

Purification of recombinant aprotinin from transgenic corn germ fraction using ion exchange and hydrophobic interaction chromatography

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Abstract Plants have attracted interest as hosts for protein expression because of the promise of a large production capacity and a low production cost. However, recovery costs remain a challenge as illustrated for recovery of recombinant aprotinin, a trypsin inhibitor, with removal of native corn trypsin inhibitor from transgenic corn (Azzoni et al. in *Biotechnol Bioeng* 80:268–276, 2002). When expression is targeted to corn grain fractions, dry milling can separate germ and endosperm fractions. Hence, only the product-containing fraction needs to be extracted, reducing the cost of extraction and the impurity level of the extract. Selective extraction conditions can reduce impurity levels to the point that low-cost adsorbents can result in relatively high purity levels. In this work, we attempted to achieve comparable purity with these lower cost methods. We replaced whole grain extraction and purification of recombinant aprotinin with sequential trypsin affinity and IMAC steps with an alternative of germ fraction extraction and purification with ion exchange and hydrophobic interaction chromatography (HIC). Using germ extraction at acidic pH supplemented with heat precipitation to remove additional host proteins resulted in a higher specific activity feed to the chromatographic steps. The cation exchange step provided 7.6× purification with

76.4% yield and no sodium dodecyl sulfate–polyacrylamide gel electrophoresis detectable native corn trypsin inhibitor. After the HIC step (2.7× step purification with 44.0% yield), the final product had a specific activity that was 75.3% of that of the affinity-purified aprotinin.

Keywords Recombinant protein · Aprotinin · Transgenic corn · Germ · Recovery · Extraction · Ion exchange · Hydrophobic interaction chromatography

Introduction

Production of recombinant proteins using plants as hosts has shown many advantages as recently reviewed (Menkhaus et al. 2004). One attractive feature of this technique is the low cost associated with production coupled with the expectation that purification will be no more costly than when using microbial hosts. When producing recombinant proteins from corn seeds, approximately 40 and 48% of the total cost is consumed in extraction and purification, respectively (Evangelista et al. 1998). A variety of extraction conditions have been studied, and the general guideline is to use acidic pH to reduce contaminant corn proteins (Azzoni et al. 2002). Case-specific purification strategies have been developed for different target proteins. Affinity-based chromatography is effective in achieving high purity when used as the only purification step or paired with other chromatography techniques (Azzoni et al. 2002; Witcher et al. 1998; Zhong et al. 1999), while a single ion exchanger is generally insufficient (Menkhaus et al. 2004; Dharmadi et al. 2003). Affinity-based chromatography may be replaced by multiple conventional chromatographies such as ion exchange, size exclusion, and hydrophobic interaction (Kusnadi et al. 1998).

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The separation task can be simplified by targeted expression to grain fractions and separation of those fractions before extraction (Zhong et al. 2006). Corn kernels are composed of two major fractions: starch-rich endosperm (83%) and protein- and oil-rich germ (11%; Pomeranz 1992). Methods to separate germ and endosperm are well established in the grain milling industry (Johnson 2000; Schenck 1992). Additionally, solubility characteristics of germ proteins are different from endosperm proteins (Johnson 2000).

The transgenic grain (supplied by ProdiGene) has targeted expression of aprotinin in the germ. With an extraction ratio of 1:5 whole grain meal to liquid (*w/v*) at pH 3.0, the overall protein content was 0.29 mg/ml, and the specific activity in trypsin inhibition units (TIU) was 0.022 TIU/mg protein, where TIU was due to both aprotinin and corn trypsin inhibitor (CTI), a host protein (Azzoni et al. 2002). The recombinant aprotinin has a specific activity of 12.2 TIU/mg and has a concentration of 0.83 µg/ml (or 0.010 TIU/ml) in the extract. The *pI* and molecular weight of the recombinant aprotinin is 10 and 6.5 kDa, respectively (Zhong et al. 1999). In a previous report (Azzoni et al. 2002), a trypsin agarose affinity column was first used to separate aprotinin from extracts, but this column also adsorbed and co-eluted CTI. A subsequent immobilized metal affinity chromatography (IMAC) step was used to remove the CTI (CTI binds to IMAC while aprotinin does not). The second IMAC step was later replaced by combining cation exchange (CEC) that achieved similar purification goals (Azzoni et al. 2005).

