

Characterization of Chaperone-Like Activity of Small Heat Shock Protein (sHSP) Isolated from Indonesian Traditional Food (Tempoyak) *Lactobacillus plantarum* U10

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The characterization of small heat shock protein (sHSP) from tempoyak-originated *Lactobacillus plantarum* was investigated. The heat adaptive response proteins were ranging from 18 kDa to 51 kDa. Interestingly, the Intercellular Protein (IP) fraction of heat shocked-*L. plantarum* U10 exhibited chaperone like activity by the ability to prevent loss of proteinase K activity from denaturation. Furthermore, The sHSP gene that related to the predicted sHSP ±18 kDa protein were successfully identified by PCR method and this gene has 423 bp size. The sHSP gene has 140 amino acids (with unique motive at C-terminus T-L-P-K amino acid sequence) and has closely 100% identity with those of *L. plantarum* isolated from food or non-food environment. Moreover, the gene encoding sHSP ±18 kDa protein was indeed up-regulated after *L. plantarum* U10 treated by heat shocking as proven by Reverse Transcriptase-PCR. This result suggested that sHSP ±18 kDa in our study may confers a survival advantage on *Lactobacillus plantarum* and capable of protecting the cell against under temperature stress.

Key words: chaperone assay, heat shock protein, *Lactobacillus plantarum* U10, RT-PCR

Karakterisasi gen *small heat shock protein* (sHSP) dari *Lactobacillus plantarum* U10 yang diisolasi dari makanan tradisional Indonesia tempoyak telah diteliti. Protein yang berhubungan dengan respon adaptif panas mempunyai ukuran yang bervariasi antara 18 kDa sampai 51 kDa berdasarkan pemisahan SDS-PAGE. Fraksi interselular protein (IP) dari *L. plantarum* yang telah diberikan kejutan panas menunjukkan bahwa protein pendamping (chaperon) dibuktikan oleh kemampuan untuk mencegah hilangnya aktivitas proteinase K dari denaturasi dengan uji chaperon. Selain itu, gen sHSP yang diprediksi sHSP ±18 telah berhasil diidentifikasi dengan metode PCR dan memiliki ukuran 423 bp dengan 140 asam amino dengan motif khas pada C-terminal (urutan asam amino T-L-P-K) dan memiliki identitas mendekati 100 % dengan *L. plantarum* lain yang diisolasi dari makanan atau lingkungan. Gen penyandi sHSP ±18 dengan pemberian kejutan panas dibuktikan dengan Reverse Transcriptase PCR (RT-PCR). Hasil ini menunjukkan bahwa sHSP ±18 kDa dapat memberikan manfaat untuk kelangsungan hidup *L. plantarum* dan mampu melindungi sel dari tekanan panas.

Kata kunci: *chaperone assay*, *heat shock protein*, *Lactobacillus plantarum* U10, RT-PCR

Lactic acid bacteria (LAB) are “generally regarded as safe” (GRAS) organisms that are widely utilized as starter culture, food preservative, and flavour enhancer in the food and beverage industry. Recently, LAB have been used for unconventional purposes including production of heterologous proteins, metabolic engineering, and vaccine delivery (de Vos and Hugenholtz 2004). The vast applicability of these lactic acid producer bacteria because of their ability to resist and well adapt in variable environments (Belfiore *et al.* 2013; Song *et al.* 2014; Wu *et al.* 2014).

The principal protein that responsible for wide adaptability of LAB in different environment which is

a group protein called heat shock proteins (HSP) (Broadbent *et al.* 1997). These chaperon proteins, although not all HSP contain chaperonin activity, will correct the expressed proteins to prevent it from misfolding event, allowing resumption of normal and physiological activity and leading to a higher level of stress tolerance when LAB face environmental stress conditions (Haslbeck *et al.* 2002; Narberhaus 2002). Depend on the type of stress, expression of these chaperon proteins by LAB will also differ. Generally the small heat shock proteins (sHSP) have a major role in LAB adaptation and survival among the others (Guzzo 1996; Koponen *et al.* 2012). The molecular mass of sHSP range from the smaller 18 kDa up to 30 kDa. For instance, sHSP expressed by *Leuconostoc oenos* (heat shock 42 °C, acidic shock pH 3, and ethanol

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shock 12%) has molecular mass of 18 kDa (Guzzo *et al.* 1997), sHSPs expressed by *Lactobacillus plantarum* (heat shock 37 or 40 °C, cold shock 12°C, solvent shock ethanol 12% or buthanol 1%) have molecular mass of 18.5 kDa, 18.55 kDa and 19.3 kDa (Capozzi *et al.* 2012; Fiocco *et al.* 2007). Based on their primary structure, the sHSP is classified into two classes, A and B. The B class has longer N-terminal region and shorter C-terminal region than the A class (Münchbach *et al.* 1999).

