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Production of Recombinant Protein of Salsola Kali (Sal k1) Pollen Allergen in Lactococcus Lactis

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ABSTRACT

The *Salsola kali* pollen is considered the main cause of allergic sensitization in desert and semi-desert regions. We have constructed recombinant *Lactococcus lactis* producing Sal k1 protein with the aim of using it as a mucosal vaccine for specific immunotherapy.

The Sal k1 gene was amplified, and transferred into a PNZ 8148 plasmid. The PNZ8148-Sal k1 recombinant plasmid was transformed into competent *E.coli* strain MC1061 for replication, and then was isolated and cloned into competent *L. lactis* by electroporation. The cloning was verified by PCR and gene sequencing. The production of recombinant Sal K1 (rSal K1) protein was induced by nisin. The rSal K1 protein was purified by affinity chromatography and dialysis, and confirmed by SDS-PAGE and western blot analyses.

The recombinant *L. lactis* was successfully constructed. Production of a 40-kDa rSal k1 protein with the *L. lactis* was shown by sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE) analysis. In addition, western blot analysis using specific mouse anti-Sal k1 polyclonal antibodies and sensitive human sera verified the 40-kD protein as rSal k1 allergen.

This study demonstrated that *L. lactis* may be used as a promising live delivery system for recombinant Sal k1 protein without altering its immunoreactivity; however, its efficacy in the context of the immune system is suggested to be pursued in future studies.

Keywords: *Lactococcus lactis*; Pollen; Recombinant protein; *Salsola kali*; Sal k1

INTRODUCTION

Chaenopodiacea subfamily comprises several genera such as; Chenopodium, Salsola, etc. that are causative agents of allergic reactions in Asia, North Africa, Europe, and, the USA.[1]

Salsola kali (Russian thistle or Tumbleweed), the most well-known species of Salsola, is widely distributed in desert and semi-desert regions of the world, and *S. kali* pollen is considered as a main cause of allergic sensitization in these regions.[2-4] Although the allergenic activity of *S. kali* pollen have been ascribed to various molecules,[1,5] a 40-kDa protein called Sal k1, belonging to the pectin methylesterase family, has been identified as the major allergenic part

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of this pollen.[6] The Sal k1 protein has recently been shown to associate with the severity of allergic rhinitis in Iran.[7] It has been suggested that Sal k1 allergen-specific immunotherapy using an appropriate delivery platform might be effective in Sal k1 allergy desensitization.

Mucosal applications of recombinant lactic acid bacteria (LAB) vaccines have attracted increasing attention as a promising approach in safe allergen-specific immunotherapy.[8-10] These bacteria do not contain any lipopolysaccharide endotoxin in their cell wall, found in *E. coli* that is routinely implemented for expression recombinant protein, and have long been used as a safe additive in human food processing and preservation.[11-13] In addition, accumulating evidence acknowledges the health-promoting properties of LABs as probiotics in human.[14-16] For example, they have been shown to exert immunomodulatory activities, diminishing IgE production and inhibiting or alleviating the severity of allergic immune responses.[17-19] Moreover, it is possible to concurrently produce several proteins of interest in a single LAB.[8] Together, these features make these bacteria highly suitable food-grade live vectors for delivering prophylactic/therapeutic recombinant proteins in modulating allergic responses.[20] Among several potential LAB species, *Lactococcus lactis* has widely been used as a therapeutic-recombinant-protein delivery system in experimental models as well as in clinical trials,[21,22] because it has been shown to promote Th1 responses *in vitro* and in vivo.[9,23]

Accordingly herein, we have constructed Sal k1-recombinant *L. lactis* producing Sal k1 protein with the aim of being used as a mucosal vaccine in Sal k1-specific immunotherapy.

