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Secreted production of a custom-designed, highly hydrophilic gelatin in *Pichia pastoris*

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A custom-designed, highly hydrophilic gelatin was produced in *Pichia pastoris*. Secreted production levels in single-copy transformants were in the range 3–6 g/l of clarified broth and purification to near homogeneity could be accomplished by differential ammonium sulfate precipitation. Despite the fact that gelatins are highly susceptible to proteolysis because of their unfolded structure, the recombinant protein was shown to be fully intact by SDS–PAGE, N-terminal sequencing, gel filtration chromatography and mass spectrometry. Owing to its highly hydrophilic nature, the migration of the synthetic gelatin in SDS–PAGE was severely delayed. Esterification of the carboxylic amino acid side chains resulted in normal migration. The high polarity of the synthetic gelatin also accounts for its negligible surface activity in water at concentrations up to 5% (w/v), as determined by tensiometry. Circular dichroism spectrometry showed that the non-hydroxylated gelatin did not form triple helices at 4°C. The spectrum was even more representative of the random coil conformation than the spectrum of natural non-hydroxylated gelatins.

Keywords: collagen-like protein/hydrophilic gelatin/*Pichia pastoris*/proteolytic stability/synthetic gene

Introduction

Gelatin, in essence denatured and partially degraded collagen, is traditionally prepared by hot acid or alkaline extraction of animal bones and hides. Apart from its main use as a gelling agent in food (Asghar and Henrickson, 1982), gelatin is also used in medical and industrial applications such as intravenous infusions (Saddler and Horsey, 1987), matrix implants (Pollack, 1990), injectable drug delivery microspheres (Rao, 1995) and photographic film (Courts, 1980). Despite the diversity of current uses of natural gelatin, recombinant gelatins may provide benefits for specific applications, in that the chemical composition and molecular weight can be precisely controlled and reproduced (van Heerde *et al.*, 1999; de Wolf *et al.*, 2000). Furthermore, recombinant gelatins do not bear the risk of associated infectious diseases such as bovine spongiform encephalopathy (BSE).

Several reports have described the production of recombinant gelatin-like proteins in *Escherichia coli*. Analogously to the natural amino acid sequence of the collagen triple-helix forming domain, synthetic genes are constructed from repeating (Gly–Xaa–Yaa)$_n$-encoding oligonucleotides, where Xaa and Yaa are often proline (Goldberg *et al.*, 1989; Obrecht *et al.*, 1991; Gardner *et al.*, 1993; Cappello and Ferrari, 1994). Gene instability problems are commonly observed with such highly repetitive genes (Capello and Ferrari, 1994). Also, expression levels usually obtained in *E.coli* are rather low and purification of the intracellularly produced protein can be difficult. Recently, Kajino *et al.* reported the use of *Bacillus brevis* for the expression of gelatin-like proteins (Kajino *et al.*, 2000). They used sequence stretches selected from natural collagen genes and polymerized them to form semi-synthetic gelatin. Prior to their report, we reported the use of the methylotrophic yeast *Pichia pastoris* as a superior host for the secretion of recombinant gelatins having natural amino acid sequences, at up to 14.8 g/l of clarified broth (Werten *et al.*, 1999).

Having established the suitability of *P.pastoris* for the expression of natural recombinant gelatins, we set out to investigate the possibilities of producing entirely custom-designed gelatins having novel physico-chemical properties. A monomeric gene encoding a highly hydrophilic 9 kDa gelatin was designed such as to allow convenient polymerization into larger multimers. The monomeric gene is much longer than the single oligonucleotide monomers used in the expression of synthetic gelatins in *E.coli*, mentioned above. This offers more flexibility in the design of the amino acid sequence and a concomitant decrease in the overall repetitiveness of the gene. Here, we describe the high-level secretion of a fully synthetic, highly hydrophilic and non-degraded 36.8 kDa gelatin by *P.pastoris* and its characterization.

Materials and methods

Vector construction

The monomeric gelatin gene (referred to hereafter as 'P' for 'polar') was constructed by overlap extension polymerase chain reaction (PCR) (Ho *et al.*, 1989) of long oligonucleotides (underlined in Figure 1A). PCR was performed with a Perkin-Elmer GeneAmp 9700, using the proofreading enzyme *Pwo* DNA polymerase (Eurogentec). The 5′ half of the gene was constructed by overlap extension of the first and second oligonucleotides and co-amplified by outer primers directed against nucleotides 1–26 (sense) and 197–174 (antisense). Likewise, the 3′ half of the gene was constructed by overlap extension of the third and fourth oligonucleotides and co-amplified by primers directed against nucleotides 174–197 (sense) and 363–337 (antisense). The resulting PCR products were isolated from an agarose gel and were combined by another overlap extension PCR and co-amplified with the primers directed against nucleotides 1–26 (sense) and 363–337 (antisense). The resulting 0.3 kb PCR fragment was digested with *Xho*I/*Eco*RI and cloned in vector pMTL23 (Chambers *et al.*, 1988) to form vector pMTL23P. The sequence of the gene was verified by automated DNA sequencing of both strands.

