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# Purification and Characterization of a 44-kDa Recombinant Collagen I α 1 Fragment from Corn Grain

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This paper demonstrates that a fibrous, repetitive amino acid sequence collagen-related protein, a 44-kDa fragment of human collagen I α 1 (CIα1), was expressed in corn grain molecularly equivalent to that produced in recombinant yeast. The recombinant CIα1 was extracted and purified from early generation plants having low levels of recombinant protein accumulation. It was selectively extracted at low pH and purified by ion exchange and gel filtration chromatography, resulting in a 44-kDa CIα1 with >70% purity and 60% recovery. The N-terminal sequence, amino acid composition, and immunoreactivity closely matched those of an analogous 44-kDa CIα1 fragment produced by the yeast *Pichia*. The corn-derived 44-kDa CIα1 had an intact protein mass of 44088 Da, which is within 0.2% of the mass calculated from the expected sequence. Tandem mass spectrometry confirmed the primary sequence with 78% coverage. The amino acid composition analysis indicated a low level of prolyl hydroxylation. Glycoprotein staining revealed no glycosylation.

**KEYWORDS: Recombinant collagen fragment; protein extraction; purification; characterization; chromatography; transgenic corn; maize**

## INTRODUCTION

Corn grain is used as feedstock for many large-volume industrial products (e.g., ethanol, biodiesel, polylactic acid, sweeteners) and has been shown to be a suitable system for the accumulation of recombinant proteins equivalent to similar proteins obtained from eukaryotic sources (*1*−*10*). Although crop management, safety and public acceptance concerns remain to be addressed, the use of corn seed as an expression host could also be an attractive system for the production of intermediate to low-value (less than U.S. $50/kg) recombinant proteins used for industrial processing or as structural components. These applications could require thousands of tons of recombinant protein per year. The low cost requirement and high volume demand necessitate producing these proteins as coproducts of agricultural-based processes, such corn grain-based biorefining. In addition to the well-known industrial enzymes, peptides with desirable material properties are potential intermediate to low-cost/high-volume products; these include gelatin for pharmaceutical capsules, which is the market motivating this work, and advanced performance protein-based polymers (e.g., silk, elastin).

Consistently high accumulation levels (10−40 kg of recombinant protein/acre, 2.5−10 g/kg of grain) using tissue-specific promoters in high-yielding corn commercial lines producing up to 4000 kg of grain/acre will be required for the commercialization of such products. This has been achieved through extensive selection and crossing. For example, the accumulation level for recombinant avidin was increased 150-fold in eight generations to 10 g of avidin/kg of grain (*1*, *3*−*8*, *10*). However, the low early generation expression levels make it difficult to purify enough protein to determine the structural and functional parameters of the expressed recombinant protein. It is critical to be able to characterize the product at an early stage of the development program before commitment of the large amount of resources required to reach high expression levels. This paper describes for the first time the extraction, purification, and characterization of a fibrous structural mammalian protein, a recombinant 44-kDa collagen I α 1 (CIα1) chain fragment, from transgenic corn. Early screening of an analagous protein showed promising capsule-forming properties. The protein was present in corn grain at a low accumulation level (20 mg/kg) typical of that obtained in early field trials.

The currently preferred recombinant technology for collagen fragment production is based on the yeast *Pichia* fermentation (*11*). Recombinant CIα1 fragments are secreted in *Pichia* fermentation as fully intact species as determined by electrophoresis gels, N-terminal sequencing, gel filtration chromatography, and mass spectrometry. The accumulation of recombinant collagen and of collagen fragments has also been demonstrated

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in other recombinant expression systems such as other yeasts [*Sacchromyces cerevisias* (*12, 13*), *Hansenula polymorpha* (*14, 15*)], mammalian cells (*16*−*18*), insect cell culture (*19, 20*), *Escherichia coli* (*21, 22*), *Bacillus* (*23*), silkworms (*24*), milk of transgenic animals (*25, 26*), tobacco cell culture (*11*), and tobacco plants (*27, 28*). Recent studies indicated that transient and stable expression of CIα1 can be achieved in barley and rice suspension cell culture (*29, 30*) and in barley and corn seeds (*31, 32*).

The corn grain-derived CIα1 extraction was patterned after studies showing that pH and ionic strength have the greatest effects on protein extraction from grain. The total extractable corn endogenous protein concentration increased 10-fold when the pH was increased from 3 to 10 (*1, 33*). The total extractable corn endogenous protein concentration also increased with addition of NaCl up to 300 mM (*1*). Collagen fragments are acid soluble (*28*), as illustrated by the *Pichia*-derived CIα1 having maximum solubility at pH 1−2, offering the possibility for selective extraction of CIα1 at low pH while minimizing the extraction of corn grain-related protein. The *Pichia*-derived collagen fragments (*34*) and tobacco-derived collagen (*35*) were purified using ion exchange chromatography (IEC) eluted with an acetate buffer. Size-based separations may be able to remove the remaining host proteins from corn extracts as most of those extractable at acidic pH are expected to be smaller than 30 kDa (*36*).

