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Purification and Cloning of a Protein Secreted from *Lactobacillus acidophilus*

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Among the proteins secreted from *Lactobacillus acidophilus* KCTC 3151, a 36 kDa and 24 kDa protein, whose amounts were relatively abundant, were purified and their N-terminal amino acid sequences determined. The N-terminal amino acid sequence of 36 kDa protein exhibited high homology with thymidine phosphorylase and glyceraldehyde-3-phosphate dehydrogenase. The N-terminal amino acid sequence of the 24 kDa protein did not show significant homology with proteins in Protein Data Base nor Gene Bank. Nucleotide sequence of the gene encoding 36 kDa protein indicates that the protein possesses the domains for α-helical, phosphate binding and pyrimidine binding sites, which are also shown in thymidine phosphorylases. Also, the protein contains conserved domains of dehydrogenase II and III. However, the activity of thymidine phosphorylase or glyceraldehyde-3-phosphate dehydrogenase could not be detected in the purified fractions of the 36 kDa protein.

A group of gram positive bacteria, lactobacilli, along with lactococci and bifidobacterium, is valuably used for the production of fermented food such as yogurt, cheese and sausage (Stackernbrandt and Teuber, 1988). Because lactobacilli are tolerable in anaerobic and acidic conditions, the bacteria are one of the major organisms in the small intestine colonizing the gastroinestinal tracts of animals including humans (Coconnier et al., 1992). As other lactic acid bacteria, lactobacilli, produce and secrete lactic acid, resulting in the inhibition of other bacterial growth (Kandler and Weiss, 1986).

Lactobacilli secrete bacteriocins, antibiotic polypeptides with molecular masses of 3,000 to 6,000 Da (Mørtvedt et al., 1991; Muriana et al., 1991; Axelsson et al., 1993). Another class of secreted proteins in lactobacilli is the S-layer protein which is bound on the cell wall (Beveridge and Graham, 1991). The basicity of the S-layer protein serves to protect against acidic surroundings (Beveridge and Graham, 1991). Also, the protein plays roles in adhesion to epithelium and uptake of macromolecules (Edward and Smit, 1991; Matuschek et al., 1994).

Compared to other lactic acid bacteria, the mechanism of protein secretion in lactobacilli has not been well addressed. To study the secretion mechanism, we purified proteins secreted from a strain of *Lactobacillus acidophilus*, the most acid tolerable among lactobacilli and originating from the human intestine, and cloned the gene from one of the purified proteins.

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Materials and Methods

Purification of 36 and 24 kDa proteins

Lactobacillus acidophilus KCTC 3151, obtained from the Korea Food Research Institute, was grown in MRS media (Difco) for 2 h at 37℃. Cells were removed by centrifugation at 8,000 rpm for 10 min at 4℃. To the obtained supernatant, 0.38 g/ml of ammonium sulfate was added, followed by centrifugation at 10,000 rpm in a Kontron A8.24 rotor centrifuge for 20 min at 4℃. The pellet was resuspended in Buffer A (50 mM KCl, 25 mM Tris-Cl (pH 7.4) and 10% glycerol) and dialyzed against Buffer A.

For the purification of 36 kDa protein, the dialyzed ammonium sulfate precipitate was loaded onto a DEAE-Sepharose (Pharmacia Biotech) column, equilibrated with Buffer A, and eluted with a gradient of 50 mM to 1 M KCl. 36 kDa protein was detected on 14% SDS-polyacrylamide gel electrophoresis. The fractions containing 36 kDa protein were pooled and dialyzed against Buffer B (25 mM KCl, 25 mM Tris-HCl (pH 7.4), and 10% glycerol). The dialyzed sample was applied to a Fast-S (Pharmacia Biotech) column equilibrated with Buffer B and eluted with 25 mM to 1 M of KCl. The fractions containing 36 kDa protein were applied to a phosphocellulose-11 (Whatman) column equilibrated with Buffer B and eluted with a gradient of 25 mM to 1 M KCl.

