"X" Denotes that an amino acid was not detected in this cycle.

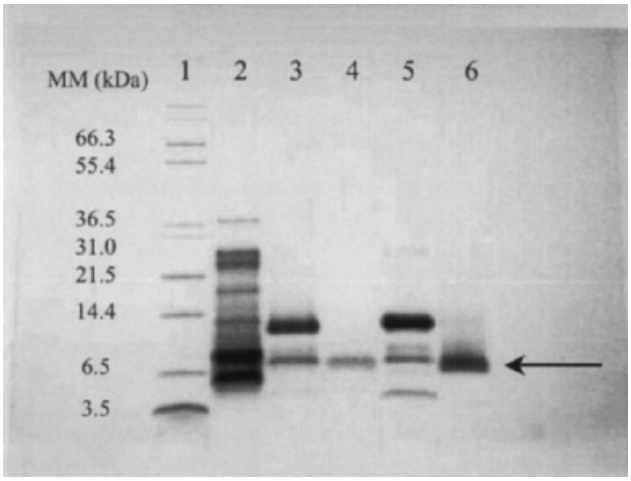

Figure 8. SDS-PAGE of the samples from the two chromatographic steps. Lane 1, molecular mass markers; lane 2, extracted corn proteins loaded into the affinity column (22 μg of proteins); lane 3, proteins eluted from the affinity column and loaded into the IMAC column (10 μg); lane 4, aprotinin flowthrough from the IMAC column (3 μg); lane 5, corn proteins eluted from the IMAC column (14 μg); and lane 6, commercial aprotinin (6.5 μg). The arrow indicates the position of aprotinin.

It is important to note that an increase of the expression level of the recombinant aprotinin should have an important impact on the economics and on the feasibility of a large scale commercial production. The expression level (0.17%) in the seed we had for this work is not as high as in two other recombinant proteins commercially produced in corn seed, avidin at 5.7% and β-glucuronidase at 0.7% (Kusnadi et al., 1998). In the meantime, ProdiGene, Inc., has increased aprotinin expression by more than 20-fold and will launch the aprotinin product at the end of 2002 (Zivko L. Nikolov, personal communication).

CONCLUSION

The proposed process for extraction and purification of recombinant aprotinin form transgenic corn extracts yielded a highly purified aprotinin with an overall recovery of 49%. An appropriate choice of extraction pH and ionic strength offers control of aprotinin and CTI extractability. A key step in the purification process studied was the IMAC step. The immobilized metal column allowed the separation of aprotinin from CTI and also captured the trypsin leakage. Thus, the use of IMAC as a second step may eliminate the need for a subsequent chromatographic polishing of the aprotinin product. Another advantage of the proposed purification process is the copurification of CTI. And finally, the use of IMAC as the CTI removal step may also be useful for the recovery and purification of other recombinant proteins produced in corn.

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References

Auerswald EA, Schröder W, Kotick M. 1987. Synthesis, cloning and expression of recombinant aprotinin. Biol Chem Hoppe-Seyler 368: 1413–1425.

Auerswald EA, Hörlein D, Reinhardt G, Schröder W, Schnabel E. 1988. Expression, isolation and characterization of recombinant [Arg^{15}, Glu^{52}]aprotinin. Biol Chem Hoppe-Seyler 369:27–35.

Austin S, Binghaîm ET, Koegel RG, Mathews DE, Shahan MN, Straub RJ, Burgess RR. 1994. An overview of a feasibility study for the production of industrial enzymes in transgenic alfalfa. Ann NY Acad Sci 721:234–244.

Azzoni AR, Miranda EA. 1999. Recovery of aprotinin from insulin industrial process effluent by affinity adsorption. Bioproc Eng 21:553–560.

Barthel T, Kula MR. 1993. Rapid purification of DesPro(2)-Val15-Leu17-aprotinin from the culture broth of a recombinant *Saccharomyces cerevisiae*. Biotechnol Bioeng 42:1331–1336.

Baufreton C, Velthuis H, Jansen P, Besnerais P, Wildevur R, Loysance D. 1996. Reduction of blood activation in patients receiving aprotinin during cardiopulmonary bypass for coronary artery surgery. ASAIO 42:M417–M423.

Beath SM, Nuttall GA, Fass DN, Oliver Jr WC, Ereth MH, Oyen LJ. 2000. Plasma aprotinin concentrations during cardiac surgery: full-dose versus half-dose regimens. Anesth Analg 91:257–264.

Cramer CL, Boothe JG, Oishi KK. 2000. Transgenic plants for therapeutic proteins: linking upstream and downstream strategies. In: Hammond J, McGarvey P, Yusibov V, editors. Plant biotechnology: new products and applications. New York: Springer. 95–115.