The monomeric gene was released by digesting pMTL23P

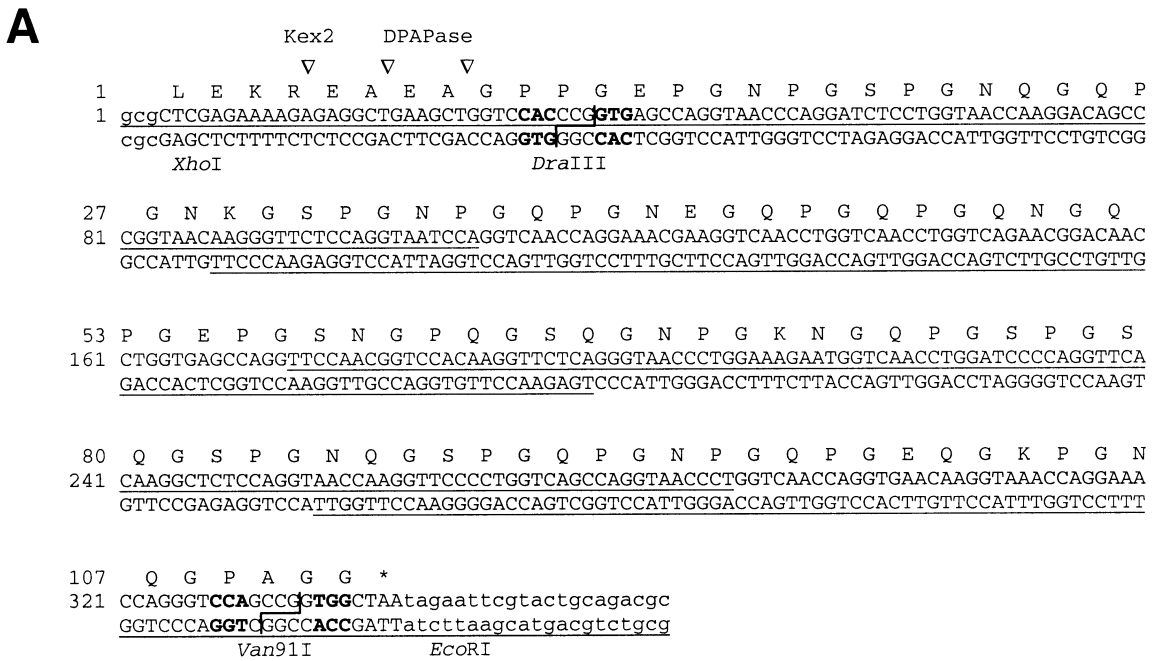

Fig. 1. Construction of the synthetic gelatin gene. (**A**) Overlapping oligonucleotides and encoded P monomer. Letters in bold type indicate the non-ambiguous nucleotides recognized by *Dra*III and *Van*91I. (**B**) Multimerization of P monomer to P4. Note that *Van*91I/*Dra*III hybrid sites are not recleavable.

with *Dra*III/*Van*91I (Figure 1B). In a separate reaction the vector was linearized with *Van*91I and dephosphorylated. The *Dra*III/*Van*91I fragment was then inserted into this linearized vector to yield vector pMTL23P2. This process of insertional doubling can in principle be repeated to form multimers of any desired length, but was repeated only once here to form the vector pMTL23P4. The tetrameric gene (referred to hereafter as 'P4') was then cloned into the *Xho*I/*Eco*RI sites

of vector pPIC9 (Invitrogen) that contains a *HIS4* selectable marker, an alcohol oxidase 1 (*AOX1*) promoter/terminator cassette and a *Saccharomyces cerevisiae* α-factor prepro secretory signal (Clare *et al.*, 1991a). The Kex2 and dipeptidylaminopeptidase (DPAPase) cleavage sites of the α-factor prepro sequence are lost from pPIC9 when using the *Xho*I site, but are restored upon ligation of the gelatin gene by the sequence between *Xho*I and *Dra*III (Figure 1A).

Transformation of P.pastoris

Plasmid pPIC9P4 was linearized with *Sal*I in order to obtain preferentially Mut^+ transformants [i.e. by integration at the *his4* locus rather than the *AOX1* locus and thus allowing normal growth on methanol (Clare *et al.*, 1991b)]. Transformation of *P.pastoris* strain GS115 [*his4* (Cregg *et al.*, 1985)] by electroporation and selection of Mut^+ transformants was as described previously (Werten *et al.*, 1999).

Fermentative production of synthetic gelatin in P.pastoris

Fermentations were performed in 1–140 l fermenters (Applikon) in minimal basal salt medium (Invitrogen) supplemented with 0.2% (v/v) PTM_1 trace salts (Invitrogen). Methanol fed-batch fermentations were performed as described previously (Werten *et al.*, 1999), with the exception that no protease-inhibiting supplements such as casamino acids were added and that the pH during methanol fed-batch was maintained at 3.0 for all fermentations.