L. plantarum becomes one of the most applicable bacterium in food industries. For instance, the ability of this bacteria to produce natural antibiotics has gradually replaced the use of synthetic antibiotic as a food preservative (da Silva Sabo *et al.* 2014). *L. plantarum* U10 from traditional Indonesia fermented food named Tempoyak (fermented durian fruit) has been isolated and characterized (Urnemi *et al.* 2010). This strain exhibited excellent antibacterial activity against broad pathogenic bacteria, thus this strain has potential application as natural antibacterial agent (Urnemi *et al.* 2010). To the best of our knowledge, there are few studies on the function of sHSP and respective gene on the growth and survival of *L. plantarum* especially those which habitating fruit-based fermented foods. Therefore, we aim to characterize the function of the chaperone-like activity containing small heat shock protein expression under in vitro heat stress condition and to elucidate its respective gene.

MATERIALS AND METHODS

Heat Shock Treatment. *L. plantarum* U10 were cultured in 100 ml MRS (*de Man, Rogosa, Sharpe*) medium and incubated at 37 °C overnight. Cells were harvested by centrifugation at 10 000 × g when the OD₆₀₀ was ~0.6. The pellets were resuspended with 20 mL MRS medium. The heat shock treatment at 42 °C : (a) Control (without heat shock), (b) heat shock 30 s, (c) heat shock 45 s. After the treatment, the cells were kept at room temperature for 20 min. The survival rate of cells was monitored by counting CFU on agar after incubation at 37 °C overnight. All experiments were carried out in triplicate (Delmas *et al.* 2001; Guzzo *et al.* 1997).

Cell-free Supernatant Extraction. The method followed as described by Birdsell and Cota-Robles 1967. Cells pellet was mixed with 20 µg µL⁻¹ of lysozyme, 0.5 M of sucrose, and 1 mM EDTA and let stand for 1 h at 37 °C to disrupt the cell membrane

enzymatically. Afterward, the disrupted cells were subjected for freeze thaw treatment to increase cell lysis for three times with each repetition for 1 h (30 min of freeze and 30 min thaw) before pellets was sonicated. Then, 100 mL of buffer B (Tris HCl 10 mM pH 8.5, NaCl 100 mM, and tween-20 0.25%) was added into the pellet, and sonicated for 15 s, interval one minutes with five times repetition. The extract was isolated by centrifugation at 17 000 × g, 4 °C for 20 min. The supernatant or extracellular fluid was move to sterile falcon then pellets subjected to SDS-PAGE.

Chaperon Activity Assay The method followed methods from Collada *et al.* 1997 and Kim *et al.* 1998 by modification. Chaperon activity assay of intracellular proteins fraction (IP) of *L. plantarum* U10 was conducted on 0.2% gelatin-containing agarose using proteinase K. A total of 0.25 grams of agarose was dissolved in 50 mM Tris-HCl pH 7.4 and then heated. Into the agarose solution then added 2.5 mL of 2% gelatin, stirred until homogeneously mixed. A 25 mL of agar solution was poured to the plate, let it harden and create wells using tips. There are several combinations for each sample: Sample 1; proteinase K mixed with IP (1:1, w/w) then denaturated for 15 min at 100 °C, Sample 2; proteinase K mixed with IP (1:1, w/w) without denaturation, Sample 3; IP without denaturation, Sample 4; protease K only (4 µg µL⁻¹) denaturated for 15 min at 100 °C, Sample 5; proteinase K only (8 µg µL⁻¹). The agarose plate was incubated for 24 h at 37 °C. The chaperon activity was detected by Proteinase K activity and was shown as clear zone formation. The statistics analysis were carried out using programs Minitab15 software.