Our starting material was a germ fraction from dry milling rather than the milled whole corn kernels that were used previously (Azzoni et al. 2002, 2005; Zhong et al. 1999). Like Azzoni et al. (2002), our first objective was to study conditions of extracting aprotinin from fractionated germ to achieve some purification at the extraction stage. An additional heating step (aprotinin is heat stable below 120°C) was adopted to reduce contaminant proteins in the feed loaded onto chromatography columns. Our second objective was to study the effectiveness of CEC and hydrophobic interaction chromatography (HIC) to purify recombinant aprotinin from transgenic corn seeds, in particular separating it from CTI.

Materials and methods

Chemicals and transgenic germ fraction

Salts, acids, and bases were from Fisher Scientific (Pittsburgh, PA). CEC and HIC resins were from Sigma-Aldrich (St. Louis, MO). Germ fraction was provided in milled form by

ProdiGene (College Station, TX). The germ meal was stored at 4°C.

Extraction of aprotinin

Germ meal suspensions were stirred continuously at room temperature for 60 min (Azzoni et al. 2002) at a ratio of 1 g solids to 5 ml deionized water with different concentrations (0–300 mM) of NaCl. The slurry was adjusted to and maintained at a pH value from 3.0 to 10.0 using either 1 M HCl or 1 M NaOH throughout the extraction. After centrifuging at 13,200×*g* for 15 min (Sorvall RC5B Plus centrifuge, DuPont, Wilmington, DE), supernatant was decanted and filtered through a Corning µStar syringe tip filter (3 cm² effective filtration area, 0.45-µm pore size, cellulose acetate membrane, Corning Incorporated, Corning, NY). According to the vendor, cellulose acetate has a “very low” protein binding (Corning Inc. 2007). Samples (permeate) were analyzed for trypsin inhibition activity and protein concentration immediately.

In another set of experiments, the supernatant was held at 80°C for 10, 20, 40, or 60 min to evaluate the possibility of precipitating contaminants. Samples were cooled by immersion in room temperature water, followed by centrifugation and filtration as above.

Chromatography experiments

The conditions for extraction and chromatography were germ meal extraction at pH 3.0 with no salt addition, followed by heating to 80°C and holding for 10 min, then filtering and adjusting pH to 7.0 for loading to the CEC column. The AKTA Prime chromatography system (Amersham Biosciences, Piscataway, NJ) and 1×20 cm Econo[®] columns (Bio-Rad, Hercules, CA) was used for both CEC and HIC. Before loading, columns were washed with a running buffer to baseline. The running buffer was also used to wash the column after loading, followed by elution. Fractions of 5 ml were collected during elution; flow rate was 1 ml/min throughout. Fractions were assayed for TIU and total protein concentration.

The CEC column was packed to a 10.5-cm bed height (CV=10 ml) with 8.0 g of wet, drained CM-Sepharose CL-6B (Sigma) suspended in running buffer (buffer A, 20 mM Tris, pH 7.0). One hundred twenty milliliters of the feed was loaded, followed by washing the column with 10 CV of buffer A. The elution was a 0–100% linear gradient from buffer A to 1 M NaCl in buffer A (buffer B) over 5 CV, followed by 5 CV of buffer B.

Fractions containing trypsin inhibition activity from CEC were pooled. Under continuous agitation, the feed for HIC was prepared by drop-wise addition of 2.19 M

$(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris, pH 7.0 into 7.8 ml of pooled fractions to a total volume of 50 ml with final minor adjustment to pH 7.0 as needed. The final concentration of $(\text{NH}_4)_2\text{SO}_4$ in the feed was 1.85 M.

Phenyl-Sepharose® 6 Fast Flow resin (5.3 g of wet, drained resin; Sigma) was suspended in running buffer [buffer C, 1.85 M $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris, pH 7.0] and packed to a 10-cm bed height. Forty-five milliliters of the feed was loaded to the column, and the column was then washed with 40 ml of buffer C. The two-step elution used 40 ml of 0.925 M $(\text{NH}_4)_2\text{SO}_4$, 20 mM Tris, pH 7.0 (buffer D) followed by 40 ml of 20 mM Tris, pH 7.0 (buffer A).