MATERIALS AND METHODS

Construction of pNZ8148-Sal k1 Recombinant Plasmid

Amplification of Sal k1 gene: a PET32-Sal k1 recombinant plasmid already available at our lab was extracted from *E.coli* strain BL21 and then used to achieve adequate segments of the *Sal k1 gene* by polymerase chain reaction (PCR).[1] A pair of specific primers introduced NcoI and HindIII restriction sites at respectively, 5' and 3' end of the *Sal k1* gene; the oligonucleotide sequence of the primers follows as: forward, 5'-CTCACCATGGGCAGCTGATTCCCCC TAATCCAGC-3' and reverse, 5'-AAGAAAGCTTCACTTTCGGTGGTGGAGGTAGC C-3'. The gene was then amplified using Pfu (Pars Tus, Mashhad, Iran). Thermal cycling profile of the reactions included; starting at 96^{o}C for 180 seconds, followed by 33 amplification cycles, and termination at 72^{o}C for 180 seconds. The PCR product was run onto 1.5% agarose gel, and visualized using ethiduim bromide under an Ultraviolet transilluminator (UVP., USA).

Transfection Sal k1 Gene into pNZ 8148 Plasmid

The PCR-amplified DNA (*Sal k1* genes fragments) was purified using Bioneer's PCR Purification Kit (Bioneer, Korea), according to the manufacturer's instructions. Both pNZ 8148 plasmid and PCR products were digested with NcoI and HindIII in Tango buffer (Fermentas, Vilnius, Lithuania) at 37^{o}C for 3 hours, and then the accurate digestion was confirmed by 1.5 % agarose gel analysis.

Replication of pNZ8148-Sal k1 Recombinant Plasmids

Competent *E. coli* strain MC1061 was prepared with the Inoue method[24] in Luria-Bertani (LB), and was used as host for the replication of pNZ8148-Sal k1 recombinant plasmids. The appropriate colonies were selected and subcultured on solid and liquid LB media containing proper antibiotics; meanwhile ten grown colonies were tested for successful plasmid transformation by PCR as described above. The recombinant plasmid was extracted using Pars Tus extraction kit (Mashhad, Iran), according to the manufacturer's instructions. The final extraction solution was subjected to 1% agarose gel analysis. In addition, the extraction product was digested with NcoI and HindIII restriction enzymes, and was then subjected to agarose gel analysis, again. In addition, the pNZ8148-Sal k1 recombinant plasmid was further verified by gene sequencing (Macrogen, Seoul, South Korea) using a pair of primers specific for the insertion region of Sal k1 fragment into the pNZ8148 plasmid and nearby regions. The oligonucleotide sequence of these primers follows as: forward, 5'-TCGATAACGCGAGCATAATAAACG-3' and reverse, 5'-GGCTATCAATCAAAGCAACACGTG-3'.

Cloning of Sal k1 Gene into L. Lactis

Preparation of competent L. Lactis

The *L. lactis* strain NZ9000 was obtained from MoBiTec Company (Germany) and was cultivated on glucose-containing M17 agar at 30°C for 24 hours. To prepare competent cells, *L. lactis* was cultivated in 10 mL G/L-SGM17B liquid medium at 30°C for 48 hours, diluted in additional 10 mL G/L-SGM17B, incubated at 30°C for 3 hours, centrifuged at 6000×g, 4°C for 20 minutes, the pellet was re-suspended in 80 mL sucrose 0.5 M and glycerol 10%, centrifuged and re-suspended in 40 mL sucrose 0.5 M, glycerol 10% and EDTA 50mM, incubated on ice for 15 minutes. Again centrifuged and re-suspended in 20 mL sucrose 0.5 M and glycerol 10%, centrifuged and re-suspended in 80 µL sucrose 0.5 M and glycerol 10%, and finally saved at -80°C in 40-µL aliquots.

Electroporation of pNZ8148-Sal k1 Recombinant Plasmids into L. Lactis

Electroporation was used to incorporate pNZ8148-Sal k1 recombinant plasmids into competent *L. lactis*. Briefly, 1 µl of pNZ8148-Sal k1 recombinant plasmid was admixed with 40 µL of competent *L. lactis* into a pre-chilled electroporation cuvette on ice and subjected to pulsing using BioRad Gene Pulser (USA) set to 2000V, 25 µF, 200 Ω, and 4.5-5 milliseconds. Afterwards, 1 mL G/L-SGM17B, 20 mM $MgCl_2$, and 2 mM $CaCl_2$ were added to the cuvette, incubated on ice for 5 minutes and then at 30° C for 60-90 minutes, and were finally cultivated on M17 agar supplemented with glucose and antibiotics, incubated at 30°C for 48 hours.