Previous publications of corn grain-derived recombinant proteins have focused on the production and downstream processing, providing only limited protein characterization data for nonglycosylated 6.5-kDa aprotinin (*1*), β-glucuronidase (GUS), and avidin (*7*). The most extensive characterization was conducted for corn-derived trypsin, for which Western blotting, MS, and glycosylation staining were used (*10*). All analytical results of the corn grain-derived trypsin were equivalent to the native protein with the exception of the molecular weight. The differences in molecular weight were attributed to O-linked glycosylation of the corn-derived protein. Karnoup et al. (*37*) showed that O-linked glycosylation found in a corn grain-derived human IgA1 was through hydroxylated proline residues, indicating that a maize prolyl hydroxylase was able to convert proline to hydroxyproline and subsequently glycosylate IgA1. Our cloned CIα1 fragment does not contain the consensus sequences for N-linked glycosylation, leaving only O-linked glycosylation, which could occur through serine, threonine, or hydroxylated proline or lysine residues as the O-linked glycosylation has no consensus motif (*37*). Our characterization of the corn-derived 44-kDa CIα1 includes immunoreactivity, amino acid composition, glycosylation staining, and mass spectrometry combined with peptide mapping.

## MATERIALS AND METHODS

**Materials.** The transgenic corn grain expressing the 44-kDa CIα1 was provided by the former ProdiGene Inc. (College Station, TX). This grain was harvested in the fall of 2004 from a field trial in Nebraska growing second-generation transgenic plants containing the gene for the expression of a 44-kDa fragment of the triple-helical region of human CIα1. The CIα1 fragment has 498 amino acids resulting in a calculated MW of 44002.8 Da, assuming proper cleavage of the signal sequence. Amino acids 372−869 of CIα1 are included in the 44-kDa CIα1. Its estimated p*I* is 8.1. Upon receipt at Iowa State University (ISU), the seeds were stored in a 4 °C cooler, sealed in moisture-proof containers. A lyophilized 44-kDa CIα1 of the same sequence produced in *Pichia pastoris* (*Pichia*-derived 44-kDa CIα1) was provided by FibroGen Inc. (South San Francisco, CA) to be used as reference material.

**Vector Construction and Transformation of Corn-Derived 44-kDa CIα1.** ProdiGene used the corn expression vector PGN9004 to clone the gene for the 44-kDa CIα1 fragment. The expression of the 44-kDa CIα1 gene was regulated by an embryo-specific maize globulin-1 promoter (PGNpr2) (*38*). The expression vector also provided a signal sequence—barley α-amylase signal sequence—to direct the 44-kDa CIα1 to the endoplasmic reticulum (ER).

The resultant vector was introduced into corn immature embryos using *Agrobacterium* transformation (*39*). Embryos were cultured to recover 20 independent transgenic events and regenerated to recover transgenic first-generation (T0) plants. The first-generation seeds (T1) produced from the T0 plants were transferred to a greenhouse to produce T1 plants that were pollinated using pollen donors from elite high-yielding inbred lines. The T2 seeds were collected and planted in the field trial, generating the grain (T3) used for this study.

**Corn Grain Fractionation and Defatting.** The 44-kDa CIα1-containing corn grain was separated into germ-rich and endosperm-rich fractions using a dry milling procedure established at the Center for Crops Utilization Research (CCUR) at ISU (*40*). The germ-rich fraction was milled into flour using a household coffee mill (GE model 169028) and then defatted. Defatting was carried out by mixing the ground germ-rich corn flour with hexane (1:5 w/v) using a magnetic stir bar for 60 min at 0 °C in an ice bath. The supernatant was decanted after centrifugation (15 min, 2000*g*, 23 °C) and the flour then extracted a second time as above. The residual hexane in the flour was removed by air-drying at room temperature.

**Recombinant 44-kDa CIα1 Recovery.** The defatted germ-rich corn flour was extracted twice by mixing with extraction buffer (0.1 M phosphoric acid, 0.15 M sodium chloride, pH 1.8) in a ratio of 1:5 w/v for 3 h at room temperature. The extracts were clarified by centrifugation (∼3000*g*, 10 min at 23 °C), then pooled and dialyzed (Pierce SnakeSkin T Dialysis Tubing, 3.5K MWCO) overnight at 4 °C against the chromatography buffer (50 mM sodium acetate, pH 4.6), diluted 1:1 with the same buffer and filtered through a 0.45 μm membrane (GV Durapore PVDF, Millipore) before chromatography.

