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Heterologous Expression and Optimized One-Step Separation of Levansucrase *via* Elastin-like Polypeptides Tagging System

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Abstract Elastin-like polypeptides (ELPs) undergo a reversible inverse phase transition upon a change in temperature. This thermally triggered phase transition allows for a simple and rapid means of purifying a fusion protein. Recovery of ELPs-tagged fusion protein was easily achieved by aggregation, triggered either by raising temperature or by adding salt. In this study, levansucrase has been used as a model enzyme in the development of a simple one-step purification method using ELPs. The levansucrase gene cloned from *Pseudomonas aurantiaca* S-4380 was tagged with various sizes of ELPs to functionally express and optimize the purification of levansucrase. One of two ELPs, ELP[V-20] or ELP[V-40], was fused at the C-terminus of the levansucrase gene. A levansucrase-ELP fusion protein was expressed in *Escherichia coli* DH5α at 37°C for 18 h. The molecular masses of levansucrase-ELP[V-20] and levansucrase-ELP[V-40] were determined as 56 kDa and 65 kDa, respectively. The phase transition of levansucrase-ELP[V-20] occurred at 20°C in 50 mM Tris-Cl (pH 8) buffer with 3 M NaCl added, whereas the phase transition temperature (T_t) of levansucrase-ELP[V-40] was 17°C with 2 M NaCl. Levansucrase was successfully purified using the phase transition characteristics of ELPs, with a recovery yield of higher than 80%, as verified by SDS-PAGE. The specific activity was measured spectrophotometrically to be 173 U/mg and 171 U/mg for levansucrase-ELP[V-20] and levansucrase-ELP[V-40], respectively, implying that the ELP-tagging system provides an efficient one-step separation method for protein purification.

Keywords: Elastin-like polypeptide (ELP), levansucrase, protein purification, inverse phase transition, tagging system

Advances in genetic engineering have made it possible to produce recombinant proteins, extending their market size annually. Despite of the developments in the area of advanced genetic engineering, purification and separation account for 60% to 70% of production costs [3, 21]. In addition, the design of effective methods for purification of recombinant proteins persists as a major problem. A variety of systems have been developed to simplify the purification process. Notably, affinity systems, such as poly His [1, 24], GST [22], MBP [23], and T7 tags have been developed and applied to chromatography for better purification [3, 10, 18, 25]. Although affinity chromatography methods are considered powerful techniques in selective purification, there preclude many disadvantages including the activity loss of the purified target proteins owing to extreme pH for protein desorption from affinity packing materials [25]. Some fusion tags interfere with the folding of the target protein or are toxic to the host cells, and peptide-based affinity tags have a low binding capacity [2]. Furthermore, chromatographic separation methods are time-consuming, difficult to scale-up, and comprise a bottleneck step in high throughput systems [10]. Given these disadvantages, more economical and simple methods have been sought.

Elastin-like polypeptides (ELPs) consist of the repeating pentapeptide Val-Pro-Gly-Xaa-Gly, where Xaa is termed a "guest residue" and can be any amino acid with the exception of proline. To distinguish different ELPs constructs, the nomenclature of ELPs tag is given as ELP[X_iY_j-n]. The bracketed capital letters are single-letter amino acids with the frequency of that guest residue in the repeat unit. The total length of the ELPs in number of pentapeptides is given by n [15]. ELPs undergo a reversible inverse phase transition from soluble forms into aggregates as the temperature increases [13–15, 26]. Aggregation is driven by hydrophobic interactions of the side chain of the amino acids and β-turn structure by repeated proline [27]. These ELP aggregates are easily removed by centrifugation and resolubilized in buffer at below the transition temperature. The phase transition temperature of ELPs can be controlled by the hydrophobicity of the guest residue amino acid,

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Table 1. List of synthetic oligonucleotides used for gene construction.