Confirmation of Sal k1 Gene Cloning

Several grown colonies of *L. lactis* were randomly chosen and subjected to PCR assay to examine the presence of *Sal k1* gene (successful cloning), as described above. The confirmed *Sal k1-possessing* colonies of *L. lactis* were then subcultured on M17 agar and broth at 30°C for 24 hours to be used for the production of recombinant Sal k1 (rSal k1) protein.

Production and Purification of rSal K1 Protein

Nisin-Induced Expression of Sal k1 Protein

Fifteen colonies confirmed to contain PNZ8148-Sal k1 recombinant plasmids were grown in M17 broth supplemented with glucose and Chloramphenicol at 30°C for 24 hours; 10 µL of which were then transferred into 990 µL fresh M17 medium, and allowed to reach OD600 of 1-5. Afterwards, Nisin was added to the culture medium at concentrations of 1 and 5 ng/mL, followed by incubation at 30°C for 3 hours.

Purification of rSal k1 Protein

The M17 medium containing bacterial cells (recombinant *L. lactis)* was centrifuged at 2000 rpm, 4°C for 20 minutes, re-suspended in appropriate volume of lysis buffer (Tris-HCl 50 mM, NaCl 200 mM, tritone x100 5%, glycerol 10%) subjected to ultra-sonication, and centrifuged again at 6000 rpm, 4°C for 20 minutes. The supernatant containing the expressed allergens was then purified by metal affinity chromatography on a Ni-IDA column (Pars Tous Biotechnology, Iran) according to the manufacturer's instructions and dialysis. Protein concentration was determined by following the Bradfords Method.[37]

Immunoreactivity of rSal k1 Protein

Production of Anti-Sal k1 Polyclonal Antibodies

A recombinant *E. coli* stain BL21 (Novagen, Gibbstown, NJ, USA) already available at our lab which had been confirmed to accurately express Sal k1 protein was used as a source of Sal k1 protein to elicit anti-Sal k1 polyclonal antibodies in mice. Briefly, *E. coli* BL21 was cultivated in LB liquid medium containing ampicillin in the presence of isopropyl β-D-1-thiogalactopyranoside (IPTG), as a protein-expression inducer, at 37°C for 24 hours. The produced 40-kD Sal k1 protein was purified and confirmed by affinity chromatography, dialysis, and SDS-PAGE analysis, as described. Weekly, 200 µL of Sal k1 protein (50µg/L) was intraperitoneally administrated to two mice for 4 weeks, with complete Freund's adjuvants on day zero and incomplete Freund's adjuvants on days 7, 14, 28. Finally, animals were sacrificed and their sera containing anti-Sal k1 polyclonal antibodies were collected and saved at -20°C until use.

Western Blot Analysis

The protein content of the SDS-PAGE gels were transferred onto poly vinylidene difluoride, PVDF, (Immobilon-p, Miliportcorp, Bedford, MA, USA), which were cut into vertical strips, incubated with blocking solution containing bovine serum albumin 2%

at 4°C overnight. Following washing with phosphate-buffered saline (PBS), the strips were incubated with anti-Sal k1 polyclonal antibodies (1:1000) at room temperature for 2 hours, washed and incubated with streptavidin-horseradish peroxidase (HRP)-conjugated anti-mouse-Ig antibodies (1:50000; Bio-Rad, CA, USA), incubated at room temperature for 1 hour, washed and exposed to 3, 3'-diaminobenzidine for chromogenic detection of HRP activity. Then we repeated the western blot analysis by using sensitive human sera as described above. We prepared serum from 20 people that suffered from allergy to Sal k1 pollen; the serums were pulled and used for western blot. The allergy of patients had previously been determined by skin test for Sal k1.