For the purification of 24 kDa protein, the dialyzed ammonium sulfate precipitate was applied to a CM-

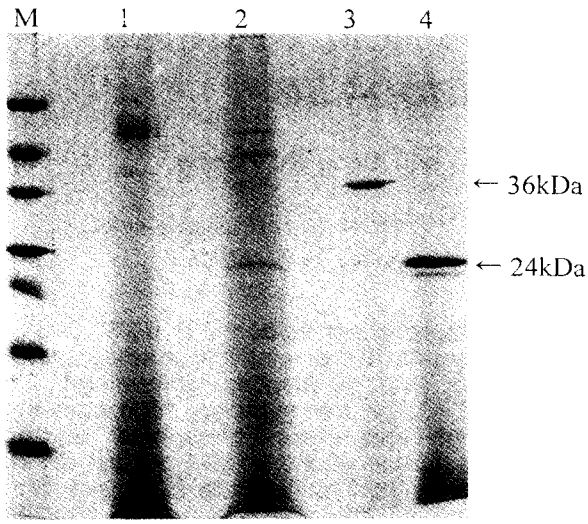

Fig. 1. Identification and purification of the proteins secreted from *Lactobacillus acidophilus* KCTC 3151. Proteins were analyzed on a 15% SDS-polyacrylamide gel followed by Coomassie brilliant blue staining. Lane 1 contained 10 μg of fresh MRS media which was precipitated with 10% trichloroacetic acid. Lane 2 contained 50 μg of ammonium sulfate precipitate of two days culture supernatant. Lane 3 and 4 contained 1.6 and 2.7 μg of purified 36 and 24 kDa protein, respectively. Molecular weight markers (M) were bovine albumin, 66 kDa; egg albumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 29 kDa; trypsinogen (bovine pancreas), 24 kDa; trypsin inhibitor, 20 kDa; and lactalbumin, 14.2 kDa.

Sepharose (Pharmacia Biotech) column, followed by chromatography on Fast-Q and phosphocellulose-11 columns as above.

Determination of N-terminal amino acids of 36 and 24 kDa proteins

The purified proteins were further separated on SDS-PAGE and electroblotted to Immobilon P membrane (Millipore). The protein band visualized by staining with Coomassie brilliant blue was cut out and used for N-terminal amino acid sequencing at Korea Basic Science Institute, Seoul.

Cloning of the gene encoding 36 kDa protein

Genomic DNA isolated from *Lactobacillus acidophilus* KCTC 3151 as previously described (Sambrook et al., 1989) was partially digested with *BamH*I. DNA fragments of 8 to 15 kbp were fractionated by a 10 to 40% sucrose gradient and precipitated with ethanol (Ausubel et al., 1989). The DNA was ligated to the *Xho*I site, partially filled with the λ FixII (Stratagene) as the vendor recommended. The ligation mixture was infected to *E. coli* XL1-Blue (P2). Independent recombinants were obtained. To screen the λ genomic library, the 5'-^{32}P-labeled degenerated oligonucleotides for 36 kDa protein (Fig. 2) were used. Subcloning was performed into vector pBluescript II KS(+) (Stratagene) with an *E. coli* host DH5α (Sambrook et al., 1989). Nucleotide sequencing was carried out with *Exo*III-deletion mutants by Sequenase™ v 2.0 (USB).

Results

Identification and purification of proteins secreted from Lactobacillus acidophilus

Some proteins synthesized in lactobacilli are secreted to its surroundings. The proteins of *Lactobacillus acidophilus* KCTC 3151 secreted to culture medium were identified (Fig. 1). Cells grown in MRS media for two days were removed by centrifugation. Then proteins remaining in the supernatant were recovered by trichloroacetic acid precipitation and analyzed by SDS-

A

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1  MIVKIGINEFGRIGRLAFRRIYEL  24    thymidine phosphorylase
     *  ****  ************
1   TVFIGINGFGRIGRLAFRRIMDGE 24    36kDa
      *   ****************
1   SVKVGINGFGRIGRLAFRRIQNV  23    glyceraldehyde-3-phosphate dehydrogenase
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B

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1 AIVQNDASVVTVNTVSNNSISVYNN 25
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C

