

Expression and characterization of a low molecular weight recombinant human gelatin: development of a substitute for animal-derived gelatin with superior features

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Abstract

Gelatin is used as a stabilizer in several vaccines. Allergic reactions to gelatins have been reported, including anaphylaxis. These gelatins are derived from animal tissues and thus represent a potential source of contaminants that cause transmissible spongiform encephalopathies. We have developed a low molecular weight human sequence gelatin that can substitute for the animal sourced materials. A cDNA fragment encoding 101 amino acids of the human $\alpha 1$ (I) chain was amplified, cloned into plasmid pPICZ α , integrated into *Pichia pastoris* strain X-33, and isolates expressing high levels of recombinant gelatin FG-5001 were identified. Purified FG-5001 was able to stabilize a live attenuated viral vaccine as effectively as porcine gelatin. This prototype recombinant gelatin was homogeneous with respect to molecular weight but consisted of several charge isoforms. These isoforms were separated by cation exchange chromatography and found to result from a combination of truncation of the C-terminal arginine and post-translational phosphorylation. Site-directed mutagenesis was used to identify the primary site of phosphorylation as serine residue 546; serine 543 was phosphorylated at a low level. A new construct was designed encoding an engineered gelatin, FG-5009, with point mutations that eliminated the charge heterogeneity. FG-5009 was not recognized by antigelatin IgE antibodies from children with confirmed gelatin allergies, establishing the low allergenic potential of this gelatin. The homogeneity of FG-5009, the ability to produce large quantities in a reproducible manner, and its low allergenic potential make this a superior substitute for the animal gelatin hydrolysates currently used to stabilize many pharmaceuticals.

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Gelatins are widely used in the pharmaceutical industry as stabilizers in vaccines and other biopharmaceuticals. Live attenuated viral vaccines used to immunize against measles, mumps, rubella, varicella, influenza, Japanese encephalitis, as well as rabies,

diphtheria, tetanus toxoid, and pertussis vaccines, all contain gelatin as a stabilizer [1,2]. The gelatins used in these vaccines are hydrolysates, prepared from high molecular weight gelatin by exposure to elevated temperature, treatment with proteases, or other processes to decrease the size of the constituent polypeptide chains [3–5]. Gelatin hydrolysates are heterogeneous mixtures of hundreds of different sized peptides. The heterogeneous nature of these protein mixtures creates

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a significant challenge from the analytical characterization standpoint. Furthermore, the hydrolysates, like all gelatins in use today, are derived from bovine or porcine bones or hides, tissues enriched in type I collagen [3]. Thus, gelatin hydrolysates represent a potential source of contaminants that cause transmissible spongiform encephalopathies [6].

Several cases of allergic reactions to vaccines containing animal-derived gelatin have been reported. Analysis of the vaccine components has identified gelatin as the allergen in these cases [7]. The types of allergic reactions documented range from non-immediate to immediate types reactions, including anaphylaxis [8–10]. The sera from the children with immediate type reactions to gelatin have been analyzed and found to contain antigelatin IgE antibodies [11]. The epitope recognized by these IgE antibodies was shown to reside on the $\alpha 2$ chain of type I collagen [12]. Further detailed analysis of the $\alpha 2$ chain identified the sequence Ile-Pro-Gly-Glu-Phe-Gly-Leu-Pro-Gly-Pro, corresponding to residues 485–494 of the helical domain, as the epitope [13].

The availability of a well-characterized, homogeneous, human sequence gelatin that can be manufactured under Good Manufacturing Practices (GMP) yielding a reproducible product will eliminate many of the challenges associated with the use of the currently available gelatins. The purpose of the work described here was to design and produce a homogeneous, low molecular weight human sequence gelatin that would not form a gel at high protein concentrations or low temperatures and thus would be suitable for use as a stabilizer/excipient in pharmaceutical applications.

To create such an excipient, we expressed various fragments of the human type I collagen $\alpha 1$ chain in the yeast *Pichia pastoris*. This system was chosen since this organism can be fermented to high cell density in completely defined media, is capable of high level recombinant protein expression, and because we have previous experience expressing collagen and gelatin in *Pichia* [14–16]. A purification process was established and a series of analytical assays were developed to characterize the gelatin and establish the high purity of the material. During the course of our studies we discovered this gelatin underwent several post-translational modifications during expression in *P. pastoris* including proteolysis, C-terminal truncation, and phosphorylation. The sites of these modifications were characterized and new constructs were designed to optimize the production of high levels of a homogeneous human sequence gelatin fragment.

We demonstrate that an 8500 Da recombinant human gelatin functioned as a vaccine stabilizer, maintaining the titer of a live attenuated influenza strain as well as a commercially available gelatin hydrolysate, and was not recognized by specific antigelatin IgE antibodies from children with gelatin allergies.

Materials and methods

All restriction enzymes and calf intestinal phosphatase were purchased from New England Biolabs. Plasmid DNA and PCR purification kits were from Qiagen. Source 15S, Q-, and SP-Sepharose resins were from Amersham Pharmacia Biotech. *P. pastoris* strain X-33, plasmid pPICZ α A, pPIC9K, zeocin, yeast nitrogen base without amino acids, biotin, and 10–20% Tricine gels were from Invitrogen Life Sciences. Gelcode Blue stain was from Pierce Chemical. Carboxypeptidase B, LysC, and the Expand High Fidelity PCR kit were purchased from Roche Biochemicals. Oligonucleotides were purchased from Sigma–Genosys. All other chemicals were of the highest quality available.

Cloning and plasmid construction

A cDNA encoding amino acids 531–631 of the human $\alpha 1$ (I) procollagen gene was amplified by PCR using a human skin fibroblast cDNA library (Clontech; Palo Alto, CA) as template. The primers used in the PCR were A1531F and A1631R, these and all other primers were designed based on the published human type I procollagen cDNA sequence [17]. The sequence of the primers used in this study is shown in Table 1. The PCR was performed using the Expand High Fidelity PCR System according to the manufacturer's recommendations. The cycling parameters were 1 cycle of 3 min at 96 °C followed by 30 cycles of 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 3 min. PCR DNA was purified, digested with *Xho*I–*Xba*I, and run on a 1% low melt agarose gel. The 329 bp *Xho*I–*Xba*I digested PCR product was excised from the gel and ligated to *Xho*I–*Xba*I digested plasmid pPICZ α A to create an in-frame fusion to the yeast alpha mating factor prepro sequence for expression and secretion in *P. pastoris*. Transformants were selected on LB plates containing 50 μ g/mL zeocin. Plasmid DNA purified from several transformants were confirmed to have the desired nucleotide sequence by DNA sequence analysis. A plasmid encoding human pro $\alpha 1$ (I) amino acids 531–630 was constructed the same way using primers A1531F and A1630R.