RESULTS

pNZ8148-Sal k1 Recombinant Plasmid

A 1017-bp *Sal k1* gene was successfully amplified from a pET32-Sal k1 recombinant plasmid by PCR assay using a specific pair of primers, and Pfu (Figure 1A). Following digestion with the NcoI and HindIII restriction enzymes to generate sticky ends (Figure 1B), the *Sal k1* gene fragment was successfully incorporated into pNZ 8148 plasmid by means of T_4 Ligase. Agarose gel electrophoresis analysis revealed 3167 bp and 1017 bp DNA fragments indicating pNZ 8148 plasmid and *Sal k1* gene, respectively (Figure 2). In addition, the result of gene sequencing further confirmed the accurate replication of Sal k1 fragment (Figure 3).

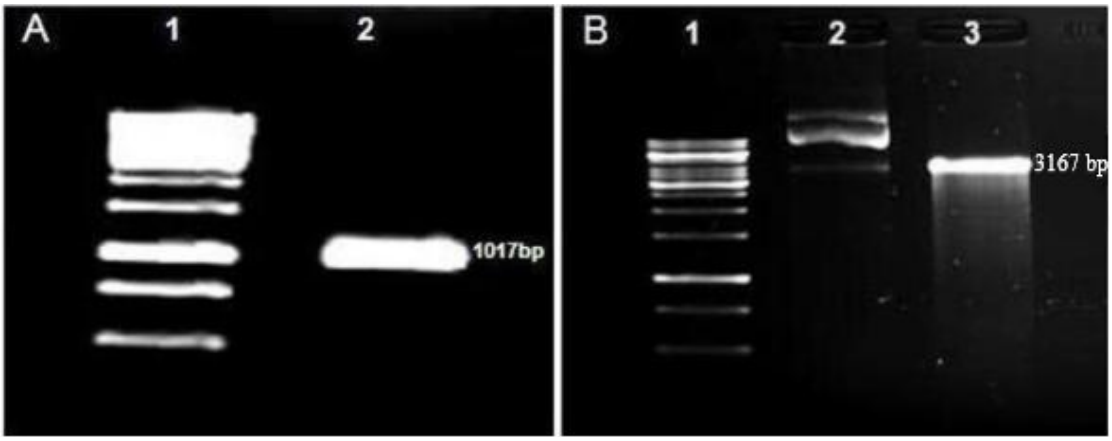

Figure 1. Agarose 1.5 % gel electrophoresis analysis of *Sal k1* gene DNA and pNZ 8148 plasmid to study the production of recombinant protein of Salsola Kali (Sal k1) pollen allergen in Lactococcus Lactis. A) lane 1; DNA ladder (10 Kbp) (Fermentas) and lane 2; 1017-bp *Sal k1* DNA fragment amplified by Pfu High-Fidelity DNA polymerase, and B) lane 1; DNA ladder (10 Kbp), lane 2; intact PNZ 8148 plasmid and lane 3; PNZ 8148 plasmid following double-cut by NcoI and HindIII enzymes (The length of the piece that is separated by two restricted enzymes is too short so it can be ignored)

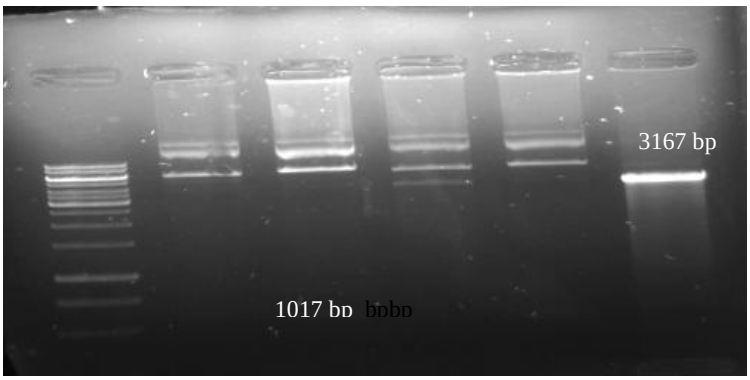

Figure 2. Agarose gel electrophoresis analysis 3165 bp and 1017 bp DNA fragments indicating pNZ 8148 plasmid and *Sal k1* gene to evaluate the production of recombinant protein of Salsola Kali (Sal k1) pollen allergen in Lactococcus Lactis.