Name	Sequences (5'→3')
Primer 1	TAGCGAATTCATATGGGCCACGGCGTGGGTGTTCCGGGCGTAGGTGTCCCAGGTGTGGGCGTA (63mer)
Primer 2	ATTTCAGCCCGCCCGGCACGCCGACACCAGGAACACCAACGCCCGGTACGCCCACACCTGGGA (63mer)
Primer 3	CGTCAAAGCTTATCATTTCAGCCCGCCCGGCA (32mer)
Primer 4	CGTAGCGAATTCATATGGGCCA (22mer)
Primer 5	ATGTGCAAGCTTGATCGCCTCCCTCCTTTC (30mer)
Primer 6	GCTTCCGGATCCCTTGAGCGTTACATCGAG (30mer)
SFI 1	GATCC**CTGGTGCCACGCGGT**TCTGGGCCAGGCGGGCC*ATAA*G (42mer)
SFI 2	AATTC*TTAT*GGCCCGCCTGGCCCAGA**ACCGCGTGGCACCAG**G (42mer)

*Restriction sites are underlined, the thrombin cleavage site is in bold, and the stop codon is in italics.

chain length, and ionic strength [14, 28]. This characteristic transition allows a simple and rapid means for non-chromatographic purification of large quantities of industrial protein. In previous works, Meyer and Chilkoti [14, 15] successfully purified thioredoxin using ELP[V-20] and ELP[$V_5A_2G_3$-90]. Kostal *et al.* [8] demonstrated the removal of a heavy metal using modified ELPs. Shimazu *et al.* [20] accomplished the separation of organophosphorus hydrolase and self-immobilization of ELPs onto hydrophobic surfaces by phase transition.

Levan is a natural homopolymer of fructose with β-2,6 linkages. It offers a variety of industrial applications in the fields of cosmetics, food sweeteners, pharmaceuticals as a cholesterol lowering agent, and an antitumor agent as a plasma substitute. Levansucrase is an enzyme that catalyzes the synthesis of levan from sucrose [4–6]. It has both hydrolytic activity and fructosyl transfer activity and is produced by bacteria such as *Zymomonas mobilis*, *Bacillus subtilis*, *Rahmella aquatilis*, *Microbacterium laevariformans*, *etc.* [4, 7, 8].

In this study, levansucrase was used as a target protein to apply ELPs tags for simple one-step purification based on temperature-triggered precipitation. In addition, ELPs tags composed of valine as Xaa were investigated for the optimization of the purification conditions, and the effects of tags length and ionic strength were studied.

Materials and Methods

Materials

pUC19 vector and all restriction enzymes were purchased from New England BioLabs Inc. (Beverly, U.S.A.). *E. coli* DH5α competent cell and *Taq* polymerase were from Takara Co. (Shiga, Japan). *Pfu* polymerase, employed as an enzyme for PCR, was purchased from Promega Co. (Carlsbad, U.S.A.). Protein markers in the SDS-PAGE were purchased from Invitrogen Co. (St. Louis, U.S.A.) and Promega Co. (Madison, U.S.A.). All oligonucleotides were synthesized by Bioneer Co. (Seoul, Korea). A GeneAmp PCR System 2400 purchased from Applied Biosystems (Foster City, U.S.A.) was used for the PCR procedures. The glucose kit for levansucrase activity measurement was purchased from Sigma Co. (St. Louis, U.S.A.).

Synthesis of ELP[V-5] and Oligomerization

DNA coding for $(VPGVG)_5$ and the restriction enzyme site was constructed using four synthetic oligonucleotides (Table 1). Two oligonucleotides, Primer 1 and Primer 2, with 3' overlapping complementary ends, were annealed and extended with *Taq* polymerase by PCR. Using the other oligonucleotides, Primer 3 and Primer 4, complementary strands of 125 bases were synthesized. It was digested with EcoRI and HindIII, and then inserted into pUC19 for amplification. ELP[V-20] and ELP[V-40] were then oligomerized by a recursive directional ligation method using the restriction enzymes PflMI and BglI of which the detailed procedure is described elsewhere [16].