Isolation of Genomic DNA and PCR. The total genomic DNA of *L. plantarum* U10 was isolated from 5 mL culture in MRS broth (Oxoid) grown at 37 °C overnight. Bacterial cells were collected by centrifugation at 11 000 × g for 10 min. The genomic DNA was obtained according to the method with modification (Zhu *et al.* 1993). The pellet was resuspended with 500 µL TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) containing 60 µg µL⁻¹ lysozyme and then incubated at 37 °C for 1 h. After incubation, 200 µL of 10 % sodium dodecyl sulfate, 100 µL of 5 M NaCl and 80 µL of 10 % CTAB were added. The mixture was then incubated at 68 °C for 30 min and an equal amount of chloroform (1:1, v/v) was added. Centrifugation was conducted at 13 000 rpm for 10 min. The supernatant was collected and 1:1 (v/v) ethanol was added and then centrifuged at 13 000 rpm for 10 min. DNA was dissolved in TE buffer containing

10 $\mu\text{g } \mu\text{L}^{-1}$ RNase. The sHSP gene was amplified using nested-PCR with first round PCR using primer 5'TGAAATTTGAAAGGGGA'3 and 5'GGGCCGCTCACTTGTTACT'3 and for the second round PCR using primer 5'ATGGCTAATACTTTAATGAATCGG'3 and 5'TTATTGAATTTTCGATTTGA CCG'3. Those primers were retrieved from the nucleotide sequences of putative small heat shock gene on *L. plantarum* WCFSI (GenBank accession number AL935259) (Spano *et al.* 2004). The sHSP gene was amplified in 20 μL volumes each containing 20 ng template DNA, 1 unit μL^{-1} of genomic DNA were isolated from *L. plantarum* and added to a 20 μL PCR mixture containing 0.5 μL Kapa Tak polymerase, 0.5 μL of 10 mM dNTP mix, 1x PCR buffer 4 μL , 2 mM MgCl_2 1 μL , 0.5 μL of each primer, and ddH₂O 12.5 μL . The PCR was run following this program: pre-denaturation 5 min at 94 °C followed by 35 cycles of 1 min at 94 °C, primer annealing temperature for 1 min at 55 °C, and 72 °C extension for 30 s, followed by a final extension step at 72 °C for 5 min. PCR products were analyzed on gel electrophoresis carried out by applying 20 μL of sampel to 1.5 % agarose gel. Gel was run for 30 min at 100 V in TBE 1x buffer. The standard marker was used 1kb DNA ladder promega. After electrophoresis the gel was stained with ethidium bromide for 10 min, and then wash with aquadest and thereafter visualized using UV light source. Analyses of DNA and amino acid sequences were carried out using BLAST programs on NCBI (www.ncbi.nlm.nih.gov).

Total RNA Isolation and RT-PCR Analysis. The total RNA of heat shocked-*L. plantarum* U10 was obtained using Ribo-Pure Bacteria Kit Ambion as manual instruction. *L. plantarum* U10 were cultured in MRS medium and grown for 13 h at 30 °C. Afterward, the cell was transferred into fresh MRS medium then incubated at the same condition until OD₆₀₀ reached 0.6. The cells were harvested by centrifugation at 13 000 \times g. The heat shock treatment was done with added 1 mL fresh MRS to the pellets and then heated in 42 °C for 15 min and control without heat shock treatment, then allowed at room temperature for 20 min. Two hundreds fifty μL of ice-cold Zirconia Beads was poured to each sample. The cells were centrifuged, and then the supernatant was removed. The next step, the cells were resuspend in 350 μL RNAWIZ by vortexing vigorously for 10-15 s. The cells were transferred in RNAWIZ to a tube containing 250 μL Zirconia Beads, then it was vortexed for 10 min to lyse the cells. The zirconia beads were separated by centrifugation at 13 000 \times g, 5 min at

