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Cryoprotective Effect of the Serine-Rich Repetitive Sequence in Silk Protein Sericin[1]

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The silk proteins, fibroin and sericin, are produced in the silk gland of *Bombyx mori*, and hydrophilic sericin envelops fibroin with successive sticky layers in the formation of a cocoon. To study the biological functions of sericin, we focused on the serine-rich sericin peptide consisting of 38 amino acids, which is a highly conserved and internally repetitive sequence of a sericin protein. The corresponding gene was chemically synthesized, and the PCR-amplified gene was ligated to oligomerize sericin peptide and fused at the amino terminus to a His-tagged and proteolytic cleavage sequence in an inducible expression vector. When the dimers of sericin peptides were overexpressed in *Escherichia coli*, the transformants showed a prominent increase in cell viability after freezing in medium. Further, the purified dimeric sericin peptide from *E. coli* was found to be effective in protecting lactate dehydrogenase from denaturation caused by freeze-thaw. Both of these protective effects against freezing stress in cells and proteins were also observed with sericin hydrolysate. These results indicate that this unique sericin peptide, like sericin, has a high cryoprotective activity and will be valuable as a new biomaterial for industrial use.

Key words: *Bombyx mori*, cryoprotection, freezing tolerance, repetitive sequence, sericin.

Two silk proteins of *Bombyx mori* have been identified as major components of the cocoon (*1*). Fibroin and the linked subunits, which are synthesized in the posterior silk gland, are assembled into an insoluble silk thread. Sericins, secreted in the middle silk gland, ensure the cohesion of the cocoon by gluing twin silk threads together. Proteins of the sericin family have a high serine content (30%) and are thought to protect other proteins in the cocoon and the pupa of the silkworm from various stresses, possibly due to their extreme hydrophilicity and solubility in heated water (*2*).

Sericin was recently found to suppress lipid peroxidation and to inhibit tyrosinase (polyphenol oxidase) activity *in vitro* (*3*). However, little study has been reported regarding the biologically functional property of sericin at the molecular level, because of the difficulty of preparing sericin molecules which show the diversity generated by alternative splicing of the transcripts of two sericin genes, *Ser1* and *Ser2* (*4–6*). A *Ser1* gene, which was cloned and in part sequenced, transcribes at least four major mRNAs of 10.5, 9.0, 4.0, and 2.8 kb, with apparent molecular masses of the sericins ranging from 65 to 400 kDa (*1, 7*). The *Ser2* gene produces two major mRNAs of 6.4 or 5.0 and 3.1 kb, while the corresponding polypeptides show variations from 164 to 227 kDa (*5, 8*). It is noteworthy that the *Ser1* mRNAs contain the highly conserved and internally repetitive unit of 114 base pairs encoding the deduced peptide of 38 amino acids, more than 12 times (*4*). The sequence is characterized by its unusually high hydrophilic amino acid residue content, serine (45%) and threonine (11%), and might therefore act as the cement which holds the hydrophobic fibroin fibers together during cocoon formation (*4*). However, the possible contribution of this unique sequence is unknown.

In the present study, in order to understand the biological functions of sericin, we focused on the repetitive sequence of 38 amino acid residues, termed "sericin peptide." The corresponding gene was chemically synthesized and the PCR-amplified gene was overexpressed in *Escherichia coli*. We first examined the role of sericin peptide in the freezing tolerance of *E. coli* cells. We then analyzed the *in vitro* cryoprotective activity of the purified sericin peptides from the recombinant *E. coli* for a model enzyme (lactate dehydrogenase) (*9*).

MATERIALS AND METHODS

Materials—An *E. coli* strain JM109 [*recA1* Δ(*lac-proAB*) *endA1 gyrA96 thi-1 hsdR17 relA1 supE44*/(F′*traD36 proAB*⁺ *lacI*ᵠ *ZΔM15*)] and the isopropyl-β-D-thiogalactopyranoside (IPTG; Wako Chemicals, Osaka)–inducible vector pQE30 (Qiagen, Hilden, Germany) with the sequence cod-

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Abbreviations: BSA, bovine serum albumin; HPLC, high performance liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; LDH, lactate dehydrogenase; LEA, late embryogenesis-abundant; NTA, nitrilotriacetic acid; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; TFA, trifluoroacetic acid.

ing six consecutive histidine residues at the 5′ end of cloning sites were used for the expression of the synthetic sericin peptide gene. The plasmid vector pBluescript II SK+ (Toyobo Biochemicals, Osaka) was used for the subcloning and the DNA sequencing of sericin peptide gene. All enzymes for DNA manipulations were obtained from Takara Shuzo (Kyoto) and used under conditions recommended by the supplier. Factor Xa from bovine plasma and lactate dehydrogenase (LDH) from pig heart were purchased from Novagen (Madison, USA) and Oriental Yeast (Tokyo), respectively. A molecular weight marker for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was purchased from Daiichi Pure Chemicals (Tokyo).

