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Full Length Research Paper

Expression of T4 Lysozyme Gene (*gene e*) in *Streptococcus salivarius* subsp. *Thermophilus*

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In present study, we aimed to express T4 Lysozyme gene (*gene e*) in *Streptococcus salivarus* subsp. *thermophilus* to create better probiotics for poultry. The *Esherichia coli* plasmid, Bluescript II SK +/- harboring *gene e* named pL1, was converted to a new *E. coli-Streptococcus* sp. shuttle vector (pL2) by cloning and inserting *Streptococcal* replication origin of pTRW10 vector into pL1. pL2 plasmid isolated from *E. coli* was introduced into *S. salivarius* subsp. *thermophilus* and *Lactococcus lactis* cells by electro-transformation. The lysozyme enzymes expressing by these bacteria were found to be active on *Micrococcus luteus* cells and thereby preventing their growth on assay plates. Thermostability of these enzymes from the recombinant bacteria was also found different from each other. The lysozyme expressed by *S. salivarius* subsp. *thermophilus* cells seemed to increase its capacity for thermoresistance and was not denaturated at 70°C for 15 min. In contrast, the enzyme expressed by *L. lactis* and *E. coli* cells were easily denaturated when exposed to the same temperature treatment.

Key words: Expression, lysozyme, *Streptococcus salivarius* subsp. *thermophilus*, thermostability.

INTRODUCTION

Lysozyme (EC 3.2.1.17) is an enzyme that catalyzes the hydrolysis of glycosidic bonds between N-acetyl glucosemine (NAG) and N-acetyl muramic acid (NAM), which are present in peptidoglycan heteropolymers of prokaryotic cell wall (Nicklin et al., 1999). T4 lysozyme is a muramidase which facilitates lysis of T4 phage-infected cells, thereby releasing replicated phage particles (Tsugita et al., 1968; Tsugita, 1971). Although it is similar to hen egg white lysozyme in the structure of its active site and its site of attack on the peptidoglycan cell wall (Mathews et al., 1981), T4 lysozyme presumably lyses T4-infected cells by attack on the cell wall from the cytoplasmic side (Perry et al., 1985). The phage lysozyme exhibits a much greater specific activity when assayed with *Escherichia coli* as a substrate than egg white lysozyme (Tsugita et al., 1968). In addition to its anti-bacterial action, lysozyme may have analgesic, anti-tumor, anti-metastatic and anti-inflammatory activities (Sava et al., 1989). Lysozyme is of widespread distribution in prokaryotes and eukaryotes such as bacteriophages, bacteria, plant and animals (Schindler et al., 1977). The gene encoding lysozyme is cloned from mammalian tissues and secretions, insects, plant, protozoa, bacteria and viruses (Schindler et al., 1977; Eschenlauer et al., 2000) and then expressed in some bacteria, fungi, yeast and plants (Eschenlauer et al., 2000; Oberto and Davison, 1985; Castanon et al., 1988; Cui et al., 1990; Tsuchia et al., 1992; Kim et al., 2004; Re-Vega et al., 2004). *Gene e* encoding lysozyme E cloned from bacteriophage T4 has been expressed in *E. coli* (Owen et al., 1983; Perry et al., 1985) and then its nucleotide sequence has been determined (Owen et al., 1983). Recombinant *E. coli* cells harboring pUC18L and pRS16L, which both expressing *gene e*, are grown in L and LB medium without lysis but burst out easily in hypotonic environment such as distilled water or 10 mM Tris-HCl, pH 8 buffer (Guzel et al., 2002).

Probiotics are defined as viable microorganism which, upon ingestion in sufficient amounts, exert health benefits include alleviation of symptoms of lactose intolerance, enhancement of the immune system, reduction of the duration of rotavirus diarrhea, a decrease of faecal bacte-

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rial enzyme activity and mutagenicity, prevention of recurrence of superficial bladder cancer, and prevention of atrophic diseases (Fuller, 1992). Probiotic Lactic Acid Bacteria (LAB) are commercialized mainly in dairy food and feed supplements (Salminen and Wright, 1993; Wallace and Chesson, 1995).