Site-directed mutagenesis

Alanines were substituted at residues 541, 543, 546, and 553 by PCR mediated mutagenesis. The introduction of these mutations was performed using a two-step PCR strategy with primers shown in Table 1. In each mutagenesis reaction, a forward or reverse mutagenic primer was used that flanked the nucleotide(s) to be changed. One set of PCRs employed forward primer A1531F and a reverse mutagenic primers while the second PCR used a forward mutagenic primers and

Table 1
List of primers used for PCR

Primer	Sequence (5'–3')
A1531F	GTATCTCTCGAGAAGAGAGAGGCTGAAGCTGGTCTGCCTGGTGCCAAGGGT
A1631R	TGCTCTAGACTATTATCTCTCGCCAGCGGGACCAGCAGGGCC
A1630R	TGCTCTAGACTATTACTCGCCAGCGGGACCAGCAGGGCC
T541AF	CCTGGTGCCAAGGGTCTGGCTGGAAGCCCTGGCAGCCCT
T541AR	AGGGCTGCCAGGGCTTCCAGCCAGACCCCTTGGCACCAGG
S543AF	GCCAAGGGTCTGACTGGAGCCCCTGGCAGCCCTGGTCCT
S543AR	AGGACCAGGGCTGCCAGGGGCTCCAGTCAGACCCCTTGGC
S546AF	CTGACTGGAAGCCCTGGCGCCCCTGGTCCTGATGGCAAAA
S546AR	TTTGCCATCAGGACCAGGGGCGCCAGGGCTTCCAGTCAG
T553AF	CCTGGTCTGTATGGCAAAGCTGGCCCCCTGGTCCCGCC
T553AR	GGCGGGACCAGGGGGGCCAGCTTTGCCATCAGGACCAGG
S543/546AF	CTGCCTGGTGCCAAGGGTCTGGCTGGAGCCCCTGGCGCCCCTGGTCCTGAT
S543/546AR	GACGGACCACGGTTCCAGACCGACCTCGGGGACCGCGGGGACCAGGACTA
VM579PPF	GGTGCCCGTGGTCAGGCTGGTCCGCCGGGATTCCCTGGACCTAAAGGT
VM579PPR	ACCTTATAGTCCAGGGAATCCCGCGGACCAGCCTGACCACGGGCACC

reverse primer A1630R. These two reactions amplify the 5' and 3' portions of the gelatin cassette, respectively. The products from each of these reactions were purified using a QIAGEN PCR cleanup column, mixed together in equimolar amounts and a second round of PCR was performed with primers A1531F and A1630R, amplifying the entire gelatin cassette containing the desired mutation. This strategy was used to generate each of the four individual mutant DNAs. The PCR product from each of these reactions was purified, digested with *XhoI*–*XbaI*, and cloned into the *XhoI*–*XbaI* sites of plasmid pPICZαA, as described above. The sequence of the PCR product in each plasmid was confirmed by DNA sequence analysis and shown to contain only the desired changes. The plasmid containing all four mutations was created using the plasmid with the mutation at position 553, and two PCRs were performed with primers A1531F and T553AR or T553AF and A1630R. The reaction products were mixed together, and re-amplified with A1531F and A1630R, the DNA was cloned and sequenced. The construct created from these reactions contained alanine substitutions at positions 541 and 553, and was used in a third round of PCR mutagenesis with primers S543/546AF and S443/546AR, and primers A1531F and A1630R as before, to create the four mutation constructs.

The introduction of proline residues at positions 579 and 580 to eliminate a proteolytic cleavage site was done using the same PCR strategy. The plasmid containing the PCR product with the alanines at positions 541, 543, 546, and 553 was used as a template and two reactions were run. One reaction used primers A1531F and VM579PPF while a second reaction used VM579PPR and A1630R. The PCR products were mixed, amplified with primers A1531F and A1630R, cloned, and sequenced. A formula name was assigned to each gelatin and the corresponding sequence is summarized in Table 2.

Table 2
Formula numbers and amino acid sequence of recombinant human gelatins

Formula number	Sequence
FG-5001	α1 (I) 531–631
FG-5002	α1 (I) 531–630
FG-5003	α1 (I) 531–630 T541A
FG-5004	α1 (I) 531–630 S543A
FG-5005	α1 (I) 531–630 S546A
FG-5006	α1 (I) 531–630 T553A
FG-5009	α1 (I) 531–630 T541A, S543A, S546A, T553A, V579P, M580P

Construction of *P. pastoris* strains expressing gelatin

Plasmids were digested with *PmeI* to linearize the DNA for integration into the *P. pastoris* genome at the alcohol oxidase 1 (*AOX1*) loci. The linearized DNA was recovered by ethanol precipitation and resuspended in diH₂O at ~1 μg/mL. Five micrograms of linearized DNA was electroporated into *P. pastoris* strain X-33 using a Bio-Rad Micropulser Electroporator using the fungi setting. Transformants were selected on YPD plates containing 2 mg/mL zeocin to enrich for strains containing multiple copies of the integrated DNA. Strains secreting recombinant human gelatin were identified following growth in shake flasks in buffered minimal methanol medium (BMM, 0.1 M potassium phosphate, pH 6.0, 1.3% yeast nitrogen base without amino acids, 4 × 10^{−5}% biotin, and 0.5% methanol). Gelatin expression was evaluated by SDS–PAGE, using 10–20% Tricine gels, and proteins were visualized by staining with Gelcode Blue.

Fermentation of gelatin strains

One vial of the frozen *P. pastoris* cells (10 OD₆₀₀, 1.8 mL) was used to inoculate a 1 L baffled shake flask

containing 250 mL of BMG medium (0.1 M potassium phosphate, pH 6.0, 1.3% yeast nitrogen base without amino acids, $4 \times 10^{-5}\%$ biotin, and 0.5% glycerol) and grown at 30 °C and 250 rpm. After ~16 h, this culture was used to inoculate a NBS Bioflow 3000 5 L reactor containing 2.5 L of sterile medium consisting of 40 g/L glycerol, 30 mL 85% phosphoric acid/L, 4.23 g potassium hydroxide/L, 14.9 g magnesium sulfate heptahydrate/L, 18.2 g potassium sulfate/L, 0.93 g calcium chloride/L, 2.4 mL PTM1 trace salts/L and 1.1 mL Struktol anti-foam/L. The pH of the media was adjusted to 5.0 with ammonium hydroxide prior to inoculation. The fermentation was run at 30 °C and the dissolved oxygen concentration was maintained at 30%. The pH of the reactor was allowed to decrease to pH 3.0 during the fed-batch phase and was maintained at pH 3.0 by addition of ammonium hydroxide. Glycerol feeding started when the batch phase of growth was completed and was added as a 50% (w/v) solution containing 12 mL/L of PTM1 trace salt solution. Once the biomass in the reactor reached 250 g/L wet cell weight (~24 h) a methanol feed was initiated. Methanol, containing 12 mL PTM1 salts/L, was initially fed at a rate of 2 g/h/L and was gradually ramped up to 6.8 g/h/L. The fermentor was typically fed methanol for 2–3 days. Samples were taken periodically, centrifuged, and wet cell weight was determined and recorded. The run was harvested by centrifugation and the supernatant was stored at –20 °C.