**Recombinant 44-kDa CIα1 Purification.** The dialyzed extract was fractionated [ÄKTAprime plus (GE Healthcare) system controlled by PrimeView software with elution monitored by $A_{280}$] by cation exchange chromatography (CEC) using an XK16/20 column (GE Healthcare) packed with 12 mL of SP Sepharose Fast Flow resin (Sigma) equilibrated with 50 mM sodium acetate (pH 4.6). The elution was carried out over 15 column volumes using a salt concentration gradient from 0 to 0.5 M sodium chloride in 50 mM sodium acetate (pH 4.6) at a 3 mL/min flow rate. Fractions were collected in 8-mL aliquots. The collected fraction with highest 44-kDa CIα1 content as determined by ELISA was concentrated five times using a Centriplus YM3 (Amicon, 3K MWCO) filter before proceeding to the next chromatographic step. The concentrated CEC pool was further purified by gel filtration chromatography (GFC) using an XK 16/40 column (GE Healthcare) packed with 70 mL of Sephacryl S-200HR GFC medium (GE Healthcare). Protein elution into 5-mL fractions was carried out with 50 mM sodium acetate, 0.15 M sodium chloride (pH 4.6) buffer at a flow rate of 0.5 mL/min. The two fractions with highest 44-kDa CIα1 content as determined by ELISA were used for the characterization studies.

**Total Soluble Protein Assay.** Total soluble protein concentration was determined using the Coomassie Plus - The Better Bradford Assay Kit (Pierce). Bovine serum albumin (BSA) was used as standard. Protein concentration was the average of three replicate assays.

**Enzyme-Linked ImmunoSorbent Assay (ELISA).** A competitive ELISA, which was developed and validated by FibroGen, was transferred to ISU to determine the 44-kDa CIα1 concentration. A 96-well plate (Corning Costar High Binding catalog no. 3590) was coated with 2 μg/mL of streptavidin (Zymed 43-4301) in PBS (Mediatech 21-040-CV) and incubated at 4 °C overnight. Biotinylated *Pichia*-derived 44-kDa CIα1 [125 ng/mL in assay buffer (100 mM PBS, 0.05% Tween 20, 0.1% BSA, 0.1% Kathon CG/ICP, pH 7.0] was then immobilized to the plate by incubation with the streptavidin-coated plate at 4 °C overnight to provide the competitive binding against either *Pichia*-derived 44-kDa CIα1 of known concentration (diluted in the assay buffer and used to establish the standard curve) or corn grain-

derived 44-kDa CIα1 for the primary antibody. CIa1 samples were added to the plate followed immediately by the primary antibody, which was a rabbit polyclonal antibody (FibroGen) raised against a *Pichia*-derived 25-kDa CIα1 and used at 1:4000 dilution. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (H+L) (Invitrogen) was used as the secondary antibody at a 1:5000 dilution. Both primary and secondary antibodies were diluted in the assay buffer. A SureBlue TMB substrate solution (Kirkegarrd & Perry Laboratories) was used as the substrate for the HRP conjugate and incubated with it at ambient temperature (23 °C) for 30 min. The plate was then read at 450 nm on a microplate reader (EL340, BioTek Instruments).

**SDS-PAGE, Western Analysis, and Glycosylation Detection.** SDS-PAGE was carried out by following the manual of the Ready Gel Precast Gels Application Guide (Bio-Rad) using a Mini-PROTEAN II Electrophoresis Cell (Bio-Rad) and 4−15% Tris-HCl precast gels (Bio-Rad). The *Pichia* 44-kDa CIα1 was used as reference material.

For Western analysis, the protein bands from the gel were transferred to a poly(vinylidene difluoride) (PVDF) membrane (Invitrogen) after SDS-PAGE. The membrane was incubated with a rabbit polyclonal antibody, followed by incubation with HRP-conjugated goat anti-rabbit IgG (H+L) (Invitrogen) (secondary antibody with 1:5000 dilution) for 1 h at 23 °C. An ECL plus Western blotting detection system (Amersham Biosciences, RPN 2132) was used to visualize the immunoreactive protein bands.

A Gelcode Glycoprotein Staining Kit (Pierce) was used to detect the presence of glycosylation in the purified corn 44-kDa CIα1. After SDS-PAGE, the gel is stained with the kit reagents resulting in only the protein bands containing glycoproteins developing a magenta color. A replicate gel was stained with Coomassie Brilliant Blue to visualize all of the proteins.

**Intact Protein Mass Determination by Mass Spectrometry (MS).** Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) MS was used to measure the mass of the intact protein. The GFC pooled fractions with the highest 44-kDa CIα1 content were further purified to remove impurities by reverse phase high-pressure liquid chromatography (RP-HPLC) (Phenomenex, Jupiter C4, 5 μm, 300 Å, 250 × 4.6 mm) before the MS analysis. Chromatography was performed using Waters (Milford, MA) HPLC system controlled by Empower software. The elution was carried out using a linear gradient of 30−60% acetonitrile in 0.1% trifluoroacetic acid over 30 min at a 1 mL/min flow rate. The isolated 44-kDa CIα1 fraction, matched to the elution time of a calibration injection of *Pichia*-derived 44 kDa CIa1, was freeze-dried, reconstituted with DI water, and then mixed with 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) used as the matrix. The mixture was spotted on a MALDI sample plate and air-dried. The external calibration was performed using BSA. The analysis was done using a Voyager-DE STR MALDI-TOF MS (PerSeptive Biosystems) in the Protein Facility at ISU.

