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Cloning and Characterization of a Gene Encoding Phosphoketolase in a *Lactobacillus paraplantarum* Isolated from *Kimchi*

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Abstract A gene coding for phosphoketolase, a key enzyme of carbohydrate catabolism in heterofermentative lactic acid bacteria (LAB), was cloned from a *Lactobacillus paraplantarum* C7 and expressed in *Escherichia coli*. The gene is 2,502 bp long and codes for a 788-amino-acids polypeptide with a molecular mass of 88.7 kDa. A Shine-Dalgarno sequence (aaggag) and an inverted-repeat terminator sequence are located upstream and downstream of the phosphoketolase gene, respectively. The gene exhibits an identity of >52% with phosphoketolases of other LAB. The phosphoketolase of *Lb. paraplantarum* C7 (LBPK) contains several highly conserved phosphoketolase signature regions and typical thiamine pyrophosphate (TPP) binding sites, as reported for other TPP-dependent enzymes. The phosphoketolase gene was fused to a glutathione S-transferase (GST::LBPK) gene for purification. The GST::LBPK fusion protein was detected in the soluble fraction of a recombinant *Escherichia coli* BL21. The GST::LBPK fusion protein was purified with a yield of 4.32 mg/400 ml by GSTrap HP affinity column chromatography and analyzed by N-terminal sequencing. LBPK was obtained by factor Xa treatment of fusion protein and the final yield was 3.78 mg/400 ml. LBPK was examined for its N-terminal sequence and phosphoketolase activity. The K_M and V_{max} values for fructose-6-phosphate were 5.08±0.057 mM (mean± SD) and 499.21±4.33 μmol/min/mg, respectively, and the optimum temperature and pH for the production of acetyl phosphate were 45°C and 7.0, respectively.

Keywords: *Kimchi*, *Lactobacillus paraplantarum*, phosphoketolase

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Kimchi, a traditional fermented vegetable dish in Korea, is produced by natural fermentation carried out by lactic acid bacteria (LAB) [10]. *Leuconostoc* and *Lactobacillus* species are considered the dominant bacteria in *kimchi* fermentation and are believed to be responsible for the unique *kimchi* taste. The transition from leuconostocs to lactobacilli as the dominant organisms is suspected to be the major cause of overripening or acidification during the late stage of *kimchi* fermentation. *Lactobacillus plantarum* is one of the dominant species and is responsible for the overacidification of *kimchi* when the number of *Leuconostoc* species decreases [11, 15]. The market for commercially produced *kimchi* is steadily expanding, which necessitates the development of methods for mass production of *kimchi* and retard of acidity caused by *Lb. plantarum* [5]. To effectively control the fermentation and preservation, some food microbiologists are turning to the screening of starter strains that can control the fermentation of *kimchi* [2, 7, 8, 12]. However, few studies have been done on the regulation of enzymes in the sugar metabolism of LAB during *kimchi* fermentation [9, 17, 18]. The regulation of correlate enzymes involved in catabolic metabolism could be used to control product uniformity in industrial *kimchi* production.

Our group has isolated a bacteriocin-producing *Lactobacillus paraplantarum* C7, which inhibits the growth of *Lb. plantarum*, from *kimchi* [12]. *Lb. paraplantarum* is a heterofermentative organism, producing lactic acid, acetate, ethanol, and CO_2 as major products via the pentose phosphoketolase pathway. In particular, phosphoketolase plays a role in a major branch point in this pathway. Nevertheless, only a few studies on phosphoketolases in LAB have been reported [1, 6, 13, 23], and none of them have

involved *Lb. paraplantarum*. In the present study, we cloned the phosphoketolase gene from *Lb. paraplantarum* C7 (LBPK) and characterized it based on phosphoketolase activity.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lb. paraplantarum C7 was used in this study as the bacterial source of the phosphoketolase gene [12]. Cells were grown in an MRS (deMan-Rogosa-Sharpe) broth (Difco, Franklin Lakes, U.S.A.) at 30°C. *Escherichia coli* BL21 (DE3) cells were used as the *E. coli* expression host and were routinely grown at 37°C in LB (Luria-Bertani) media supplemented with ampicillin (50 μg/ml) as required.

pGEM-T Easy vector (Promega, Madison, U.S.A.) was used for subcloning the phosphoketolase gene. pGEX-5X-3 (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as an expression vector for expression of the glutathione S-transferase::LBPK fusion protein (GST::LBPK).