Gelatin purification

Fermentation broth from the 5 L fermenter was clarified by centrifugation at 8000g at 4 °C. The supernatant was dialyzed into 50 mM Tris–HCl, pH 9.0, 50 mM NaCl and any precipitate that formed during dialysis was removed by centrifugation. The dialyzed cell-free broth was fractionated on a Q-Sepharose Fast Flow column (10 mL broth/5 mL resin). The column was equilibrated in 50 mM Tris–HCl, pH 9.0, 50 mM NaCl, and run at a flow rate of 3 mL/min at room temperature. Bound proteins were eluted from the column using same buffer supplemented with 1.0 M NaCl. The column was monitored for absorbance at 215 nm.

Analytical cation exchange chromatography

Fractionation of gelatin charge isoforms was accomplished by cation exchange chromatography using Source 15S resin (Amersham–Biotech) in a XK16 column (30 mL bed volume). The column was equilibrated and run in 40 mM Na acetate, pH 4.5, using an AKTA Explorer 100. Approximately, 10 mg of gelatin was loaded onto the column for analysis. Bound proteins were eluted using a linear gradient from 0 to 0.1 M NaCl over 50 CV. The flow rate was 3 mL/min and the column was monitored at 215 nm.

Reversed-phase HPLC and peptide mapping

Gelatin was fractionated on a Zorbax 300SB C18 column (2 × 150 mm) using a gradient of 2–60% acetonitrile in 0.05% trifluoroacetic acid over 60 min at a flow rate of 0.2 mL/min. The column was maintained at 40 °C and monitored at 215 nm. Gelatin peptides were generated using LysC in 50 mM Tris–HCl, pH 8.7, at an enzyme to substrate ratio of 1:100. The digests were incubated at 30 °C for 5 h. The peptides were resolved on the same reversed-phase column except the gradient was 2–24% acetonitrile.

Mass spectroscopy and protein sequencing

Gelatin or gelatin peptides were desalted on a C18 reversed-phase column, lyophilized, resuspended in dH_2O , and diluted to a final protein concentration of 1 mg/mL in 25% acetonitrile and analyzed on a Thermo-finnigan LCQ electrospray ion trap mass spectrometer by direct infusion. N-terminal sequence analysis was determined by automated Edman degradation using an ABI Procise Model 494 sequencer. C-terminal sequencing was performed at the Karolinska Institute, Stockholm, Sweden.

Host cell protein (HCP) ELISA

A fermentation run was performed with a *P. pastoris* strain that was constructed by transformation of GS115 with a non-recombinant plasmid (pPIC9K, Invitrogen) as described above. Following fermentation, the cell-free broth was collected by centrifugation, concentrated by lyophilization, resuspended, and used to immunize rabbits to generate antisera that recognized the proteins secreted and released into the extracellular media during the fermentation process. Antisera were shown to recognize the vast majority of the proteins present in the cell-free broth based on a comparison of silver stained gels and Western blots performed with the crude rabbit sera on the immunizing antigen preparation. The antibodies were used to develop a sandwich ELISA.

IgE binding assay

An in vitro binding assay was used to evaluate the reactivity of antigelatin IgE antisera with various collagens and gelatin. This assay and the sera have been described previously [7,11].

Virus stabilization assay

Influenza strain A/Sydney CAZ-002 was used for these studies. A high titer stock containing 9.0 log tissue culture infectious dose 50 mL^{-1} ($\text{TCID}_{50}/\text{mL}$) was prepared in chicken eggs and formulated in phosphate-buffered saline,

pH 7.2, supplemented with sucrose, L-arginine, and L-glutamate (SPGAG formulation) at a titer of $7.3 \log \text{TCID}_{50}/\text{mL}$. One set of formulations was supplemented with 1.0% porcine gelatin hydrolysate (Kind and Knox, Sioux City, Iowa) and a second set was supplemented with FG-5001 expressed in *P. pastoris* at 1.0% as well. A third set of samples was not supplemented with gelatin. Aliquots of 100 μL were placed into 0.5 mL microfuge tubes and incubated at 15°C for 15 days. Aliquots of each formulation were assayed for $\text{TCID}_{50}/\text{mL}$ dose using MDCK cells at days 0, 4, 7, 10, and 15.

Results

PCR was used to amplify various regions of the human pro $\alpha 1$ (I) cDNA to construct expression plasmids encoding human collagen fragments, or gelatin, as they will be referred to here. Although the sequence of the $\alpha 1$ (I) chain has the typical Gly–X–Y repeat motif characteristic of collagen [17], our preliminary experiments indicated different regions of the $\alpha 1$ (I) chain were not expressed equally well in *P. pastoris*. This work describes a 101 amino acid region of the human pro $\alpha 1$ (I) chain corresponding to residues 531–631, a portion of the middle third of the helical domain. The 300 bp fragment was amplified with primers that added a *Xho*I site at the 5' end of the PCR product, as well as the amino acids Lys–Arg–Glu–Ala–Glu–Ala, to create an in-frame fusion to the *Saccharomyces cerevisiae* alpha mating factor prepro sequence. The PCR product was cloned into plasmid pPICZ α A, linearized for targeted integration into the *AOX1* loci of *P. pastoris* strain X-33. Transformants were selected on YPD plates containing 2 mg/mL zeocin, to enrich for multi-copy strains, and were initially screened in small scale roller tube cultures in BMM. The cultures were fed fresh methanol every 24 h to a final concentration of 0.5%. Following 72 h of growth the medium was separated from the cells by centrifugation, and analyzed by SDS–PAGE for gelatin expression and secretion.

Several strains were identified that expressed and secreted detectable levels of gelatin encoding amino acids 531–631 of the $\alpha 1$ (I) chain (Supplementary material, Fig. 1). The gelatin expressed by these strains was designated FG-5001. The molecular weight of FG-5001 should be 8748 Da, based on the sequence encoded by the cDNA. The gelatin band migrates just below the 14 kDa globular protein standard. This result was not unexpected since collagenous proteins migrate more slowly in SDS–PAGE than globular proteins of comparable mass due to their high proline content [18]. Expression of one isolate (Supplementary material; Fig. 1, lane 2) was tested in a 5 L fermentor.

The strain was grown in the 5 L fermentor as described under Materials and methods. The pH of the fermentor was maintained at pH 3 to minimize proteolysis of gelatin. Following an initial batch and fed-batch phase the strain was fed methanol to induce gene expression for 3 days. Analysis of the cell-free fermentation broth indicated the strain expressed high levels of gelatin (Supplementary material, Fig. 1).

