Strain improvement of *Aspergillus sojae* for increased L-leucine aminopeptidase and protease production

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Abstract Conventional random mutagenesis was implemented to improve L-leucine aminopeptidase (LAP) and protease production in *Aspergillus sojae*. Through successive mutagenesis by ethyl methanesulfonate (EMS), UV, and 1-methyl-2-nitro-1-nitrosoguanidine (NTG), EMS25, EU36, and EUN13 mutants from each mutagenesis process were screened using a newly developed quick and easy screening method. The mutant EUN13 exhibited a 9.6-fold increase in LAP [50.61 ± 4.36 U/g-initial dried substrate (IDS)] and a 3.8-fold increase in protease production (13.36 ± 0.31 U/g-IDS) on solid-state fermentation. This mutant showed more frequent branching and higher *lap1* mRNA expression as compared to the parent strain SMF 131, which at least in part contributed to the increased LAP and protease production. The mutant EUN13 can be used as a starter organism for diverse industrial soybean fermentation processes for the production of conventional products such as *meju*, *doenjang*, and *ganjang* as well as for the production of new fermented soybean-based sauces.

Keywords L-leucine aminopeptidase (LAP) · Protease · *Aspergillus sojae* · Random mutagenesis · Soybean fermentation

Introduction

Soybean fermentation products such as *meju* (soybean brick), *doenjang* (soybean paste), and *ganjang* (soy sauce) have been consumed for a long in Korea. Such products have been produced traditionally depending on natural inoculation of various microorganisms. These traditional processes have several disadvantages with regard to difficulties in mass production, standardization, and safety concerns; therefore, starters have gained popularity for the industrial production of soybean fermentation products. In fact, recently, a new sauce, *yongdu*, was developed and commercialized by applying soybean fermentation technology to traditional soybean products such as *doenjang* and *ganjang* (Sempio, 2016). Therefore, although several starters have been developed and used for soybean fermentation in the food industry, the demand for appropriate starters, especially those that offer the advantages of flavor development/enhancement is continuously increasing.

Aspergillus oryzae is widely used as a fungal starter in industrial soybean fermentation of *meju*, *doenjang*, and *ganjang* in Korea. However, recent studies have suggested that *Aspergillus sojae* also possess merits as a starter for soybean fermentation. For example, this species has high proteolytic activity, which is an important characteristic for more effective breakdown of soybean proteins to peptides and various amino acids during fermentation (Kim et al., 2017; Sato et al., 2011). In addition, *A. sojae* has a high L-leucine aminopeptidase (LAP, EC 3.4.11.1) activity (Kim et al., 2017; Nampoothiri et al., 2005), which contributes to flavor development in soybean fermentation products. LAP plays an important role in the production of low molecular weight peptides (Toldrá et al., 2000). LAP also decreases the bitterness of soybean fermentation products by

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hydrolyzing hydrophobic amino acid residues such as leucine from the amino-terminal regions of the peptides.

In a previous study, we isolated several *A. sojae* strains from *meju*, collected from across Korea. Among them, *A. sojae* SMF 131 showed the highest LAP and protease production (Kim et al., 2017). Although its LAP and protease production capability is sufficient for industrial applications, strain improvement is believed to optimize its industrial applicability. Presently, molecular genetic manipulation techniques are frequently adopted for strain improvement. However, the conventional random mutagenesis processes using physical and/or chemical mutagens are widely used for strain application in the food industry because of critical issues involved with the use of genetically modified organisms (GMOs) (Abdullah et al., 2013; Adrio and Demain, 2006; Chand et al., 2005; Heerd et al., 2014; Javed et al., 2010).

In this study, we developed a fast and convenient screening method to select a strain with both high LAP and protease production capability. With the application of this screening method, we could successfully select mutants demonstrating significantly improved production of LAP and protease after EMS, UV irradiation, and NTG treatments. The selected mutants were characterized to understand the mechanisms that increased the production of LAP and protease.

Materials and methods

Strain and media

Several *A. sojae* have been previously isolated from *meju* (Korean fermented soybean brick) (Kim et al., 2017). Among them, *A. sojae* SMF 131 showed the highest LAP and protease activities and was thus subjected to mutagenesis to further improve its LAP and protease production capabilities. *A. sojae* SMF 131 and mutants were routinely cultured on potato dextrose agar (PDA) plates, and their spores were collected with 0.1% Tween 80 after culturing them on PDA plates for 3 days at 30 °C. For screening mutants, lactose minimal media (LMM) (Lee et al., 2009) supplemented with 1% skim milk (LMM-S) or LMM supplemented with 1% skim milk and 0.02% leucine- β -naphthylamide (L β NA) (LMM-SN) was used. The soybean solid-state fermentation (SSF) medium was prepared by mixing 20 g of crushed soybeans with 13 mL of 0.22% ZnSO₄·7H₂O solution, as described elsewhere (Kim et al., 2017).