4 °C, then the supernatant was kept to a fresh 1.5 μL tube. Into the supernatant 0.2 volumes choloform was added, mixed well and incubated 10 min at room temperature. The mixtures was spun 5 min at 4 °C, 16 000 \times g and then the supernatant was transferred to fresh tube. Finally, RNA purification was done by adding 0.5 volumes of 100 % ethanol to the supernatant, and the sample was moved to the filter cartridge and was centrifugated for 1 min, the flow through was discarded and filter was washed with 700 μL wash solution 1 (warm at 37 °C before used), then centrifuged for 1 min. After that the filter sample was washed with 2x500 μL wash solution 2/3, then centrifuged for 1 min. The sample was centrifuged for 1 min to remove excess wash solution from the filter. RNA was eluted by applying 25-50 μL Elution Solution (preheated to 95-100 °C) to the center of the filter. The DNase treatment was done with addition of 10x DNase buffer to the RNA, and then incubation for 30 min at 37 °C. The quality of RNA samples was checked on 1.2% agarose gel, and the concentration was determined spectrophotometrically at 260 nm. About 150 ng of total RNA were used in a final volume of 25 μL for the RT-PCR experiments. The RT-PCR program was as follow: 45 °C, 30 min (reverse transcriptase reaction); 94 °C, 5 min; 94 °C, 30 s (denaturation); 52 °C, 1 min (annealing); 72 °C, 1 min; 72 °C, 5 min (extention). The PCR fragments were visualized on 1.2% agarose gel.

RESULTS

The Effect of Heat Shock Treatment on Profile of the Protein Expression in *L. plantarum* U10. To elucidate the diversity and function of heat shock protein on the growth and survival of *L. plantarum* U10, the bacteria was treated by incubation at different temperature counted for its cell viability after treatment. The prominent effect of heat stress (42 °C) on protein expressions was observed for 30 min induction that clearly shown the abundant protein expressed with diverse molecular weight (18.16 kDa, 34 kDa, 40.48 kDa and 51.93 kDa) (Fig 1A). While prolonged induction time reduced significantly some of protein expressed, it may due to the difference in turn-over of protein degradation within cell. To confirm whether heat stress could reduce cells viability that impact on the quantity of protein, we have done cells counting for each treatment. There is no significantly differences between heat-treatments groups (3.3×10^{10} CFU μL^{-1} for 30 min induction, and 3.2×10^{10} CFU μL^{-1} for 45 min

induction) and control group (3×10^{10} CFU μL^{-1}) (Fig 1B). This result confirm that the elevated protein expression in *L.plantarum* U10 is due to solely the effect of heat stress treatment.

Chaperone Activity of Cell-free Extract from Heat Shocked-*L. plantarum* U10. To confirm the involvement of sHSP in *L.plantarum* U10 resistency to heat stress, we tested the chaperone activity within cell-free extract (IP) of heat shocked-*L.plantarum* U10 by observing the proteinase K activity. IP fraction exhibited chaperonin-like activity as proven by residual proteinase K activity that could be still observed on sample no 1 after denaturation treatment

while sample no 4, proteinase K alone with denaturation, loss its protease activity (1.3 ± 0.11 cm vs 0.73 ± 0.11 cm) (Fig 2). Proteinase K alone without denaturation showed broad clear zone formation (3.36 ± 0.11 cm). On the other hand, protease activity was only slight observed in sample 3 (1.06 ± 0.11 cm). This result indicated that the heat resistance of *L.plantarum* U10 may due to chaperonin activity within its cells.

sHSP Gene Identification of *L.plantrarum* U10 and Its Identity to Other *L.plantarum* Strains. Here, we focused to identify ± 18 kDa protein at gene level with assumption that the targeted protein may belong to