FG-5001 was purified from the cell-free broth by a two-step process. Following dialysis into 50 mM Tris–HCl, pH 9.0, 50 mM NaCl, the gelatin was fractionated on a Q-Sepharose column. The chromatography conditions used captured the majority of the yeast contaminants while the gelatin was in the non-bound fraction (Supplementary material, Fig. 2). FG-5001 obtained after this single chromatography step was greater than 95% pure as judged by SDS–PAGE; no other contaminating yeast protein were seen by Gelcode Blue staining. To illustrate the unique nature of this recombinant gelatin it was compared to animal sourced gelatin hydrolysates used as stabilizers in commercially available vaccines. FG-5001 migrates as a single band of discrete molecular weight following fractionation by SDS–PAGE while the gelatin hydrolysates contain hundreds of different sized polypeptides and appear as a large smear on the gel (Fig. 1A).

To determine whether this recombinant human gelatin of defined molecular weight exhibits the same biological activity as an animal-derived gelatin hydrolysate, we performed a virus stabilization experiment. A high titer stock of an influenza strain was diluted to $10^{7.3} \text{TCID}_{50}/\text{mL}$ in a sucrose–phosphate based formulation, with or without 1.0% animal gelatin hydrolysate or FG-5001. The virus formulations were incubated at 15°C for 2 weeks and the titer was measured using the TCID_{50} assay with MDCK cells (Fig. 1B). The formulation without gelatin lost over 1 log of titer after 4 days at 15°C and continued to decrease over the course of the experiment. The formulations with FG-5001 or animal gelatin both retained their titer; no significant loss could be measured over the experimental period. These results demonstrate the animal-derived gelatin hydrolysate and FG-5001 performed equally well at stabilizing the virus.

There are several documented cases of immunologic reactions to animal sequence gelatins [7]. We took advantage of the availability of these antigelatin IgE antibodies from the sera of children with gelatin allergies to probe the allergenicity of our recombinant gelatin. Binding assays were carried out with the sera from four different children. In our initial binding experiments, FG-5001 was tested and no reactivity with the sera was observed (data not shown). In later experiments, we tested the reactivity of FG-5009 (an engineered gelatin described below), bovine and human collagen preparations, and purified $\alpha 1$ and $\alpha 2$ chains isolated from recombinant human type I collagen expressed in *P. pastoris* [19] with the antigelatin

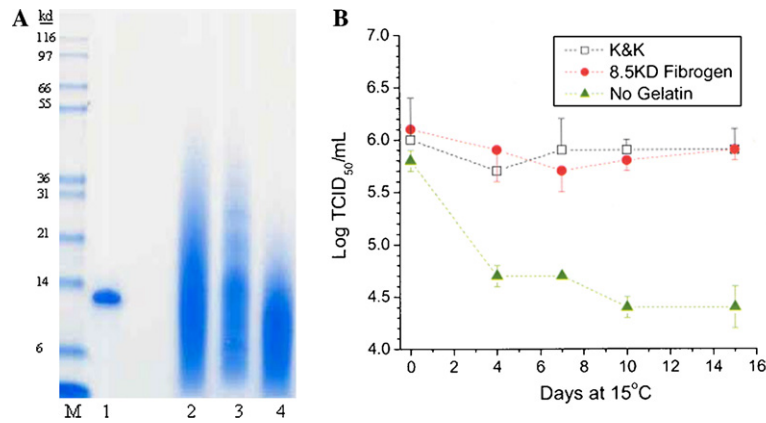


Fig. 1. SDS–PAGE analysis of gelatins and stabilization of a live-attenuated influenza virus. (A) FG-5001 (lane 1) and a porcine gelatin hydrolysate from Kind and Knox, Sioux City, Iowa (lane 2) as well as two different lots of porcine gelatin hydrolysate from Dynagel, Calumet City, Illinois (lanes 3 and 4) were analyzed by SDS–PAGE using a 10–20% Tricine gel. Proteins were visualized by staining with Gelcode Blue. Mark12 molecular weight markers (M). (B) Influenza strain A/Sydney CAZ-002 was diluted to 7.3 Log TCID₅₀/mL in PBS (No gelatin), PBS + 1.0 % animal gelatin hydrolysate (K&K), or PBS + 1.0% FG-5001 (8.5 KD FibroGen) and incubated at 15 °C for 15 days. Titers were measured at the indicated time points using the TCID₅₀ assay.

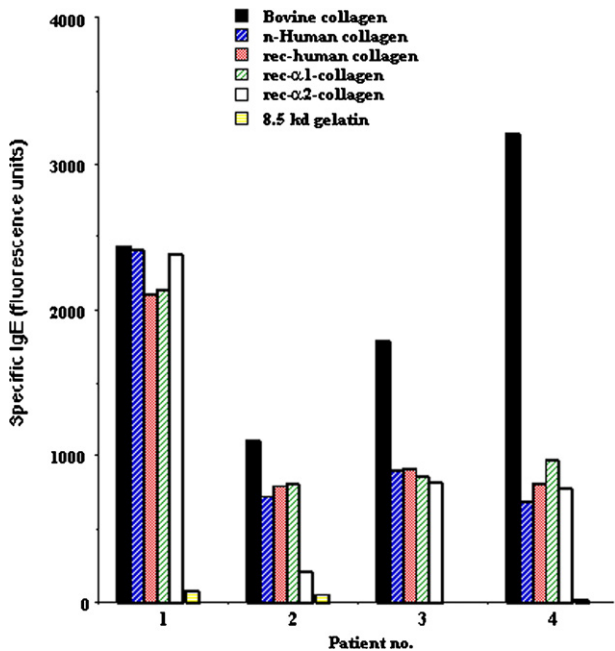


Fig. 2. In vitro binding of antigelatin IgE antibodies to collagen and gelatin. Various collagens and gelatin (1 µg/ml) were coated on microtiter plates at 4 °C for ~18 h. Sera were diluted 1:10, added to the plate, and incubated at 25 °C for 3 h. Bound antibody was measured by the addition of antihuman IgE conjugated to β-D-galactosidase and assaying for β-galactosidase activity as described [11]. *n*-Human collagen-type I collagen from placenta, rec-human collagen-type I collagen produced in *P. pastoris*, rec-α1 collagen-α1 (I) chain isolated from recombinant human type I collagen, rec-α2-collagen-α 2 (I) chain isolated from recombinant human type I collagen, 8.5 kDa gelatin-FG-5009.

sera. All four sera reacted with bovine and human type I collagen (Fig. 2). Sera 3 and 4 appeared to preferentially recognize bovine collagen. Sera 2 reacted more strongly with the α1 chain than the α2 chain of human type I collagen. No detectable binding to FG-5009 was found with

either of the sera. This lack of reactivity with the antigelatin IgEs demonstrated the low allergenic nature of these recombinant gelatins.