Small-scale purification of synthetic gelatin by differential acetone precipitation

Differential acetone precipitation was as described previously (Werten *et al.*, 1999). Chilled acetone was added to fermentation supernatant at 40% (v/v), after which endogenous proteins were pelleted by centrifugation. The acetone concentration in the supernatant was then increased to 80% (v/v) and the pellet obtained after centrifugation was washed with 80% acetone and air-dried.

Preparative purification of synthetic gelatin by differential ammonium sulfate precipitation

Preparative purification of synthetic gelatin from fermentation supernatant consisted of twice-repeated ammonium sulfate precipitation at 40% saturation (4°C) and subsequent washing of the precipitate with 60% saturated ammonium sulfate. Depending on the scale of the purification, separation of the precipitate from the liquid was either by centrifugation or by depth filtration using AKS-4 sheets (USF Seitz-Schenk). The protein was subsequently desalted by diafiltration and lyophilized.

Bicinchoninic acid protein assay

A commercially available bicinchoninic acid (BCA) protein assay was used according to the manufacturer's recommendations (Pierce). The reaction was performed at 60°C for 30 min. The calibration curve was prepared gravimetrically from lyophilized, desalted P4 gelatin, purified by differential ammonium sulfate precipitation (purity at least 98%).

SDS–PAGE

SDS–PAGE (Laemmli, 1970) was performed in a Mini-PROTEAN II system (Bio-Rad) under reducing denaturing conditions. Gels consisted of a 5% stacking and a 12.5% separating zone (2.7% cross-linking). Gels were stained using Coomassie PhastGel Blue R-350 (Amersham-Pharmacia Biotech) and were destained by heating in water using a microwave oven, similarly to Faguy *et al.* (Faguy *et al.*, 1996).

Gel filtration chromatography

Protein in 0.1 M sodium chloride was loaded on a 10×250 mm column packed with Superose 12 (Amersham-Pharmacia Biotech). Elution was carried out with 0.1 M sodium chloride at a flow-rate of 0.2 ml/min, collecting 2 ml fractions and monitoring the absorbance at 214 nm.

Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed at the Department of Biochemistry, Wageningen University, The Netherlands, using a Voyager DE-RP delayed extraction mass spectrometer (PerSeptive Biosystems). Samples were prepared by the dried droplet method, using sinapinic acid dissolved in 30% (v/v) acetonitrile, 4% (v/v) trifluoroacetic acid as matrix. Measurements were made in the positive, linear mode and the accelerating voltage was 25 000 V. Cytochrome *c* and bovine serum albumin were used as external calibrants.

Chemical modification of gelatins

Esterification of carboxylic amino acid side chains was adapted from Wilcox (Wilcox, 1967). A 100 µg amount of protein was incubated in 500 µl of methanol, 0.1 M hydrochloric acid at 4°C for 72 h. The methanol was exchanged for 1 mM hydrochloric acid by diafiltration in a 3 kDa Microcon (Millipore). Removal of ester groups was performed by incubating the esterified protein in 100 mM Tris–HCl, pH 8.8 at 20°C for 72 h.

Hydrazination of carboxylic amino acid side chains was performed according to Matagne *et al.* (Matagne *et al.*, 1991). A 50 µg amount of protein was dissolved in 20 µl of 50 mM sodium phosphate buffer, pH 7. After addition of 170 µl of 8 M urea, 1 M hydrazine and 0.1 M 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide, pH 4.5, the mixture was incubated at room temperature for 2 h.

Surface tension measurements

Surface tension at the liquid–air interface was measured according to the du Noüy ring method (Lecomte du Noüy, 1919) using a Krüss K6 tensiometer. The temperature of the sample vessel was maintained at 20°C. The actual measurements were performed 5 min after lowering of the ring on to the liquid surface, as suggested by Clarkson *et al.* (Clarkson *et al.*, 1999). Bovine serum albumin was used as a reference protein and raw data were corrected for the hydrostatic volume effect according to Harkins and Jordan (Harkins and Jordan, 1930).

Circular dichroism spectrometry

Proteins were dissolved in Milli-Q water at 0.1 mg/ml. Measurements were performed at the Department of Biochemistry, Wageningen University, The Netherlands using a Jasco J-715 spectropolarimeter. The pathlength was 0.1 cm and spectra were recorded from 190 to 260 nm at 4°C, using a scanning speed of 20 nm/min at a resolution of 0.1 nm.