Sequencing of the Phosphoketolase Gene

In order to obtain the putative phosphoketolase gene from *Lb. paraplantarum* C7, genomic DNA was purified with a DNeasy tissue kit (Qiagen, Hilden, Germany). A product of approximately 2 kb was obtained by PCR amplification with degenerate oligonucleotide primers PK-1 and PK-2, which were designed from the conserved regions GPGHGGQ and VHGYRE, respectively [13] (Table 1). For the cloning of the full-length phosphoketolase gene from *Lb. paraplantarum* C7, chromosome walking was performed by PCR with primers LBPK-3 and LBPK-4, based on the determined 2 kb sequence and the reported nucleotide sequence of the *xpk* gene from *Lb. plantarum* WCFS1. Primer pairs LBPK-1/LBPK-3 and LBPK-2/LBPK-4 were used to amplify the 5'- and 3'-flanking regions of the 2 kb fragment, respectively. PCR amplification was performed with a GeneAmp PCR system (GeneAmp 2700, Foster City, U.S.A.) and consisted of 30 cycles of denaturation at 94°C for 2 min, annealing at 55°C for 2 min, and elongation at 72°C for 3 min. The PCR products were sequenced using the dideoxy chain-termination method [20]. The BLAST program was used to determine the sequence homologies of the putative phosphoketolase gene with known phosphoketolases in the GenBank database.

Production and Purification of GST::LBPK

The phosphoketolase gene was amplified using LBPK-1 and LBPK-2 and then cloned into pGEM-T Easy, resulting in plasmid pLBC7-PK. To construct an expression vector containing the LBPK gene under the control of the *tac* promoter, the LBPK gene was amplified using LBPK-F1 and LBPK-R, which contains restriction enzyme sites SmaI and XhoI, respectively. The amplified fragment was digested with SmaI and XhoI and inserted into the same sites in pGEX-5X-3, resulting in plasmid p5X-3LBPK.

E. coli BL21 (DE3) harboring p5X-3LBPK was cultured at 37°C in 400 ml of LB broth containing ampicillin until the OD_{600} reached 0.5. The LBPK gene was induced by IPTG (1.0 mM) for 4 h at 28°C, and the cells were harvested by centrifugation (Hanil Science, Suwon, Korea) at 4,000 ×*g* for 10 min at 4°C. The pellets of recombinant *E. coli* were resuspended in 40 ml of PBS buffer, pH 7.3 (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), and disrupted by an ultrasonic oscillator (Sonics and Materials Inc., Newton, U.S.A.). The cell debris was removed by centrifugation (14,000 ×*g*, 30 min, 4°C). Cell lysates were stored at 4°C.

An ÄKTA explorer (Amersham) was used to analyze the affinity chromatography separation of the *E. coli* BL21 (DE3) cell extracts harboring p5X-3LBPK. A GSTrap HP column (1 ml, Amersham) packed with Glutathione Sepharose was used. The 50 mM Tris-HCl buffer containing 10 mM reduced glutathione (pH 8.0) was fed at a flow rate of 1 ml/min, and the GST::LBPK fusion protein was eluted with a glutathione gradient (0–10 mM).

The purified GST::LBPK was cleaved by Factor Xa (Amersham) at 10°C for 6 h in 50 mM Tris-HCl containing 150 mM NaCl and 1 mM $CaCl_2$ (pH 7.5).

The expression and purification level of GST::LBPK was analyzed by 10% tris-glycine gel. The protein bands were detected by Coomassie blue staining.

Table 1. Sequences of the oligonucleotide primers used in the amplification of the phosphoketolase gene from *Lb. paraplantarum* C7.