**Internal Peptide Sequencing by Tandem MS.** Two types of proteolytic digestions were performed for individual analysis by MS. Sequencing-grade trypsin (Promega) was added to the corn 44-kDa CIα1 at an enzyme to substrate ratio of 1:20 (w/w) in 50 mM ammonium bicarbonate buffer, pH 7.8, with the digestion carried out for 3 h at 37 °C. Sequencing-grade endoproteinase Glu-C (New England Biolabs) was added to the 44-kDa CIα1 at an enzyme to substrate ratio of 1:20 (w/w) in 50 mM Tris-HCl, 0.5 mM Glu-Glu, pH 8.0 (New England Biolabs), with the digestion carried out for 18 h at 23 °C. Both digests were diluted two times with 50% methanol and 0.5% formic acid, and the resultant solutions were further purified and concentrated using a C18 ZipTip pipet tip (Millipore). About 20 μL of each solution was loaded separately to the ESI-quadrupole-TOF tandem mass spectrometry (Qstar XL, Applied Biosystems). Analysis of the MS and tandem MS (MS/MS) spectral data was done using the Mascot computer algorithm, which allows for peptide amino acid sequence interpretation. A self-defined database with the human CIα1 amino acid sequence containing the expected sequence for the 44-kDa CIα1 fragment was selected and used for the analysis. Both MS spectra collection and amino acid sequence interpretation were carried out in the Proteomics Facility at ISU.

**N-Terminal Sequencing.** The purified corn grain-derived 44-kDa CIα1 was further separated from minor impurities by SDS-PAGE and transferred to a PVDF membrane. The 44-kDa region band was cut out and loaded onto the Procise protein sequencer (Applied Biosystems). Cycles of Edman degradation were conducted followed by injection onto RP-HPLC. The first 20 amino acids on the N terminus were sequenced. The sequencing was performed at the ISU Protein Facility with confirming results provided by FibroGen Inc.

**Amino Acid Composition Analysis.** For the amino acid composition analysis, the PVDF membrane excised 44-kDa CIα1 band from SDS-PAGE was subjected to hydrolysis under vacuum (6 N HCl, 150 °C, 65 min). The resultant amino acids were then derivatized with phenylisothiocyanate (PTC) using a PTC derivatizer (Perkin-Elmer Applied Biosystems model 420A) and analyzed by a PTC Amino Acid Analyzer (Perkin-Elmer Applied Biosystems model 130A). A *Pichia*-derived 44-kDa CIα1 (in liquid solution instead of a PVDF extracted band) was also submitted and analyzed for comparison. *Trans*-4-Hydroxy-L-proline (Sigma H5534) was used as standard for quantifying the hydroxyproline content. The amino acid composition of the 44-kDa CIα1 samples was performed at the ISU Protein Facility.

## RESULTS AND DISCUSSION

**Protein Extraction.** The extraction was designed to recover the corn grain-derived 44-kDa CIα1 with minimal coextraction of the endogenous corn grain components, thus reducing the complexity of the extract for the subsequent chromatographic steps. The total soluble protein (TSP) extracted from 5 g of corn germ-rich flour was 25.5 mg (with total protein concentration ~0.6 mg/mL), representing about 10% of the maximum TSP (taken as that extracted at pH 10) in corn germ due to the low pH of the extraction buffer. This value was similar to that obtained by other researchers using acidic pH for extraction (*33*). About 580 μg of 44-kDa CIα1 was recovered in the extract, corresponding to 120 mg of 44-kDa CIα1/kg of corn germ fraction. The 44-kDa CIα1 comprised 2% of the TSP in this acidic extract. The two-stage extraction and the 3-h extraction time were chosen to obtain a reasonable balance of yield and concentration, and further optimization was not pursued. Analysis of the whole grain before germ fractionation indicated a concentration of 20 mg of 44-kDa CIα1/kg of grain, consistent with values seen in the seed used for planting the field trial. To achieve commercially acceptable levels it will be necessary to increase the CIα1 accumulation level in corn grain at least 50−250-fold to achieve 1−5 g of 44-kDa CIα1/kg of grain.

**Corn-Derived 44-kDa CIα1 Purification.** A summary of the results of the purification process is provided in **Table 1**.

**Figure 1** shows the elution profile of the 44-kDa CIα1 from the first chromatographic step, the cation exchange column. No significant amount of the 44-kDa CIα1 was detected during the sample loading and washing steps, indicating that the 44-kDa CIα1 was effectively captured by the column. The 44-kDa CIα1 was eluted within the first main peak, as indicated by the arrow in **Figure 1**, and collected mainly in three fractions. One of these fractions was an order of magnitude richer in 44-kDa CIα1 than the other two, and it was carried forward for the second chromatographic step. This fraction was 11 times enriched, 23% pure, and contained 73% of the 44-kDa CIα1 loaded to the column (**Table 1**).