Results

Design and construction of the synthetic P4 gelatin gene

The basic structure of natural gelatins consists of repeating Gly–Xaa–Yaa triplets, where Xaa and Yaa are often proline and hydroxyproline, respectively (the latter being post-translationally modified proline). This structure maintains the open, unfolded conformation characteristic of gelatin. Owing to this unfolded conformation, gelatin is fairly hydrophilic

Table I. Composition and basic physico-chemical parameters of synthetic and natural gelatins[a]

Amino acid	P4	Col3a1[b]	Cattle bone gelatin[c]
Ala	1.0	7.9	12.0
Arg	0.0	4.8	5.0
Asn	12.0	3.5	–
Asp	0.0	3.1	–
Asx	–	–	4.3[d]
Cys	0.0	0.0	0.0
Gln	16.0	3.5	–
Glu	4.0	3.5	–
Glx	–	–	6.7[d]
Gly	33.7	33.8	33.4
His	0.0	1.8	0.5
Ile	0.0	1.3	1.2
Leu	0.0	1.8	2.4
Lys	3.0	3.5	2.6[e]
Met	0.0	0.4	0.7
Phe	0.0	0.4	1.3
Pro	22.4	21.1	22.2[e]
Ser	8.0	6.1	3.4
Thr	0.0	1.8	1.6
Trp	0.0	0.0	0.0
Tyr	0.0	0.4	0.2
Val	0.0	1.3	2.4
GRAVY	–1.77	–1.08	–0.75 to –1.09[e]
Isoelectric point	4.9	9.7	4.7 to 5.4[f]
Molecular weight (kDa)	36.8	20.6	65 to 300[f]

[a]Theoretical amino acid composition (mol%), GRAVY values, isoelectric point and molecular weight were calculated using the ProtParam tool available at the Expasy WWW server (Appel *et al.*, 1994). Experimental values were obtained as indicated below.
[b]Natural non-hydroxylated recombinant gelatin produced in *P.pastoris* (Werten *et al.*, 1999).
[c]The amino acid composition (mol%) of limed cattle bone gelatin is the mean of four measurements.
[d]Asn/Asp and Gln/Glu cannot be distinguished by amino acid analysis and are given as Asx and Glx, respectively. This does not affect the GRAVY calculation.
[e]Values for Lys and Pro include hydroxylysine and hydroxyproline, respectively. The hydropathy indices of these modified amino acids were assumed to lie between those of the respective unmodified amino acids and the lowest value of the Kyte and Doolittle hydropathy scale. The range of GRAVY values indicated for cattle bone gelatin represents both extremes.
[f]Natural cattle bone gelatin is a heterogeneous mixture of molecules of different molecular weights and isoelectric points within the indicated ranges (Alleavitch *et al.*, 1988).

because its hydrogen bonds are highly exposed. Furthermore, only a small fraction of the protein is occupied by hydrophobic amino acids such as Trp, Tyr, Phe, Leu, Ile, Val and Met.

Our synthetic P4 gelatin design also provides the (Gly–Xaa–Yaa)$_n$ structure. To increase its hydrophilicity relative to that of natural gelatins, we designed a gelatin without any hydrophobic amino acids other than proline and with a high content of the hydrophilic amino acids asparagine and glutamine (Table I). To illustrate the high hydrophilicity of this synthetic gelatin compared with natural gelatins, the GRAVY values [grand average of hydropathy (Kyte and Doolittle, 1982)] of P4, natural recombinant Col3a1 gelatin (Werten *et al.*, 1999) and cattle bone gelatin are indicated in Table I.

The content of acidic and basic residues was modulated to give an isoelectric point similar to that of common limed bone gelatin (Table I). Only Lys was used as a basic residue, because we anticipated proteolysis of mono-arginylic sites based on previous work (Werten *et al.*, 1999).

The P4 gene was constructed from four identical P monomers

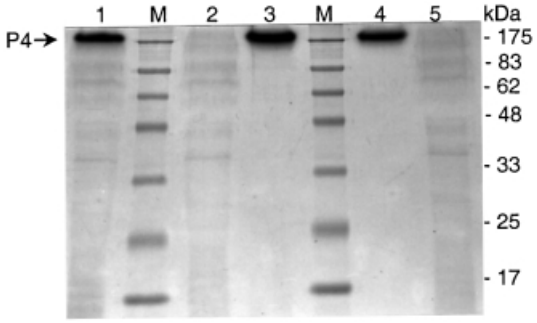

Fig. 2. Purification of synthetic gelatin. Lane 1, fermentation supernatant; lane 2, precipitate obtained at 40% (v/v) acetone; lane 3, precipitate obtained by increasing the acetone concentration of the 40% (v/v) supernatant to 80% (v/v); lane 4, precipitate obtained at 40% of ammonium sulfate saturation; lane 5, supernatant obtained at 40% of ammonium sulfate saturation; lane M, molecular weight marker.

that were designed to have the codon usage of *P.pastoris* highly expressed genes (Sreekrishna and Kropp, 1996). The monomeric gene contains restriction sites for *Dra*III and *Van*91I. These enzymes allow the design of mutually complementary, non-palindromic overhangs that enable convenient elongation of the gene by insertional doubling in a fixed orientation (Figure 1). The process can be repeated until the desired polymer length has been achieved. This modular design offers flexibility in the construction of future gelatins by allowing the combination of different types and lengths of polymerized gelatins via the *Dra*III/*Van*91I sites. The *Xho*I and *Eco*RI sites provided by the sequence allow the direct insertion of the final synthetic gene into *P.pastoris* expression vector pPIC9, resulting in a fusion to the alpha-mating factor prepro secretion signal. Thus, the combined four P modules were cloned into pPIC9 to yield vector pPIC9P4.

