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High-yield Production of Functional Human Lactoferrin in Transgenic Cell Cultures of Siberian Ginseng (*Acanthopanax senticosus*)

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Abstract Human lactoferrin (hLf) is an iron-binding glycoprotein that has been considered to play many biological roles in the human, including the stimulation of the immune system, antimicrobial and anti-inflammatory effects, and regulation of iron absorption. We generated transgenic Siberian ginseng (*Acanthopanax senticosus*) cell cultures producing a functional hLf protein using the signal peptide sequence from the endoplasmic reticulum and driven by an oxidative stress-inducible *SWPA2* promoter which is highly expressed in plant cell cultures. The production of hLf increased proportionally to cell growth and showed a maximal level (up to 3.6% of total soluble protein) at the stationary phase in suspension cultures. Full-length hLf protein was identified by immunoblot analysis in transgenic cell cultures of Siberian ginseng. Recombinant hLf (rhLf) was purified from suspension cells of Siberian ginseng by ammonium sulfate precipitation, cation-exchange and gel filtration chromatography. N-terminal sequences of rhLf were identical to native hLf (nhLf). The overall monosaccharide composition of rhLf showed the presence of plant specific xylose while sialic acid is absent. Antibacterial activity of purified rhLf was higher than that of nhLf. Taken together, we anticipate that medicinal Siberian ginseng cultured cells, as demonstrated by this study, will be a biotechnologically useful source for commercial production of functional hLf not requiring further purification.

Keywords: cell culture, high expression promoter, human lactoferrin, medicinal plant, recombinant protein

INTRODUCTION

Lactoferrin is an 80 kDa iron-binding glycoprotein which was originally found in milk. Human lactoferrin (hLf) is a major protein component of breast milk and has significant protective roles, including antimicrobial [1], antifungal [2], anti-endotoxin [3], and antiviral [4]. Recombinant hLf (rhLf) has been expressed in a variety of systems, *e.g.*, fungi [5], yeast [6], and mammalian (cow) [7]. Animal and fungal production systems, however, require extensive purification processes and are at risk to harbor harmful mammalian disease-causing viruses, microbes, fungi, and prions of animal origin [8].

Transgenic plants as plant bioreactors have been actively developed for the production of economically important biomolecules such as vaccines and vitamins. Recently, the use of transgenic plants as protein factories was pursued and resulted into a cost-effective alternative for large-scale production. Plant cell cultures, therefore, have become attractive systems for producing secondary metabolites and recombinant proteins [9]. A great advantage of plant cell suspension culture systems is that recombinant proteins can be produced under controlled conditions with large scale-up and at reduced cost [10].

RhLf is expressed in transgenic plants and plant cell cultures. Thus, Mitra and Zhang first reported expression of the hLf gene under the control of the CaMV 35S promoter in tobacco cultured cells, which contained approximately 1.8% of total cellular protein [11]. Low levels of hLf have also been expressed in tobacco leaves, rice, and potato plants, with approximately 0.3% of the total cellular protein [12-14] and with strong antibacterial activity. Recently, transgenic rice was developed that expressed hLf at very high levels [15-18] which may foretell

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commercial availability involving infant formula [16,17].

Use of a strong promoter is important for a high level of expression of foreign genes in transgenic plants and plant cell cultures. The *SWPA2* promoter is a strongly oxidative stress-inducible peroxidase promoter, which was cloned from the sweetpotato (*Ipomoea batatas*) [19]. In transgenic tobacco, the *SWPA2* promoter highly induced the expression of GUS protein during suspension cultures after the stationary stage was reached. Therefore, the *SWPA2* promoter is biotechnologically useful tool for the development of particular transgenic cell lines engineered to produce key pharmaceutical proteins. We have reported in our previous studies that transgenic tobacco and ginseng cell lines can express hLf protein at a high level when using the *SWPA2* promoter [20,21].

Because it eliminates the need to purify the transgenic protein from the original organism before feeding, the expression of recombinant proteins in edible tissues, such as banana, rice, or ginseng, is advantageous when compared to other systems, *e.g.*, inedible tobacco. Such an edible tissue system as Siberian ginseng (*Acanthopanax senticosus*), a woody medicinal plant found only in northeast Asia, can be used for the industrial production of medicinal raw materials and pharmacologically active components [22]. Medicinal plant culture cells have been used in commercial applications of various health foods and teas. With respect to medicinal plants, mass production of plant cells and tissues through large-scale suspension cultures may be applied directly to the production of pharmacologically active components and raw materials. In this report, we develop transgenic cell cultures of Siberian ginseng that produce elevated levels of rhLf and characterize by biochemical analysis and biological function.