**Figure 2** shows the elution profile of the 44-kDa CIα1 from the second chromatographic step (GFC). The 44-kDa CIα1 eluted mainly over two fractions before the elution of the majority of the native corn proteins as shown by the arrow in **Figure 2**. Because of the very low protein concentration (~0.02 μg of CIa1/mL) and the fact that CIa1 lacks amino acid residues with an aromatic ring such as tryptophan or tyrosine that has a strong absorbance in the range of 280 nm wavelength, the 44-kDa CIα1 cannot be resolved as a defined peak. The yield of

**Table 1.** Purification of the Corn Grain-Derived 44-kDa CIα1

| step | vol (mL) | protein (μg) | 44-kDa CIα1 (μg) | purity by ELISA[a] (%) | purity by gel[b] (%) | step purifn factor[c] | yield (%)[d] |
|---|---|---|---|---|---|---|---|
| IEC | | | | | | | |
| load | 67 | 20971 | 433 | 2.06 | 1.2 | 1 | |
| pooled | 8 | 1370 | 316 | 23.10 | 10 | 11.2 | 73 |
| GFC | | | | | | | |
| load | 0.9 | 1119 | 259 | 23.10 | 10 | | |
| pooled | 10 | 76.5 | 213 | 100 | 70 | 4.3 | 82 |

[a] Purity by ELISA was based on the amount of 44-kDa CIα1 as determined by ELISA and total protein as determined by Coomassie Blue protein assay using BSA as a reference standard. This value overestimates purity at higher levels because the total protein contribution of the 44-kDa CIα1 is underestimated in the total protein assay because of its color yield being lower than that of the BSA standard. [b] Purity was estimated by the densitometric analysis of SDS−polyacrylamide gel stained with Coomassie Brilliant Blue, where lower dye-binding by 44-kDa CIα1 results in low estimates of purity. [c] Step purification factor was based on the purity by ELISA values. The overall purification factor is 48.5. [d] Yield was estimated by the ratio of the amount of 44-kDa CIα1 collected in the eluate fractions to the amount loaded to the chromatographic column.

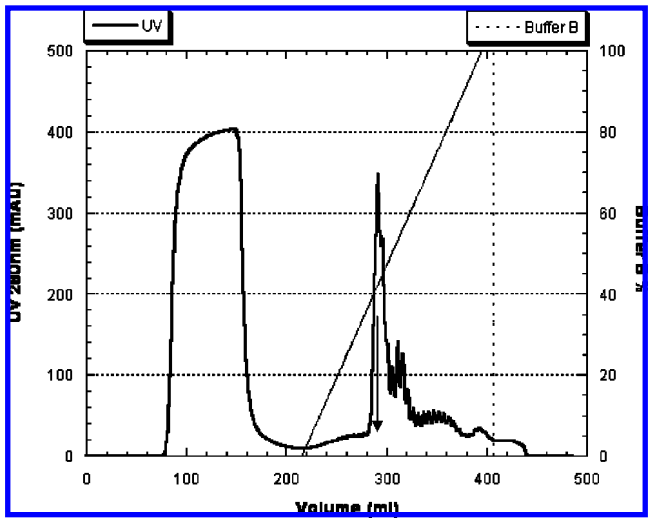


**Figure 1.** Elution profile of corn grain-derived 44-kDa CIα1 from SP-Sepharose Fast Flow cation exchange chromatography. The elution was carried out over 15 column volumes in a linear NaCl gradient from 0 to 0.5 M NaCl in 50 mM sodium acetate (pH 4.6) at 3 mL/min. The arrow shows the region where 44-kDa CIα1 was eluted. Buffer A, 50 mM sodium acetate; buffer B, 0.5 M NaCl + 50 mM sodium acetate.

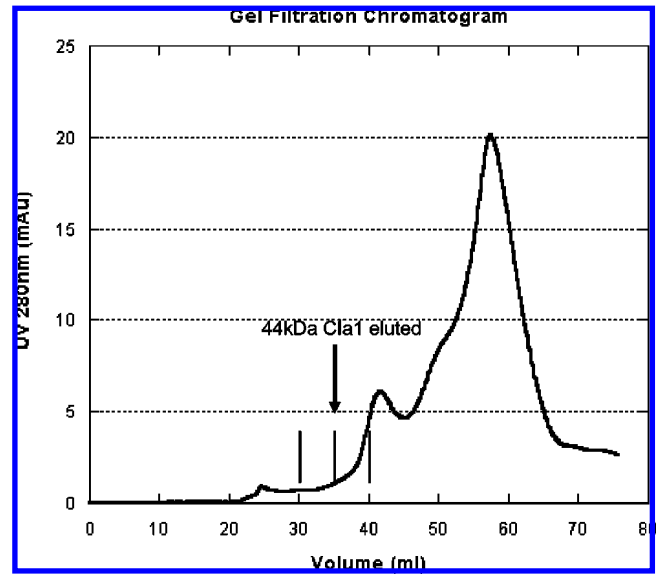


**Figure 2.** Elution profile of corn grain-derived 44-kDa CIα1 from Sephacryl S-200HR gel filtration chromatography. Elution was carried out with 50 mM sodium acetate plus 0.15 M sodium chloride (pH 4.6) at 0.5 mL/min. The arrow indicates the region where 44-kDa CIα1 was eluted. Two 5 mL fractions were collected.

the 44-kDa CIα1 for this purification step was 82%, with a purity of 100% based on the ratio of 44-kDa CIα1 content to the total protein content in the pooled fractions. This purity is based on ELISA for the CIα1 fragment and the total protein assay by using BSA as a standard. An alternative estimate based on densitometry using the SDS-PAGE gels (**Figure 3**, lane 4) yielded a purity value of 70%. The discrepancy between the two purity results could be caused by the low binding of the dye to collagen in both assays.