Analytical assays

The assay protocol from Sigma for aprotinin (product A4529) was used. The assay measures the inhibition of trypsin hydrolysis of the substrate $\text{N}\alpha$ -benzoyl-DL-arginine-*p*-nitroanilide to $\text{N}\alpha$ -benzoyl-DL-arginine and *p*-nitroaniline. One TIU is defined as a 50% decrease of the hydrolysis rate observed in the presence of two trypsin units. A trypsin unit is cleavage of 1.0 μmol of BAPNA per minute at pH 7.8 and 25°C. The activity of trypsin is also inhibited by CTI, so TIU represents combined inhibition by both aprotinin and CTI when both are present.

To approximate aprotinin and CTI contributions to TIU content, a selective adsorption on an ion exchanger based on the *pI* difference between aprotinin (*pI* of 10, Zhong et al. 1999) and CTI (*pI* of 8.16 <http://ca.expasy.org>) was used to estimate the relative contributions. One milliliter of extract adjusted to pH 8.5 was mixed with 0.05 g of ion exchange resin in a 1.5-ml microcentrifuge tube and continuously agitated for 30 min on an end-to-end shaker (Lab Industries, Berkeley, CA) at room temperature. Two sets of tests were used to adsorb aprotinin using cation exchange adsorbents or CTI using anion exchange adsorbents. Besides the aforementioned CM-Sepharose, another cation exchange adsorbent was SP-Sepharose Fast Flow (Sigma), while anion exchange adsorbents were DEAE-Sepharose (Sigma) and Q-Sepharose Fast Flow (Sigma). After mixing, the suspensions were centrifuged at 10,000×*g* for 15 min. The supernatant was carefully removed using a plastic pipette and assayed for TIU and protein concentration. To elute the adsorbed protein, the remainder sediment resin was resuspended with 1 ml of 1 M NaCl in 20 mM Tris at pH 8.5, mixed for 60 min, and centrifuged as above. The supernatant (eluent) was transferred and assayed for TIU and protein concentration immediately.

The Bradford method (Bradford 1976) was used to determine the total protein concentration of samples using an assay kit containing Coomassie (Bradford) reagent and bovine serum albumin standard from Pierce Biotechnology

(Rockford, IL). All analytical assays were performed in duplicate, and results presented are averages of two tests.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed under a reducing condition with 16.5% Tris–Tricine gels from Bio-Rad (Hercules, CA). Samples were diluted with a sample buffer that contained 2% β -mercaptoethanol following the Laemmli sample buffer product manual (catalog number 161-0737, Bio-Rad), followed by heating at 95°C for 5 min. A purified (as reported by Azzoni et al. 2002) aprotinin sample from ProdiGene, referred to hereafter as the “the ProdiGene aprotinin”, was loaded for reference. The gel was stained with Coomassie blue dye (G-250, Bio-Rad), followed by destaining and scanning of the gel (Image Scanner™ II, GE Healthcare Bio-Sciences, Piscataway, NJ). The image was converted to a gray scale using Adobe® Photoshop® CS software (version 8.0, Adobe Systems, San Jose, CA).

Statistical analyses

Analysis of variance (ANOVA) was used to evaluate the data from extraction studies. Two independent variables, pH and salt concentration, were analyzed with the assistance of a JMP® statistical software (version 6.0, SAS Institute, Cary, NC).

Results

Extraction

ANOVA for salt and pH influences on extraction showed only the pH effects to be significant and those are detailed in Table 1. The TIU of extracts was strongly affected by pH during extraction (Fig. 1). Extracts at a pH lower than 6 had a higher TIU than those at higher pH values (Fig. 1a). On the other hand, more proteins were extracted at a higher pH (Fig. 1b), similar to the results from whole grain (Azzoni et al. 2002). As a result, the specific activity for trypsin inhibition increased with decreasing pH (Fig. 1c). Thus, extraction at pH 3.0 with no salt addition was used for the purification portion of this work. Samples showed no decrease in TIU after heating, while the total protein concentration dropped approximately 10% after heating for 10 min; prolonged heating did not reduce host protein content further.