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3   F    I    G    I    N    G    F    G  10
   TTC  ATA  GGA  ATA  AAC  GGA  TTC  GG
     T    C    G    C    T    G    T
          T    C    T         C
               T              T
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Fig. 2. N-terminal amino acid sequence of 36 and 24 kDa proteins. A, N-terminal amino acid of 36 kDa protein was compared with that of glyceraldehyde-3-phosphate dehydrogenase (*Streptomyces arenae*) and thymidine phosphorylase (*Lactobacillus casei*) (Avraham et al., 1990). B, N-terminal amino acid sequence of 24 kDa protein. C, For the cloning of gene of 36 kDa protein, a degenerated oligonucleotide was designed from N-terminal amino acid sequence of 36 kDa protein.

Fig. 3. Nucleotide sequence and deduced amino acid sequence of the gene encoding the 36 kDa protein and its flanking region. The junction site with the λ-left arm was marked as an arrow. The underlined regions, a α-helical region, a phosphate binding domain and a pyrimidine binding domain, are conserved in thymidine phosphorylases of various organisms (Mushegian and Koonin, 1994, Fig. 4). The wave-lined regions, dehydrogenase domain II and III, show low homology with L-2-hydroxyisocaproate dehydrogenase (Lerch et al., 1989).

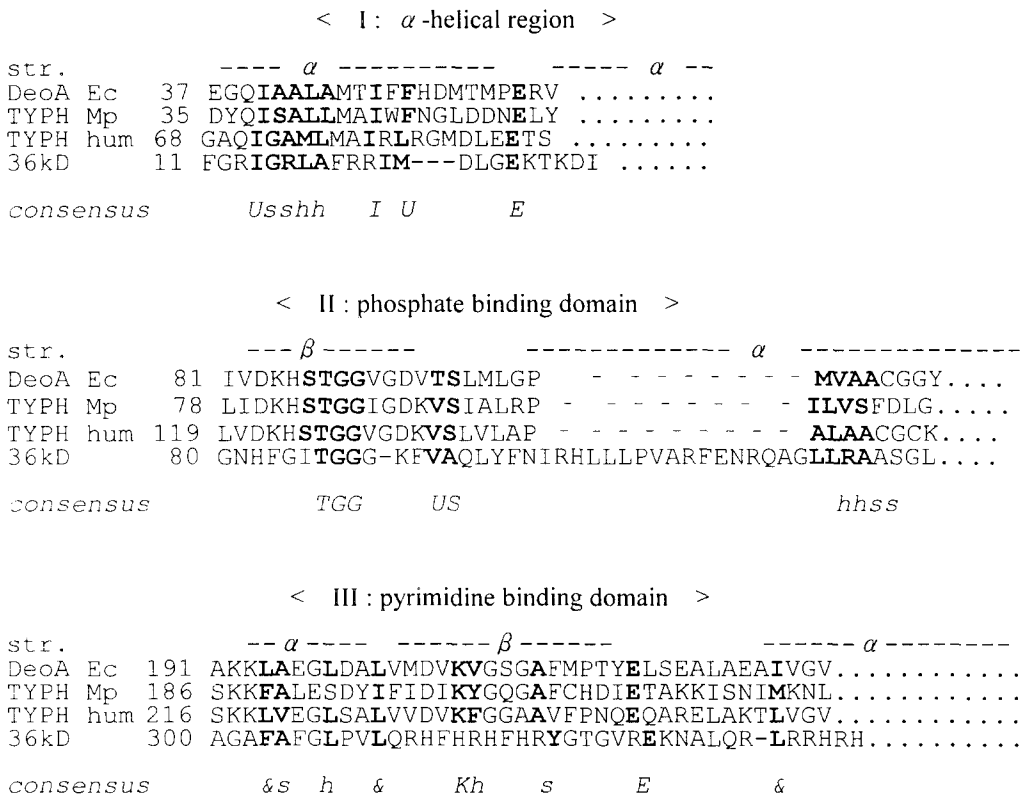

Fig. 4. Conserved sequences in 36 kDa protein and thymidine phosphorylase of various organisms. DeoA Ec, thymidine phosphorylase of *E.coli*; TYPH Mp, thymidine phosphorylase of *Mycoplasma pyrum*; TYPH hum, thymidine phosphorylase of human. *U*, a bulky aliphatic residue (I, L, V, M); *&*, a bulky hydrophobic residue (I, L, V, M, F, Y, W); *h*, any hydrophobic residue (I, L, V, M, F, Y, W, C, A); *s*, a small residue (G, A, S) (Mushegian and Koonin, 1994).

polyacrylamide gel electrophoresis (Fig. 1; lanes 1 and 2). Several distinct proteins, which did not exist in fresh medium, appeared.