Mutagenesis

Spores (100 μ L, 1×10^7) were inoculated into 50 mL of potato dextrose broth (PDB) and cultured at 30 °C with shaking at 200 rpm until the length of the germ-tubes reached about 5 times the spore diameter. The incubation time was different for each strain (average: 6–7 h). The germlings were recovered by centrifugation at 6000 $\times g$ and resuspended in 50 mM of sodium phosphate buffer. For EMS mutagenesis, the germlings were treated with EMS at concentrations of 0–2 M. For UV mutagenesis, the resuspended germlings were exposed to UV for 0–180 min. For NTG mutagenesis, the germlings were treated with NTG at concentrations of 0–3 μ g/mL. The samples exhibiting 10–20% survival were analyzed for screening mutants with improved LAP and protease production. The UV-treated germlings were immediately cultured on the selection media. Germlings treated with EMS or NTG were washed with 50 mM sodium phosphate buffer (pH 7.5) to remove chemical mutagens by discarding supernatants after centrifugation at 6000 $\times g$. This washing step was repeated 3 times.

Mutant screening

The mutants were screened for improved protease production first and then for improved LAP production. First, the mutagen-treated germling suspension (100–200 μ L) was spread onto LMM-S plates. Colonies that formed a larger clear zone as compared with the untreated strain (control) during the first 2 days of incubation were selected as mutants for the further analysis of protease activity. For screening improved LAP producers, the LMM-SN plates containing L β NA were prepared as per the LAP zymography method (Finlay and McCloud, 1990). L β NA was used to visualize the LAP activity: Naphthylamine was released from L β NA by LAP and subsequently stained with the Fast Black K dye to develop purple color as a result of the interaction with the released naphthylamide (Manildi, 1972). Therefore, the intensity of the purple color surrounding a colony indirectly indicated the extent of LAP activity. The primarily screened mutants of high protease activity were pure-cultured on PDA plates for 2 days. The agar plug of the isolated mutants were punched out from PDA plates using a 200 μ L micropipette tip and then transferred to LMM-SN plates, and followed by incubation for 24–48 h at 30 °C. L β NA showed antifungal activity; therefore, a colony transferred from PDA did not grow but produced the enzyme on the media. After formation of a clear zone around the transferred plug, the plates were stained with a 0.06% Fast Black K solution at 37 °C for 15 min. The colonies were selected on the basis of the intensity of the purple color and the size of the purple zone.

Enzyme production and assay

To evaluate enzyme production by SMF 131 and mutant strains, 1 mL of spores (1×10^7) was inoculated into 20 g of soybean SSF media and then incubated for 72 h at 30 °C and 50% relative humidity (RH). The sample (10 g) was then removed and homogenized with 190 mL of 2% NaCl solution. The homogenized samples were incubated for 15 min at 25 °C with shaking at 200 rpm to extract the enzymes. The crude enzyme extracts were prepared by filtration of the extracts through Whatman No. 2 filter paper (GE Healthcare, Little Chalfont, UK).

The LAP assay was performed as described previously with slight modifications (Kim et al., 2017). The reaction mixture comprised of 1.5 mL of 100 mM sodium phosphate buffer (pH 7.0), 1.3 mL of 3-distilled water, 0.1 mL of substrate, and 0.1 mL of crude enzyme extracts in a Falcon tube. The substrate L-leucine-*p*-nitroanilide (LpNA) was dissolved in methanol to yield a 24 mM solution. After incubation at 50 °C for 10 min in a water bath, the reaction was terminated by adding 50 µL of acetic acid and placing each Falcon tube on an ice bath. For blank preparation, 50 µL of acetic acid was added to the enzyme reaction mixture before adding crude enzyme extracts to stop the enzyme reaction, followed immediately by absorbance measurement. Before and after the reaction, 200 µL of each sample was dispensed into a 96-well plate, and the absorbance was measured at 405 nm using a spectrophotometer (Infinite M200 PRO; Tecan, Männedorf, Switzerland). One enzyme unit of LAP activity is defined as the amount of enzyme that can hydrolyze 1 µmol of LpNA in 1 min (Chien et al., 2002).