FG-5001 in the flow-through fraction of the Q-Sepharose column was homogeneous with respect to molecular weight (Supplementary material, Fig. 2). Based on the amino acid sequence of this gelatin the predicted isoelectric point is 9.4 and thus the protein should bind to a cation exchange column. FG-5001 was fractionated on several different cation exchange resins (data not shown) and a gradient was developed that demonstrated the presence of charge variants. Source 15S resin gave the best resolution, fractionating FG-5001 into five major and three minor charge variants, designated by roman numerals in Fig. 3A. Each of the peaks eluting from the Source 15S column were desalted and analyzed by SDS–PAGE, electrospray mass spectroscopy (ES-MS), and N-terminal sequencing. N-terminal sequence analysis demonstrated each fraction contained the intact N-terminal sequence G-L-P-G-A-K, predicted from the encoded cDNA.

The results of the ES-MS analysis are shown in Table 3. The predicted mass of FG-5001 is 8748 Da, based on the amino acid sequence encoded by the cDNA. The mass of the gelatin in peak VI matched the calculated mass, indicating this fraction contained the unmodified form of the molecule. The gelatin eluting in peak V was 157 mass units smaller than predicted. The C-terminal residue of this gelatin is arginine, removal of this residue would decrease the mass by 157. Loss of an arginine from the protein would make this species less basic causing it to elute earlier than the unmodified gelatin (peak VI) in the NaCl gradient as shown in Fig. 3A. The mass of peak IV was 80 mass units larger than expected. This mass change could be the result of the addition of a single phosphate group. This hypothesis is consistent with the

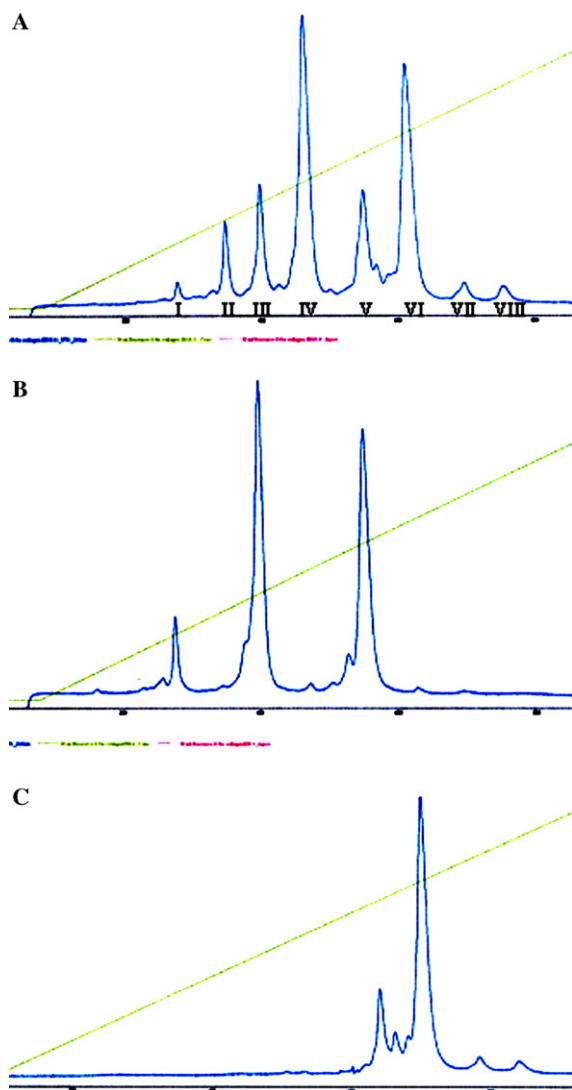


Fig. 3. Analysis of gelatin charge heterogeneity by chromatography on Source 15S before and after treatment with carboxypeptidase B or alkaline phosphatase. FG-5001 was fractionated on a Source 15S column (A) and was resolved into eight peaks (I–VIII), fractions corresponding to each peak were collected, desalted, lyophilized, and resuspended in diH₂O for ES–MS analysis (Table 3) to identify charge isoforms. FG-5001 was also treated with carboxypeptidase B (B) or alkaline phosphatase (C) and fractionated on the same column.

elution of this isoform before the unmodified gelatin (peak VI) due to the additional negative charge from the phosphate group. Peak III was found to have a mass of 8671, 77 mass units lower than predicted. Such a mass could be accounted for by the deletion of the C-terminal arginine and the addition of a single phosphate group. Both of these modifications would increase the negative charge on the gelatin consistent with its earlier elution from the column. The mass of peak II was found to be 160 U larger than predicted and most likely represents a gelatin that has been phosphorylated at two sites. The elution position of this isoform is consistent with this type of modification. We were not able to obtain good

Table 3

Analysis of FG-5001 charge isoforms by mass spectroscopy

Peak #	Mass	Mass Δ	Theoretical modification
I	ND	–	+2 phosphates, deletion of C-terminal Arg
II	8907	+160	+2 phosphates
III	8671	–77	+1 phosphate, deletion of C-terminal Arg
IV	8828	+80	+1 phosphate
V	8591	–157	Deletion of C-terminal Arg
VI	8748	0	No modification
VII	8729	–18	Succinimide intermediate of Asp
VIII	8729	–18	Succinimide intermediate of Asp

ND, mass could not be accurately determined.

ES–MS data from peak I. Peaks VII and VIII both had mass values of –18 relative to the predicted mass. The exact nature of the change leading to this mass reduction is unknown, but it is consistent with the loss of a water molecule during the formation of a succinimide intermediate at aspartic acid residues. Such an intermediate is known to form during the non-enzymatic conversion of aspartic acid to isoaspartate [20]. The gelatin sequence contains three aspartic acid residues each followed by a glycine, a sequence context that favors succinimide formation [21]. Furthermore, succinimides are known to be stabilized at pH 5.0, close to the pH at which the chromatography is performed.

To experimentally demonstrate these predictions were correct, purified FG-5001 was treated with carboxypeptidase B and alkaline phosphatase, and analyzed on the Source 15S column. The chromatogram from the carboxypeptidase B treated gelatin had three peaks (Fig. 3B). The elution position of these peaks corresponded to the elution position of the peaks that were predicted to have the C-terminal arginine removed (peak V) and C-terminal truncation, and phosphorylation at one or two sites (peaks III and I, respectively). The loss of the C-terminal arginine on peaks V and III from the non-treated gelatin was confirmed by C-terminal sequencing of these fractions. The sequence of peak VI (unmodified gelatin) was determined to be G-E-R, while peaks V and III had the sequence G-E.

The chromatogram from the phosphatase treated FG-5001 was also markedly different from the control in that the first four peaks were eliminated (Fig. 3C). The chromatogram contained one major peak and one minor peak, eluting at the same positions as the unmodified gelatin, and the isoform with the C-terminal arginine removed, respectively. This was the expected result since removal of the phosphate groups from peaks I–IV would make them less negatively charged causing them to bind tighter to the column and shifting their elution position later in the gradient.