Among the identified proteins, two proteins of 36 and 24 kDa, whose amounts were relatively abundant (Fig. 1), were purified. The supernatant obtained from two days of cultivation was precipitated by the addition of 0.38 g/ml of ammonium sulfate. The dialyzed precipitate was applied to column chromatographies. Under the chromatographic conditions, the 36 kDa protein did not bind to either weak anion-exchange column of DEAE-Sepharose or weak cation-exchange column of Fast-S. Flow-through of Fast-S column, onto which the flow-through of DEAE column was loaded, was applied to a phosphocellulose-11 column. The 36 kDa protein was eluted with 100 to 200 mM of KCl. The 24 kDa protein was purified by chromatographies of CM-Sepharose, Fast-Q and phosphocellulose-11. The purified proteins were visualized by SDS-polyacrylamide gel electrophoresis (Fig. 1; lanes 3 and 4).

N-terminal amino acid sequences of 36 and 24 kDa protein

To understand the function of purified 36 and 24 kDa proteins, N-terminal amino acid sequences were determined. The first 24 amino acid sequence of N-terminus of purified 36 kDa protein exhibited high homologies with glyceraldehyde-3-phosphate dehydrogenase of various organisms and thymidine phosphorylase of *Lactobacillus casei* (Fig. 2A). However, the activity of neither glyceraldehyde-3-phosphate dehydrogenase nor thymidine phosphorylase could be detected with the purified 36 kDa protein.

The first 25 amino acid sequence of 24 kDa protein was determined, but the homology with other proteins could not be found in Protein Data Base nor Gene Bank (Fig. 2C).

The gene encoding 36 kDa protein

To clone the gene encoding 36 kDa protein, genomic DNA isolated from *Lactobacillus acidophilus* KCTC 3151 was partially digested with *BamH*I restriction enzyme and the genomic library was constructed with λ FixII kit (Stratagen). As a probe for library screening, synthetic oligonucleotide was designed whose sequence contained relatively low degeneracy to the 24 amino acid sequence of the 36 kDa protein N-terminus (Fig. 2C). Plaque hybridizations were performed, then eight λ phage were finally selected. Restriction and Southern hybridization analysis indicated that all eight phage

were identical. Among the eight phage, SY36-8 was used to clone the gene encoding the 36 kDa protein. 3.5 kbp the *Sal*I/*Bgl*II fragment of SY36-8 containing a region responding to the degenerated probe was subcloned into the *Sal*I/*Bgl*II site of the pBluescript II KS(+) vector and yielded a plasmid pLG1. To localize the gene for the 36 kDa protein, *Exo*III deletion mutants were generated and used for nucleotide sequencing (Fig. 3). The left edge of 3.5 kbp fragment contained the sequences of the left arm of the λ vector. Except for the first three amino acids, the remaining 21 N-terminal amino acid sequence of the 36 kDa protein identically matched the sequences deduced from the nucleotide sequence (Fig. 3). The molecular weight of 38 kDa, which was calculated from the amino acid sequence, was close to 36 kDa as determined by SDS-polyacrylamide gel electrophoresis. The translational start codon, ATG, could not be found in the left region of the sequences from the 3.5 kbp fragment. This result indicates that the purified 36 kDa protein was processed prior to secretion by cleavage of a leader sequence.

Discussion

The entire amino acid sequence of the 36 kDa protein was deduced from the nucleotide sequences (Fig. 3).