Construction of Levansucrase-ELPs Fusion Gene

All cloning steps were performed according to standard molecular biology protocols [19]. The plasmid pLK1 containing the *Pseudomonas aurantiaca* S-4380 levansucrase gene (*lsc*A) was supplied from RealBiotech Co. (Jochiwon, Korea) and used as a template for PCR [5, 6]. The *lsc*A gene was amplified with forward primer "Primer 5" and reverse primer "Primer 6". After appropriate purification, the PCR products containing HindIII and BamHI restriction sites were restricted with these two restriction enzymes, and then inserted in pUC19 vector for amplification. ELPs tag oligomerization and levansucrase-ELP fusion gene construction were carried out in a different pUC19 vector. Plasmid pUC19 was digested with the same enzymes and ligated with the *lsc*A gene (pUC-lsc). The pUC-lsc vector was subsequently modified to introduce a thrombin cleavage site and a unique SfiI restriction site on the C-terminus of the *lsc*A gene for insertion of the ELPs gene. Introduction of the sequence encoding the thrombin cleavage site and SfiI restriction site was performed by annealing of oligonucleotides, SFI 1 and SFI 2. These oligonucleotides (SFI fragment) contained a BamHI, SfiI, EcoRI restriction site, the sequence encoding the thrombin cleavage site

represented in bold letters in Table 1, and a stop codon. An SFI fragment was then partially digested with BamHI and EcoRI. Plasmid pUC-lsc was digested with the same enzymes and ligated with the SFI fragment. Previously synthesized ELPs coding genes were digested by restriction enzymes PflM I and BglI. Then, ELP[V-20] and ELP[V-40] were fused separately at the C-terminus of the levansucrase using the SfiI restriction site, resulting in pUC-lsc-ELP[V-20] and pUC-lsc-ELP[V-40], respectively. All cloning products were identified by DNA sequencing.

Expression of Fusion Protein

The plasmids were transformed into *E. coli* DH5α. One colony with a mucous morphology, which is indicative of levan formation activity, in LB solid media containing 2% sucrose and 50 μg/ml ampicillin, was inoculated and grown overnight in 5 ml of LB media containing 100 μg/ml ampicillin [5]. The overnight culture was transferred to 200 ml of LB media supplemented with 100 μg/ml of ampicillin. After 18 h, the culture was harvested and resuspended in 50 mM Tris-Cl buffer, pH 8.0. Cells were disrupted by ultrasonication (5s/5s burst) to prepare the soluble and insoluble fractions. Cell debris was removed by centrifugation at 16,000 ×*g* for 20 min at 4°C. The expression of ELPs-tagged levansucrases was identified in 10% SDS-polyacrylamide gel using Coomassie blue staining dye.

Determination of Phase Transition Temperature (T_t)

The phase transition temperature (T_t) was determined by measuring the optical turbidity of an aqueous solution of the recombinant protein as a function of temperature. NaCl of 1, 2, and 3 M was added separately into 1 ml of 50 mM Tris-Cl buffer containing 5 mg/ml total soluble protein. Temperature was increased from 16 to 40°C with 2°C increments. The turbidity at 4°C was set as the blank, and increased with temperature, identifying the onset of the inverse temperature transition. The value of T_t is defined as the temperature at which 50% relative turbidity occurs. The relative turbidity of 100% was set as an absorbance value of 5 mg/ml total protein at 60°C that was denatured.

Purification by Inverse Phase Transition Cycling (ITC)

After NaCl was added to the soluble cell lysate to make 2 M and 3 M, respectively, they were incubated at the phase transition temperature for 10 min. The aggregated fusion protein was separated by centrifugation at 16,000 ×*g*, 30°C for 5 min. The supernatant was discarded, and the pellet was dissolved in 50 mM Tris-Cl (pH 8.0) buffer at 4°C. The solution was centrifuged at 4°C for 15 min [14, 15]. Each step of purification was monitored by SDS-PAGE and measurement of the levansucrase activity.

Levansucrase Activity Assay and Measurement of Protein Concentration

Levansucrase activity was determined spectrophotometrically at 450 nm according to the method of O'Mullan *et al.* [6, 17]. Since levansucrase releases glucose from sucrose, glucose concentration was measured using a glucose oxidase-peroxidase coupled colorimetric kit. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmole of glucose per minute.