Protein engineering was performed to create a gelatin that lacked the modified residues leading to the formation of these charge isoforms. First, a new construct was made by PCR that was truncated by one residue at the C-terminus. The C-terminal residue of this new gelatin,

FG-5002, is glutamic acid and should not be subject to truncation. A strain was constructed that expressed this gelatin, and it was fermented and purified by Q-Sepharose chromatography, and analyzed on the Source 15S column. The chromatogram contained three peaks (Fig. 4B). Based on the elution position compared to the original construct (Fig. 4A), the first peak eluting from the column was gelatin containing two phosphorylated residues, the second peak was phosphorylated at one position, and the third peak contained unmodified gelatin.

FG-5002 contains two serine and two threonine residues, potential sites of phosphorylation [22]. No tyrosine residues are present in the sequence. The identification of the phosphorylated residues was accomplished by site-directed mutagenesis of the sequence encoding FG-5002. The mutant constructs were created by PCR, strains were produced as described above, fermented at 5 L scale, gelatin was purified and analyzed on the Source 15S column.

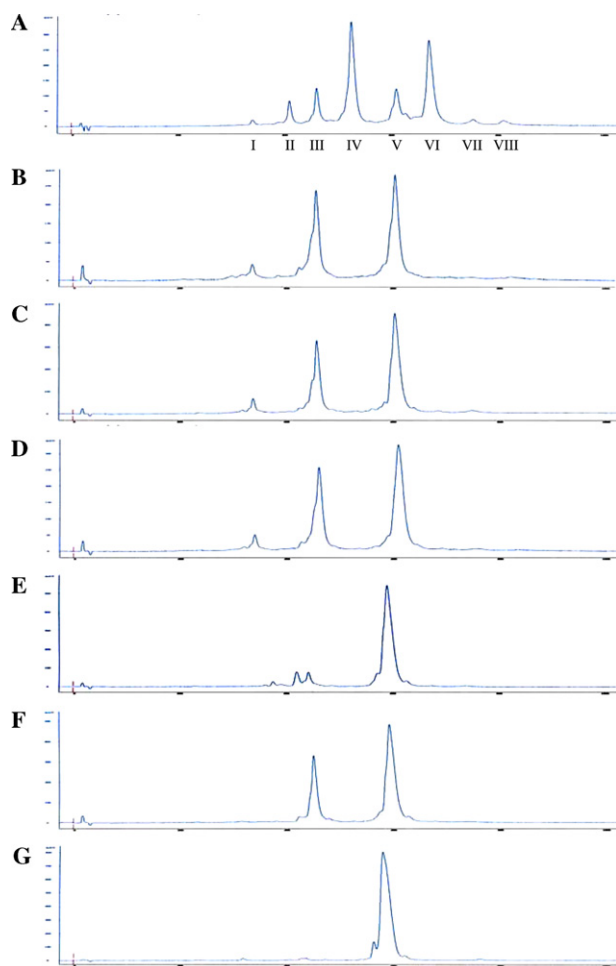


Fig. 4. Analysis of charge heterogeneity of gelatin point mutants by cation exchange chromatography. Ten milligrams of purified FG-5001 (A), FG-5002 (B), FG-5003 (C), FG-5006 (D), FG-5004 (E), FG-5005 (F), and FG-5009 (G) was fractionated on the Source 15S column. The eight charge isoforms present in the wild-type gelatin are indicated below (A) in Roman numerals.

Changing the threonine residues at positions 541 (FG-5003) and 553 (FG-5006) to alanine did not change the pattern of charge heterogeneity relative to the parental construct, suggesting neither of the threonine residues were phosphorylated (Figs. 4C and D). Mutation of serine 543 to alanine (FG-5004) eliminated the first peak eluting from the column, corresponding to the gelatin isoform that had been phosphorylated at two positions (Fig. 4E). Mutation of serine 546 to alanine (FG-5005) resulted in a very significant decrease in the size of the peak corresponding to the gelatin with a single phosphate (Fig. 4F). Additionally, the first peak eluting from the column was eliminated. This elution pattern suggests serine 546 is the primary site of phosphorylation and serine 543 is where a second phosphorylation event occurs in a small fraction of the material. The small amount of gelatin eluting at the position of the single phosphorylated form in panel F corresponds to the fraction of gelatin phosphorylated only at position 543 when position 546 is altered by mutagenesis. This result indicates that elimination of the primary phosphorylation site does not affect the degree to which the secondary site of phosphorylation is utilized, that is, no increase in phosphorylation at position 543 was observed in the S546A mutant. These experiments identified serines at position 546 and 543 as the primary and secondary sites of phosphorylation.

Proteolysis of animal sequence gelatin expressed in *P. pastoris* has been reported and could be minimized by employing low pH fermentation runs [23]. We performed our fermentation runs at pH 3 and found that proteolysis was also minimized, but not completely eliminated (Fig. 5, lane 2). The site of proteolytic cleavage in FG-5001 purified from a pH 3 fermentation was identified by N-terminal sequencing of gelatin bands fractionated by SDS-PAGE after transfer to a PVDF membrane. The upper band corresponded to the N-terminus of the intact gelatin and the lower band (indicated by the arrow) corresponded to the N-terminus of the proteolytic fragment. This analysis identified the site of cleavage between methionine residue 580 and glycine residue 581. N-terminal sequence analysis also demonstrated efficient processing of the secretory leader since no mating factor α prepro sequences were detected at the N-terminus.

To produce a gelatin that would not contain multiple charge isoforms, we used the construct encoding amino acids 531–630 (FG-5002), and changed both serine residues and threonine residues to alanine. In this same construct, we mutated the amino acid sequence at the proteolytic cleavage site. The valine and methionine residues preceding the identified proteolytic cleavage site were changed to proline. These residues were chosen because Gly-Pro-Pro is the most abundant triplet found in type I collagen [17]. All of these changes were introduced by PCR and confirmed by DNA sequence analysis. The resulting plasmid encoding the engineered gelatin, FG-5009, was