Gene Synthesis of Sericin Peptide—We constructed the sericin peptide gene, which codes for two repeats of 38-amino-acid sericin peptide (SerD). To remove all amino terminal vector-encoded sequences following purification by digestion with a site-specific protease, the cleavage sequence (Ile-Glu-Gly-Arg) of factor Xa was added at the 5′ end of the sericin peptide gene. For this purpose, eight synthetic oligomers, ranging in size from 61 to 69 nucleotides (numbered from SP1 to SP8) were chemically synthesized. Oligonucleotides were then assembled as follows. The 5′ termini of all oligomers were phospholylated using T4 polynucleotide kinase, then complementary pairs of individual oligomers were constructed by annealing. The annealed oligomer pairs were ligated, and the predicted DNA fragment was recovered by 2.0% agarose gel electrophoresis. The 268-bp *Pst*I-*Eco*RI fragment containing the entire synthetic sericin peptide gene was first subcloned into pBluescript II SK+, and the nucleotide sequence was confirmed with a Model 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, USA) using dideoxy chain termination sequencing. The resultant plasmid was designated as pBSserD.

Construction of Expression Plasmids—pBSserD was digested with *Bcl*I and *Hin*dIII, and the entire SerD gene obtained was ligated into the *Bam*HI and *Hin*dIII sites of pQE30 to construct pQEserD. Secondly, to introduce the *Xho*I site on the 3′ end of the SerD gene, polymerase chain reaction (PCR) was carried out with pQEserD as a template and primers TetraF (5′-AAGGTCGCTCGAGTACCG-GT-3′) and TetraR (5′-CGCTCAGACTCGA GACAGAT-3′) using a Gene Amp PCR system 2400 (Perkin-Elmer Applied Byosystems). After the PCR, the unique amplified band of 250 bp was digested with *Xho*I and ligated to the large fragment of partially *Xho*I-digested pQEserD. The resultant plasmid containing four repeats of sericin peptide (SerT) was named pQEserT, and the nucleotide sequence was confirmed. Plasmid pQEserH, which encoded six repeats of sericin peptide (SerH), was further constructed as follows. To introduce the *Bam*HI and *Sca*I sites on the 5′ end of the SerD gene, PCR was performed with pQEserD as a template and primers HexaF (5′-ATCGGATCCGTC-TCGAGTACTGGTT-3′) and HexaR (5′-GTTCTGAAGGTC-ATTACTGG-3′). The expected 310-bp band of PCR products was digested with *Bam*HI and ligated to the *Bam*HI site of pQEserT to construct pQEserH. The nucleotide sequence was confirmed by DNA sequencing. Finally, we constructed the expression plasmid pQEserO, which encoded eight repeats of sericin peptide (SerO), as follows. To introduce the *Sca*I site on both ends of the SerD gene, PCR was carried out with pQEserD as a template and primers OctaF (5′-GAAGACGTCTCGAGTACTGGTTCT-3′) and OctaR (5′-TGAATTCGCTAGTACTCG AAACGGA-3′). After the PCR, the expected 250-bp band was digested with *Sca*I and then ligated to pQEserH partially digested with *Sca*I to construct pQEserO. The nucleotide sequence was confirmed by DNA sequencing.

Expression of the Sericin Peptide Gene in E. coli—The *E. coli* strain JM109 was transformed with four expression plasmids (pQEserD, pQEserT, pQEserH, and pQEserO), respectively. Each recombinant strain was grown at 37°C in M9 medium (*10*) containing 2% casamino acids and 50 μg/ml ampicillin (M9CA). When absorbance at 600 nm reached 0.5, IPTG was added to the culture medium to a final concentration of 1 mM to induce gene expression. After cultivation for 4 h at 37°C, the cells were harvested by centrifugation and cell-free extracts were prepared by sonic oscillation (200 W; Kubota, Tokyo) for 10 min under cooling. The total cellular proteins were subjected to SDS-PAGE on a 15% (w/v) polyacrylamide gel, and then either stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Hercules, USA) or electrotransferred to a poly(vinylidene difluoride) membrane (Bio-Rad). The His-tag was detected with horseradish peroxidase-conjugated Ni/nitrilotriacetic-acid (Ni-NTA; Qiagen). Protein bands were also excised for NH_2-terminal amino acid sequences analysis by automated Edman degradation with a Model 476A pulsed liquid protein sequencer (Perkin-Elmer Applied Biosystems).

Freeze-Tolerance Test—E. coli JM109 cells harboring pQEserD were grown at 37°C in M9CA medium. When absorbance at 600 nm reached 0.5, the gene expression was induced with 1 mM IPTG. After cultivation for 4 h at 37°C, culture samples were diluted with M9CA medium to an absorbance of approximately 1.0 at 600 nm. Aliquots (0.1 ml) of cells were transferred into 1.5-ml microcentrifuge tubes and immediately stored at −20°C. Under these conditions, it took about 1 h for cells to become frozen, assuming that the cooling rate was slow (approximately 0.5–1.0°C/min). Samples of frozen cells were thawed at 25°C for 10 min, serial dilutions were made in 0.9% NaCl, and aliquots plated immediately on M9CA plates. Survival rates were expressed as percentages of the number of colonies after incubation at 37°C for 24 h relative to the number of colonies before freezing.