The protein concentration was measured using 0.1 ml of 0–1.4 mg/ml protein sample. Pre-mixed Bradford reagent (Sigma Co.) using Coomassie blue was prepared with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Construction and Expression of ELPs-tagged Levansucrase

The transition temperature and reversible character of the ELPs tag and ELP fused recombinant protein are influenced by the hydrophobicity of the guest residue and ELPs molecular mass [28]. The transition temperature is reduced by the composition of high hydrophobic scale amino acids at the guest residue and repeat number of pentapeptides. With valine as the only guest residue to fix the hydrophobicity, the effect of ELPs size (molecular mass) on purification of levansucrase was investigated. In previous Chilkoti's results, the transition temperature of ELP[V-20] with thioredoxin fusion protein in PBS with 1.3 M NaCl was 33°C. It showed the successful separation property and the suitable temperature for protein stability. The ELP[V-20] and ELP[V-40] were selected to give appropriate separation properties to target protein, levansucrase, and to compare transition temperature change dependent upon target protein. ELP [V-5], consisting of five repeated pentapeptides, was used as a template for ELPs tags of varying sizes. To construct the ELP[V-5] monomer gene, four synthetic oligonucleotides were annealed to form double-stranded DNA using PCR. Table 1 lists the oligonucleotides sequences used for gene construction. Oligonucleotides Primer 1 and Primer 2 complementarily served as a template and a primer. Using the Primer 3 and Primer 4 containing the EcoRI, HindIII restriction enzyme site, complementary strands of 125 bases were synthesized. It contained not only EcoRI and HindIII but also PflMI, BglI restriction enzyme sites. The PCR product was inserted into the EcoRI and HindIII site of pUC19. It was then transformed into *E. coli* JM109 for amplification. ELP[V-5] gene was excised with PflMI and BglI. ELPs tags were oligomerized through the compatible cohesive ends of PflMI and BglI. ELP[V-15] was constructed by the first ligation because the size of the monomer gene containing ELP[V-5] and restriction enzyme sites was relatively smaller than the vector. Various sized ELPs tags from ELP[V-15] to ELP[V-80] were synthesized

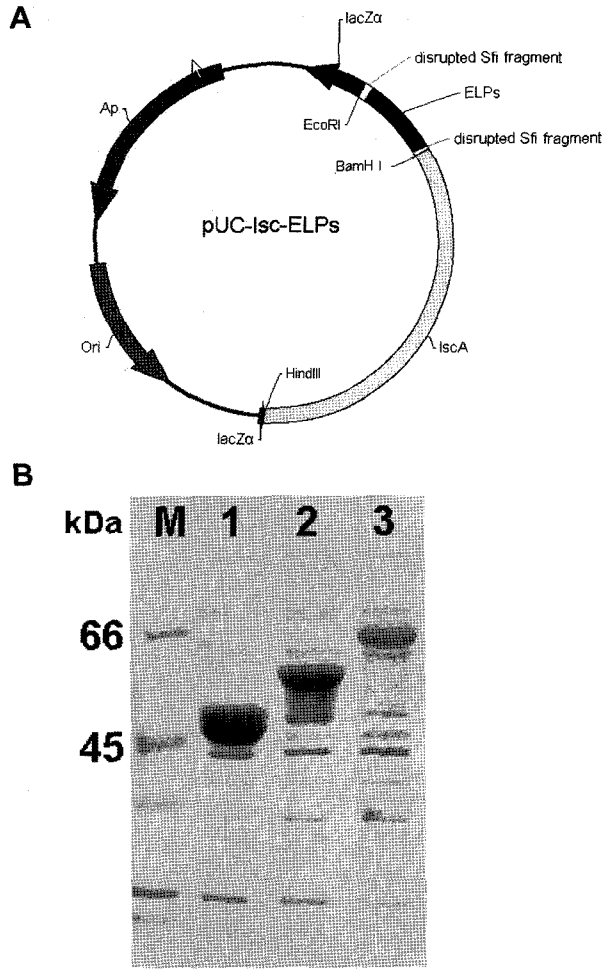

Fig. 1. Plasmid map and SDS-PAGE analysis of the levansucrase-ELPs fusion protein expressed in *E. coli*.