Protease activity assay was performed as per the method described by Kim et al. (2017), which is based on the Sigma's nonspecific protease assay method (Sigma-Aldrich Chemical Co., St. Louis, USA).

Fungal biomass measurements

During soybean SSF, the fungal biomass was measured by ergosterol analysis, as described by Seitz (1979). Freeze-dried sample was ground using a mortar and pestle and the ground sample (500 mg) was then transferred to a 50-mL conical tube. After vortexing with 10 mL methanol (HPLC grade) for 1 min, ergosterol was extracted by rotating the tube in a hybridization oven at 50 °C for 30 min. The mixture was centrifuged at $1500 \times g$ and then settled for 5 min at the room temperature. The clean extract (5 mL) was removed and transferred to a new 25 mL screw-capped vial containing 1 g potassium hydroxide. The mixture was vigorously vortexed to dissolve thoroughly and then settled and cooled to the room temperature for 15 min. Next, 7 mL of n-hexane was added to the vial, vortexed, and

incubated at 75 °C in a water bath for 30 min, with shaking at 150 rpm, followed by cooling to the room temperature. The upper hexane layer (4 mL) was decanted with a pipette and transferred to a test tube. Then, 5 mL of n-hexane was added to the remnant in a screw-capped vial and mixed vigorously. The upper layer (5 mL) was again removed and added to the earlier aliquot. This procedure was repeated once more. The three pooled hexane extracts (totaling 14 mL) were evaporated in a water bath maintained at 75 °C. The residue was re-dissolved in 700 µL of methanol (HPLC grade) and filtered through a 0.45 µm PVDF filter (PALL, New York, USA). The ergosterol content was determined by HPLC on a C18 reverse column (Supelco, 3 µm pore size, 4.5, 15 cm). The mobile phase of methanol:water (96:4 v/v) was flowed at the rate of 1 mL/min, and injection volume was 20 µL. The column temperature was maintained at 30 °C, and the absorbance of the eluted ergosterol was detected at 282 nm. The standard ergosterol (Sigma Aldrich Corp., St. Louis, MO, USA) had a retention time of 11 min. The formula for converting the ergosterol content to biomass weight was established by measuring the ergosterol contents of known mycelial weight. Using this formula, the ergosterol contents of the soybean SSF products were converted to their respective mycelial weights.

Morphological observation

For the observation of colony morphology, 10 µL of spores (1×10^7 spores/mL) of each strain was 1-point inoculated onto the center of a PDA plate and incubated for 7 days at 30 °C. The shape, color, and sporulation of the colony obtained were noted during incubation. The diameter of each colony was measured every 24 h, and the radial extension rate (mm/h) was calculated as increase in colony diameter per hour. The shape of the conidia was observed microscopically.

For the observation of detailed hyphal structure, the spore suspension (1×10^7 spores/mL) was applied to the four sides of a PDA cube ($1 \times 1 \times 0.5 \text{ cm}^3$) which was placed on a slide glass. This slide glass was placed on the top of two toothpicks that were placed on a PDA plate. Moistened tissue paper was kept inside a plate to prevent drying of the agar cube. This plate was incubated for 48 h at 30 °C. Hyphal branching was observed every 24 h using a microscope until the hyphae grew tightly.

RNA isolation and real-time RT-PCR analysis

Real-time RT-PCR was used to measure the relative expression levels of *lap* genes in SMF 131 and mutants as described previously (Hong et al., 2015) with some modifications. Samples of soybean SSF prepared by the same

method as for enzyme extraction were lyophilized. RNA was isolated from the lyophilized powder using TRIzol[®] reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) as per the manufacturer's manual and then purified using the Qiagen Cleaning Kit (Qiagen, Hilden, Germany). The isolated RNA was treated with DNase I (Thermo Fisher Scientific Inc.), and the cDNA was synthesized by using the Thermo's cDNA Synthesis Kit (Thermo Fisher Scientific Inc.). RT-PCR was performed using the CFX96 (BIO-RAD, Hercules, CA, USA) with the primers for *lap1* (RT-lap1F: 5'-GAGCGCATCAGGACTCCATC-3' and RT-lap1R: 5'-CCTCTCCGGCATAGAAGTGG-3'), *lap2* (RT-lap2F: 5'-CGCTTTTGTGGGCTTCGTTG-3' and RT-lap2R: 5'-TAGCCAGTCTTCTTCAGCTC-3') and *lap3* (RT-lap3F: 5'-TCCACAGCAGGCACTGTTG-3' and RT-lap3R: 5'-ACTAGCCTGGTGCAGTGTGG-3'). The amplification mixture comprised of 10 µL of the SoFast[™] EvaGreen[®] Supermixes (BIO-RAD), 10 pmol of each primer, and 10 ng of DNA template in a final volume of 20 µL. After pre-denaturation at 95 °C for 10 min, amplification was performed in 45 cycles, as follows: denaturation at 95 °C for 10 s, annealing at 70 °C for 2 s, and elongation at 72 °C for 20 s. The relative expression levels were calculated using the Bio-Rad CFX Manager[™] Software (version 1.6) by using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001). All the values were normalized using the expression level of the β -tubulin gene as a reference and the SMF 131 *lap1* expression level as a calibrator.