Batch adsorption results developed to differentiate relative contributions from aprotinin and CTI are presented in Table 2. The adsorbent type in each category of ion

Table 1 ANOVA of extracts at different pH conditions^a

Extraction pH	3.0	4.0	5.0	6.0	7.0	8.0	10.0
Trypsin inhibition unit (TIU/ml)	AB	B	AB	A	C	C	D
Total protein concentration (mg/ml)	A	A	B	C	B	D	E
Specific activity (TIU/mg protein)	A	B	C	D	E	E	E

^a In each category, levels not connected by the same letter are significantly different ($p < 0.05$).

exchange gave similar results. For cation exchange, ~25% of TIU remained in the sample after adsorption at pH 8.5, most likely representing the content of CTI (pI of 8.13). After elution with 1 M NaCl at pH 8.5, ~70% of the extracted TIU was recovered and would represent the aprotinin content (pI of 10, Zhong et al. 1999). For anion exchange, 70% TIU did not adsorb to the resin at pH 8.5, providing a complementary indication that this is the aprotinin contribution to the total TIU of the extract.

Cation exchange chromatography

Although the batch adsorption at pH 8.5 followed by elution at pH 8.5 with 1 M NaCl enabled the recovery of ~70% TIU using either SP- or CM-Sepharose (Table 2), a preliminary chromatography run at pH 8.5 using CM-Sepharose resulted in poor recovery of TIU (~1%) after elution with 1 M NaCl at the same pH. Other screening runs included two experiments using SP-Sepharose at pH 7.0 that gave yields of ~50% (details not shown). All these preliminary experiments had a poorer recovery of TIU than the conditions detailed in the experiments section (CM-Sepharose at pH 7.0) and reported in the following.

When the heat-treated extract was loaded onto the CM-Sepharose column, the majority of proteins were in the flow-through (70 to 190 min in Fig. 2). The washing step removed some loosely bound corn protein. During elution, there was a single peak, within which, two adjacent fractions showed inhibition activity. Elution of those two fractions occurred over the conductivity range of ~47 and 62.7 mS/cm corresponding to NaCl concentrations of ca 0.56–0.76 M. The first fraction had the majority of TIU. The combined fractions accounted for 76.4% of the total feed TIU (Table 3). Examination of this product by SDS-PAGE (Fig. 3, lane 4) showed no band at 14 kDa (Azzoni et al. 2002, 2005) for CTI, thus indicating essentially complete recovery of the aprotinin portion of the TIU of the feed. The purification factor after this CEC step was 7.6 based on TIU (Table 3).