A. Expression of the ELPs-tagged *lscA* gene is regulated by the *lac* promoter. The *lscA* gene was inserted into the HindIII and BamHI site of pUC19 vector, and ELPs tag was fused at the C-terminal of *lscA*. The thrombin cleavage site was inserted between the *lscA* gene and ELPs tag. **B.** Levansucrase-ELPs fusion proteins were detected on 10% SDS-PAGE. The molecular mass of recombinant protein was gradually increased with increased molecular mass of fused ELP. Lane M, protein size marker; lane 1, levansucrase (control) (47 kDa); lane 2, levansucrase-ELP[V-20] (56 kDa); lane 3, levansucrase-ELP[V-40] (65 kDa).

by the serial ligations of ELP[V-5] and ELP[V-15]. Two kinds of ELPs gene, ELP[V-20] and ELP[V-40], were tested for levanscurase purification.

The levansucrase gene was amplified by PCR from the pLK1 vector containing the *Pseudomonas aurantiaca* S-4380 *lscA* gene. The levansucrase gene was composed of 1,275 bp coding for a protein of 424 amino acids, with a molecular mass of 47 kDa [5, 6]. The *lscA* gene was inserted into the HindIII and BamHI site of a pUC19 vector. The pUC-lsc vector was modified to introduce an SFI fragment for insertion of the ELPs sequence, a thrombin cleavage site, and a stop codon. ELP tags were fused at the C-terminus of the *lscA* gene (pUC-lsc-ELPs, Fig. 1A). The pUC-lsc-sfi vector without the ELP tag was constructed as a control (pUC-lsc-sfi) to confirm the effect of phase transition.

The protein expression in DH5α harboring the pUC-lsc-ELPs and pUC-lsc-sfi vectors was optimized at 37°C using LB medium containing 100 μg/ml of ampicillin. Since the expression of the *P. aurantiaca lscA* gene under control of the *lac* promoter in *E. coli* is growth dependent, the cell culture was maintained without induction for 18 h. After harvesting, the cell pellet was resuspended in 50 mM Tris-Cl (pH 8.0) and sonicated, yielding the supernatant containing ELPs-tagged levansucrase. Recombinant levansucrase in the soluble fraction of cell lysate was detected on 10% SDS-acrylamide gel (Fig. 1B). This gel showed a gradual increase of the 9 kDa ELP[V-20]. The molecular masses of levansucrase without the ELPs tag, levansucrase-ELP[V-20], and levansucrase-ELP[V-40] were 47 kDa, 56 kDa, and 65 kDa, respectively. The expression level of protein was calculated using an image analyzer. The apparent amount of target protein was greater than 70%, 50%, and 30% of the total soluble protein, respectively. However, ELPs could not be visualized by Coomassie blue staining because of a lack of aromatic side chains [12]. Therefore, it should be noted that the density of gel in Fig. 1B indicates the amount of expressed levansucrase without the influence of ELPs.

Determination of Optimum Conditions for Levansucrase Purification

The phase transition temperature of ELP-tagged levansucrase was optimized by salt concentration. The inverse phase transition of ELPs was investigated by comparing levansucrase with levansucrase-ELPs fusion protein. Salt concentration was set to 1, 2, or 3 M by adding NaCl into the soluble cell lysate to test the change of transition temperature with increasing NaCl concentration. The aggregation of levansucrase with ELPs at different salt concentrations is

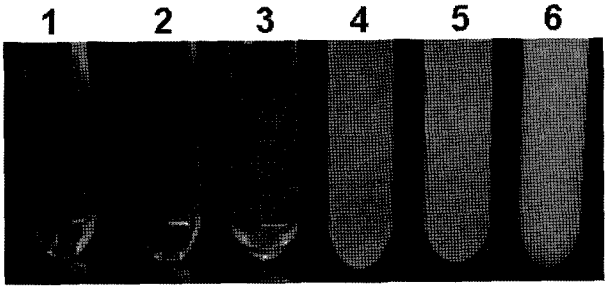

Fig. 2. Effect of concentrations of added NaCl on aggregation at room temperature.