Evangelista DJ, Suttnar J. 1997. Separation used for purification of recombinant proteins. J Chromatogr B 699:383–401.

Giddings G, Allison G, Brooks D, Carter A. 2000. Transgenic plants as factories for biopharmaceuticals. Nat Biotechnol 18:1151–1156.

Hojima Y, Pierce JV, Pisano JJ. 1980. Hageman factor fragment inhibitor in corn seeds: Purification and characterization. Thromb Res 20: 149–162.

Hood EE, Howard JA. 1999. Protein products form transgenic plants. Agro-Food-Ind Hi-Tech May/June: 35–36.

Kassel B. 1970. Bovine trypsin-kallikrein inhibitor (Kunitz inhibitor, basic pancreatic trypsin inhibitor, polyvalent inhibitor from bovine organs). Methods Enzymol 19:844–852.

Kunitz M, Northrop JH. 1936. Isolation from beef pancreas of crystalline trypsinogen, trypsin inhibitor, and an inhibitor trypsin compound. J Gen Physiol 19:991–1007.

Kusnadi AR, Nikolov ZL, Howard JA. 1997. Production of recombinant proteins in transgenic plants: Practical considerations. Biotechnol Bioeng 56:473–484.

Kusnadi AR, Hood EE, Wicher DR, Howard JA, Nikolov ZL. 1998. Production and purification of two recombinant proteins form transgenic corn. Biotechnol Prog 14:149–155.

Langmuir I. 1916. The constitution and fundamental properties of solids and liquids. J Am Chem Soc 38:2263–2295.

Lei MG, Reeck GR. 1986. Combined use of trypsin-agarose affinity chromatography and reversed-phase high-performance liquid chromatography for the purification of single-chain protease inhibitor from corn seeds. J Chromatogr 363:315–321.

Leite A, Kemper EL, Silva MJ, Luchessi AD, Siloto RMP, Bonaccorsi ED, El-Dory HF, Arruda P. 2000. Expression of correctly processed human growth hormone in seeds of transgenic tobacco plants. Mol Breed 6:47–53.

Mahoney WC, Hermonson MA, Jones B, Reeck GR. 1984. Amino acid and

secondary structural analysis of the corn inhibitor of trypsin and activated Hageman factor. Biol Chem Hoppe-Seyler 259:8412–8416.

Norris K, Norris F, Bjorn SE, Diers I, Petersen C. 1990. Aprotinin and aprotinin analogues expressed in yeast. Biol Chem Hoppe-Seyler 371: 37–42.

Swartz MJ, Michell HL, Cox DJ, Reeck GR. 1977. Isolation and characterization of trypsin inhibitor from opaque-2 corn seeds. J Biol Chem 252;22:8105–8107.

Sulkowisk E. 1989. The saga of IMAC and MIT. BioEssays 10:170–175.

Tamagawa RE, Azzoni AR, Miranda EA, Vijayalakshmi, M. 1999. Aprotinin recovery: Comparison between biospecific and pseudobiospecific affinity adsorptions. Braz J Chem Eng 16;2:119–127.

Vedvick T, Buckholz RG, Engel M, Urcan A, Kinney J, Provow S, Siegel RS, Thill GP. 1991. High-level secretion of biologically active aprotinin from yeast *Pichia pastoris*. J Ind Microb 7:197–202.

Wen L, Huang J, Zen KC, Johnson BH, Muthukrishnan S, Mackay V, Manney M, Reeck GR. 1992. Nucleotide sequence of a cDNA clone that encodes the maize inhibitor of trypsin and activated Hageman factor. Plant Mol Biol 18:813–814.

Wilson CM. 1987. Proteins of the kernel. In: SA Watson, PE Ramstad, editors. Corn: chemistry and technology. St. Paul: American Association of Cereal Chemists, Inc. p 273–305.

Zhang C, Glatz CE. 1999. Process engineering strategy for recombinant protein recovery from canola by cation exchange chromatography. Biotechnol Prog 15:12–18.

Zhong GY, Peterson D, Delaney DE, Bailey M, Wicher DR, Register JC, Bond D, Li CP, Marshall L, Kulisek E, Ritland D, Meyer T, Hood EE, Howard JA. 1999. Commercial production of aprotinin in transgenic maize seeds. Mol Breed 5:345–356.

Zurek C, Kubis E, Keup P, Hörlein D, Beunink J, Thömmes J, Kula MR, Hollemberg CP, Gellissen G. 1996. Production of two aprotinin variants in *Hansenula polymorpha*. Proc Biochem 31;7:679–689.