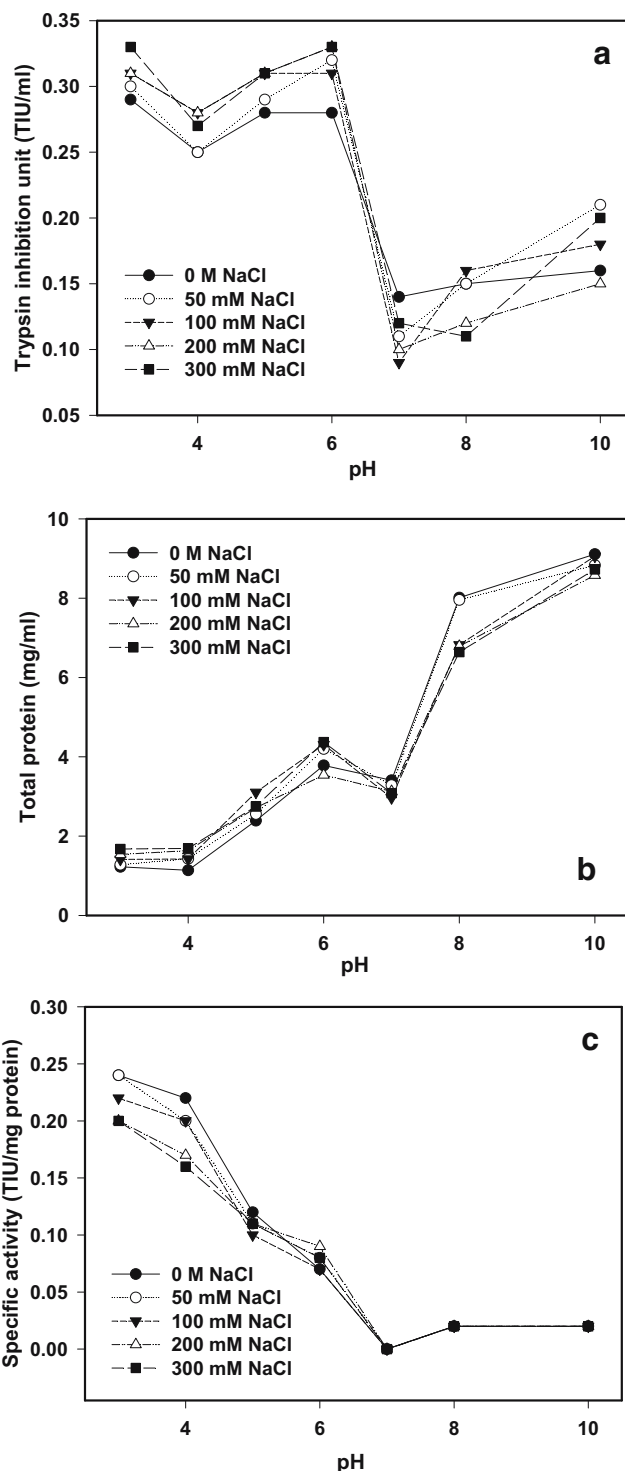


Fig. 1 Effects of pH and salt concentration on extracting recombinant aprotinin from transgenic corn germ fraction: **a** trypsin inhibition units, **b** total protein concentration, and **c** specific activity in extracts from germ

Hydrophobic interaction chromatography

The HIC chromatogram (Fig. 4) showed protein elution at each of the two steps. The inhibition activity was in three adjacent fractions within the first peak at 0.925 M

Table 2 Estimation of relative amounts of CTI and aprotinin in extracts

Sample	Adsorbent	TIU/ ml	Percent of TIU relative to extract
Extract	–	0.30	100
Remaining extract after adsorption at pH 8.5	CM- Sephacrose	0.08	27
	SP- Sephacrose	0.07	23
	DEAE- Sephacrose	0.21	70
	Q- Sephacrose	0.21	70
Sample after elution with 1 M NaCl at pH 8.5	CM- Sephacrose	0.21	70
	SP- Sephacrose	0.20	67

ammonium sulfate. The step yield in the combined three fractions was 44.0%, with a specific activity of 9.19 TIU/mg protein and a step purification factor of 2.7 (Table 3).

SDS–PAGE results (Fig. 3) illustrate the progression of purification at each of the steps. Lanes 2 and 3 (heat-treated vs initial unheated extract) are similar. Most larger proteins, as well as the 14 kDa CTI, were removed with CEC (lanes 4–6). The pooled fractions from HIC showed two bands (lane 7), corresponding to those seen in the ProdiGene aprotinin (lane 8). While both our final product and the ProdiGene aprotinin each had two bands, there was less of the lower molecular weight band in the ProdiGene aprotinin. This is reflected in the lower specific activity of our final product (9.19 vs 12.2 TIU/mg).

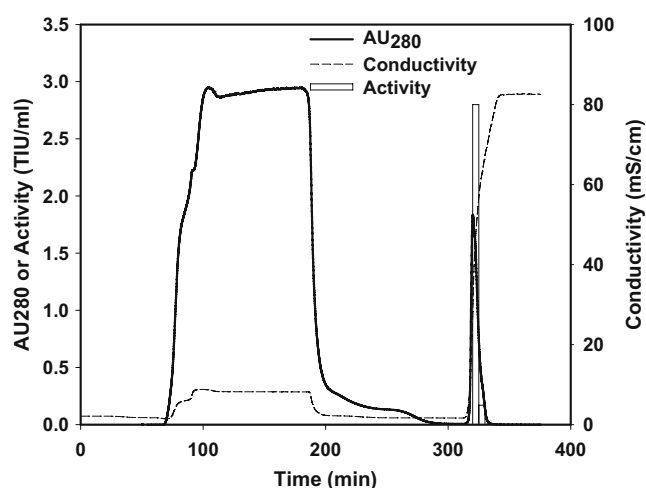


Fig. 2 Chromatogram for binding and elution of germ extract applied to the CM-Sephacrose cation exchange column. The flow rate was 1 ml/min, and the fractions were 5 ml during a linear gradient elution. Details of CEC are described in the text