Higher NaCl concentration and large ELPs tag showed easier phase transition. The result confirmed that the aggregation of ELPs was not the salting out of the proteins. 1, Levansucrase (control) with 1 M NaCl; 2, levansucrase (control) with 2 M NaCl; 3, levansucrase-ELP[V-20] with 1 M NaCl; 4, levansucrase-ELP[V-20] with 2 M NaCl; 5, levansucrase-ELP[V-40] with 1 M NaCl; 6, levansucrase-ELP[V-40] with 2 M NaCl.

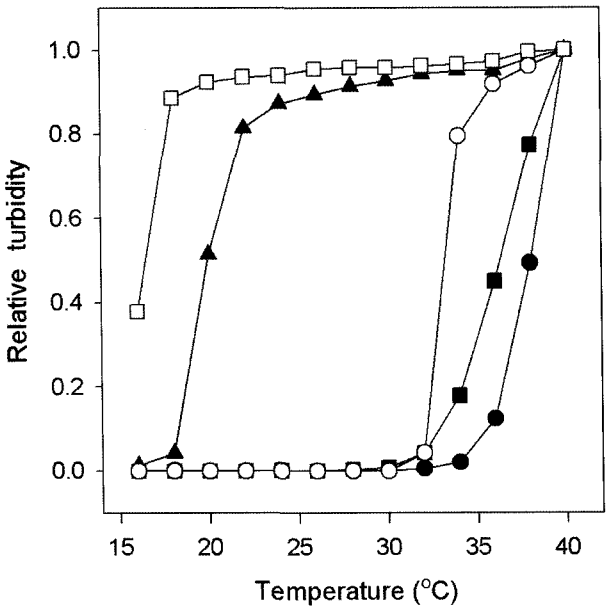

Fig. 3. Turbidity change of levansucrase-ELP[V-20] and levansucrase-ELP[V-40] with varying temperature. The turbidity profiles were obtained for ELPs-tagged levansucrase in Tris-Cl buffer with 1, 2, and 3 M NaCl from 20°C to 40°C.
●, Levansucrase-ELP[V-20] with 1 M NaCl; ■, levansucrase-ELP[V-20] with 2 M NaCl; ▲, levansucrase-ELP[V-20] with 3 M NaCl; ○, levansucrase-ELP[V-40] with 1 M NaCl; □, levansucrase-ELP[V-40] with 2 M NaCl.

shown in Fig. 2. The results verify that the precipitation resulted from phase transition, not from salting out by high NaCl concentration. Specifically, levansucrase-ELPs fusion protein showed the phase transition with the addition of salts, whereas levansucrase without an ELPs tag showed no change in turbidity.

The transition temperature for purification was determined by turbidity changes at OD_{600} with increasing temperature from 16 to 40°C. Fig. 3 shows the relative turbidities of each sample of levansucrase-ELPs with different salt concentrations. The phase transition of levansucrase-ELP[V-20] occurred at 38, 36, and 20°C in Tris-Cl supplemented with 1, 2, and 3 M NaCl, respectively. The phase transition temperature of levansucrase-ELP[V-40] was 33 and 17°C with 1 and 2 M NaCl, respectively. As the molecular mass of the ELPs tag and ionic strength increased, the transition temperature decreased. In previous studies, the T_t of 0.225 mg/ml and 40 mg/ml free ELP[V-20] was found to be 76°C and 48°C [12, 16]. The T_t of thioredoxin-ELP[V-20] was 37°C with 1 M NaCl [15]. The phase transition temperature could change depending on the size and characteristics of the fusion protein, as well as the sample purity and concentration. As described above, the T_t of ELPs was decreased by fusion with different proteins. In the cases of thioredoxin and levansucrase, two proteins would be analogous in terms of similar T_t despite different molecular masses. The size of levansucrase (47 kDa) is 4 times larger than thioredoxin (12 kDa). It is supposed that the different factors, for example, hydrophobicity, 3-dimensional structure, and molecular weight of target protein, affect the aggregation properties of the ELPs tag.