The present study is aimed to express the *gene e* in *Streptococcus salivarius* subsp. *thermophilus* to create recombinant probiotic for poultry, which combines beneficial effects of both probiotic and lysozyme enzyme.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth media

S. salivarius subsp. *thermophilus* FI8976, *L. lactis* IL1403 strains and pTRW10 plasmid DNA (*E. coli-Streptococcus* sp. shuttle vector) were obtained from M. Sait EKİNCİ (University of Kahramanmaras Sütcü Imam, Kahramanmaras, Turkey). The host bacterium *E. coli* DH5α and the indicator bacteria, *Micrococcus luteus,* were purchased from Stratagene and Sigma-Aldrich, respectively. Recombinant plasmid DNA pL2 was created from pL1 (pBluescript II SK +/- plus *gene e,* not published) in this study. *S. salivarius* subsp. *thermophilus* and *Lactococcus lactis* were cultured in M17 at 42°C and 37°C, respectively. *E. coli* and *M. luteus* cells were cultured in LB (Luria Bertani) at 37°C. Growth media were supplemented with 10 µg/mL ampicillin to select recombinant *S. salivarius* subsp. *thermophilus* and *L. lactis* and 50 µg/ml ampicillin for recombinant *E. coli* cells. All recombinant techniques were performed according to Sambrook et al. (1989) if otherwise is stated.

Construction, transformation and isolation of plasmid DNA

To construct pL2 plasmid, the coding sequences of the *Streptococcal* origin of replication from pTRW10 were inserted into HindIII restriction site of the pL1 plasmid. The plasmid pL2 constructed was transformed into *S. salivarius* subsp. *thermophilus* FI8976 (Wei et al., 1995), *L. lactis* IL1403 (McIntyre and Harlender, 1989; Holo and Nes, 1989) and *E. coli* DH5α (Dower et al., 1988) by electroporation. Plasmid DNA was isolated from *E. coli*, *S. salivarius* subsp *thermophilus* and *L. lactis* according to Birnboim and Doly (1979).

Plate growth inhibition assay

The lysozyme activity of recombinant bacteria was screened by growth inhibition of lysozyme sensitive *M. luteus* on the plates. All the recombinant bacteria were grown until 1.2 (OD_{600nm}). The cultures were harvested by centrifugation. The bacterial supernatant containing lysozyme was exposed to 37, 50 and 70°C for 15 min. Indicator strain *M. luteus* (500 µl) was grown until 0.6 (OD_{450}) (Anonymous, 2007) and then added into the supernatant (1000 µl). The mixture was incubated at 37°C for 1 h. After incubation, the mixture was spread on the surface of LB plates and incubates overnight at 37°C. The growing *M. luteus* colonies on the plates were then accounted.

Thermostability of lysozyme enzyme on SDS-PAGE

To determine the thermostability of lysozyme enzymes, the supernatant of recombinant bacteria is exposed to various temperatures (37, 40, 50, 60, 70, 80, 90 and 100°C) for 15 min before centrifugation at 15.000 rpm for 15 min to remove denaturated proteins. The supernatant was then mixed with equal volume of tricholoroacetic acid. Protein was then collected by centrifugation. Protein analysis was performed by using a denaturating polyacrylamide gel (SDS-PAGE, 12% (w/v)). After electrophoresis, protein bands were visualized by comassie blue staining procedure (Laemli, 1970).

RESULTS

To construct pL2 plasmid vector, 2300 bp HindIII cut DNA fragment of *E. coli-Streptococus* shuttle vector (pTRW10), carrying *Streptococcus* origin of replication, was transfered into HindIII site of pL1 vector harboring of pBluescript II SK +/- and lysozyme gene (*gene e*) of Bacteriophage T4 (Figure 1).

Ligation mixture was used to introduce into *E. coli* cells and resulting *E. coli*/pL1 and *E. coli*/pL2 transformants were grown as mixed population in LB-Agar medium including 50 µg/mL of ampicillin. Plasmid DNA was isolated from mixed population of *E. coli* harboring either pL1 or pL2 was directly used to transform *S. salivarius* subsp. *thermophilus* and *L. lactis* cells by electrotransformation. *S. salivarius* subsp. *thermophilus*/pL2 and *L. lactis*/pL2 colonies could grow on M17 agar medium supplemented with 10 µg/mL ampicillin because of its *Streptococcal* origin of replication carried on the plasmid. The pL2 plasmid was isolated from *S. salivarius* subsp. *thermophilus* and *L. lactis* cells. It was then re-electrotransformed into *E. coli* to construct *E. coli*/pL2 cells. Lysozyme E expressed by three recombinant bacteria (*S. salivarius* subsp. *thermophilus*/pL2, *L. lactis*/pL2 and *E. coli*/pL2) were tested for lysis on assay plates. The recombinant bacteria yielded clear zones around colonies by lysing *M. luteus* cells grown on all the surface of the test plates.