Primer	Oligonucleotide sequence (5'→3')	Specificity	Reference
PK-1	GGYCCHGGWCATGGTGGHCAA	Degenerate primer, forward	[13]
PK-2	TTCRCGGTARCCATGMAC	Degenerate primer, reverse	[13]
LBPK-1	GTTAAAATAGAACTTGTAAACGAAAACA	*Lb. plantarum*, *xpk*, forward	This study
LBPK-2	CTGAGTTGACAGTATCCAATCG	*Lb. plantarum*, *xpk*, reverse	This study
LBPK-3	CCGGTTAAGGTGCGCGTAGATAGAGTTTTG	Determined sequence in this study, reverse	This study
LBPK-4	CTACACGACAGTTTCCCAGAGATGA	Determined sequence in this study, forward	This study
LBPK-F1	CCCCGGGTCATGACAACAGATTACTCATCACCAG	*Lb. paraplantarum* C7, LBPK, forward	This study
LBPK-R	CCTCGAGTTTTCAAACCTTTCCATTGCCAGTCGTTA	*Lb. paraplantarum* C7, LBPK, reverse	This study

Underlines indicate nucleotides participating in restriction sites.

Amino Acid Sequence Analysis

N-terminal amino acid sequences of GST::LBPK and LBPK were determined at the Korea Basic Science Institute (Seoul, Korea) using an Applied Biosystems model 491 precise protein sequencer, after desalting with Prosorb sample preparation cartridges (Applied Biosystems).

Measurement of Phosphoketolase Activity

Phosphoketolase activity was measured spectrophotometrically according to the method of Orban and Patterson [16] with some modifications, where ferric acetyl hydroxamate, produced from acetyl phosphate, was measured. A solution containing 71 mM sodium fluoride and 24 mM sodium iodoacetate was mixed with 270 mM fructose-6-phosphate (F6P). After the addition of cell extract, the solution was mixed well and then incubated at 37°C for 30 min. Following incubation, 13% hydroxamine·HCl was added and the solution allowed to incubate at room temperature for 10 min. The reaction was stopped by adding 15% trichloroacetic acid, 4 M hydrochloric acid, and 5% ferric chloride and centrifuged (10,000 ×*g*, 15 min, 4°C). The absorbance of supernatant at 505 nm was measured. One unit of phosphoketolase activity was defined as the amount of protein required to liberate 1 μmol of product in 1 min.

Nucleotide Sequence Accession Number

The determined nucleotide sequence of the phosphoketolase gene from *Lb. paraplantarum* C7 was deposited in the GenBank database under the accession number AY365027.

Results and Discussion

Sequence Determination and Homology Analysis

The putative phosphoketolase gene from the *Lactobacillus paraplantarum* C7 is 2,502 bp long and encodes a 788-amino-acids polypeptide with a calculated total mass of 88.7 kDa (Fig. 1). The phosphoketolase is preceded by a potential Shine-Dalgarno (SD) sequence (aaggag) and has free-energy SD-anti-SD bindings (ΔG^0) of −12.8 kcal/mol [22]. An inverted repeat with the potential to form a stem-loop structure was found downstream of the phosphoketolase gene and could act as an intrinsic terminator. The deduced polypeptide sequence of the *Lb. paraplantarum* C7 phosphoketolase gene along with those of functionally

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the phosphoketolase from *Lb. paraplantarum* C7.
The SD sequence is indicated with asterisks underneath the nucleotides. Inverted repeats capable of forming a stem-loop structure are indicated by arrows.