Purification by Inverse Transition Cycling (ITC) and Activity Assay

In order to assess its potential for application in industrial processes, ELP-levansucrase was purified using one-step centrifugation at mild temperatures from 30 to 40°C. Three and 2 M NaCl were added for levansucrase-ELP[V-20] and levansucrase-ELP[V-40], respectively, at 30°C. Note that the phase transition temperatures of each case were 20°C and 17°C, respectively. The precipitated levansucrase-ELPs protein was isolated by centrifugation at 16,000 ×*g* for 5 min after incubation for 10 min. In this case, centrifugation after incubation for 10 min provided better purification yield than the case without incubation (data not shown). Each step of purification by ITC was monitored by 10% SDS-PAGE gel (Fig. 4). It appeared that ELPs-tagged levansucrase was purified from contaminant proteins by one-step centrifugation. These results clearly demonstrate that the ELPs tag has a functioning property.

Utilizing methods outlined by O'Mullan *et al.* [17], the activity of levansucrase and ELPs-tagged levansucrase was

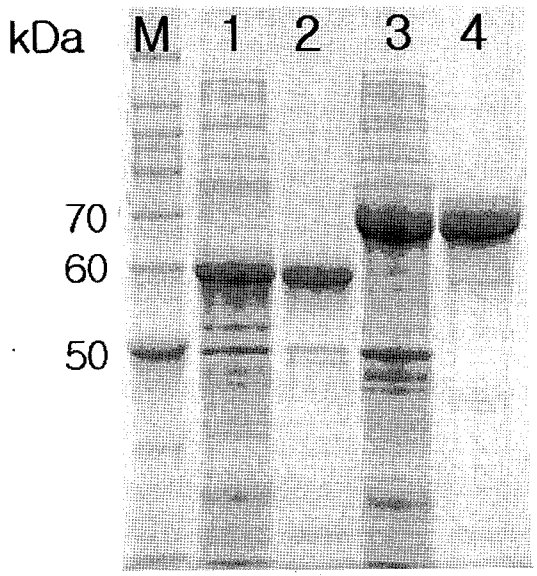

Fig. 4. SDS-PAGE analysis of ITC purification.
After ITC purification, the purity of ELPs-tagged levansucrase was compared with nontreated sample. More than 80% of recovery yield was achieved by one-step centrifugation. Lane M, protein size marker; lane 1, levansucrase-ELP[V-20] soluble fraction (control); lane 2, levansucrase-ELP[V-20] with 3 M NaCl after ITC; lane 3, levansucrase-ELP[V-40] soluble fraction (control); lane 4, levansucrase-ELP[V-40] with 2 M NaCl after ITC.

Table 2. Purification profile of ELPs-tagged levansucrase.

Sample	Levansucrase-ELP[V-20] with 3 M NaCl		Levansucrase-ELP[V-40] with 2 M NaCl	
	Before ITC	After ITC	Before ITC	After ITC
Activity (U)	498.75	400.11	340.36	332.32
Protein (mg)	5.00	2.31	5.00	1.94
Specific activity (U/mg)	99.75	173.21	68.07	171.30
Activity recovery (%)	80.2		97.6	

assayed. Sucrose conversion by levansucrase yields glucose in a 1:1 molar ratio to the amount of sucrose used. The amount of released glucose allows determination of the overall enzyme activity. Glucose concentration was measured with a Sigma glucose kit. Because other contaminating proteins from *E. coli* and added NaCl were removed by the phase transition and centrifugation of ITC step, an additional desalting step was not needed. Both levansucrase without the ELPs tag and levansucrase with the ELPs tag appeared to be active. The activities in each step of ITC are listed in Table 2. The recovery yield of levansucrase-ELP[V-20] with 3 M NaCl and levansucrase-ELP[V-40] with 2 M NaCl was about 80.2% and 97.6%, respectively. Specific activity of levansucrase with ELP[V-20] and ELP[V-40] was 173 and 171 U/mg, respectively. Host protein contamination was assured to be negligible. These results show that the protein activity was not interfered by the ELPs tag. Therefore, the ELPs-tag system is considered to be applicable not only for purification, but also for recycling of levansucrase, because the levansucrase-ELPs fusion protein showed activity after reverse phase transition.

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