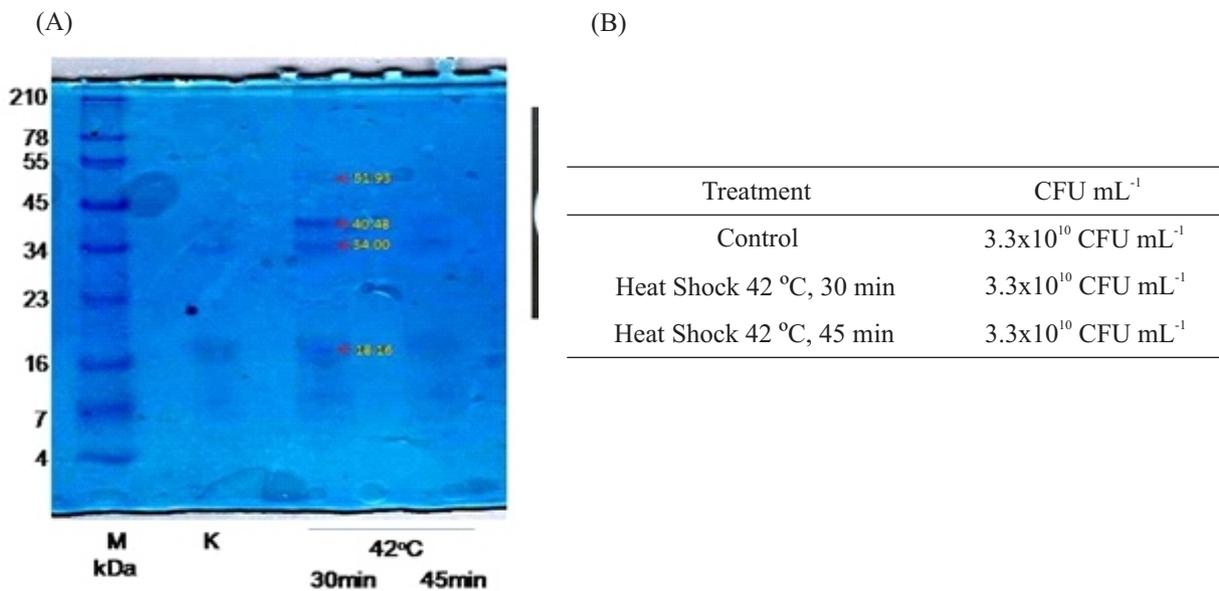


Fig 1 Profile of *L. plantarum* intracellular protein expression after heat shock treatment.(A) Bacteria was incubated in 42 °C for 30 or 45 min, then the intracellular protein was collected and the proteins profile (50 μg of total protein for each samples) was separated on 12% SDS-PAGE (B) The population of bacteria was counted before and after heat shock treatment and shown as CFU mL⁻¹.

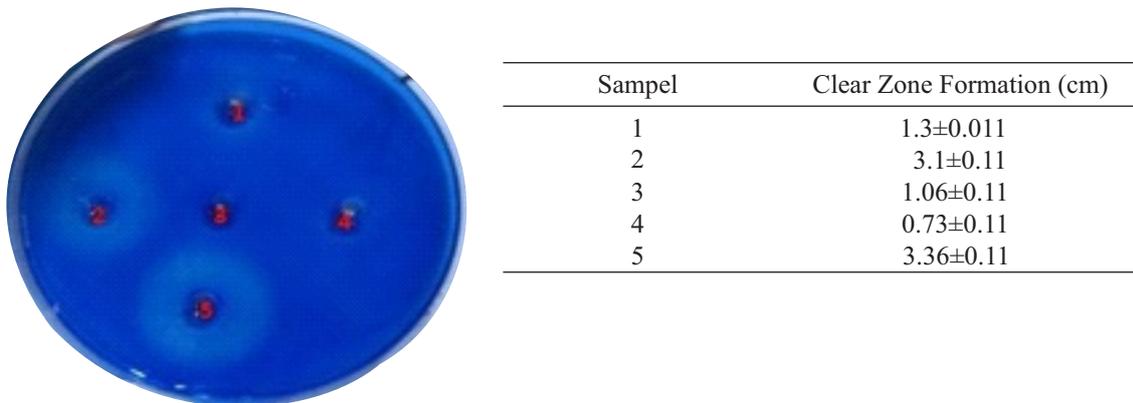


Fig 2 Chaperon activity assay of intracellular proteins fraction (IP) of (42 °C, 30 min) heat shocked- *L. plantarum* on 0.2% gelatin-containing agarose using proteinase K. Sample 1: proteinase K mixed with IP (w/w) then denaturated for 15 min at 100 °C; Sample 2: proteinase K mixed with IP (w/w) without denaturation; Sample 3: IP without denaturation; Sample 4: proteinase K only ($4 \mu\text{g} \mu\text{L}^{-1}$) denaturated for 15 min at 100 °C; Sample 5: proteinase K only ($8 \mu\text{g} \mu\text{L}^{-1}$) only. The agarose plate was incubated for 24 h at 37 °C. proteinase K activity was shown as clear zone.

sHSP protein based on their molecular mass. The sHSP gene was obtained by PCR with size of 423 bp (140 amino acid) and theoretically molecular mass of respective protein was 15.44 kDa, respectively (Fig 3A). Furthermore, our sHSP has 100 % protein identity with *L.plantarum* JDM1, *L.plantarum* ZJ316 and *L.plantarum* 16 while has 99 % with *L.plantarum* WCSF1 and the amino acid sequence (T-L-P-K) that shows identity of sHSP also observed at 124 to 127 position (Fig 3B).