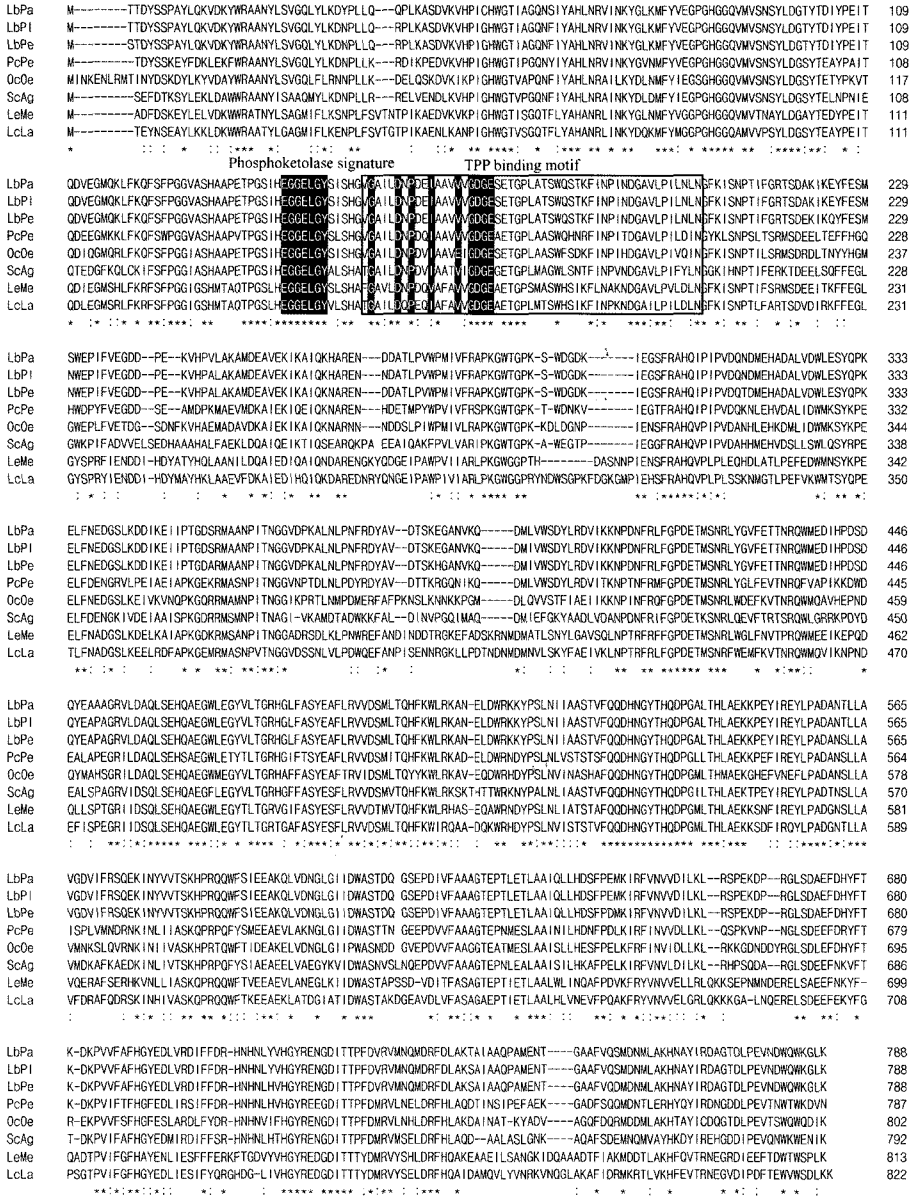

Fig. 2. Alignment of the deduced amino acid sequences of the phosphoketolase gene.
LbPa, *Lb. paraplantarum* C7; LbPl, *Lb. plantarum* WCFS1; LbPe, *Lb. pentosus* MD363; PcPe, *Pediococcus pentosaceus* ATCC 25745; OcOe, *Oenococcus oeni* PSU-1; ScAg, *Streptococcus agalactiae* 2603V/R; LeMe, *Leuconostoc mesenteroides* LMC7; LcLa, *Lactococcus lactis* IL1403.

related proteins are shown in Fig. 2, which reveals conserved regions throughout the sequence. The alignment of the *Lb. paraplantarum* C7 phosphoketolase amino acid sequence with sequences of related proteins showed extensive identities, as summarized below and in Table 2.

Alignments of the amino acid sequences of several phosphoketolases revealed several highly conserved regions including two signature patterns. The deduced phosphoketolase of *Lb. paraplantarum* C7 contains these two highly conserved regions. The function of the first consensus pattern, E-G-G-E-L-G-Y (PROSITE PS60002, amino acids 142–148 in the phosphoketolase protein), is not known, whereas the second corresponds to the thiamine pyrophosphate (TPP, vitamin B_1) enzyme signature sequence (amino acids 155–204 in the phosphoketolase protein), which matches the consensus pattern G-x(3)-D-x-P-x(2)-[LIVF]-x(3)-[LIVM]-G-D-G-E (PS60003) (Fig. 2).

Phosphoketolase contains a protein motif common to transketolases and other TPP enzymes. TPP is involved in many aspects of intermediary metabolism and serves as the coenzyme for several types of carbohydrate cleavage reactions, in addition to participating in well-known decarboxylation mechanisms [4]. In *Lb. paraplantarum* C7, the slightly modified form G-D-G-E-x(30)-N (amino

Table 2. The percent amino acid identities between pairs of phosphoketolases from different Gram-positive microorganisms.