MATERIALS AND METHODS

Expression Vector and Transformation

Embryogenic calli induced from mature Siberian ginseng (*A. senticosus*) seeds were maintained in the dark in MS medium [23] that contained 30 g/L sucrose, 1 mg/L 2,4-D with subculturing every 3 weeks. Transformation of the Siberian ginseng callus was carried out by *Agrobacterium tumefaciens* EHA105 harboring the SWPA2pro:: ER-hLf/pCAMBIA2300 expression cassette as described previously [24]. Kanamycin-resistant calli were isolated and transferred to MS medium containing 1 mg/L 2,4-D, 150 mg/L kanamycin, and 300 mg/L cefotaxime (selection medium) every 3 weeks.

Quantification of hLf Protein Levels by ELISA

The hLf protein levels expressed in transgenic Siberian ginseng calli were determined using a quantitative enzyme linked immunosorbent assay (ELISA). Transgenic or nontransgenic (control) Siberian ginseng calli (1 g of fresh weight) were ground in liquid nitrogen and homogenized in an equal volume of ice-cold extraction buffer (50 mM potassium phosphate, pH 7.0). The homogenates were centrifuged at 12,000 g for 15 min at 4°C. The soluble protein concentration in the homogenate supernatant was determined using a Bio-Rad protein analysis kit [25]. The recombinant hLf content was determined using BIOXYTECH® Lacto*f*-EIA™ (*Oxi*Research™).

Northern Blot Analysis

Total RNA from Siberian ginseng cell lines with high levels of hLf protein was extracted with TRIzol™ (GIBCO/BRL) for northern blot analysis, according to the instructions of the manufacturer. Approximately 15 μg of total RNA was electrophoresed on a 1% agarose gel containing 0.67 M formaldehyde, and blotted onto a Zeta membrane (Bio-Rad) and hybridized to 1.0 kb of the hLf DNA-probe. Hybridization was carried out in 0.25 M sodium phosphate (pH 7.2) containing 7% SDS at 60°C. After hybridization, the blot was washed once with 20 mM sodium phosphate (pH 7.2), 1% SDS at room temperature for 10 min, then twice with the same solution at 60°C.

Western Blot Analysis

Total soluble protein was extracted from transformed Siberian ginseng cultured cells as described above. Protein extracts (50 μg) were separated on a 10% (w/v) acrylamide gel, along with 1 μg of commercially available lactoferrin (Sigma) as a standard. The resolved proteins were transferred to a PVDF nylon membrane (Millipore Co.) and the membrane immersed in a blocking solution (1% BSA and 10 mM Tris-HCl, pH 7.4, 150 mM NaCl) for 1 h at 4°C. The membrane was incubated overnight in a 1:20,000 dilution of commercially available polyclonal antibody conjugated with peroxidase (Rabbit anti-Human Lactoferrin, BIODESIGN International). After washing five times with TBST buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20), the blot was detected with the ECL plus Western Blotting Detection System (Amersham Pharmacia Biotech) according to the instructions of the manufacturer.

Purification of the rhLf Protein and its Biochemical Analysis

The total soluble protein was extracted from the transgenic Siberian ginseng suspension cultures (100 g of fresh weight) by homogenization in an equal volume of ice-cold extraction buffer (50 mM potassium phosphate, pH 7.0) containing a cocktail of protease inhibitors (Roche, Mannheim, Germany) and centrifuged at 12,000 g for 15 min at 4°C. The supernatant was concentrated by ammonium sulfate precipitation (50%, w/v), and was purified and captured on an SP Sepharose Fast Flow column (Amersham Pharmacia Biotech). After washing, the rhLf was eluted with a linear salt gradient (0 to 2.0 M NaCl in 0.2 M sodium acetate, pH 7). Fractions containing only rhLf were concentrated on Stirred cells (Ultrafil-

tration membrane YM 10, Amicon), and purified by gel filtration chromatography using Superdex 200 HR 16/60 (Amersham Biosciences). The presence of hLf was analyzed by ELISA and SDS-PAGE.