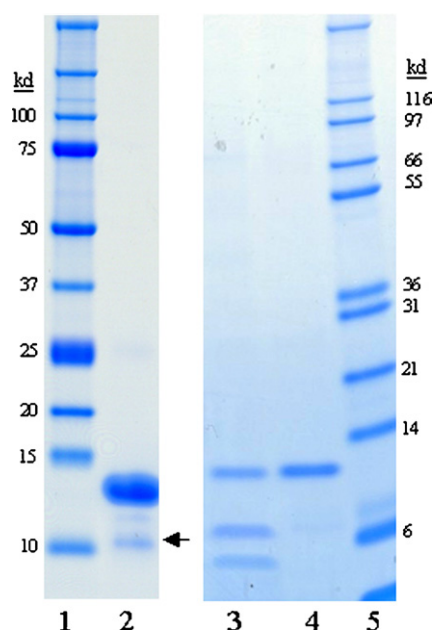


Fig. 5. Identification of protease cleavage site and expression of protease resistant recombinant gelatin. Ten micrograms of purified FG-5001 was fractionated on a 10–20% Tricine gel, and transferred to a PVDF membrane and stained with Coomassie blue R250 (lane 2). Bio-Rad Precision Plus molecular weight markers were run in lane 1. The major component and the band indicated with the arrow were excised from the membrane and sequenced by automated Edman degradation. FG-5001 (lane 3) and FG-5009 (lane 4) were expressed in shake flasks at pH 6.0, and conditioned media was analyzed on a 10–20% Tricine gel. Proteins were visualized by staining with Gelcode Blue. Mark12 molecular weight markers are shown in lane 5.

integrated into *P. pastoris* strain X-33 and gelatin producing strains were identified. The wild-type and engineered strains were grown in expression media buffered at pH 6.0 to determine if the V-M to P-P substitution prevented proteolysis (Fig. 5, lanes 3 and 4, respectively). Analysis of the conditioned media by SDS-PAGE confirmed this amino acid change eliminated proteolytic modification of the expressed gelatin. Similar levels of expression were seen with FG-5009 both in small-scale shake flasks and in the 5 L fermentor, indicating the amino acid substitutions we introduced did not alter productivity.

FG-5009 was purified from cell-free fermentation broth and analyzed on the Source 15S column. One major peak eluted from the Source 15S column indicat-

ing the charge heterogeneity was eliminated as a result of engineering the molecule (Fig. 4G). FG-5009 was analyzed further by reversed-phase HPLC (rp-HPLC), peptide mapping with endopeptidase LysC, and ES-MS.

ES-MS analysis was used to determine the mass of intact FG-5009 as well as the mass of the peptides generated by LysC digestion. FG-5009 had a mass of 8464 ± 1.6 in very close agreement with the theoretical mass (Table 4). Analysis of purified FG-5009 by rp-HPLC demonstrated one major peak with several very minor components eluting both before and after the main component (Fig. 6A). The nature of the minor components was investigated by N-terminal sequence analysis. The components eluting before the main peak were degradation products and their sequence corresponded to internal sequences. The minor components eluting after the main peak had the same N-terminus as the main component. Digestion of purified FG-5009 with LysC followed by rp-HPLC analysis revealed six peaks as expected based on the sequence (Fig. 6B). Each of the peaks was identified by ES-MS. The results of these analyses are shown in Table 4. The mass of each of the peptide matched the expected mass. These analyses indicated FG-5009 was homogeneous and no other modifications occurred during expression.

During the construction of these strains we developed a sensitive ELISA to measure the levels of yeast components in our gelatin preparations and to monitor their removal by various purification steps. High titer antibodies were obtained from rabbits immunized with non-fractionated cell-free fermentation broth from a non-recombinant *P. pastoris* strain and a sandwich ELISA was developed. These antibodies were also tested in Western blots and detected nearly all of the proteins present in the antigen preparation that could be seen by silver staining (data not shown). The assay had a limit of detection of 0.05 ng/mL.

FG-5009 was expressed at 1.47 g/L of cell-free broth using a fed-batch fermentation process utilizing a 120 h methanol feed. The purification and recovery of FG-5009 is summarized in Table 5. The process consisted of a cation exchange chromatography step (IEX I) to capture the product and a chemical extraction step to remove impurities, followed by recovery of FG-5009 by salt precipitation. The precipitate was solubilized, buffered exchanged,

Table 4
Mass spectroscopy analysis of the engineered gelatin and gelatin peptides

Sample	Residues	Calculated mass	Observed mass	Peptide sequence
Intact gelatin	1–98	8464.0	8462.6	—
L1	1–6	541.3	541.2	GLPGAK
L2	7–20	1163.6	1163.4	GLAGAPGAPGPDGK
L3	21–54	3052.5	3052.7	AGPPGPAGQDGRPGPPGPPGARGQAGPPGFPGPK
L4	55–62	685.3	685.2	GAAGEPGK
L5	63–80	1572.8	1572.6	AGERGVPGPPGAVGPAGK
L6	81–98	1533.7	1533.4	DGEAGAQQPPGPAGPAGE

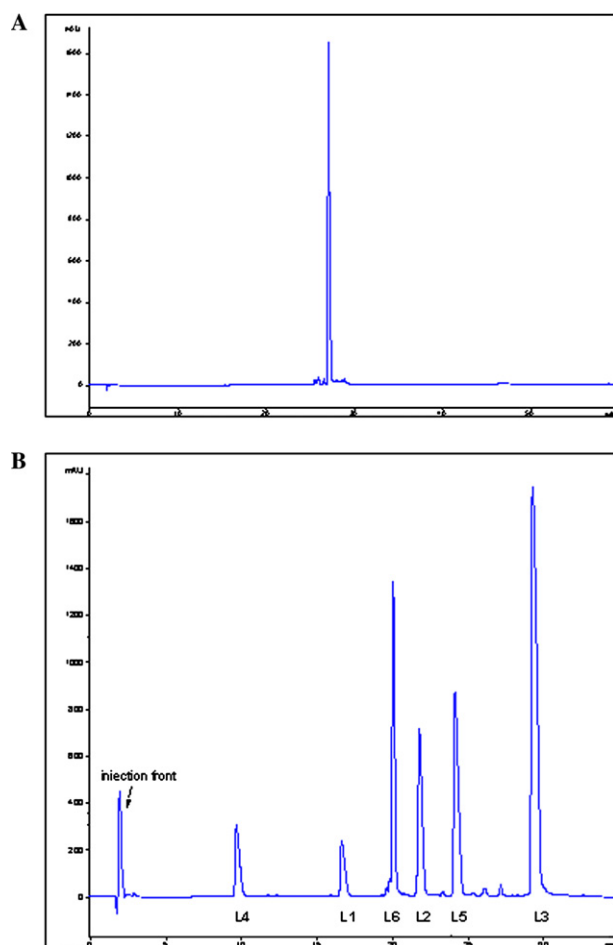


Fig. 6. Analysis of FG-5009 by reversed-phase HPLC. (A). Purified FG-5009 (50 µg) was fractionated by reversed-phase HPLC on a C18 column and bound protein was eluted with an acetonitrile gradient from 2 to 60%. (B) Two hundred micrograms of FG-5009 was digested with LysC at a 1:100 ratio (enzyme:substrate) in 50 mM Tris-HCl, pH 8.7. The peptides were separated by reversed-phase HPLC on a C18 column with a gradient of 2–24% acetonitrile

and fractionated on an anion exchange column (IEX II). FG-5009 from IEX II was again processed by chemical extraction and subjected to a final diafiltration step in to distilled water. FG-5009 obtained with this process contained less than 0.05 ppm HCP. The overall recovery of gelatin through the process was 63%.