Strain	LbPa	LbPl	LbPe	PcPe	OcOe	ScAg	LeMe	LcLa	Accession no.
LbPa	100								AAQ64626.1
LbPl	98.7	100							NP_786060.1
LbPe	97.5	98.5	100						CAC84393.1
PcPe	70.9	71.4	71.8	100					ZP_00323020.1
OcOe	66.2	66.8	66.5	62.1	100				ZP_00319693.1
ScAg	59.8	60.7	61.0	59.5	55.2	100			AAN00662.1
LeMe	54.5	55.1	55.2	56.5	53.0	50.3	100		AAV66077.1
LcLa	52.5	53.2	52.9	53.2	51.9	46.5	70.0	100	NP_267658.1

Pair alignments between homologous proteins were made with the CLUSTAL W program. LbPa, *Lb. paraplantarum* C7; LbPl, *Lb. plantarum* WCFS1; LbPe, *Lb. pentosus* MD363; PcPe, *Pediococcus pentosaceus* ATCC 25745; OcOe, *Oenococcus oeni* PSU-1; ScAg, *Streptococcus agalactiae* 2603V/R; LeMe, *Leuconostoc mesenteroides* LMC7; LcLa, *Lactococcus lactis* IL1403.

acids 170–204 in the phosphoketolase protein) of the conserved region G-D-G-x(24-27)-N-N was also present, which appears in TPP-binding enzymes such as acetolactate synthase, transketolase, the E1 component of 2-keto acid dehydrogenase, and enzymes homologous to Xfp, xylulose-5-phosphate (X5P) and F6P specific phosphoketolase, from *Bifidobacterium lactis* [3, 14].

Expression and Purification of the GST::LBPK

Phosphoketolase expression was induced by IPTG addition in BL21 cells harboring pLBC7-PK containing the phosphoketolase gene. SDS-PAGE analysis of *E. coli* extracts revealed a band of approximately 88.7 kDa in size, which agreed well with the expected size of phosphoketolase, though at a low level (data not shown).

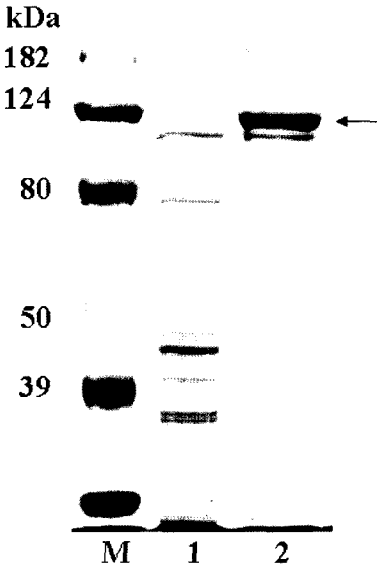

Fig. 3. Expression of GST::LBPK under the tac promoter of pGEX5X-3.
Lane: M, molecular weight marker; 1, *E. coli* BL21 cells harboring p5X-3LBPK without 1 mM IPTG; 2, *E. coli* BL21 cells harboring p5X-3LBPK with 1 mM IPTG.

To express a high level of the LBPK protein, the LBPK gene was fused with the glutathione S-tranferase gene. To determine whether GST::LBPK could be expressed, *E. coli* BL21 cells harboring p5X-3LBPK (containing GST::LBPK) were treated with 1 mM IPTG. A dominant protein band at 117 kDa, GST::LBPK, was induced (Fig. 3). The amount of the expressed GST::LBPK was estimated to be 32.2% of the total cell protein in *E. coli* BL21 harboring p5X-3LBPK, when analyzed by the ImagJ densitometer program, and GST::LBPK was soluble (Table 3).

GST::LBPK was purified by affinity chromatography (Fig. 4). Approximately 4.3 mg of GST::LBPK was purified from 122.8 mg of total cell protein (Table 3). A single peak was obtained for glutathione at 2.4 to 3.8 mM (Fig. 4A). The purification degree of GST::LBPK was assessed by SDS-PAGE (Fig. 4B, lane 1). N-Terminal sequencing of the purified GST::LBPK confirmed that the first 15 residues (MXPILGYWKIKGLVQ) were identical to the GST::LBPK sequence, deduced from the DNA.