Genomic DNA preparation, cloning, and sequencing

Genomic DNA was isolated from freeze-dried mycelial powder as described previously (Kim et al., 2017) using the Promega Wizard DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's manual.

The *lap1* gene was amplified with primers (lap1F: 5'-GTGCCTATGGATCATGCATC-3' and lap1R: 5'-CAACTACCTTAGCCTCTTGG-3') by PCR. PCR was performed using the C1000[™] Thermal Cycler (BIO-RAD). The amplification mixture comprised of 10 µL of the GoTaq Green Master Mix (Promega), 10 pmol of each primer, and 10 ng of DNA template in a final volume of 20 µL. After pre-denaturation at 95 °C for 3 min, amplification was performed in 30 cycles of reaction (denaturation at 95 °C for 1 min, annealing at 55 °C for 2 min, and elongation at 72 °C for 30 s), followed by a final extension at 72 °C for 10 min. The PCR amplified products were cloned by using the pTOP_Blunt_V2 Kit (Enzymomics, Daejeon, Korea). Sequence analysis was performed by primer walking by Macrogen Inc. (Seoul, Korea). The *lap* promoter region was amplified with primers (Promoter-lap1F: 5'-GAAGAGGTGCCAAGTGTTGC-3' and Promoter-lap1R: 5'-GCAGCCACTCGGATGATTTC-3')

using *pfu* polymerase, and the PCR products were directly sequenced by Macrogen Inc. The amplification mixture comprised of 10 µL of the GoTaq Green Master Mix (Promega), 10 pmol of each primer, and 10 ng of DNA template in a final volume of 20 µL. After pre-denaturation at 95 °C for 3 min, amplification was performed in 30 cycles of reaction (denaturation at 95 °C for 1 min, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s), followed by a final extension at 72 °C for 1 min.

Results and discussion

Sequential mutagenesis for the improvement of LAP and protease production

Previously, we selected *A. sojae* SMF 131 that showed the highest LAP and protease production from among the *A. sojae* wild-type strains isolated from various *meju* (Kim et al., 2017). To improve the applicability of *A. sojae* in the food industry for soybean fermentation, conventional mutagenesis was implemented (Fig. 1). Because a large number of mutant colonies are generated during mutagenesis, it is important to select mutants efficiently and quickly. Therefore, we developed a quick method for screening both protease and LAP activities in the same plate, as described in the Materials and Methods section.

Briefly, we first treated SMF 131 with EMS at concentrations of 1–1.4 M to yield a survival rate of 10–20%. EMS25 exhibited the highest LAP activity [5.28 U/g-initial dried substrate (IDS)] on soybeans for 72 h SSF from among 10 mutants that were primarily selected by plate screening. Then, EMS25 was subjected to UV irradiation for 100–140 min, which resulted in approximately 10–20% survival rate. We primarily selected 263 mutants showing a large clear zone on LMM-S plates and then selected 33 mutants that showed a strong intensive purple color after Fast Black K staining on LMM-SN plates. EU36 was finally selected by quantitative activity measurements of 10