E. coli JM109 cells were also grown in M9CA medium in the absence of ampicillin for 12 h at 37°C. After dilution with the same medium to an absorbance of 1 at 600 nm, approximately 1×10^8 cells (0.1 ml) were harvested, washed and resuspended in 0.1 ml of 0.5% (w/v) hydrolyzed sericin powder (*3*), 0.5% (w/v) BSA, or sterile water. Freeze-thaw treatment was the same as described above.

Purification of the Recombinant Sericin Peptide—Harvested cells harboring the sericin peptide expression plasmid (pQEserD) from 300 ml of culture were suspended in 30 ml of ice-cold sonication buffer [50 mM NaH_2PO_4, 50 mM Na_2HPO_4 (pH 7.8) and 300 mM NaCl], and disintegrated by sonication using a sonicator (200 W). The sonicated cells were boiled for 5 min, then centrifuged at 10,000 ×*g* for 10 min. The soluble fraction in the supernatant was then purified using Ni-NTA agarose (Qiagen). The His-tagged fusion proteins were bound to the Ni-NTA resin. The resin was then collected on a column, washed with 50 mM Na-phosphate (pH 6.0), 300 mM NaCl, and 10% (v/v) glycerol, and eluted with 300 mM imidazole in the buffer.

The eluted fractions were dialyzed exhaustively against 100 volumes of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM $CaCl_2$. To release mature sericin peptide from the fusion protein, 1 μg of factor Xa was added to 200 μg of the fusion protein (an enzyme-substrate ratio of 1:200 w/w) in 200 μl of 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 1 mM $CaCl_2$, and the reaction mixture was incubated for 12 h at 16°C under the conditions recommended by the supplier. The sericin peptides were then purified using reverse-phase high performance liquid chromatography (HPLC) on a C8 column (Amersham Pharmacia Biotech, Buckinghamshire, England) connected to a SMART system (Amersham Pharmacia Biotech). The peptide peak eluted with a 0–70% (v/v) acetonitrile gradient containing 0.1% trifluoroacetic acid (TFA) in a period of 60 min was collected manually by monitoring the absorbance at 214 nm. Protein concentrations were determined using bicinchoninic acid (*11*) with a BCA Protein Assay Kit (Pierce, Rockford, USA).

Mass Spectrometry—Mass spectrometric analyses were carried out with a Voyager Elite mass spectrometer (PerSeptive Biosystems, Framingham, USA) using a 25 kV accelerating voltage. Mass spectra were obtained by adding the individual spectra from 32 laser shots. The samples were run in the linear mode. The protein solutions were diluted 1:1 (v/v) with the matrix solution, 10 mg/ml α-cyano-4-hydroxycinnamic acid (Sigma, St. Louis, USA) in 50% CH_3CN in 0.1% TFA, and then allowed to air-dry on the sample target before analysis. Insulin B chain (3,496 Da; Sigma) and thioredoxin (11,674 Da; Sigma) were used as external standards.

Cryoprotection Assay for LDH—A freeze-labile enzyme LDH (3 IU/ml) was dissolved in 50 mM potassium phosphate buffer, pH 7.5, and supplemented with sericin peptide or bovine serum albumin (BSA) at the indicated concentrations (w/v). The LDH solutions were frozen in liquid nitrogen for 60 s, then thawed for 5 min in a 30°C waterbath. This cycle was repeated eight times, and the enzyme activity was measured after each cycle. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), 0.8 mM Na-pyruvate, 0.2 mM NADH, and 0.316 μg of LDH in 3.17 ml. The reaction was initiated by the addition of 20 μl of LDH solution (15.8 μg/ml) and followed by measuring the decrease in absorbance at 340 nm. The residual activities of LDH after freeze-thaw treatment were measured at 25°C for at least 2 min.

RESULTS

Design of the Synthetic Sericin Peptide—We designed a synthetic sericin peptide gene based on the known amino acid sequence of sericin 1 deduced from the *Ser1* gene (*4*). The *Ser1* gene of the silkworm *Bombyx mori* is known to encode four major mRNAs (*7*). By conceptual translation of the cDNA sequence, it was proposed that the serine-rich peptide consisting of 38 amino acids represents at least a part of an internally repeated motif of sericin protein. The residues at each position were then compared, and a consensus 38-mer repetitive sequence, termed "sericin peptide," was established (Fig. 1). Taking into account the stability of transcriptional and translational products in the host cells, we chemically synthesized the gene corresponding to two repeats of sericin peptide (SerD), which consists of 76 amino acid residues. The amino acid sequence of SerD and the synthetic gene fragments are shown in Fig. 2. The amino acid codons were selected from those that most frequently increase the translational efficiency in *E. coli*. To release the original sericin peptide from the fusion protein, the additional DNA sequence containing the cleavage sequence (Ile-Glu-Gly-Arg) of factor Xa was fused to the amino terminus. For experimental convenience, sequences for the restriction enzymes *Pst*I, *Bcl*I, *Xho*I, *Bam*HI, *Bpu*1102I, and *Eco*RI were placed at unique sites at the 5′ and 3′ ends. The designed SerD gene was assembled from eight component fragments (SP1–SP8).