SDS-PAGE was used to monitor the purification process performance (**Figure 3**). The extract (lane 2) mainly consists of proteins smaller than 25 kDa, assuming that there are not many multimeric proteins. The 44-kDa CIα1 was partially enriched after the cation exchange step (lane 3) and further purified after the GFC step (lane 4). Whereas some native corn proteins with MW similar to the 44-kDa CIα1 remained in the GFC product fraction, most small corn proteins were removed during this step. The *Pichia*-derived 44-kDa CIα1 (first band in lane 5) is shown for comparison and shows that the corn-derived 44-kDa CIα1 had a MW similar to that of its *Pichia* counterpart. The MWs of both corn- and *Pichia*-derived 44-kDa CIα1 estimated from the gel were about 60 kDa, 36% higher than the expected MW of 44 kDa. A similar phenomenon was reported by Werten et al. (*41*) when they analyzed a 36.8 kDa recombinant collagen fragment produced from *P. pastoris*. Their explanation was that the collagen fragment's high hydrophilicity resulted in low binding to the SDS, leading to slower migration.

**Immunorecognition.** Western analysis demonstrated the immunoreactivity of the corn grain-derived 44-kDa CIα1 (**Figure 4**). The *Pichia*-derived 44-kDa CIα1 was also included in this analysis for comparison. The results showed that the corn grain-derived CIα1 had a molecular weight similar to that of its *Pichia* counterpart and that both the corn- and *Pichia*- derived 44-kDa CIα1s were reactive to the same 25-kDa CIα1 specific antibody, confirming the reactivity seen in the ELISA. A faster migrating corn grain-derived 44-kDa CIα1 immunoreactive protein band was observed when the sample loading to the gel was higher (lane 4, **Figure 4**). A similar phenomenon was reported by others producing CIα1 fragments. Werten et al. (*42*) reported that CIα1 fragment produced by *P. pastoris* was susceptible to proteolytic degradation. Other recombinant proteins expressed in corn had also shown susceptibility to proteolytic degradation. A rootworm-control recombinant protein expressed in genetically modified corn plants exhibited a proteolytic fragment with MW slightly smaller than that of the target protein in Western blotting (*43*). Therefore, it is reasonable to suspect that the observed faster migrating protein is a

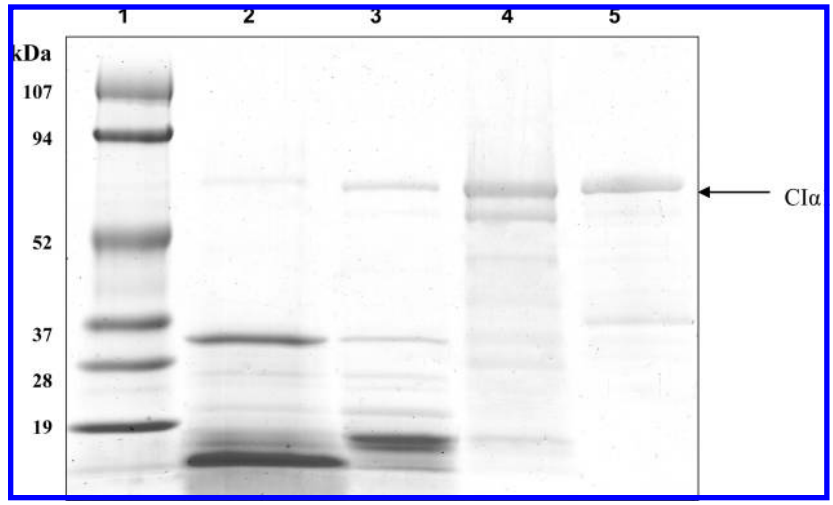


**Figure 3.** Coomassie Brilliant Blue-stained SDS-PAGE: lane 1, protein marker; lane 2, corn grain-derived 44-kDa CIα1 extract; lane 3, IEC fraction; lane 4, GFC pooled fractions; lane 5, *Pichia*-derived 44-kDa CIα1. The arrow indicates the position of the 44-kDa CIα1.

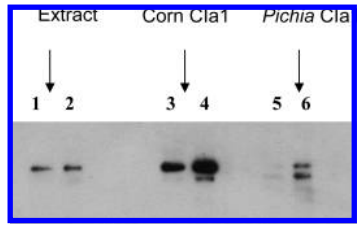


**Figure 4.** Western blotting: lanes 1 and 2, corn grain-derived 44-kDa CIα1 extract; lanes 3 and 4, purified corn grain-derived 44-kDa CIα1; lanes 5 and 6, *Pichia*-derived 44-kDa CIα1 supplied by FibroGen. The paired lanes of the same material differ in sample loading with the first lane having 10 μL applied sampe (~1 μg of total proteins for extract, 0.03 μg for corn CIα1, and 0.02 μg for *Pichia* CIα1) and the second lane having 20 μL of applied sample.