Purification of Recombinant LBPK

The GST::LBPK fusion protein consisted of the following regions sequentially from its N-terminus: the GST domain, Factor Xa cleavage site, and the LBPK domain. Thus, the recombinant LBPK was cleaved from GST by enzymatic digestion with Factor Xa using various enzyme concentrations and reaction times. The efficiency of this cleavage reaction was 85% using 10 U of Factor Xa/mg of the fusion protein at 10°C for 6 h (data not shown). Cleavage resulted in a single band corresponding to approximately 89.9 kDa, which contained LBPK and the remaining amino acids of the GST N-terminal residue (RNSRVKECFE) (Fig. 4B, lane 2). The identity of purified LBPK was confirmed by N-terminal sequencing (RNSRVKECFEMTTDY). Thus, the recombinant LBPK could be obtained by enzymatic digestion with Factor Xa. The final yield of purified LBPK per 400 ml of culture was approximately 3.8 mg (Table 1), and the overall yield of the enzyme in the purification procedure was 73.1%, with a specificity of 147.3 U/mg.

Table 3. Expression and purification of LBPK in *E. coli*.

Purification step	Total protein (mg)[a]	Total activity (U)[b]	Specific activity (U/mg)[c]	Purification (fold)	Yield (%)
Crude extract[d]	122.8	761.9	6.2	1.0	100
Soluble extract	107.8	744.6	6.9	1.1	97.8
GSTrap HP	4.3	557.4	129.0	20.8	73.1
Factor Xa digestion	3.8	556.9	147.3	23.7	73.1

[a]Total protein concentration was determined by BCA protein assay (Pierce) using bovine serum abumin (BSA) as a standard.
[b]One unit of phosphoketolase activity was defined as the amount of enzyme that converted 1 μmol of acetyl phosphate into 1 μmol of fructose-6-phosphate per minute under the assay conditions.
[c]The specific activity is expressed in units of enzyme activity per milligram of protein in an activity assay.
[d]The starting material was crude extract from the lysis of 400 ml of induced *E. coli* culture.

Kinetics Characterization and Phosphoketolase Activity of LBPK

The effect of temperature on phosphoketolase activity is shown in Fig. 5A, which indicates that the optimal temperature for enzyme activity is 45°C. The phosphoketolase activity increased rapidly as the temperature increased to 45°C, and then decreased at temperatures higher than 50°C. The enzyme activity was maximal at pH 7.0 (Fig. 5B). The K_M value estimated from a Lineweaver-Burk plot was 5.08±0.057 mM (mean±SD) for fructose-6-phosphate, with a maximun rate (V_{max}) of 499.0±4.33 μmol/min/mg.

A phosphoketolase catalyzes the conversion of F6P and X5P into the glyceraldehydes 3-phosphate and acetyl phosphate, with both activities measurable based on the acetyl phosphate formed in the presence of P_i and thiamine PP_i [23]. The produced acetyl phosphate forms a ferric acetyl hydroxamate, a product with a bright purple color, upon the addition of ferric chloride. This conversion can be monitored by spectrophotometry and colorimetry [19]. *E. coli* transformants harboring p5X-3LBPK developed a light-yellow to reddish-violet color immediately upon the addition of ferric chloride. Although we did not test the utilization of X5P by the *Lb. paraplantarum* C7 phosphoketolase, the cloned putative phosphoketolase exhibited a high homology with Xfp from other LAB. Xfp belongs to the class of nonspecific enzymes and uses both X5P and F6P as substrates [14], and the F6P-specific enzyme F6PPK is also known to act on X5P [21]. This suggests that the phosphoketolase of *Lb. paraplantarum* C7 acts on both F6P and X5P, although X5P was not tested as a substrate in this study.

In conclusion, a gene encoding a putative phosphoketolase, a key enzyme in carbohydrate catabolism by heterofermentative LAB, has been cloned from *Lb. paraplantarum* C7. The deduced amino acid sequence of the gene shows high homology with phosphoketolases, especially Xfp from other LAB, and contains two highly conserved signature patterns found in phosphoketolase genes. One of the signature sequences, a TPP-binding site, is also found in transketolase genes. F6P was transformed into acetyl phosphate and acetic acid in *E. coli*, indicating that the enzyme encoding the gene exhibits phosphoketolase activity.