Construction of the E. coli Expression Plasmid—The SerD gene was excised with *Bcl*I and *Hin*dIII from pBSserD and ligated to the *Bam*HI and *Hin*dIII sites of pQE30 (Fig. 3). The resultant plasmid pQEserD carries the entire SerD gene with the sequence coding six consecutive histidine residues and the cleavage site of factor Xa at the amino terminus under the control of the *T5* promoter/*lac* operator. To examine the relationship between the biological function and the length of the sericin peptide, expression plasmids pQEserT, pQEserH, and pQEserO including four, six, and eight repeats of a sericin peptide, respectively, were constructed by PCR amplification based on pQEserD (Fig. 3).

Expression of the Sericin Peptide Gene in E. coli—*E. coli* JM109 was transformed with the expression plasmids pQEserD, pQEserT, pQEserH, and pQEserO, and the gene expression was induced by IPTG. SDS-PAGE revealed large amounts of products at positions corresponding to a higher molecular mass than the predicted sizes of about 9, 18.4, 25.6, and 32.7 kDa (Fig. 4A). The NH_2-terminal amino

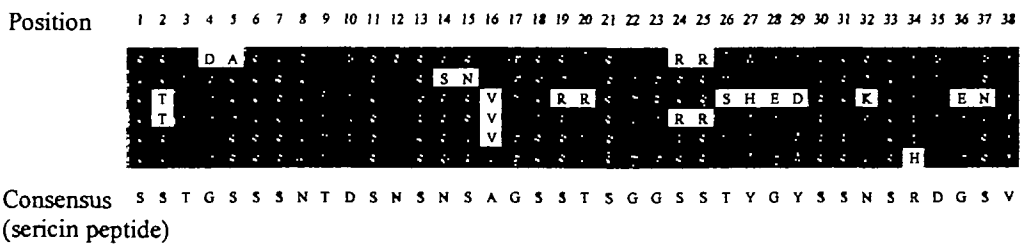


Fig. 1. **The Ser-rich internally repetitive 38-mer sequence of the silk protein sericin 1.** All the sequences shown are cited from Okamoto *et al.* (*4*). Identical amino acid residues are shaded. By comparison of these sequences, a general consensus sequence termed sericin peptide was determined.

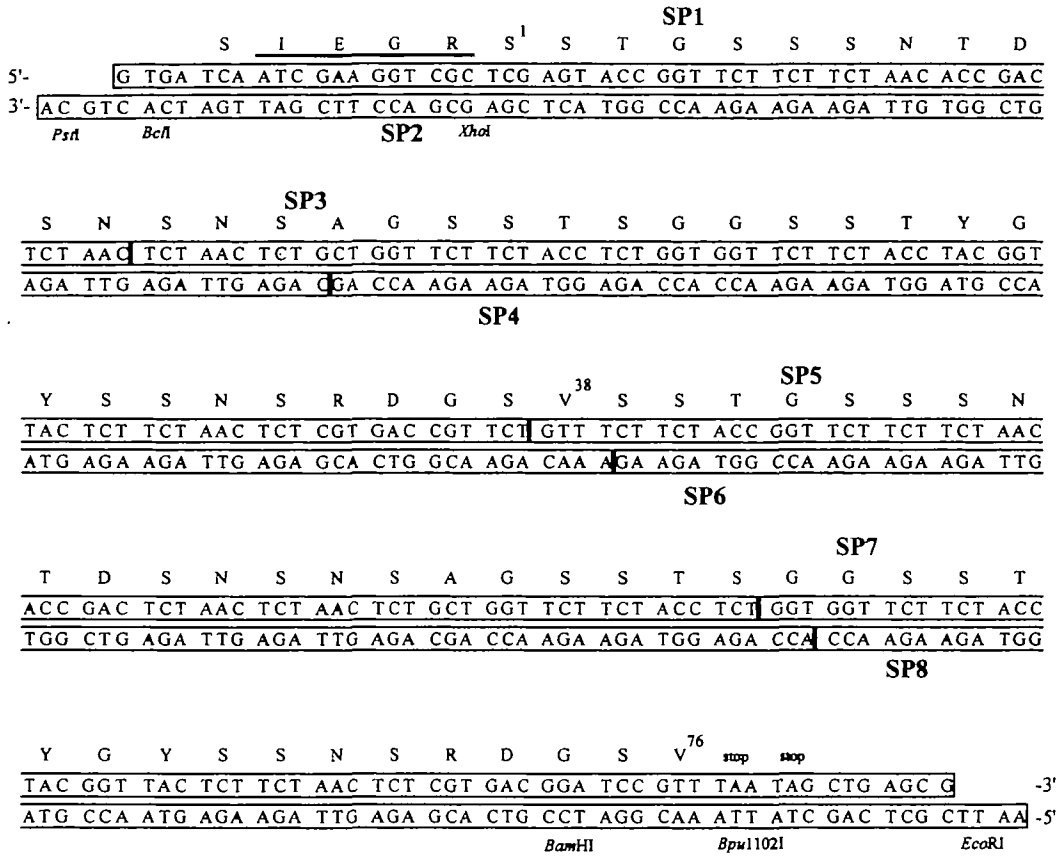


Fig. 2. **Nucleotide and amino acid sequences of the synthetic gene encoding two repeats of sericin peptide (SerD).** The amino acid sequence is shown above the DNA sequence. Each box (SP1–SP8) indicates the synthetic oligonucleotide used to assemble the sericin peptide gene. The vertical lines show the ligated positions of the synthetic gene fragment. The restriction enzyme sites used to oligomerize the gene are indicated. The recognition amino acid sequence for factor Xa is underlined.