Purified rhLf protein (30 μg) was subjected to amino acid sequence analysis using a Procise Sequencer (Applied Biosystems) and the analysis of rhLf monosaccharide were carried out using the facilities of the Korea Basic Science Institute. Purified rhLf (100 μg) was hydrolyzed in 6 N HCl at 100°C for 4 h. The hydrolyzate was evaporated to dryness using a SpeedVac, the residue resuspended in distilled water, injected into a Bio-LC DX-600 (Dionex, Sunnyvale, CA, USA), and detected by PED2 with integrated amperometry. To separate the monosaccharides, 300 mM NaOH was used at a flow rate of 0.25 mL/min on a CarboPac PA-1 column (Dionex, 4 ×250 mm) with CarboPac PA1 cartridge (4.5 × 50 mm).

Assay of Antibacterial Activity

Two different bacterial strains, *Staphylococcus aureus* (KCTC1916) and *Escherichia coli* DH5α, were selected for the growth inhibition assay and grown to an A_{600} of 0.4. Aliquots of 1.0 mL were transferred to culture tubes. Purified rhLf protein (500 μg) was added to each culture tube and incubated at 37°C on a rotary shaker for 2 h. Commercially available native lactoferrin (500 μg) (Sigma) and protein extraction buffer were used as positive and negative controls, respectively. After the incubation, the total number of colony-forming units was determined by serial dilution on LB plates and by counting the number of colonies [14].

RESULTS

Eight weeks after *Agrobacterium*-mediated transformation, kanamycin-resistant Siberian ginseng calli were generated and selected by PCR [24]. The levels of hLf protein in 11 randomly selected lines with high cell growth at 20 days after subculture were determined by ELISA and ranked according to expression levels (Fig. 1). Quantitative analysis revealed that the hLf content was ranged from 19.4 μg/g fresh weight (FW) in cell line 3 to 141.5 μg/g FW in cell line 5. Transgenic cell lines expressed the hLf contents in a range of 0.2 to 2.3% of total soluble protein (TSP) (Fig. 1). Three transgenic cell lines (5, 6, and 23) that produced a high level of hLf protein were selected for further study. The transformed Siberian ginseng calli had similar growth characteristics to the non-transformed calli.

To examine the expression of the hLf gene in transgenic Siberian ginseng cells, total RNA isolated from three cell lines (5, 6, and 23) with high hLf contents, in terms of their total soluble protein, were analyzed by the northern hybridization using an hLf-specific probe. The northern blot analysis indicated the presence of hLf transcripts (Fig. 2A). The hLf gene was highly expressed in transgenic cell line 23, whereas gene activity was detected

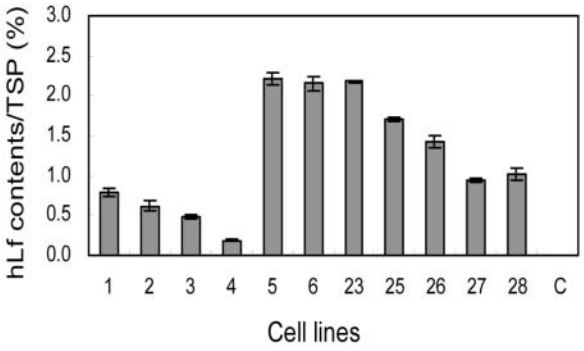

Fig. 1. Human lactoferrin content (%) of the total soluble protein (TSP) in transformed Siberian ginseng calli. Eleven PCR-positive Siberian ginseng cell lines were selected and protein extracts were prepared. The expression levels of human lactoferrin were estimated by ELISA. Data are means ± S.E. of 3 independent replicate experiments.

at a low level in the other two transgenic cell lines (5 and 6). The expression of the hLf gene was not detectable in non-transformed control cells.

Three transgenic Siberian ginseng cell lines (5, 6, and 23) were tested for hLf protein expression using SDS-PAGE (Fig. 2B) and Western blot analysis (Fig. 2C). Recombinant hLf protein synthesized in three transgenic cell lines (5, 6, and 23) is shown in Figs. 2B and 2C. The immunoreactive hLf levels differed among the tested transgenic cell lines. Thus, the transgenic Siberian ginseng culture cells produced hLf protein at approximately 80 and 35 kDa, but extracts of non-transformed calli (c) did not react with the anti-hLf antibody. Western blot analysis showed that the relative molecular mass of hLf was similar to that of native hLf (nhLf). Additionally, we detected partial-length hLf as a result of degradation during the process of soluble protein extraction or premature translation termination due to extreme overexpression in plant cells.