Figure 3. The full length sequence of rSal k1 (results of determination of recombinant plasmid sequence) to investigate production of recombinant protein of Salsola Kali (Sal k1) pollen allergen in Lactococcus Lactis.

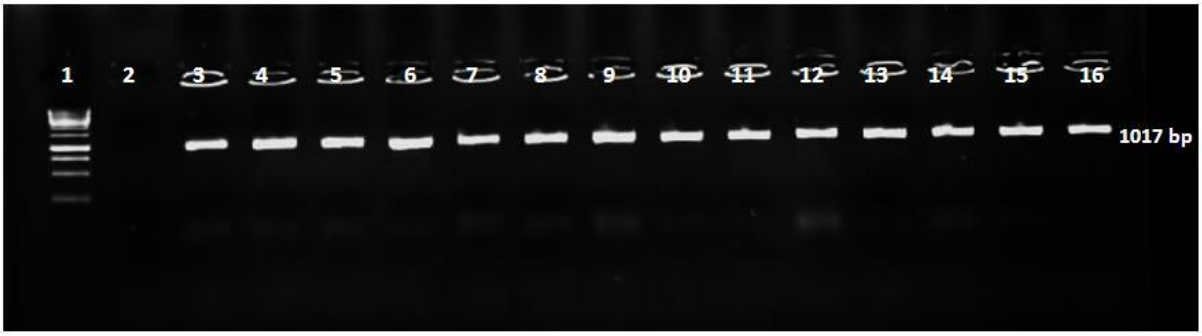

Figure 4. Agarose 1.5% gel electrophoresis analysis of PCR product for detection of *Sal k1* gene in grown colonies of recombinant Lactococcus Lactis to study the production of recombinant protein of Salsola Kali (Sal k1) pollen allergen in *L. lactis*. Lane 1; DNA ladder (10 Kbp), lane 2; negative control, and lanes 3-16; 1017-bp *Sal k1* gene fragments

The verified pNZ8148-Sal k1 recombinant plasmids were transformed into competent *L. lactis* strain NZ9000 by electroporation. Following cultivation on M17 agar at 30°C for 48 hours, several grown colonies were tested by PCR assay to confirm the recombinant *L. lactis*. As demonstrated in Figure 4, the presence of 1017-bp *Sal k1* gene fragments on agarose 1.5% gel electrophoresis analysis confirmed successful cloning of the gene into the *L. lactis*.

The rSal K1 Protein Production and Characterization

The verified recombinant *L. lactis* were used for the expression of rSal k1 protein by incubation with 1 and 5 ng/mL nisin at 30°C for 3 hours. There was no difference between the two concentrations. The produced rSal k1 protein was purified by affinity chromatography and dialysis. Results of the SDS-PAGE analysis on the purification product revealed protein bands that resolved at 40 kDa indicating successful expression of the rSal k1 protein (Figure 5). In addition, western blot analysis using specific mouse polyclonal antibodies (Figure 6A) and human sensitive sera (Figure 6B), produced in the present study confirmed immunoreactivity of the 40-kDa rSal k1 protein.