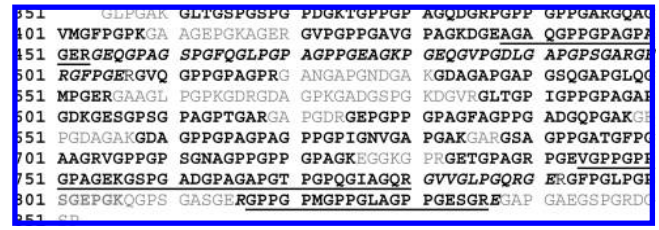


**Figure 5.** Combined sequence coverage of 79% resolved by tandem MS with both trypsin and Glu-C digestion and by N-terminal sequencing. The 44-kDa CIα1 starts at 355G and ends at 852P. Peptides in bold are derived from trypsin digestion (64% coverage), peptides in bold italic are from Glu-C digestion (28% coverage), and peptides in bold and underlined are the overlapped sequences.

proteolytic fragment resulting from the activity of corn grain-derived endogenous proteases during crop growth, grain storage, or protein extraction. The proteolytic fragment was not observable until the protein was purified and enriched at the later stage of the purification process (lane 4, **Figure 3**).

**Intact Protein Mass Determination by MS.** The MW of the intact corn grain-derived 44-kDa CIα1 was measured by MS. The measured intact protein mass of the corn grain-derived 44-kDa CIα1 was estimated from MS to be 44088 Da, which is within 0.2% of the mass (44002 Da) calculated from the amino acid sequence expected from the gene used for transformation. The accuracy for this MS analysis as provided by the manufacturer is ±0.05% for a 16 kDa protein. However, for proteins with larger size, accuracy would be worse. Adams et al. (*44*) reported an average difference of 0.43% between measured intact mass by MS and the expected mass when using the same type of MALDI-TOF to analyze zein proteins (ranging from 10 to 30 kDa) in corn kernel. Therefore, the 0.2% mass difference seen in this work is estimated to be within the accuracy range of the equipment and should not be considered as evidence for protein modification. The intact protein mass of the 44-kDa CIα1 provided reliable evidence that the corn-derived 44-kDa CIα1 was correctly expressed in the corn without significant post-translational modification.

**Peptide Sequencing by Tandem MS.** Tandem MS can be used to verify that a particular protein is being produced with as little as two to three peptides corresponding to 10−15% sequence coverage (*45*) on the basis of the low probability of another protein containing the same combination of peptides. Complete sequence coverage is recommended to identify all possible post-translational modifications on amino acid residues. To yield high overlapping sequence coverage for 44-kDa CIα1, two proteolytic enzymes, trypsin and endoproteinase Glu-C, were used to perform digestions. Trypsin predominantly cleaves proteins at the carboxyl side of lysine and arginine, except when either is followed by proline, whereas Glu-C cleaves peptide bonds C-terminal to glutamic acid residues. Theoretical analysis of these digestions for the expected amino acid sequences with the two enzymes was performed by an online program (Protein Prospector http://pospector.ucsf.edu), predicting complementary coverage for the 44-kDa CIα1. Sequence coverage of the corn grain-derived 44-kDa CIα1 resolved by tandem MS with both trypsin and Glu-C digestion is shown in **Figure 5**. Less sequence coverage was obtained from the Glu-C digest (28%) than from the trypsin digest (64%). Glu-C digestion generated longer peptides because it had fewer cleavage sites on the 44-kDa CIα1 than trypsin. The longer peptides often contained more basic amino acid residues, which could lead to the formation of ions with higher charge state. The highly charged peptides are usually more difficult to fragment further in generating the MS/MS spectrum data (*46*), thus resulting in reduced sequence coverage. The large 54 amino acid tryptic fragment from 454G (glycine) to 507P (proline) in **Figure 5**, which was not resolved by tandem MS, contained only two potential cleavage sites for trypsin. The theoretical digestion of the fragment with Glu-C by the program Protein Prospector yielded 10 short fragments, ranging from 11 to 23 amino acids. This range represents an ideal peptide length for MS fragmentation and analysis (*45*). The actual digestion of the fragment with Glu-C yielded four peptides, from

**Table 2.** Amino Acid Composition of the Corn Grain-Derived 44-kDa CIα1

| amino acid | *Pichia* 44-kDa CIα1 (%) | corn 44-kDa CIα1 (%) | human type I homotrimer[a] (%) |
|---|---|---|---|
| hydroxyproline | 0 | 2.01 | 10.8 |
| proline | 23.71 | 16.35 | 12.4 |
| glycine | 29.56 | 29.39 | 33.3 |
| aspartic acid | 4.91 | 5.99 | 4.2 |
| glutamic acid | 8.45 | 8.07 | 7.3 |
| serine | 3.02 | 4.47 | 3.4 |
| histidine | 0 | 0.53 | 0.3 |
| arginine | 4.84 | 4.53 | 5.0 |
| threonine | 1.56 | 2.00 | 1.6 |
| alanine | 13.38 | 13.08 | 11.5 |
| tyrosine | 0.11 | 0.66 | 0.1 |
| valine | 2.17 | 3.09 | 2.1 |
| methionine | 0.49 | 0.76 | 0.7 |
| isoleucine | 0.70 | 1.35 | 0.6 |
| leucine | 2.20 | 2.71 | 1.9 |
| phenylalanine | 1.32 | 1.45 | 1.2 |
| lysine | 3.61 | 3.57 | 2.6 |

[a] Values based on the literature (*49*).

which a 54-amino acid fragment was totally resolved as shown in bold italics in **Figure 5**.