The lysozyme of *S. salivarius* subsp. *thermophilus* was found to be active between pH 6.0 and 9.0 on assay plates (data is not shown). Lysozyme E expressed by *S. salivarius* subsp. *thermophilus/*pL2 cells maintained its activity on test plates (data is not shown) and SDS-PAGE when subject to the temperature at 70°C for 15 min. In contrast, the enzyme expressed by both *E. coli*/pL2 and *L. lactis*/pL2 cells could not withstand temperatures above 50°C (Figures 2, 3 and 4). Live *M. luteus* cells counts on assay plates with heat-treated lysozyme was also consistent with the results of the SDS-PAGE (Figure 5).

DISCUSSION

The expression in *S. salivarius* subsp. *thermophilus* of bacteriophage T4 lysozyme gene was studied. To express lysozyme gene in the bacterium, a shuttle vector called pL2 was constructed using Streptococcal replication origin. Bacteriophage T4 lysozyme gene was actively expressed in the three bacteria. It was not known that

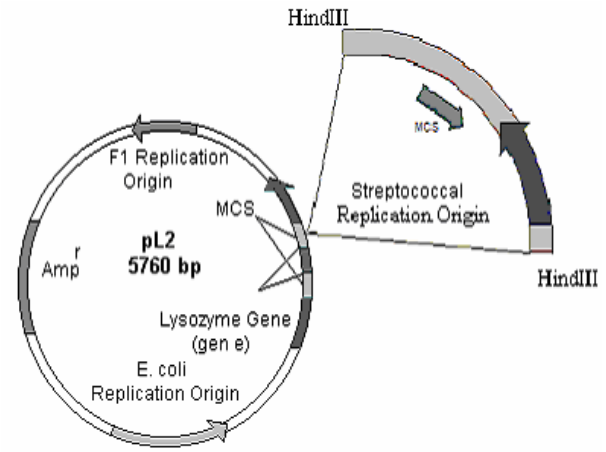

Figure 1. Construction of pL2 plasmid (pBluscript II SK +/- plus lysozyme plus replication origin of pTRW10).

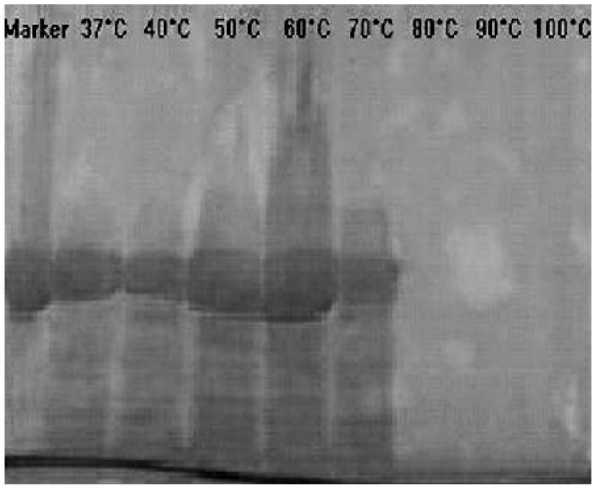

Figure 2. Thermostability of lysozyme E from *S. salivarius* subsp. *thermophilus*/pL2 in supernatant at different temperatures on SDS-PAGE.

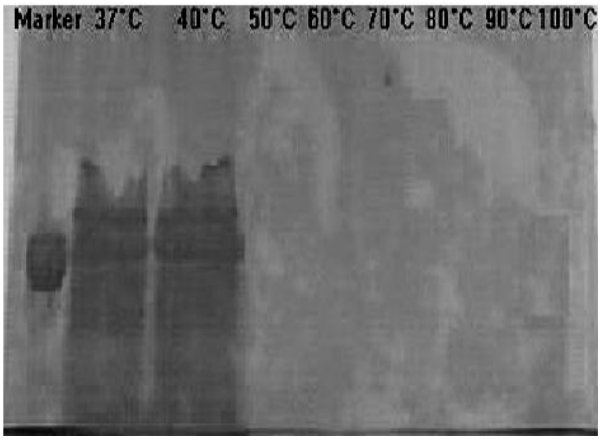

Figure 3. Thermostability of lysozyme E from *E. coli*/pL2 in supernatant at different temperatures on SDS-PAGE.