Fig. 1 Sequential mutagenesis for the improvement of LAP and protease production

selected mutants after soybean SSF because it exhibited the highest LAP activity (15.91 U/g-IDS) from among the UV-treated mutants of EMS25. Finally, EU36 mutant was treated with 0.05–0.1 µg/mL NTG to yield a survival rate of 5–10%. In the same way for EU13 screening, 315 and 27 mutants were selected at primary and second screenings, respectively. EUN13 was finally screened from the NTG-treated mutants of EU36 as it exhibited the highest LAP activity (50.61 U/g-IDS). The mutants showing high LAP activity also showed high protease activity probably because we used a plate assay designed for the screening of both LAP and protease production. In summary, EUN13, which was selected through sequential mutagenesis and screening, exhibited 50.61 U/g-IDS of LAP and 13.36 U/g-IDS of protease production during soybean fermentation, which showed 9.6- and 3.8-fold increases in comparison with the parent strain SMF 131, respectively (Table 1). These levels of LAP and protease activities were the highest among the isolated *A. sojae* and some *kaji* strains used in Japanese soy sauce production. According to Kim et al. (2017), naturally found *A. sojae* and the Japanese *kaji* strains produce 1.35–10.64 U/g-IDS protease and 0.63–6.06 U/g-IDS LAP. Because we used conventional mutagenesis, which is an accepted method for strain improvement in the food industry, the mutants can be used for food production without any limitations. Therefore, EUN13 is believed to serve as an economically advantageous starter organism in soybean fermentation.

The increases in LAP and protease production in mutants may be attributed to the faster biomass increase. In fact, these mutants had more mycelia than the parent strain, with the highest recorded for the EUN13 (Table 2). The mutants EMS25 and EU36 showed similar LAP activity/biomass, indicating that the high LAP activity in these mutants may have resulted from excessive mycelial biomass. However, EUN13 showed over two-fold increased LAP activity/biomass in comparison with the parent strain SMF 131, suggesting that some other factors also affected the LAP activity other than the biomass increase. Conversely, protease activity/biomass increased in all mutants, suggesting that while mutations affected protease

production positively, the mechanisms for such an increase remain to be elucidated.

Morphological characteristics of mutants

The mutagenesis process is often accompanied by morphological changes, which are sometimes related to enzyme production (Te Biesebeke et al., 2005; Xu et al., 2011). The colony morphology of the mutants was similar to that of the parent strain SMF 131; however, the mutant colonies were slightly smaller than those of the parent strain, with delayed conidiation (Fig. 2). Quantitative analysis of the colony growth revealed that the radial extension rates of EMS25, EU36, and EUN13 were 0.261, 0.265, and 0.236 mm/h, respectively, which are slightly lower than that of the parent strain SMF 131 (0.299 mm/h). However, the decreased radial extension rate did not necessarily mean slow mycelial growth because the dried mycelial weight was greater in mutants than in the parent strain (Table 2). These observations suggest that the mutants probably formed more compact colonies.

Spores appeared after 2 days in SMF 131, EMS25, and EU36, whereas they appeared after 3 days in EUN13. In addition, EUN13 produced lesser spores than the parental strain after 48 h of culturing. However, the numbers of conidia produced after 7 days of culturing on soybeans were indistinguishable between SMF 131 and EUN13, suggesting that conidiation of EUN13 was similar to that of the parent strain, albeit delayed. The growth rate and sporulation are important criteria for the industrial applications of a fungus. The slightly slower growth and delayed conidiation in EUN13 does not limit its application as a starter for soybean fermentation, because it grew and generated spores comparably well after 72 h of soybean fermentation as compared to that by the parent strain SMF 131.

Interestingly, mutants exhibited more frequent hyphal branching than the parent strain (Fig. 2B). Hydrolytic enzymes are mainly secreted at the tip of filamentous fungi, and a fungus with more frequent branching has the tendency to secrete more proteins (Müller et al., 2002; Wösten et al., 1991). Therefore, frequent branching in mutants is

Table 1 Protease and LAP production of *A. sojae* SMF 131 and selected mutants

Strain	LAP activity (U/g-IDS)	Relative LAP activity ^a	Protease activity (U/g-IDS)	Relative protease activity ^a
SMF 131	5.28 ± 0.81	1.00	3.55 ± 0.16	1.00
EMS25	15.91 ± 1.33	3.01	8.68 ± 0.50	2.44
EU36	29.10 ± 1.32	5.51	11.55 ± 1.05	3.25
EUN13	50.61 ± 4.36	9.58	13.36 ± 0.31	3.76

^aValues represent the relative activity of mutants to the parent strain *A. sojae* SMF 131

Table 2 Dry mycelium weight and LAP activity/biomass of *A. sojae* SMF 131 and selected mutants after 3 days of fermentation

Strain	Dry mycelium weight (mg)	LAP activity/biomass (U/g-IDs/mg)	Protease activity/biomass (U/g-IDs/mg)
SMF 131	35.12 ± 16.16	0.287 ± 0.097	0.150 ± 0.023
EMS25	57.04 ± 6.203	0.235 ± 0.032	0.278 ± 0.023
EU36	60.52 ± 10.10	0.320 ± 0.065	0.480 ± 0.021
EUN13	68.41 ± 12.56	0.677 ± 0.152	0.739 ± 0.063