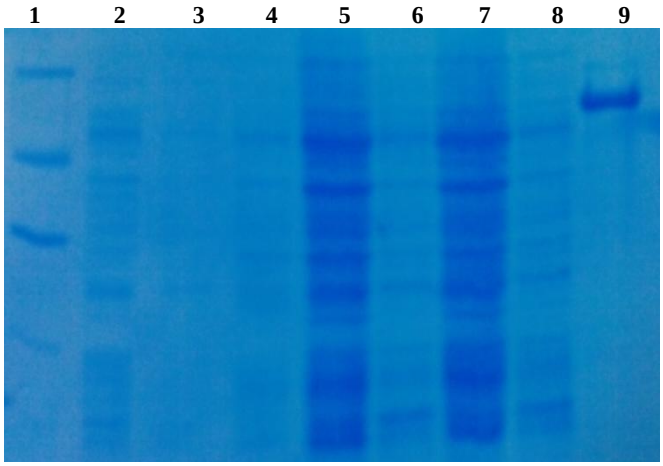

Figure 5. The SDS-PAGE analysis of 40-KD rSal k1 protein expression to study the production of recombinant protein of Salsola Kali (Sal k1) pollen allergen in Lactococcus Lactis.: Lane 1; protein size marker (Pars Tous, Iran), lane 2; supernatant recombinant *L.lactis* without nisin, lane 3; sediment recombinant *L. lactis* without nisin, lane 4; sediment recombinant *L. lactis* with 1ng/mL nisin, lane 5; supernatant recombinant *L. lactis* with 1ng/mL nisin, lane 6; sediment recombinant *L. lactis* with 5ng/mL nisin, lane 7; supernatant recombinant *L. lactis* with 5ng/mL nisin, lane 8;supernatant wild *L. lactis* (without Sal k 1), and lane 9; rSal k 1 protein.

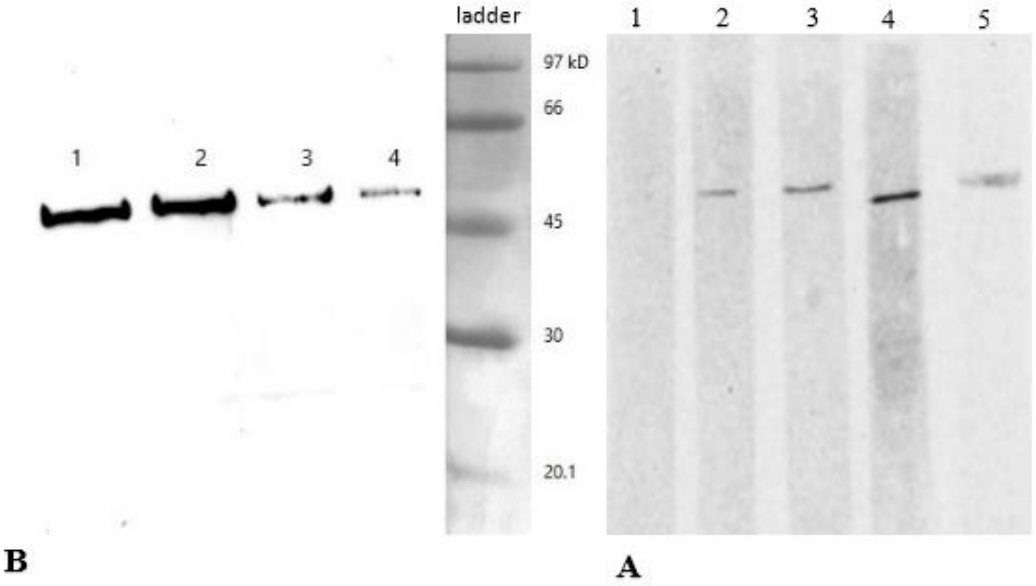

Figure 6. The western blot analysis of 40-KD rSal k1 protein to investigate the production of recombinant protein of Salsola Kali (Sal k1) pollen allergen in Lactococcus Lactis .using: A) specific mouse polyclonal antibodies: protein size marker, lane 1; supernatant recombinant *L. lactis* without nisin, lane 2; sediment recombinant *L. lactis* with 1ng/mL nisin, lane 3; supernatant recombinant *L. lactis* with 1ng/mL nisin, lane 4; supernatant recombinant *L. lactis* with 5ng/mL nisin, and lane 5; sedimentrecombinant *L. lactis* with 5ng/mL nisin.B) sensitive human sera: Lane 1; supernatant recombinant *L. lactis* with 1ng/mL nisin, Lane2; supernatant recombinant *L. lactis* with 5 ng/mL, Lane 3; sediment recombinant *L. lactis* with 1ng/mL nisin, Lane 4; sediment recombinant *L. lactis* with 5 ng/mL nisin