To investigate the hLf production levels in suspension cultures, cell line 23, which expressed high levels of hLf, was cultured in the same liquid medium. After the establishment of suspension cultures, the hLf level was investigated during cell growth. Cell growth in suspension cultures shows a sigmoidal growth curve (Fig. 3A). After an initial lag period of up to 4 days after subculture, the cell mass increased linearly, until reaching a maximum. The growth patterns of two cell lines (the non-transformed cell line and cell line 23) were nearly similar, except that the non-transformed cell line (c) and cell line 23 reached their maximum levels at 20 and 28 days after subculture, respectively. The hLf content in cell line 23 gradually increased in a culture-dependent manner during cell growth. The hLf content in cell line 23 showed a marked increase from 16 days after subculture to reach a maximum level at 28 days after subculture and yielding 3.6% of total soluble protein. Following day 28, however, the hLf content decreased (Fig. 3B).

The rhLf from suspension cultures of transgenic Siberian ginseng (cell line 23) at 28 days of liquid culture was

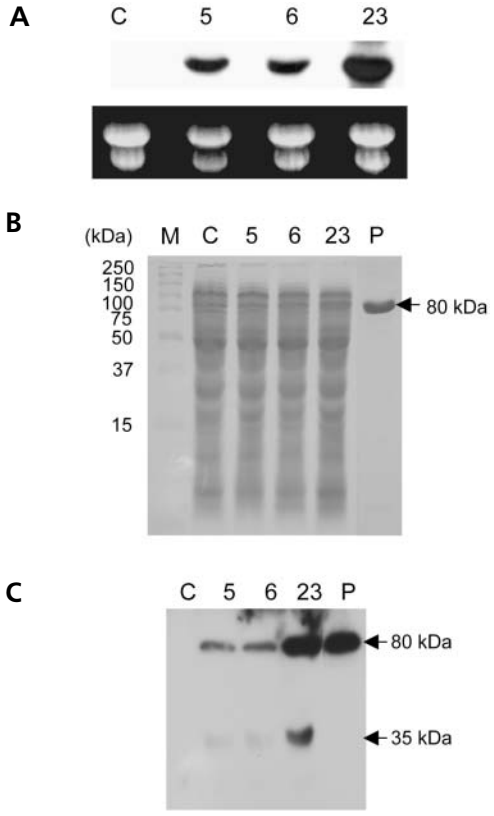

Fig. 2. Analysis of transgenic Siberian ginseng cells with the gene encoding a human lactoferrin. A: Northern blot analysis of three transgenic cell lines (5, 6, and 23) and the non-transformed control (c). Total RNA (15 μg) of each sample was fractionated on 1% agarose gel, transferred to a membrane, and hybridized with a ^{32}P-labeled human lactoferrin cDNA probe. Ethidium bromide staining of the gel is shown as a loading control. B and C: SDS-PAGE (B) and Western blot analysis (C) of transgenic Siberian ginseng cell lines expressing human lactoferrin. Lane C: protein extract from non-transformed calli; Lanes 5, 6, 23: protein extracts (50 μg) from transgenic cell lines; Lane P: commercially available native lactoferrin protein (1 μg).

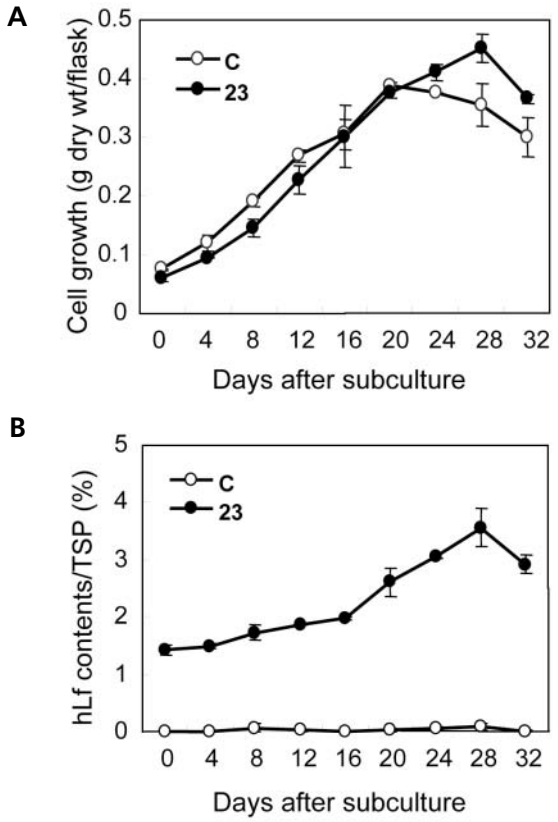

Fig. 3. Changes in cell growth (A) and human lactoferrin contents (B) in suspension cultures of cell line 23. Cells were grown in a 250 mL Erlenmeyer flask; c (open circle) and 23 (close circle) represent non-transformed and transgenic cell 23, respectively.

purified by cation-exchange using SP-Sepharose Fast Flow and gel filtration chromatography using Superdex 200 HR 16/60 (data not shown). SDS-PAGE and Western blot analyses were performed on samples at different stages of protein purification (Fig. 4). As shown in Fig. 4, the majority of the Siberian ginseng proteins appeared in the flow-through, while the rhLf eluted with 1.1 M NaCl. Highly purified rhLf was separated by gel filtration where the recovery achieved by this purification scheme approximated 10%.