It should be noted that the MS-MS data could not rule out the possibility of errors in the unmatched sequences. The instrument sensitivity limit and the incomplete MS spectra make it difficult to obtain 100% coverage (*47*). However, the 79% sequence coverage confirmed by tandem MS and N-terminal sequencing and the agreement of intact MW obtained in this work provide a high degree of confidence that the 44-kDa CIα1 produced from the corn was consistent with the gene used for expression with minimal post-translation modification.

**N-Terminal Sequencing.** The first 20 amino acid residues at the N terminus, GLPGAKGLTGSPGSPGPDGK, matched the expected sequence from the cloned gene, confirming the identity of the 44-kDa CIα1 and indicating that the signal sequence was properly cleaved.

**Amino Acid Analysis.** Amino acid analysis (**Table 2**) showed that the corn grain-derived 44-kDa CIα1 was similar to its *Pichia* counterpart for all of the amino acids measured except in the proline and hydroxyproline content. Whereas *Pichia* is not able to hydroxylate prolines (*11*), corn seed seems to be capable of prolyl hydroxylation, although to a limited degree compared with the level present in human collagen. The 2% hydroxyproline content in the corn grain-derived 44-kDa CIα1 suggested the presence of an endogenous prolyl hydroxylase in corn seed but also that mammalian prolyl 4-hydroxylase (P4H) coexpression will be necessary to obtain levels of prolyl hydroxylation in corn seed similar to human collagen I. The accumulation of prolyl hydroxylated CIα1 fragments has been reported earlier in both constitutive, glucose-fed fermentations and methanol-induced yeast *Hansenula polymorpha* fermentations, but only when a complex nutrient source (peptone) was added to the media (*15*). No prolyl hydroxylation was observed when the fermentation was conducted with a medium containing only salts and methanol (*48*). In contrast, when the same fragment was expressed in *Pichia* in the presence of peptone, no prolyl hydroxylation occurred. Hydroxyproline was also absent in all other CIα1 fragments expressed in *Pichia*.

**Glycosylation and Phosphorylation.** The amino acid analysis showed 2% hydroxyproline in the corn-derived 44-kDa CIα1. Hydroxylated proline residues represent potential sites for glycosylation. However, neither corn- nor *Pichia*-derived 44-kDa CIα1s showed any bands (**Figure 6**) indicating glycosylation. This glyscosylation staining method can detect the

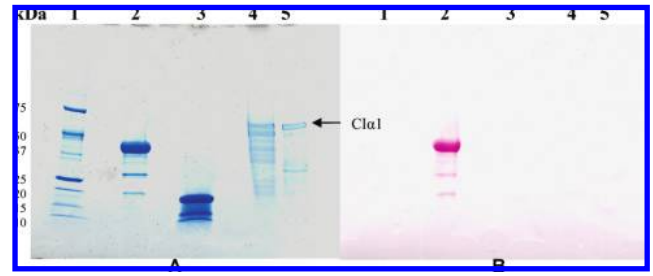


**Figure 6.** SDS-PAGE gels stained by Coomassie Brilliant Blue (**A**) or Pierce GelCode glycoprotein staining kit (**B**): lane 1, MW markers; lane 2, HRP (positive control of glycoprotein, 40 kDa); lane 3, soybean trypsin inhibitor (negative control, 20 kDa); lane 4, corn grain-derived 44-kDa CIα1 (GFC fraction 2); lane 5, *Pichia*-derived 44-kDa CIα1.

presence of carbohydrate groups in as little as 0.625 ng of avidin and 0.16 μg of HRP. The amounts of corn- and *Pichia*-derived 44-kDa CIα1 loaded to the gel were estimated to be about 2.5 μg, which was about 4000 times that of the detectable avidin and 16 times the detectable HRP.

The Mascot computer algorithm used to process the MS spectrum data allowed us to search for any potential phosphorylated amino acid residues. None were identified in the corn-derived 44-kDa CIα1.

**Composite Characterization.** The combined analyses provided reliable evidence that the corn grain-derived 44-kDa CIα1 was faithfully expressed with proper cleavage of the signal sequence, a small degree of proline hydroxylation, and no further detectable post-translational modification. This demonstrates that a mammalian-origin fibrous-type recombinant structural protein with a highly repetitive amino acid composition can accumulate in corn seed with the expected amino acid composition and sequence.

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