Production of synthetic P4 gelatin

Plasmid pPIC9P4 was used to transform *P.pastoris* GS115. Randomly chosen transformants were fermented and culture supernatants harvested throughout the fermentation were subjected to SDS–PAGE. The theoretical molecular weight of P4 is 36.8 kDa. Because collagenous proteins migrate in SDS–PAGE at an apparent molecular weight ~40% higher than the true molecular weight (Butkowski *et al.*, 1982; Werten *et al.*, 1999), one would expect a band of ~52 kDa. Instead, however, the Coomassie Blue-stained SDS–PAGE gel showed a faint blurry band at the top of the separating gel that had a tendency to diffuse from the gel during methanol–acetic acid destaining. Migration of the gelatin into the gel was improved by running the gel at 4°C at twice the voltage recommended by the manufacturer of the electrophoresis system (i.e. 400 instead of 200 V). Diffusion of the protein from the gel during destaining was reduced by destaining the gel in water heated in a microwave oven [similarly to Faguy *et al.* (Faguy *et al.*, 1996)], rather than performing the common lengthy incubations in methanol–acetic acid. Figure 2, lane 1 shows fermentation supernatant analyzed in this manner. N-Terminal protein sequencing of this band (Sequencing Centre Utrecht, The Netherlands) revealed the expected amino acid sequence (GPPGEPGNPG). There was no indication of incomplete processing of the α-factor derived Glu–Ala repeats by dipeptidylaminopeptidase, such as is occasionally observed when using this prepro sequence for secretion of heterologous proteins (Vedvick *et al.*, 1991; Briand *et al.*, 1999; Werten *et al.*, 1999; Goda *et al.*, 2000).

Recombinant gelatins with natural amino acid sequences can be purified from the fermentation broth by using differential acetone precipitation (Werten *et al.*, 1999). Endogenous extracellular proteins are precipitated at 40% (v/v) acetone, after which the gelatin is precipitated from the supernatant by addition of acetone to 80% (v/v). Figure 2, lanes 2 and 3 show that acetone precipitation was equally effective for the purification of synthetic gelatins. Amino acid analysis was performed (in triplicate) to estimate the purity of the precipitated gelatin. By linear least-squares fitting of the observed data in terms of the P4 amino acid composition, the average relative contributions (vector moduli) of the P4 component and the residuals component were found to be 96.2 and 3.8%, respectively (±1.4% SD). The purity of the preparation at the protein level is thus estimated to be >96%, as stochastic fluctuations of measured values contribute to the residuals component. BCA protein analysis of acetone precipitates of different fermentations showed gelatin yields in the range 3–6 g/l of clarified broth.

We previously found that recombinant gelatins with natural amino acid sequences can also be purified from *P.pastoris* fermentations using differential ammonium sulfate precipitation (unpublished data). Gelatinous proteins precipitate at 40% saturation, whereas endogenous extracellular *P.pastoris* proteins surprisingly do not precipitate at up to 80% saturation. In agreement with this, readily precipitable proteins such as β-lactoglobulin are rendered virtually unprecipitable upon mixing them with fermentation supernatant. We investigated whether it was possible to purify P4 gelatin by differential ammonium sulfate precipitation. Indeed, Figure 2, lanes 4 and 5 show that synthetic gelatin is quantitatively precipitated at 40% ammonium sulfate saturation, while no endogenous proteins are visible. Based on amino acid analysis (in triplicate) and subsequent linear least-squares fitting of the observed data, the purity at the protein level was estimated to be >98.1% (±0.7% SD). Two-dimensional electrophoresis followed by silver staining showed virtually no contaminants (not shown). Gelatin yields determined by BCA analysis of ammonium sulfate precipitates of several fermentations [possible only after thorough desalting because ammonium sulfate reduces BCA reactivity (Smith *et al.*, 1985)] were within 0.3 g/l of the values determined by acetone precipitation. This small difference (<10%) is largely due to interference of the BCA assay by the small amount of reducing exopolysaccharides that co-purifies in the acetone precipitation procedure, while exopolysaccharides are virtually eliminated when using ammonium sulfate precipitation (data not shown). Compared with differential acetone precipitation, the overall higher purity obtained and the higher amenability to scale-up render differential ammonium sulfate precipitation the method of choice for preparative purification of P4 gelatin.

Establishing the molecular weight of synthetic P4 gelatin

A possible explanation for the aberrant molecular weight observed in SDS–PAGE could be that P4 is glycosylated. N-Linked glycosylation can be ruled out because no susceptible sites are present in the amino acid sequence. However, *P.pastoris* is also able to perform *O*-glycosylation and the structural determinants for such an event are unclear (Duman *et al.*, 1998). To rule out this possibility, periodic acid–Schiff staining (Zacharius *et al.*, 1969) and Alcian Blue staining (Wardi and Michos, 1972) were performed on ammonium sulfate-purified P4. No glycosylation was observed (not shown).