After rhLf was purified to homogeneity, several biochemical characterizations were carried out for comparison to commercially available nhLf. The N-terminal sequence of purified rhLf was determined to be GRRRR-SVQW, identical to that of nhLf. These results indicated that the signal peptide of the rhLf product was correctly processed in the transgenic Siberian ginseng culture cells and showed that the N-terminus of the protein was intact. Monosaccharide compositions of purified rhLf are provided in Table 1. In the rhLf, the monosaccharides were fucose, galactose, mannose, xylose, and glucosamine, at ratios of 1.2, 0.9, 3.0, 1.4, and 4.2, respectively. Compared to the nhLf, rhLf was characterized by lesser amounts of galactose. Additionally, analysis shows that the purified rhLf contains xylose which is specific to a plant-type sugar chain, but lacks N-acetyl-neuraminic acid; this observation is consistent with other plant post-translational modification patterns.

The biological activity of purified rhLf from Siberian ginseng culture cells was assessed according to antibacterial properities. As shown in Table 2, purified rhLf (500 μg) showed a reduction in colony formation after all treatments compared with the no protein controls. Additionally, commercially available nhLf (500 μg) inhibited the growth of two tested bacterial strains. It is of interest that the plant-derived rhLf showed a higher antibacterial activity than did nhLf.

DISCUSSION

We have established an efficient functional hLf protein production system in transgenic cell cultures of the Sibe-

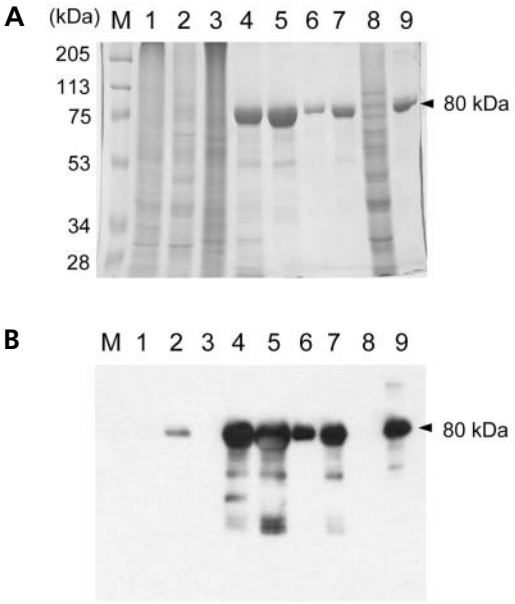

Fig. 4. SDS-PAGE (A) and Western blot analysis (B) of different fractions obtained during purification of human lactoferrin from the transgenic Siberian ginseng cell line 23. M: molecular weight markers; Lane 1: crude extract of non-transformed cells; Lane 2: crude extract of the transgenic cell line 23; Lane 3: unbound fraction from SP-Sepharose column; Lanes 4 and 5: purified human lactoferrin from SP-Sepharose column; Lanes 6 and 7: purified human lactoferrin from gel filtration chromatography; Lane 8: unbound fraction from gel filtration chromatography; Lane 9: commercially available human lactoferrin as standard. Arrows indicate hLf protein (80 kDa).

rian ginseng (*A. senticosus*). Transformation of Siberian ginseng culture cells with an hLf cDNA under the control of an oxidative stress-inducible *SWPA2* promoter led to the production of full-length 80 kDa hLf. This promoter has been shown to be oxidative stress-inducible, and is highly expressed in plant culture cells [19]. The introduction of hLf cDNA into the Siberian ginseng culture cells, under the control of this promoter, was an appropriate strategy for the production of transgenic plants to express hLf in cultured cells.