DISCUSSION

S. kali pollen allergy is a growing issue worldwide.[25] Desensitization by means of allergen-specific immunotherapy seems to be efficacious based on some evidence.[26] To date, four Sal k-type allergens including; Sal k1, Sal k2, Sal k3, and Sal k4 have been isolated from S. kali pollen, among which the Sal k 1 protein is reportedly the pollen's major allergen.[2,6,27]

In 2007, Barderaset al[2] isolated, purified and characterized an IgE-reactive protein from *S. kali* pollen. Assarehzadegan .et al[27] cloned and produced rSal k1 protein as thioredoxin and His-tags fusion protein, using a pET-32b(+) vector in *Escherichia coli* as a host. Mas et al[28] also produced rSal k1 protein using pET41b vector in *BL21 E. coli*, and showed that the immunological properties of the expressed protein were preserved compared to its natural counterpart.

In the present study, we constructed a PNZ8148-Sal k1 recombinant plasmid and incorporated it into *L. lactis*, which successfully expressed rSal k1 protein using nisin-controlled expression system. We purified the expressed rSal k1 protein by affinity chromatography and dialysis to obtain maximal yield. Also, the gene cloning process was confirmed by PCR and gene sequencing, and the expressed rSal k1 protein was verified and characterized by SDS-PAGE and western blot analyses. To the best of our knowledge, we are the first to clone and produce rSal k1 protein in *L. lactis*. Similar to our study, Daniel et al[9] had previously constructed recombinant *Lactobacillus plantarum* and *L. lactis* producing Bet v1 pollen allergen (1–2% and 0.7–1% of total soluble cellular proteins, respectively), another member of the pectin methylesterase family. They found that allergen-specific IgG1/IgG2a ratio was decreased in birch pollen allergy animal models immunized with both Bet v1-recombinant Lactobacilli, while IgG1 and IgE responses were pronounced in animals immunized with purified Bet v 1 protein.[9]

One of the prominent features of the present study was that it used *L. lactis* as a vehicle for rSal k1 protein, which can be used for future studies on *S. kali* pollen allergy. Beneficial health-related effects of probiotics are well known in the context of allergies as well as autoimmune diseases, probably through making

improvements in the intestinal microbiota.[16,29,30] Accordingly, recombinant LAB have drawn increasing attentions as safe drug carriers in mucosal immunotherapy of allergic diseases.[15,31-33] Charng et al, for instances,[34] demonstrated that recombinant allergen-producing LABs were able to decrease the allergen-induced airway inflammatory responses. Among LABs, *L. lactis* has received extensive attention as a recombinant carrier of oral vaccines for allergy desensitization.[21,35] The recombinant protein yield is a determinative factor in stimulation of the mucosal immune system.[8] The protein expression level obtained with a recombinant *L. Lactis* has been shown to be higher than those achieved with other LAB such as *Lactobacillus* species.[36] Although the present study did not assessed the rSal k1 protein yield produced by the devised recombinant *L. Lactis*, the use of PNZ8148 plasmid is assumed to provide an inducible gene expression system. This vector is food grade approved, which can express the allergen in an inducible way (with nisin inducer). In cases that react to immunotherapy by oral vaccination, the use of this inducible system can prevent allergen expression by deleting nisin. Following the elimination of allergen expression, the probiotic isn't a problem alone. Therefore the allergic reaction can be omitted. This finding can be mentioned as the most important achievement and also the novelty of this study. We used an already-verified rSal K1 protein produced by BL21 *E. coli* to elicit anti-Sal K1 polyclonal antibodies. We also used sensitive human sera for western blot analysis of the rSal K1 protein newly produced by *L. Lactis*. The western blot results confirmed the immunoreactivity of the rSal K1 protein and showed the produced protein could be recognized by IgE antibodies found in Salsola-sensitive patients' sera.

Thus, taken together and regarding the importance of Sal k1 protein, the recombinant *L. lactis* constructed in the present study is expected to serve as a more effective and safer carrier of this protein in patients sensitized to the *S. kali* pollen. Nonetheless, the efficacy of the rSal K1 protein in the context of immune system, in vivo, is suggested to be studied in the future.

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