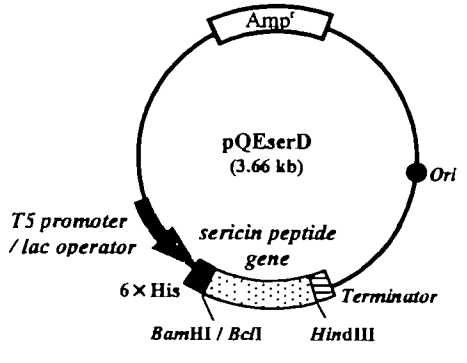
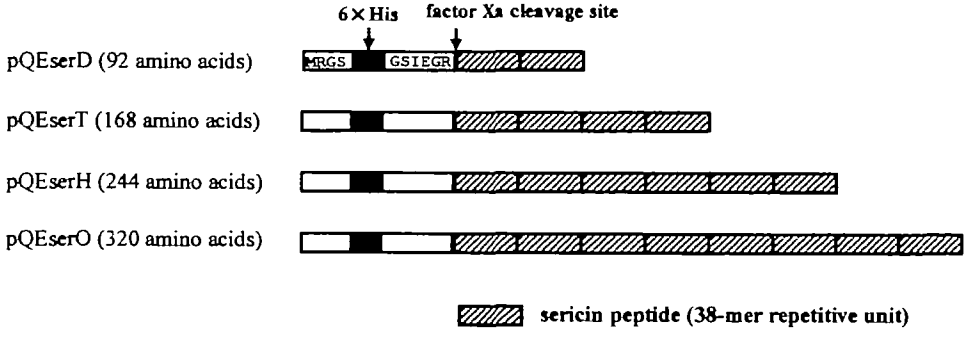


Fig. 3. **Structures of expression plasmids pQEserD, pQEserT, pQEserH, and pQEserO for sericin peptide genes.** The *Bcl*I–*Hin*dIII fragment of the SerD gene excised from pBSserD (this study) was ligated to the *Bam*HI and *Hin*dIII sites of pQE30, and resultant plasmid was designated pQEserD. The plasmid contains a *T5* promoter/*lac* operator and a terminator sequence to regulate the expression level of the sericin peptide gene. Expression plasmids pQEserT, pQEserH, and pQEserO were constructed based on pQEserD, as described in "MATERIALS AND METHODS." The SerD, SerT, SerH, and SerO genes include two, four, six, and eight repeats of the sericin peptide, respectively, and each has six consecutive His residues and the factor Xa cleavage site at the amino terminus.



acid sequence of the higher molecular mass products was MRGSHHHHHH-, which coincides with that of the designed fusion peptides (data not shown). Detection of the His-tag with Ni-NTA showed that each band arose from the His-tagged fusion protein (Fig. 4B). This mismatch of the predicted and measured molecular mass may be due to the biased amino acid composition of the sericin peptide. The growth of *E. coli* cells harboring pQEserH and pQEserO was significantly inhibited, while little effect was observed on the growth of cells expressing pQEserD and pQEserT (data not shown). This is considered to be the reason for the accumulation of larger proteins. Therefore, for the following experiments, we used the recombinant *E. coli* expressing the SerD gene.

Effect of Sericin Peptides Produced in E. coli on Freezing Stress—Sericin peptide has a high proportion of hydrophilic amino acid residues (Ser, 45%; Gly, 16%; Thr, 11%; Asn, 11%), and is extremely hydrophilic. In general, hydrophilic proteins are considered to protect cells from dehydration stress, such as freezing, desiccation, and high osmolarity, due to the maintenance of water in the cells (*12*). Therefore, we expected that the intracellular sericin peptide would also act as a cryoprotectant in *E. coli* against freezing stress, which causes cytoplasmic dehydration.

To test the freezing tolerance of the *E. coli* cells expressing the SerD gene, the recombinant and control strains were cultured in M9CA medium at 37°C. When cell suspensions were exposed to freezing at –20°C for 3 days after 4 h of induction by IPTG, the cells expressing the SerD gene showed an approximately 2-fold higher survival rate than control cells harboring the vector only (Fig. 5). Prolonged storage of the cells at –20°C for up to 5 days caused a gradual loss of freeze-tolerance in the cells expressing the SerD gene, although a significant cryoprotective effect remained (data not shown).