Discussion

Several differences were observed between our extraction results and those in literature, which may have been caused by different starting materials: Azzoni et al. (2002) used whole grain, while this work used the germ fraction of the grain. The first difference was the effect of pH on extractable TIU. Azzoni et al. (2002) observed a monotonic increase in TIU with extraction pH (Azzoni et al. 2002); our data showed a lower pH facilitated the extraction (based on TIU, Fig. 1a). A lower pH is desirable to extract a target protein from corn because it results in lower extraction of host cell proteins (Fig. 1b). The second difference was the relative contribution of CTI to the total TIU. When extracting the whole grain meal of the same transgenic corn at pH 3.0 with no salt addition, Azzoni et al. (2002) concluded that ca 50% of the total TIU was contributed by CTI, compared to our 25%. The CTI exists mostly in endosperm and secondarily in germ, but inhibitors isolated from these two locations are different (Liener and Kakade 1980; Mosolov and Valueva 2005). Therefore, a relatively smaller contribution of CTI to the total TIU would be expected with our starting material, germ. Initial processing to separate the germ-rich fraction thus reduces the amount of mass (endosperm) and CTI (mostly in endosperm) during extraction.

Germ is the fraction richer in soluble protein and is, thus, not as ideal as endosperm for the expression of recombinant proteins. Our recent work showed that germ proteins had a wide *pI* distribution between 3 and 10 for extracts at pH 4.0 and pH 7.0 (Gu and Glatz 2007), indicating there is no direct correlation between levels of soluble host proteins and *pI* distributions. However, extraction at low pH reduced the level of soluble host proteins while still extracting the aprotinin. Our extraction of the germ fraction only did increase the extract total protein concentration: 1.226 mg/ml compared to 0.29 mg/ml in Azzoni et al. (2002), but the enrichment of aprotinin was achieved. Specific activity and TIU was improved from 0.022 TIU/mg protein and 0.02 TIU/ml in Azzoni et al. (2002) to 0.24 TIU/mg protein and 0.29 TIU/ml in ours, respectively.

The enrichment of a target protein was also reported in our previous extraction of recombinant dog gastric lipase (rDGL) expressed in endosperm (Zhong et al. 2006). The dry milling strategy to separate the endosperm-rich fraction for extraction enabled a good recovery of rDGL, with a much higher specific activity than for direct extraction of whole grain meal. Extracting the endosperm-rich fraction only also reduced the amount of detergent required for a good recovery, which, together with the reduction in host protein contamination, should simplify downstream purification steps.

Table 3 Summary of the performance of chromatography columns

	Feed			Pooled fractions			Yield (%)	PF
	TIU/ml	SA	Total TIU	TIU/ml	SA	Total TIU		
Cation exchange	0.18	0.45	21.60	1.65	3.43	16.50	76.4	7.6
Hydrophobic interaction	0.26	3.43	11.58	0.34	9.19	5.10	44.0	2.7

SA Specific activity in TIU/mg protein, PF purification factor (the ratio of SA of the product to that of the feed)

The first chromatographic step, CEC, in addition to separating aprotinin from a large number of bound proteins, serves to eliminate the CTI, which was not detected (by SDS-PAGE) in the eluted fractions. The pI difference between the two trypsin inhibitors was also used by Azzoni et al. (2005) to separate CTI and aprotinin using SP-Sepharose adsorbent at pH 8.5 after capture of both on a trypsin affinity chromatography. However, their CEC step yield for aprotinin with this resin was 37%, lower than that obtained in this work with CM-Sepharose at pH 7. Although our running pH (7.0) was below the CTI pI (8.16), the CTI did not bind, so separation was achieved, but with higher aprotinin yield. It will be interesting to study the combination of the first step using CM-Sepharose at pH 7.0 (enabling an almost complete recovery of aprotinin and depletion of CTI) and the second step using trypsin affinity chromatography (binding aprotinin only). Compared to a purification route of trypsin affinity and IMAC chromatographies (Azzoni et al. 2002), the replacement of IMAC by CEC (a lower cost adsorbent), while still using the trypsin affinity adsorbent to achieve a high selectivity, should significantly reduce the purification cost.