The region from ^{80}G to ^{123}L shares homology with the catalytic domain of phosphorylase and/or kinase (Fig. 4) (Mushegian and Koonin, 1994). The sequence ^{86}TGG^{88}, which is involved in binding of phosphate, is conserved in pyrimidine phosphorylase from *E. coli* to human (Mushegian and Koonin, 1994). The upstream α-helical domain and the downstream pyrimidine binding domains, which are commonly found in pyrimidine phosphorylase (Mushegian and Koonin, 1994), appear to exist in the 36 kDa protein. Also, domains of dehydrogenase II and III (Lerch et al., 1989) are shown in the sequence of the 36 kDa protein (Fig. 3). Although the 36 kDa protein possesses homology with thymidine phosphorylase, it is not conclusive that the 36 kDa protein is a thymidine phosphorylase. We could not detect pyrimidine phosphoryase activity in the purified 36 kDa protein fractions. Whereas thymidine phosphorylase was mostly located in the cell wall even after sonication (Avraham et al., 1990), the 36 kDa protein was purified from the culture supernatant.

Acknowledgements

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References

Ausubel FM, Breut R, Kingston RE, Moore DD, Seidman JG, Smith JA, and Struhl K (1989) Current Protocols in Molecular Biology. Green Publishing Associate & John Wiley Interscience, New York, pp 5.3.1.-5.3.8.

Avraham Y, Grossowicz N, and Yashphe J (1990) Purification and charaterization of urine and thymidine phosphorylase from *Lactobacillus casei*. *Biochim Biophys Acta* 1040: 287-293.

Axelsson L, Holck A, Birkeland S, Aukrust T, and Blom H (1993) Cloning and nucleotide sequence of a gene from *Lactobacillus sake* Lb706 necessary for sakacin A production and immunity. *Appl Environ Microbiol* 59: 2868-2875.

Beveridge TJ, and Graham LL (1991) Surface layer of bacteria. *Microbiol Rev* 55: 684-705.

Coconnier M, Klaenhammer TR, Kernis S, Bernet M, and Servin AL (1992) Protein-mediated adhesion of *Lactobacillus acidophilus* BG2FO4 on human enterocyte and mucus-secreting cell line in culture. *Appl Environ Microbiol* 58: 2034-2039.

Edward P and Smit J (1991) A transducing bacteriophage for *Caulobacter crescentus* uses the paracrystalline surface layer protein as a receptor. *J Bacteriol* 173: 5568-5572.

Kandler O and Weiss N (1986) Regular, nonsporing gram positive rogs. In: Sneath PHA, Mair N, Sharpe E, and Holt JB (eds), Bergey's Manual of Systematic Bacteriology, William and Wilkins, Baltimore, pp 1535-1552.

Lerch HP, Frank R, and Collins J (1989) Cloning, sequencing and expression of the L-2-hydroxisocaproate dehydrogenase-encoding gene of *Lactobacillus confusus* in *Esherichia coli*. *Gene* 30: 263-270.

Matuschek M, Burchhardt G, Sahm K, and Bahl H (1994) Pulluanase of *Thermoanaerobacterium thermosulfurigenes* EM1 (*Clostridium thermosulfurogenes*): molecular analysis of the gene, composite structure of the enzyme, and a common model for its attachment to the cell surface. *J Bacteriol* 176: 3295-3302.

Muriana PM and Klaenhammer TR (1991) Purification and partial charaterization of lactacin F, a bacteriocine produced by *Lactobacillus acidophilus* 11088. *Appl Environ Microbiol* 57: 114-121.

Mushegian AR and Koonin EV (1994) Unexpected sequence similarity between nucleosidase and phosphoribosyltransferases of differednt specificity. *Protein Sci* 3: 1081-1088.

Mørtvedt CJ, Nissen-meyer J, Sletten K, and Nes IF (1991) Purification and amino acid sequence of lactacin S, a bacteriocine produced by *Lactobacillus sake* L45. *Appl Environ Microbiol* 57: 1829-1834.

Sambrook J, Fritsch EF, and Maniatis T (1989) Molecular Cloning. A Laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Stackernbrandt E and Teuber M (1988) Molecular taxonomy and phylogenetic position of lactic acid bacteria. *Biochimie* 70: 317-324

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