Hydrolyzed sericin powder showed a similar cryoprotective effect when *E. coli* JM109 cells were suspended and frozen at –20°C in 0.5% (w/v) solution (Fig. 6). Its cryoprotective effect was as high as that of BSA, a known cryopro-

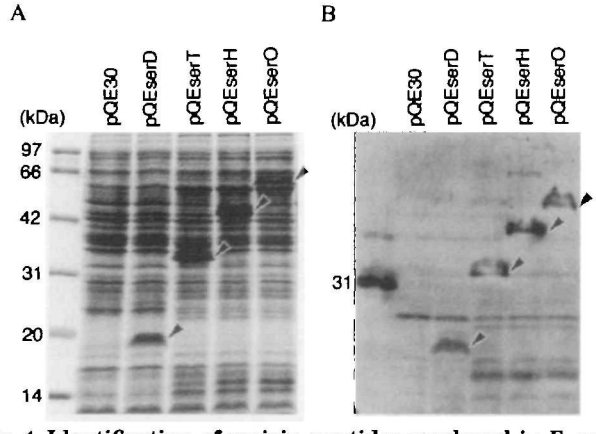


Fig. 4. **Identification of sericin peptides produced in *E. coli*.** (A) SDS-PAGE pattern visualized with Coomassie Brilliant Blue. (B) Detection of His-tagged protein with the horseradish peroxidase–conjugated Ni-NTA. Arrowheads show four recombinant proteins. *E. coli* cells harboring the expression plasmids pQEserD, pQEserT, pQEserH, pQEserO, and pQE30 (vector only) were induced with 1 mM IPTG at 37°C for 4 h, then 10 μg of total cellular proteins was subjected to SDS-PAGE on a 15% polyacrylamide gel. Molecular mass standards are shown at left. It should be noted that Ni-NTA formed a complex with a 31-kDa molecular-weight standard, bovine carbonic anhydrase.

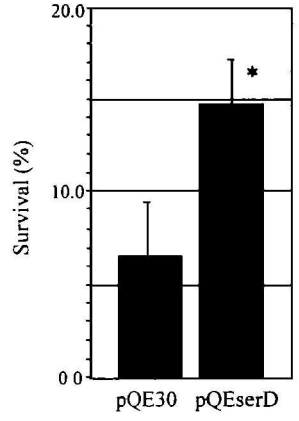


Fig. 5. **Freezing tolerance of *E. coli* cells expressing the SerD gene.** Percent survival of the *E. coli* cells harboring pQE30 (vector only) and pQEserD is shown. The *E. coli* cells were grown in M9CA medium and induced with 1 mM IPTG at 37°C for 4 h. After dilution with M9CA medium to an absorbance of 1 at 600 nm, 0.1 ml of culture sample (approximately 1×10^8 cells) was stored at –20°C for 3 days. Samples of frozen cells were thawed at 25°C for 10 min, serial dilutions were made in 0.9% NaCl, and plated immediately on M9CA plates. Survival rate was expressed by the number of colonies after incubation at 37°C for 24 h as a percentage of the number of colonies prior to freezing. The data shown are means ± SD from three independent experiments. An asterisk shows a significant difference from the corresponding control group (pQE30) by Student's *t*-test ($p < 0.01$).

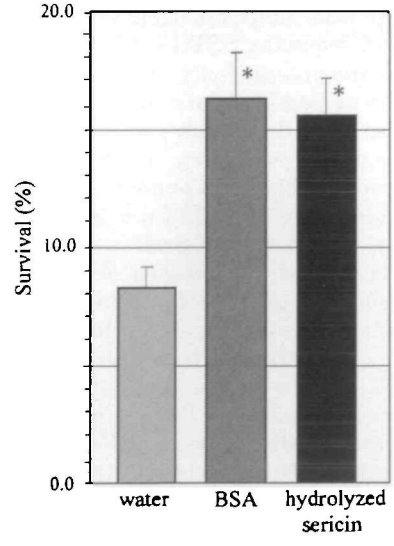


Fig. 6. **Cryoprotective effect of hydrolyzed sericin powder on *E. coli* cells.** *E. coli* JM109 cells were grown in M9CA medium in the absence of ampicillin for 12 h at 37°C. After dilution with the same medium to an absorbance of 1 at 600 nm, approximately 1×10^8 cells (0.1 ml) were harvested, washed and resuspended in 0.1 ml of 0.5% (w/v) hydrolyzed sericin powder (*3*), 0.5% (w/v) BSA, or sterile water. Freeze-thaw treatment and survival rates were the same as in Fig. 5. The data shown are means ± SD from three independent experiments. Each asterisk shows a significant difference from the corresponding control group (water) by Student's *t*-test ($p < 0.01$).

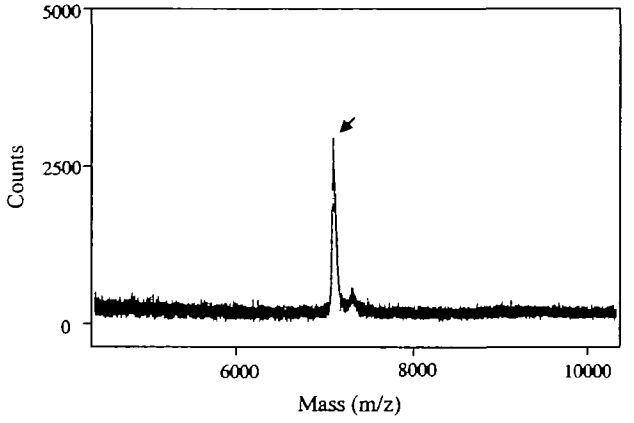


Fig. 7. **Mass measurement of sericin peptide, SerD.** Matrix-assisted laser desorption and ionization mass spectrometry was applied to determine the correct molecular mass of the mature sericin peptide. The arrowhead indicates the peak of SerD.