Discussion

We expressed a 99 amino acid fragment of the human pro α 1(I) chain in *P. pastoris*. This fragment was produced at high levels and was secreted into the extracellular media. During the purification of FG-5001, we noted that more than one charge isoform was expressed. A cation exchange separation method was developed that fractionated FG-5001 into eight charge isoforms. The modifications that lead to these charge isoforms were elucidated by

Table 5
FG-5009 purification summary

Process step	Process yield (%)	Grams/liter FG-5009
Cell-free fermentation broth	100	1.47
IEX I	98	1.44
Chemical extraction	88	1.30
Salt precipitation	82	1.20
Diafiltration	81	1.18
IEX II	71	1.05
Chemical extraction	69	1.01
Diafiltration	63	0.92

a combination of mass spectroscopy, N-, and C-terminal sequencing, carboxypeptidase B and alkaline phosphatase treatment, and cation exchange chromatography.

About 30% of the expressed protein was truncated at the C-terminus. The C-terminal residue was arginine and was most likely removed by a basic carboxypeptidase. Removal of a C-terminal lysine from endostatin expressed in *P. pastoris* has been reported and shown to result from the action of kex1, a carboxypeptidase specific for basic amino acids [24]. A new construct was made that ended at the glutamic acid residue on the N-terminal side of the arginine to eliminate this post-translational modification.

Approximately 60% of FG-5001 expressed in *Pichia* was phosphorylated. The majority of the phosphorylated species contained one phosphate moiety (~50% of the total), and a minor portion contained two phosphates (~10% of the total). The sites of phosphorylation were identified by site-directed mutagenesis. The primary site of phosphorylation was a serine residue in a Gly-Ser-Pro triplet. The site of addition of the second phosphate was also at a serine residue in a Gly-Ser-Pro triplet. These two triplets are adjacent to each other in the sequence, but for reasons that are not clear, the serine in the second Gly-Ser-Pro triplet is phosphorylated much more extensively than the first serine. During the biosynthesis of procollagen in mammalian cells, several post-translational modifications including hydroxylation of specific proline residues, hydroxylation of specific lysine residues, glycosylation of hydroxylysine, oxidative deamination of lysine, and N-linked glycosylation occurs [25]. Phosphorylation of sequences in the helical domain of collagen α chains has not been found. However, the N-propeptide of the α 1(I) chain of type I procollagen extracted from bone, but not other tissues, is phosphorylated at the only serine present in the sequence [26]. The sequence at which this phosphorylation event takes place does not show any sequence homology, other than a proline residue on the C-terminal side of the phosphorylated serine, with the site of phosphorylation identified here. The structural features or sequences that affect the degree to which the kinase involved in this reaction recognizes its substrate are unclear. Interestingly, when the primary phosphorylation

site was eliminated by site-directed mutagenesis the secondary site was not utilized more extensively, suggesting the kinase involved in this reaction is highly selective.

Review of the literature on recombinant protein expression in *P. pastoris* did not reveal evidence of any other proteins that were phosphorylated to this degree or at this type of sequence. Gelatin expressed in *Pichia* is not believed to have any significant secondary structure [27]. It is possible that the lack of secondary structure of gelatin allows this unidentified kinase to recognize, bind, and phosphorylate this sequence very efficiently. The animal gelatins that were previously expressed in *Pichia* were not analyzed to the same extent as done here and thus it is not possible to determine if they were phosphorylated [23]. However, an engineered gelatin that was designed to be more hydrophilic than native gelatin and expressed in *Pichia* was analyzed by mass spectroscopy; no evidence of phosphorylation was seen as the observed mass was 36,835 and the theoretical value was 36,818 [27]. It is interesting to note that the hydrophilic gelatin expressed in *P. pastoris* contained several Gly-Ser-Pro triplets but no evidence of phosphorylation was found. These published findings and our data suggest that the sequence we found to be phosphorylated is specifically recognized by a yeast protein kinase. Thus, the phosphorylation of the serine residues in the helical domain of collagen seen here is unique not only to the *P. pastoris* expression system but also sequence specific.

Although neither threonine residue appeared to be phosphorylated we changed both residues to alanine since they could be possible sites of O-linked mannosylation. Mannosylation of both serine and threonine residues has been documented in several proteins expressed in *P. pastoris* [28–31]. Our protein engineering strategy focused on alteration of all residues that were potential sites of phosphorylation as well as mannosylation to express a homogeneous gelatin free from unwanted post-translational modifications. To confirm FG-5009 was homogeneous with respect to both molecular weight and charge we characterized it by SDS-PAGE, ion exchange chromatography, rp-HPLC, peptide mapping, N-terminal sequencing, mass spectroscopy and by ELISA to detect host components. FG-5009 migrated as a single molecular weight species on SDS-PAGE and eluted from the Source 15S column as one major species, with a minor component eluting just ahead of the main peak. Analysis of the six LysC-derived peptides using ES-MS and N-terminal sequencing demonstrated the peptides were of the expected masses confirming no modifications of the protein had occurred. These analyses demonstrated FG-5009 was homogeneous with respect to both charge and molecular weight.

The ELISA assay we developed to detect HCP had a limit of detection of 0.05 ng/mL. Using this assay FG-5009 contained less than 0.05 ppm HCP. This result demonstrated our purification process was extremely effec-

tive at the removal of host components and confirms the high level of purity of the material.

To determine whether this gelatin preparation consisting of a single polypeptide species retained the biological activity of a gelatin hydrolysate, a virus stabilization study was performed. Many live-attenuated viruses lose infectivity when they are not stored with a stabilizer [1,32]. Gelatin hydrolysates are commonly used to stabilize vaccine preparations [32–34]. The results of our experiment demonstrated the recombinant gelatin was able to stabilize an influenza virus as well as the hydrolysate, indicating the single polypeptide contained the full biological activity of a gelatin hydrolysate. Several widely prescribed vaccines, such as MMR, varicella, rabies, and DTaP contain gelatin hydrolysates as stabilizers. The recombinant gelatin we have expressed and characterized here offers a well characterized, highly purified substitute for animal-derived gelatins, without sacrificing performance. Additionally, FG-5009 is devoid of tyrosine and tryptophan residues. This feature makes this stabilizer attractive since analytical assay employing absorbance measurements at 280 nm could be performed on formulations containing this protein without any interference. Finally, the binding studies we performed with antigelatin IgE from the sera of children with gelatin allergies demonstrated the low allergenic potential of this human sequence gelatin.

FG-5009 represents a new class of gelatin unlike any of the preparations currently available. FG-5009 can be expressed and manufactured in a GMP environment, and can be characterized as thoroughly as other recombinant proteins that are being marketed as therapeutic agents.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2004.11.016.

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