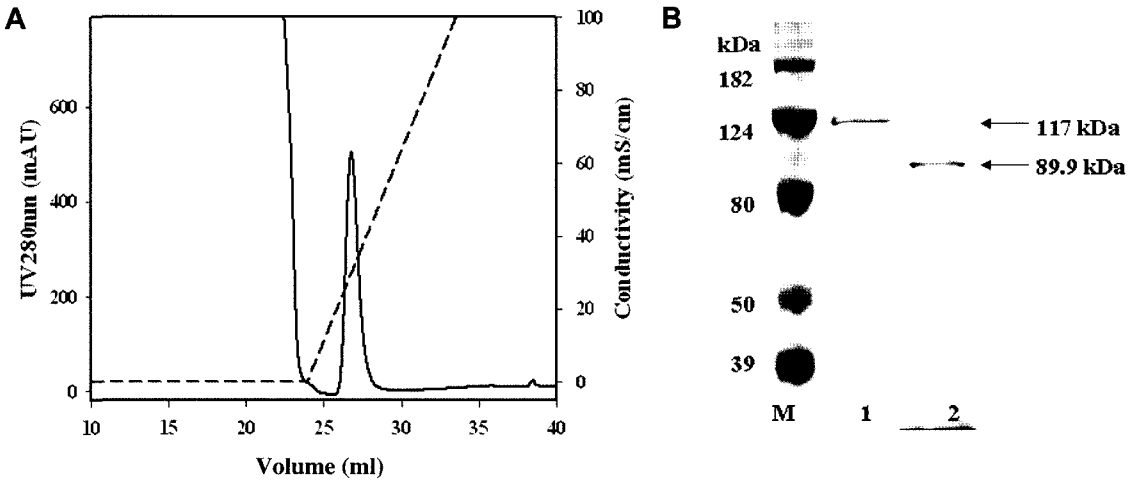

Fig. 4. Purification of GST::LBPK and cleavage with Factor Xa.
A. Chromatogram from GSTrap HP column for analysis of recombinant GST::LBPK. **B.** SDS-PAGE analysis of purified GST::LBPK and LBPK obtained by Factor Xa cleavage. Lane: M, molecular weight marker; 1, GSTrap HP column-purified fraction of GST::LBPK; 2, Cleavage product of GST::LBPK by Factor Xa.

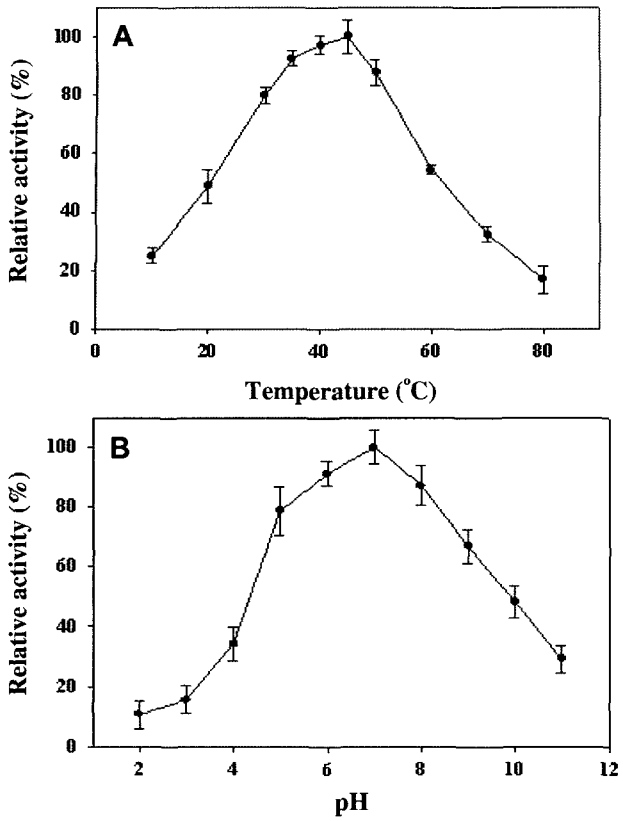

Fig. 5. Enzymatic properties of purified LBPK. The results are mean values from triplicate experiments, with bars indicating ±SD.

A. Temperature dependency of LBPK activity. The activities are shown as percentages relative to that at 45°C. **B.** pH dependency of LBPK. The activities are shown as percentages relative to that at pH 7.0.

These results will advance recombinant technology for commercial applications in the dairy industry and in the fermentation of foods such as *kimchi* by enabling regulation of the synthesis of phosphoketolase.

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