The Gene Expression of Heat Shocked-*L.plantarum* U10 Protein. The expression of the gene encoding sHSP ±18 of *L. plantarum* U10 was expressed after the environmental temperature for cell growth was shifted from 30 °C to 42 °C as shown by RT-PCR result with size was ±423 bp and no band related to sHSP ±18 in non-heat shock cell (Fig 4). This result indicated the heat shock protein gene was expressed under heat stress environment.

DISCUSSION

Several studies have shown the role of sHSP in LAB adaptation and survival under certain rush enviromental condition. Factors such as abiotic stress (heat or cold shock, densitty of cells, ethanol and buthanol solvents) greatly impact on level expression of sHSP mRNA and further quantity of sHSP produced (Fiocco *et al.* 2007). In agreement with this finding, we also detected some of proteins to be upregulated after *L.plantarum* was heat-shocked at 42 °C for 30 min (OD₆₀₀ ~0.6). Interesting finding was that the heat-treatment in our study could up-regulated several proteins with protein mass ranging from 18 kDa up to 50.93 kDa, which might be predicted as group of heat shock proteins. Guzzo (1996) revealed that the proteins pattern of cells-free extract of *L.oenos* has molecular



Fig 3 PCR result of sHSP gene (sHSP 18.55) from genomic DNA of *L.plantarum* and its amino acid sequence similarity with other *Lactobacillus plantarums* HSPs. The red box is sHSPs conserve motif (T-L-P-K amino acid sequence).

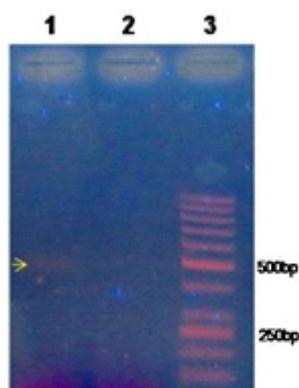


Fig 4 RT-PCR analysis of *L. plantarum* hsp ±18 gene expression, line 1; sample obtained from heat shocked-*L.plantarum* U10, line 2; sample without heat shock treatment, M; 100 bp molecular marker. The band corresponding to sHSP±18 mRNA was pointed by yellow arrow.

mass of 75, 66, 64, 24, 18, and 14.5 kDa which was previously treated by heat shock (42 °C, OD₆₀₀ ~0.4) using 2D-SDS PAGE. Among proteins expressed only protein with molecular mass of 18 kDa, named Lo18, showed remarkable expression under heat, acid, and solvent treatment. Moreover, cross reactivity study (using antisera against DnaK, GroEL and E18.5 stress proteins) to the other proteins also pointed out that only protein with 64 kDa gave positive result against GroEL. In our study, the protein expression could also be classified into one of group SHP. But, we need to verify this classification.

It is well known that heat shock proteins contain chaperone activity in which these proteins under stress condition will help in preventing intracellular protein from action of cellular degradation mechanism (Ehrnsperger *et al.* 1997; Veinger *et al.* 1998; Lee and Vierling 2000). Here, we developed a simple method to detect chaperonin activity within IP fraction *L.plantraum* using proteolytic enzyme activity test on agarose. This method was based on protective action of predicted chaperonin-like proteins on proteolytic enzyme degradation due to heat treatment instead of measuring the residual enzyme activity after heat treatment in present of chaperone protein containing sample (Collada *et al.* 1997; Kim *et al.* 1998). Chaperone assay clearly shown that IP fraction contained chaperon-like protein activity based on their ability to protect proteinase K from thermal inactivation. We assume that direct interaction between chaperon-like protein (within IP fraction) with proteinase K caused this proteolytic enzyme resist to heat-denaturation. Whether the IP fraction could protect other enzymes from heat denaturation in vitro need further investigation. As proven by Leroux co-workers (1997) and Lee and co-workers (1997), direct interaction of several sHSP with their target proteins to stabilize proteins, which agregate during heat or chemical treatment, is by forming a molecular chaperons. It seems that some of chaperone-like proteins within IP fraction co-operate each other to perform not only in preventing aggregation of proteinase K but also in reactivating the proteolytic activity of proteinase K during heat treatment. A study by Delmas and co-workers (2001) showed Lo18 protein (after sequentially purified by affinity chromatography and ion-exchange chromatography) could only reactivate catalytic function of citrate synthase after challenged with heat treatment (45 °C for 90 min) while the effect of this protein on lactate dehydrogenase only prevented it from heat-induced