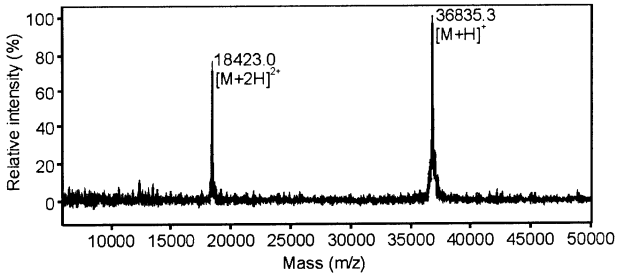

Fig. 3. MALDI-TOF mass spectrum of synthetic gelatin purified by differential ammonium sulfate precipitation. The $[M + H]^+$ and $[M + 2H]^{2+}$ peaks correspond to singly and doubly charged molecular ions, respectively.

To determine whether the synthetic gelatins have in fact the correct molecular weight, but merely exhibit aberrant behavior in SDS–PAGE, analytical gel filtration chromatography was performed. The Superose 12 column was calibrated with a mixture of natural recombinant gelatin fragments (Werten *et al.*, 1999), giving a series of molecular weights of 53, 42, 28, 16, 12 and 8 kDa. Ammonium sulfate-purified P4 was subjected to gel filtration chromatography. Only one significant peak was observed. N-Terminal protein sequencing of this fraction in solution (Sequencing Centre Utrecht) showed the correct N-terminus for P4. The molecular weight of P4 deduced from the chromatogram was 47 kDa. This is clearly much closer to the theoretical value of 36 kDa than the molecular weight apparent from SDS–PAGE, although the deviation is still significant.

Mass spectrometry was used to determine ultimately the molecular weight of P4. Materials purified by both ammonium sulfate precipitation and gel filtration chromatography were analyzed and the results were in good mutual agreement. Figure 3 shows the MALDI-TOF mass spectrum of P4 purified by ammonium sulfate precipitation. The observed molecular weight of 36 835 Da corresponds well with the theoretical value of 36 818 Da. This result shows that the apparent high molecular weight observed in SDS–PAGE is indeed the result of aberrant migration behavior. Furthermore, the SDS–PAGE and gel filtration chromatography results are confirmed, in that there is no presence of proteolytically degraded fragments.

We speculated that the aberrant migration rate of synthetic gelatin compared with normal gelatin was due to low binding of SDS in view of its high hydrophilicity, as the interaction of SDS with proteins is mainly of a hydrophobic nature (Reynolds and Tanford, 1970). Esterification of the carboxylic amino acid side chains with methanol–hydrochloric acid would increase the protein's hydrophobicity and thus its SDS binding capacity and migration rate. While the migration of natural gelatins treated in this way was only slightly affected (i.e. a decrease in apparent molecular weight of about 2 kDa), the esterified synthetic gelatin migrated much faster than the unmodified protein (Figure 4). The molecular weight observed for esterified P4 was about 50 kDa. As natural gelatins migrate about 40% more slowly than common proteins (Butkowski *et al.*, 1982; Werten *et al.*, 1999), this value is in good agreement with the value expected for natural gelatins of the same molecular weight as P4 (i.e. a 36.8 kDa natural gelatin would migrate at ~52 kDa). Removal of the ester groups at high pH restored the aberrant migration of P4 (Figure 4). Hydrazination of the carboxylic amino acid side chains of P4 did not result in an altered migration rate (not shown),

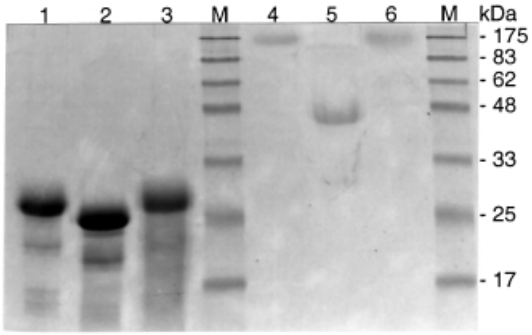

Fig. 4. Esterification of natural recombinant Col3a1 and synthetic P4 gelatin. Lane 1, unmodified Col3a1 natural recombinant gelatin; lane 2, esterified Col3a1; lane 3, esterified Col3a1 after hydrolysis of the ester groups; lane 4, unmodified P4; lane 5, esterified P4; lane 6, esterified P4 after hydrolysis of the ester groups; lane M, molecular weight marker.