Fig. 2 Morphological characteristics of *A. sojae* SMF 131 and selected mutants. **(A)** Colony morphology. Each strain was 1-point inoculated on PDA and incubated at 30 °C for 24 h (top panel) and 48 h (bottom panel). **(B)** Hyphal branching. Each strain was cultured

on a slide, and its hyphae were observed under a microscope. The arrow indicates frequent branching in the mutants as compared to that in SMF 131

believed to in partially contribute to the higher production of LAP and protease. This result was supported by high LAP and protease production per biomass in especially EUN13 (Table 2).

mRNA expression level of *lap*

RT-PCR analysis was performed to examine whether the mRNA level of *lap* had increased in mutants. Recently, our analysis revealed that the *A. sojae* genome contained three *lap* genes (unpublished data). As shown in Fig. 3, the *lap1* gene was mainly increased in mutants with by

approximately sevenfold, 18-fold, and 50-fold in EMS25, EU36, and EUN13, respectively, compared with that in the parent strain SMF 131. These increases in the mRNA expression level were presumed to be because of changes in the promoter sequence; however, no sequence change was noted for the *lap1* gene from any of the mutant (data not shown). Although the reasons for the increase in the *lap1* expression level remain to be elucidated, the enhancer element or the genes coding for the transcription factors are believed to have been affected (Foreman et al., 2003; Le Crom et al., 2009; Liu et al., 2013; Molzahn, 1977; Vitiainen et al., 2010). Complex mutations may be induced

Fig. 3 The *lap* genes expression levels of *A. sojae* SMF 131 and selected mutants. The gene expression levels were measured by RT-PCR, and the β -tubulin gene was the denominator. Values are the relative expression levels to the *lap1* gene expression of SMF 131

during 3 successive different mutagen treatments. Whole genome analysis should probably address how and where a mutagenesis process can affect the production of LAP and protease in mutants. Interestingly, LAP production increased by only about 10-fold although the mRNA expression level of the *lap1* gene increased by about 50-fold in EUN13. It is well-known that enzyme production is not always proportional to its mRNA expression level because of the possible bottlenecks at the level of translation and at the post-translational level, such as inefficient translocation, folding, transport, process, or secretion (Ward, 2012).

Soybean SSF fermentation

To evaluate LAP and protease production of mutants over fermentation time, LAP and protease production was measured during SSF using steamed soybeans. The growth patterns of SMF 131 and mutants were found to be extremely similar [Fig. 4(A)]. LAP and protease activities were not detected in the samples of 24 h culture, but the activities increased as the fermentation period progressed in all strains [Fig. 4(B), (C)]. EUN13 showed the highest LAP and protease activities for every fermentation period and reached the maximum LAP (71 ± 0.58 U/g-IDS) and protease activity (18.81 ± 0.09 U/g-IDS) after 144 h. In contrast, the SMF 131 and other mutants reached their maximum activities after 72 h of fermentation.

Genetic stability for enzyme production

Mutants sometimes have a tendency to revert to their wild types (Al-Jailawi et al., 2016; Molzahn, 1977; Simpson and Caten, 1979). Therefore, the genetic stability of a mutant is an important criterion for the application of industrial strains. The mutants EMS25, EU36, and EUN13 showed a

Fig. 4 Production of LAP and protease during soybean solid-state fermentation. Each strain was cultivated on solid-state fermentation with crushed soybeans at 30 °C. Growth was monitored by measurements of the ergosterol contents (A). Crude enzymes were prepared by extracting fermented soybeans with 2% NaCl and assayed for LAP (B) and protease (C) at every 24 h. All fermentation processes were performed in triplicate

slight increase in the levels of LAP (149% of the first culture for EMS25, 113% for EU36, and 107% for EUN13) and protease (120% of the first culture for EMS25, 112% for EU36, and 106% for EUN13) after successive subcultures for 10 generations, which suggests that they were genetically stable for enzyme production.

In this study, the mutants exhibited fermentation characteristics on soybean SSF similar to those of the parent strain SMF 131, except for increased LAP and protease

production. Therefore, mutants developed through this study, especially EUN13, will be useful as a starter for diverse soybean fermentation processes to produce conventional products such as *meju*, *doenjang*, and *ganjang* as well as to produce new fermented soybean-based sauces.

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