aggregation without restoring enzyme activity. It seems that some factors acted on refolding enzyme hence restroring its activity. In accordance with our result, The IP fraction of heat shocked-*L.plantarum* may contains such those factors in helping the proteinase K refolding.

Heat shock proteins especially those with molecular mass of ±18 kDa have been intensively studied due to their pivotal role in miroorganism growth, adaptation and survival in response to environmental stress (Sugimoto *et al.* 2008; Tsakalidou and Papadimitriou 2011; De Angelis and Gobbetti 2011). In food industry, these group of proteins exhibit significant impact on LAB strain improvement especially those which are used as starter strains (Carvalho *et al.* 2004; Ricciardi *et al.* 2012). Wine-originating *L.plantarum* sHSP genes, named *hsp18.5* (420 nucleotide long) and *hsp19.3* (444 nucleotide long) have been cloned. Their respective proteins (upregulated under heat, cold and ethanol stress) have molecular mass of 18.483 and 19.282 kDa and level of those proteins under different stress induction was strongly linked to inverted repeat sequence (TTAGCACTC-N₉-GAGTGCTAA) homologue to the CIRCE elements found to the upstream regulatory region of heat shock operons (Spano *et al.* 2004). Other sHSP gene, called sHSP 18.55, also succesfully cloned with only 27% identity with HSP18.5 and HSP19.3 though this sHSP 18.55 protein is involved in the general stress response in wine *L. plantarum* (Spano *et al.* 2005). Tempoyak-isolated *L.plantarum* contains a gene encoding sHSP 18.55 in its chromosal DNA as shown by PCR result (Fig 3) with size approximately 423 base pair and its amino acid sequence identity almost near the same to those of *L.plantarum* reference strains that habitating either food or non-food enviroments. Furthermore, T-L-P-K amino acid sequence found in Tempoyak-isolated *L.plantarum*'s sHSP ~18 protein is also found in LAB species such as *Oenococcus. oeni* and in positive-gram bacteria such as *Clostridium acetobutylicum* and *Streptococcus thermophilus* (Spano *et al.* 2005). The exact role of sHSP ±18protein on *L.plantarum* WCSF1 has been elucidated (Capozzi *et al.* 2011) Deletion effect of this gene significantly impact on cells morphology (the mutant clumped together, had rough surfaces and shrunken empty appearance), membrane fluidity and physicochemical surface properties. Interestingly, *L.plantarum* U10 in our study has an ability to express sHSP ±18 protein after heat shocked as proven by RT-PCR.

Recently, the application of sHSP protein originated from bacteria in food biotechnology gains much interest for stress biomarker in developing bacterial starter/probiotics and manufacture of foods (Guzzo *et al.* 2012). Currently, we attempt to use sHSP ± 18 gene as an alternative selectable marker to replace antibiotics genes in food grade expression vectors to answer regarding the safety issue in the recombinant protein production.

In summary, the group of sHSP which contain chaperone-like activity in tempoyak-isolated *L. plantarum* has been expressed by heat shock treatment. The sHSP mainly with molecular mass of ± 18 kDa was predicted playing an important role in heat-resistant of proteinase K *in vitro*. The gene correspondent to sHSP protein from this bacteria was successfully cloned and showed similarity properties with those of sHSP originated from other *L. plantarum*. Furthermore, the gene encoding ± 18 protein has been proven to be expressed by *L. plantarum* U10 as response to temperature shift (30 to 42 °C).

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