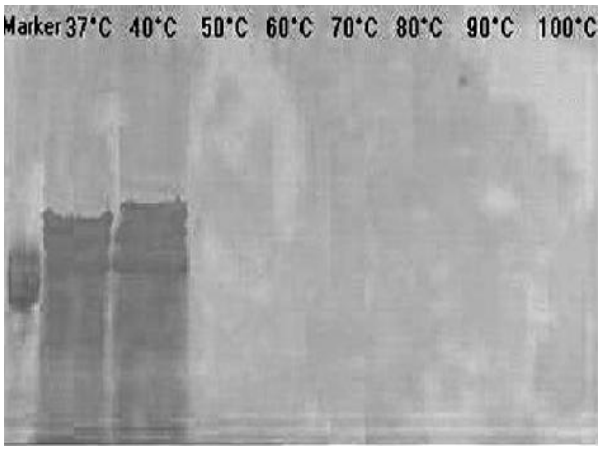

Figure 4. Thermostability of lysozyme E from *L. lactis*/pL2 in supernatant at different temperatures on SDS-PAGE.

gene e or *lacZ* promoter is recognized by the cells. Guchte et al. (1989) constructed two lactococcal expression vectors and expressed a fusion gene containing hen egg white lysozyme gene in *L. lactis* subsp. *lactis*. On the other hand, three lysozyme-encoding genes, one of eukaryotic and two of prokaryotic origin, were expressed in *L. lactis* subsp. *lactis*. Hen egg white lysozyme was not active but bacteriophage T4 and λ lysozyme were produced actively in *L. lactis* (Guchte et al., 1992).

Non-toxic expression of *gene e* was observed previously in *E. coli* (Perry et al., 1985; Guzel et al., 2002). Although both *S. salivarius* subsp. *thermophilus* and *L. lactis* colonies harboring pL2 were grown in liquid and on solidified growth media and seemed to be unaffected by the action of lysozyme, the weakened cell wall of the bacteria actually rendered the cell to burst out easily under osmotic pressure upon treated with distilled water or 10 mM Tris. HCl, pH 8.0. T4 lysozyme presumably lyses T4-infected *E. coli* cells by attack on the cell wall from the cytoplasmic side (Perry et al., 1985). However, the attack of lysozyme on LAB might be expected from both side of the cell wall.

The lysozyme E purified from Bacteriophage T4 has been reported to have a single polypeptide chain of molecular weight 19 kDa and the pH optimum between 7.0 and 8.0. The lysozyme of *S. salivarius* subsp. *thermophilus* was also found active between pH 6.0 and 9.0 on assay plates. When incubating the purified enzyme various temperature for 5 min in 0.1 M phosphate buffer, pH 6.5, it retained 20% of its original activity at 65^{o}C (Tsugita et al., 1968). However, lysozyme E expressed by *S. salivarius* subsp. *thermophilus*/pL2 cells seemed to increase its capacity for thermoresistance and was not denatured when subjected to temperature of 70^{o}C for 15 min.

The introduction, by directed mutagenesis, of a 3 - 97 disulfide bond into the normally disulfide-free enzyme T4

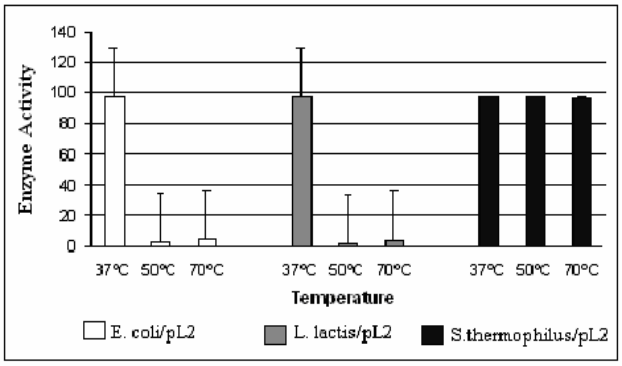

Figure 5. Bacteriphage T4 lysozyme enzyme activity on *Micrococcus luteus* cells after heat-treated from various bacterial sources.

lysozyme stabilized the protein against irreversible thermal inactivation *in vitro* (Perry and Wetzel, 1984; 1986). The crosslinked variant T4 lysozyme (13C-97C/C54V) is more stable to inactivation at 70°C and pH 6.5 than non-crosslinked T4 lysozyme (C54V/C97S) (Perry and Wetzel, 1986). However, thermostability of T4 lysozyme expressed by *S. salivarius* subsp. *thermophilus*/pL2 cells may be caused by different mechanism of protein folding in thermophilic hosts. Lysozyme was not protected and thereby degraded after incubation of the supernatant both from *L. lactis*/pL2 and *E.coli*/pL2 sources above 50°C (Figure 5). Although it is common practice to express the genes coding for thermostable proteins in mesophilic hosts such as *E. coli* and *Bacillus subtilis* for their easy purification by heat treatment, this is the first report to the our knowledge that a gene from mesophilic source was expressed in moderately thermophilic host to produce moderately thermostable protein. This moderately thermostable lysozyme may be added to milk before pasteurization and will still be active in milk to enhance immune system after pasteurization. Also, it may be used for pelleted poultry feeds to combat against pathogenic agents in digestive system.

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