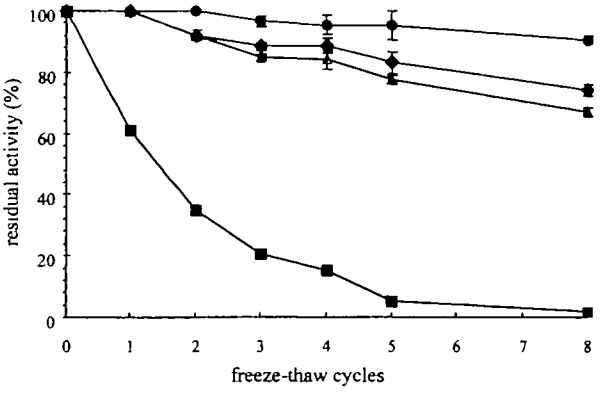


Fig. 8. **Protective activity of sericin peptide on freeze-thaw inactivation of LDH.** The residual activities of LDH after a freeze-thaw treatment in the presence of SerD (●), sericin hydrolyzate (◆), and BSA (△) are shown. All additives were applied at a concentration of 0.05% (w/v), and control (■) was performed without additives. The freeze-thaw cycle consisted of freezing in liquid nitrogen for 60 s and thawing for 5 min at 30°C. The data shown are means ± SD from three independent experiments.

tectant protein. These results indicate that both extracellular and intracellular sericin (peptide) are capable of protecting cells from freezing stress.

Purification of Sericin Peptide from the Recombinant E. coli—To further analyze the cryoprotective effect of sericin peptide, the SerD was purified from the expressed his-tag fusion proteins in *E. coli*, as described in "MATERIALS AND METHODS." It is worth noting that the fusion proteins remained soluble even after boiling, possibly due to their high hydrophilicity. This feature seemed to be advantageous for the purification from the other proteins of the host cells. The sericin peptide digested with factor Xa was separated by reverse-phase HPLC, but was unexpectedly left unstained by all the dyes tested on SDS-PAGE, probably due to its unusual amino acid composition. However, the NH_2-terminal amino acid sequence, SSTGSSNTDSNSNSAGSST-, of the separated fraction perfectly matched the designed sequence of sericin peptide (data not shown). In addition, matrix-assisted laser desorption and ionization mass spectra were obtained to accurately determine the molecular mass of the peptide (Fig. 7). A prominent peak with an apparent molecular mass of 7,112 Da was observed and was assigned to the sericin peptide, SerD.

Cryoprotective Activity of Purified Sericin Peptides—We tested the protective activity of sericin peptide on freeze-thaw inactivation of LDH (Fig. 8). In the absence of a cryoprotectant, the residual activity of LDH greatly decreased with an increase in the number of freeze-thaw cycles. Five freeze-thaw cycles resulted in the loss of over 90% of LDH activity. However, in the presence of sericin peptide, the protective effect on LDH was clearly observed. The remaining enzyme activity after eight freeze-thaw cycles was close to 100% at a concentration of 0.05% (w/v) sericin peptide. The addition of sericin hydrolyzate also led to enhanced enzyme protection against freeze-thaw treatment. The protective effects of the sericin peptide and hydrolysates were found to be expressed in a dose-dependent manner (data not shown). On a molar basis, however, the protective effect of sericin was less than that of BSA, which is known to be effective in protecting LDH against freeze-thaw inactivation (*13*). These findings reveal that the Ser-rich repetitive sequence in sericin could protect both cells and proteins under freezing stress conditions.

As described above, the sericin peptide was still soluble after boiling, suggesting that the high hydrophilicity is commonly involved in both freezing and heat resistance. However, no protective effect against heat-inactivation of LDH was observed (data not shown).

DISCUSSION

The sericin peptide (the internally Ser-rich repetitive motif of 38 amino acid residues in the silk protein sericin) was found to act as a cryoprotectant of both cells and proteins under freezing-stress conditions. The alkaline hydrolysate of the sericin protein (mean molecular mass, 12 kDa) also expressed similar activity. To our knowledge, this is the first report describing the biological role of sericin peptide.

Little attention has hitherto been paid to the functional property of the sericin protein, because sericin shows diversity and a high level of allelic polymorphism and has found no industrial use after removal from the cocoon. Its high proportion of hydrophilic amino acid residues (Ser, Gly, Asp, and Thr) suggest that sericin plays a role in enveloping fibroin with successive sticky layers. Kato *et al.* (*3*) recently reported that sericin functions as an antioxidant defense barrier against oxidative stress in order to protect the cocoon and silkworm and, as do other antioxidants such as ascorbic acid and kojic acid, inhibits the activity of tyrosinase, which is responsible for the browning of various foods and the biosynthesis of skin melanin. It is speculated that the abundant hydroxy groups in sericin might be involved in its antioxidant action by chelating trace elements such as cooper and iron. To clarify these novel actions at a molecular level, the use of the purified sericin peptide from the recombinant *E. coli* is preferable to hot-water extracted or alkaline hydrolyzed sericin containing heterogeneous molecules.