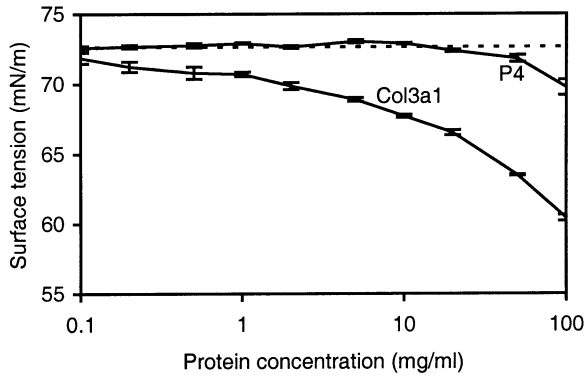

Fig. 5. Surface tension of natural recombinant Col3a1 and synthetic P4 gelatin as a function of concentration. Error bars indicate the standard deviation of five measurements. The dotted line indicates the surface tension of water.

indicating that the effect of esterification was not due to the mere removal of negative charge.

Characterization of synthetic P4 gelatin

In marked contrast with recombinant gelatins having natural sequences, we noticed that highly concentrated solutions of P4 showed essentially no foaming. A direct relationship exists between protein (surface) hydrophobicity, surface tension and foam stability (Horiuchi *et al.*, 1978). Therefore, the surface activity of P4 relative to that of Col3a1 natural recombinant gelatin was determined, using the du Noüy ring method (Lecomte du Noüy, 1919). Figure 5 shows that P4 does not show any significant lowering of the surface tension of water at concentrations up to 5% (w/v), whereas Col3a1 already has an effect at 0.01%. Within the range up to 10% of protein, it was not possible to determine the apparent critical micelle concentration (CMC; i.e. the concentration whereby the surface tension curve reaches a plateau phase) for either of the gelatin types. For comparison, bovine serum albumin has an apparent CMC of about 0.003% (Clarkson *et al.*, 1999).

We previously showed that natural, non-hydroxylated recombinant gelatins produced in *P.pastoris*, do not form collagen triple helices even at 4°C (Werten *et al.*, 1999). Synthetic P4 gelatin was also non-hydroxylated, as shown by the amino acid analyses and N-terminal sequencing described above. Circular dichroism spectrometry was performed to see whether the greater hydrophilicity of P4 somehow influences triple-

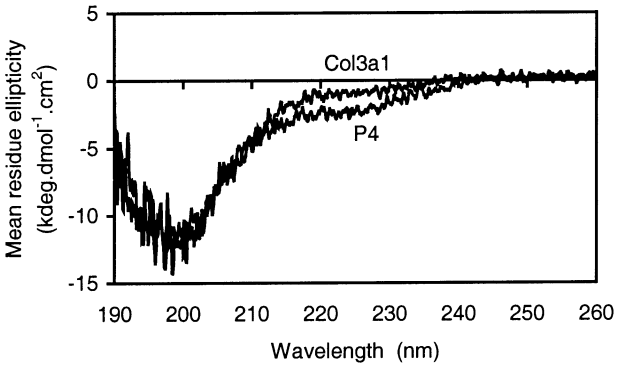

Fig. 6. Circular dichroism spectra of natural recombinant Col3a1 and synthetic P4 gelatin at 4°C.

helix formation. Figure 6 shows the spectrum of P4 and natural recombinant Col3a1 gelatin at 4°C. Both proteins show a clear absence of the positive peak at about 221 nm characteristic of the collagen triple helix (de Wolf and Keller, 1996; Rossi *et al.*, 1996). The mean residue ellipticity of P4 at that position of the spectrum is lower than that of Col3a1.

Discussion

This paper describes the extracellular production of a recombinant, custom-designed and highly hydrophilic gelatin in *P.pastoris*. Yields in single-copy transformants were 3–6 g/l of clarified broth, which is comparable to the yields obtained in single-copy transformants for natural gelatins (Werten *et al.*, 1999).

Despite the obvious physico-chemical differences, both synthetic P4 and natural recombinant gelatin could be purified from the fermentation broth by both differential acetone precipitation and differential ammonium sulfate precipitation. The universality of the two purification techniques is probably due to the hydrophilicity and unfolded structure of gelatins in general. Especially differential ammonium sulfate precipitation allowed convenient large-scale purification of P4 gelatin to near homogeneity.

Secreted synthetic gelatin was fully intact, as evidenced by SDS–PAGE, gel filtration chromatography, N-terminal sequencing and mass spectrometry. This is in contrast with natural recombinant gelatins produced in *P.pastoris*, which were partly degraded (Werten *et al.*, 1999). Apart from the occurrence of some minor background degradation, collagen type I-derived natural recombinant gelatins were cleaved into several major bands by a Kex2-like protease. Cleavage occurred C-terminal of two occurrences of the mono-arginylic sequence Met–Gly–Pro–Arg. We speculated that the amino acids occupying the –2 and –4 positions in this motif (relative to the site of cleavage) were a major factor determining the cleavage efficiency (Werten *et al.*, 1999), which is in accordance with recent data on the substrate specificity of *Saccharomyces cerevisiae* Kex2 (Bevan *et al.*, 1998; Suzuki *et al.*, 2000). It may well be that the above-mentioned minor background degradation represented a limited extent of cleavage at Arg residues having 'suboptimal' residues at the –2 and –4 positions. Therefore, in the design of the synthetic gelatin described here, only Lys was used as a basic residue to control the isoelectric point. The finding that secreted P4 was completely intact does indeed suggest a general susceptibility of Arg residues in recombinant gelatins to proteolysis and may thus have implications for the rational

design of (partially) unfolded proteins to be expressed extracellularly in *P.pastoris*.