The nature of the contaminant in the final eluent from HIC (Fig. 3) was not analyzed further in this work, but it was responsible for the limited purity obtained. However,

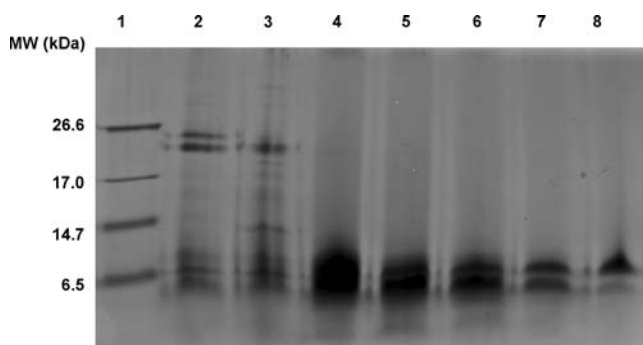


Fig. 3 SDS-PAGE of various aprotinin samples. Lane 1 Polypeptide MW standards (the band corresponding to 6.5 kDa is aprotinin); Lane 2 unheated extract with 6.5 µg protein; Lane 3 heated extract (feed to cation exchange) with 5.2 µg protein; Lanes 4–6 combined TIU-containing fractions from cation exchange with different loadings—4.8 µg (lane 4), 3.0 µg (lane 5), and 2.0 µg (lane 6); Lane 7 combined TIU-containing fractions from HIC with 0.37 µg protein; Lane 8 purified aprotinin sample from ProdiGene with 0.8 µg protein

previous studies of the recombinant protein produced in corn (Zhong et al. 1999) attributed the presence of a similar contaminant band to reduction of disulfide bonds, as sequencing showed no evidence of two proteins in their purified fraction.

In conclusion, this work demonstrated that (1) initial dry milling can be integrated into the purification strategy to reduce feed volume and enrich the target protein and (2) the combination of CEC and HIC is a promising alternative to affinity chromatography for purifying recombinant aprotinin from the germ fraction of transgenic corn seeds. Removing the endosperm fraction before extraction reduced the amount of contaminating CTI in the extract. Good recovery of aprotinin and elimination of remaining CTI was obtained with CEC. Further purification by HIC led to a final product that showed the same two bands, but with higher proportions of impurity as the ProdiGene aprotinin that had been purified by sequential trypsin affinity and IMAC. This difference was consistent with the fact that our final product had a specific activity that was 75.3% that of the ProdiGene aprotinin.

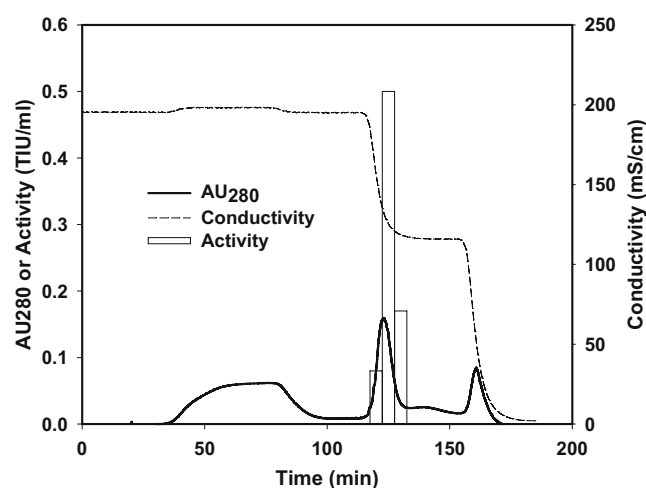


Fig. 4 Chromatogram of the binding and elution of the combined TIU fractions from IEC applied to a phenyl-Sepharose hydrophobic interaction column. The flow rate was 1 ml/min, and the fractions were 5 ml during the two-step elution. Details of HIC are described in the text

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