The partial sequence of *Ser1* cDNA coding for the 4.0 kb Ser1B mRNA has been determined and the corresponding amino acid sequence of the Ser1B protein has been deduced (molecular mass, 12.3 kDa) (*6*). The eight exons encode

eight peptide sequences, but the repeated motif in exon 8 remains unsequenced. To further analyze the alternative splicing of the transcripts in the *Ser1* gene, we recently isolated and completely sequenced a Ser1 cDNA clone from the middle silk gland. The consensus 38-amino-acid sequence proposed in this study was found twice in the amino acid sequence of exon 8 (Takahashi M, unpublished observations). Exon 8, which contains the repetitive region cited above, has no potential *N*-glycosylation site. It was previously shown that the predicted secondary structure of exon 8 exhibits mostly β structures and β turns that could form antiparallel β-sheets (*6*). The peptide sequences of exon8 are probably responsible for most of the β-sheet structure of the sericins detected by crystallography (*14*). In general, cellular freezing injury is of two types (*15, 16*). In the first, slower cooling rates cause osmotic shrinkage of a cell and freeze extracellular water. In the second, dehydration occurs and biological macromolecules and/or membrane components denature. More rapid freezing does not permit the transport of intracellular water through the membrane, and impairs membrane structure function as ice crystals form from free water in the cells. *E. coli* cells in which the antifreeze proteins (AFP) found in the winter flounder accumulated exhibited improved freezing tolerance (*17*). It was suggested that the proteins abundant in the amino acids with a negatively charged or hydroxyl-containing side chain were involved in freezing tolerance. The majority of the amino acids contained in sericin peptide have hydroxyl groups. It is therefore considered preferable for the sericin peptide as a cryoprotectant to have the capacity to form strong hydrogen-bonds with free water without interacting with macromolecules, even at lower water contents.

These observations also suggested that freezing, desiccation, and osmotic stresses, so-called dehydration stresses, cause common deleterious damages to the cell membrane and functional proteins (*18*). However, no significant osmoprotective function of the sericin peptide was observed in the presence of NaCl or sorbitol (data not shown).

During periods of water deficit, plants are known to accumulate late embryogenesis-abundant (LEA) proteins, which are thought to protect cells from stresses associated with dehydration (*19–22*). Expression of various LEA protein genes in yeast cells increased cell survival rate after freezing stress and attenuated the growth inhibition normally observed in media of high osmolarity (*23–26*). *In vitro* studies have shown that LEA proteins, which are extremely hydrophilic and remain soluble even after boiling, could also protect LDH from freeze-thaw inactivation (*27*). It was suggested that boiling-soluble proteins such as the sericin peptide or LEA proteins might act cryoprotectants. BSA is recognized as an effective cryoprotectant and widely used for biological preparations. The mechanism of its cryoprotective effect is not clear, but has been hypothetically explained (*13*). The addition of BSA raises the overall protein concentration of the enzyme solution, which might prevent the ice crystallization. On the other hand, the BSA molecules may surround the enzyme molecules and protect them from transconformation and aggregation caused by freezing. It was suggested that, as one of their possible functions, the cryoprotective effects of the sericin peptide and LEA proteins could be explained by the same principle as that of BSA (*27*). However, the cryoprotective activities seem to be related to the high hydrophilicity of the protein (*27*), so sericin peptide might be more effective to prevent the ice crystallization than BSA.

From its predicted α-helical structure including biased charge distribution, it has been proposed that LEA protein may act as an ion scavenger (*23*). It is believed that charged residues ion-coat the surface of membrane components and proteins to prevent denaturation by COOH- and NH_2-groups in the molecules. On the other hand, the amino acid sequence deduced from exon 3 in *Ser1* cDNA was found to have a high content of charged residues (Glu + Asp = 13%, and Lys + Arg = 15%) and a higher degree of water accessibility and hydrophilicity (*6*). Therefore, it can be expected that the sericin peptide engineered to introduce the charged residues or the exon 3 peptide in Ser1 protein confers salt tolerance to dehydration stresses in cells and proteins.

This sericin is of interest not only in regard to the biologically structure–functional relationship, but also for its possible industrial applications. Its antioxidant and tyrosinase-inhibitory effects make it a promising natural ingredient for cosmetic and food industries. The cryoprotective activity shown here may be valuable for the food industry. For instance, frozen-dough technology has recently been applied to the baking industry because it allows the supply of oven-fresh bakery products to consumers. Therefore, the combination of sericin with established cryoprotectants, such as trehalose (*28, 29*) and proline (*30, 31*) would contribute to the enhancement of cellular freezing resistance. Similarly, the overexpression of the sericin peptide gene may allow the construction of novel freeze-resistant baker's diploid or active dry yeast strains. In plants, drought and freezing are stresses that adversely affect the growth of plants and the productivity of crops. Such a gene transfer approach could be employed to improve the stress tolerance of transgenic plants. Investigations into these applications are currently in progress. This paper showed the cryoprotective activity of the recombinant sericin peptide in *E. coli*. A description of cDNA cloning and the expression of the *Ser1* gene in insect cells using a vaculovirus-derived vector will be published elsewhere.

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