Using a strong, tissue-specific promoter is crucial to increasing the expression of foreign genes [16,26]. The *SWPA2* promoter was isolated from culture cells of the sweetpotato and was strongly expressed in culture cells, especially following the stationary growth phase in suspension cultures [19]. In previous reports, the expression levels of hLf in transgenic tobacco or ginseng culture cells were found to be approximately 4.3 or 3%, respectively, of the total soluble protein [20,21]. In this study, we observed that the hLf yield for Siberian ginseng culture cells was higher when induced as suspension cell cultures compared to when expressed as callus. This may be due to the *SWPA2* promoter, with its known strong expression in cultured cells. Also, because suspension cultures present a larger surface area per unit cell mass, they can respond more rapidly to environmental change [27].

Table 1. Monosaccharide composition of Siberian ginseng cultured cell-derived purified human lactoferrin (rhLf) and native human lactoferrin (nhLf)

Monosaccharide	rhLf	nhLf
Fucose	1.2	1.4
Galactose	0.9	2.0
Mannose	3.0	3.0
Xylose	1.4	–
Glucosamine	4.2	3.8
N-acetyl-neuraminic acid	–	1.7

Results are expressed relatively to the amount of mannose, arbitrarily set at 3.0.

Table 2. Antibacterial activity of Siberian ginseng cultured cell-derived purified human lactoferrin (rhLf) and native human lactoferrin (nhLf)

Strains	Cell growth (%)		
	Control	nhLf	rhLf
Staphylococcus aureus	100	60.1 ± 7.0	45.2 ± 6.3
Escherichia coli (DH5α)	100	2.1 ± 0.5	33.0 ± 7.0

rhLf (500 μg) or nhLf (500 μg) was added to each culture tube and incubated for 2 h. After incubation, the cell growth was determined by serial dilution and by counting the number of colonies. Data represent the average of three experiments.

While hLf is expressed in tobacco cell culture [11], tobacco plants [12], and potato plants [14], the hLf is truncated in tobacco cell culture and the protein expressed as a full-length hLf in tobacco and potato plants and at very low levels (less than 0.3% of total soluble protein). It is not known why partial-length lactoferrin is produced in tobacco cells but it is possible that plant-produced hLf protein fails to undergo proper folding and the unfolded portion is subsequently degraded. Recently, a full-length hLf protein that yields high levels of protein expression was produced in transgenic rice [16,17], likely due to a combination of a strong tissue-specific promoter and signal peptide sequences present in the expression construct. By applying similar strategies, our results revealed that the highest hLf expression level was approximately 4% of the total soluble protein when using the stress-inducible *SWPA2* promoter and an endoplasmic reticulum-targeting signal peptide. To exploit enhancement of hLf gene expression in plants, however, experiments which substitute a synthetic gene with preferred Siberian ginseng codons, use a signal sequence to target hLf to cell organelles, and optimizes the culture conditions including elicitation for high production, are aspects to be considered for the future [28].

The biochemical properties of rhLf purified from Siberian ginseng culture cells were found to be similar to those of nhLf. Siberian ginseng-derived rhLf is glycosy-

lated and has the same N-terminus as nhLf. Additionally, it is interesting to note that the rhLf showed higher antibacterial activities than nhLf. This finding may result from differences in glycosylation, although antibacterial activity is believed to reside in the N-lobe of hLf. Nonetheless, this result is consistent with that reported by other previous reports [11,14,16,17]. Although glycosylation in plant cells is known to often be different from that in mammalian cells, the plant-derived rhLf protein retained biological activity in a manner similar to that of nhLf. N-terminal amino acids of the rhLf were identical with that of nhLf, which suggests that the processing of the amino acid sequence in plant cells is identical to that in mammalian cells. The result of N-terminal sequencing of rhLf showed that the junction between the ER signal sequence and the mature rhLf was recognized and cleaved, exposing the correct N-terminal amino acid residue.

Expression of recombinant proteins in edible plants, including medicinal plants such as banana, rice, or ginseng offers the advantage over non-edible systems that they eliminate the need to purify the transgenic protein from the original organism. In this respect, a cell culture system of Siberian ginseng expressing a high level of hLf protein can be applied to the industrial production of medicinal raw materials without purification of the pharmacologically active components.

In summary, we have demonstrated the high-yield production of a functional hLf in cell cultures of Siberian ginseng under the control of a strong oxidative stress-inducible *SWPA2* promoter. Our findings indicate that medicinal Siberian ginseng cultured cells can be biotechnologically appropriate sources for commercial production of functional hLf without additional purification. Additionally, an oxidative stress-inducible *SWPA2* promoter with high expression in cell cultures might also be useful for high production of various other recombinant proteins in plant cell cultures.

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