While the molecular weight of P4 as determined by mass spectrometry was in good agreement with the value deduced from the amino acid sequence, the molecular weight apparent from gelatin-calibrated gel filtration chromatography was about 10 kDa higher. Ionic interactions with Superose 12 are negligible in the presence of salt and only small hydrophobic peptides appear to show significant hydrophobic interactions with this matrix (Andersson *et al.*, 1985). It is therefore not very likely that a lower degree of such interactions of P4 relative to natural gelatins causes the seemingly aberrant molecular weight. Possibly the effect is due to an increased hydrodynamic size of P4 relative to natural gelatins, as a result of increased interaction of this highly hydrophilic protein with water.

Natural gelatins migrate about 40% more slowly in SDS–PAGE than expected. Several possible explanations for the aberrant migration behavior of gelatins have been suggested (Furthmayr and Timpl, 1971; Freytag *et al.*, 1979; Hayashi and Nagai, 1980; Noelken *et al.*, 1981; Butkowski *et al.*, 1982). It is most likely not the result of anomalously low SDS binding, but is at least in part due to the low average residue molecular weight of gelatin, resulting in a relatively high number of residues (i.e. molecular length) per unit of molecular weight (Freytag *et al.*, 1979; Noelken *et al.*, 1981; Butkowski *et al.*, 1982). Most other reports on aberrant protein migration rates in SDS–PAGE involve highly acidic proteins that show reduced binding of SDS due to electrostatic repulsion by the protein's high negative net charge (Ohara and Teraoka, 1987; Matagne *et al.*, 1991; Casarégola *et al.*, 1992; McGrath *et al.*, 1992). SDS–PAGE showed that P4 migrates at a highly reduced rate, even much more slowly than natural gelatins. We showed here that esterification of the carboxylic side chains of P4 restores its migration rate roughly to that expected for normal gelatins (i.e. about 40% more slowly than common proteins). In contrast, hydrazination did not affect the migration rate of P4. Hydrazination eliminates the negative charge of the same carboxylic residues as does esterification, but reduces the protein's hydrophobicity whereas esterification increases it. As the binding of SDS to proteins is primarily hydrophobic in nature (Reynolds and Tanford, 1970), the extremely slow migration of the highly polar P4 gelatin in SDS–PAGE is therefore most likely the result of insufficient SDS binding and a concomitant low negative net charge. The finding that the resolution of the SDS–PAGE was improved by increasing the field strength to twice that recommended by the manufacturer of the electrophoresis system indicates that the higher field strength aids protein migration in overcoming diffusive forces.

Surface activity is a major determinant in a protein's function as a protective colloid (e.g. in photographic emulsions). Solutions of P4 showed essentially no foaming and tensiometric analysis of solutions of P4 in water showed only negligible surface activity. P4 thus represents a novel hydrocolloid combining some of the characteristics unique to gelatins and a low surface activity commonly expected only for the most hydrophilic polysaccharide hydrocolloids.

We previously showed that non-hydroxylated, natural recombinant gelatins do not show triple-helical structure in circular dichroism spectrometry (Werten *et al.*, 1999). It is a well-established fact that hydroxyproline residues play a crucial role in the stabilization of the collagen triple helix. This role is easily recognized when examining the amino acid compositions and thermal stabilities of natural collagens from different species (Privalov, 1982). X-ray crystallography showed that the triple helix is surrounded by a cylinder of hydration (Bella *et al.*, 1994). Although recently questioned (Holmgren *et al.*, 1999; Nagarajan *et al.*, 1999), the role of hydroxyproline in the stabilization of the triple helix is generally attributed to its hydrogen bonding with this water network (Brodsky and Shah, 1995). In view of the high polarity of P4, we considered it prudent to investigate its conformation using circular dichroism spectrometry. No triple helical structure was observed at 4°C and P4 gelatin is thus an essentially non-gelling gelatin. Non-gelling gelatins permit novel applications such as low-temperature silver halide crystallization in the preparation of photographic emulsions (de Wolf *et al.*, 2000). Comparison of the circular dichroism spectrum of P4 with that of Col3a1 natural recombinant gelatin showed that the mean residue ellipticity of P4 at the discriminating wavelength of 221 nm was actually lower than that of Col3a1. Although the latter is essentially in a random coil conformation, the higher hydrophilicity of P4 probably reduces minor intramolecular and intermolecular interactions, thereby resulting in a slightly lower ellipticity.

Current research is directed towards the production of other synthetic gelatins with distinct functionalities and combining them to form chimeric tailor-made biopolymers.

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