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Full Length Research Paper

Glutaminase activity of *Lactobacillus reuteri* KCTC3594 and expression of the activity in other *Lactobacillus* spp. by introduction of the glutaminase gene

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We investigated the properties of glutaminase activity of *Lactobacillus reuteri* KCTC3594 and expressed the glutaminase gene (EC 3.5.1.2) heterologously. The enzyme activity was optimal at pH 7.5 and 40°C. High salt–tolerance of the enzyme was observed as 50% of the initial activity although it still remained at 20% (w/v) NaCl for 30 min. The glutaminase gene was cloned from *L. reuteri* KCTC3594 by PCR, and subsequently introduced into two Korean isolates of *Lactobacillus* species. All of the transformants harboring the glutaminase gene from *L. reuteri* KCTC3594 were able to elevate glutaminase activity when introduced into other lactobacilli.

Key words: *Lactobacillus* spp., glutaminase, salt-tolerance, natural flavor, glutaminase gene, heteroexpression.

INTRODUCTION

Monosodium glutamate (MSG) gives the taste “umami” which has been widely recognized as the fifth basic taste besides sweet, acid, salty and bitter. It has been widely used as a flavor enhancer in the food industry. However, there is some disagreement about the safety of MSG because some people experience side effects such as wheezing, changes in heart rate and difficulty in breathing (Farombi and Onyema, 2006).

Interest in developing a natural flavor enhancer as the alternative to MSG has been increased. Among the yeast and fungi is one of the most important enzymes used to enhance the flavor of food (Nandakumar et al., 2003).

Many organisms including mammals produce two natural flavor enhancers, glutaminase (EC 3.5.1.2) which is ubiquitous in various microorganisms including bacteria, glutaminases, which encode the isoforms known as kidney (K-type) glutaminase (KGA) and liver (L-type) is enzyme (LGA). However, in bacteria such as *Escherichia coli,* they have been specified as glutaminase A and B (Campos-Sandoval et al., 2007). On the other hand, little is not known regarding the glutaminase type in *Lactobacillus* spp. although glutaminase in lactobacilli has been characterized in several species. Also, glutaminase plays an important role in soy sauce fermentation hydrolyzing L-glutamine to produce L-glutamate which is a high flavor amino acid in foodstuffs (Masuo et al., 2004). Especially, salt tolerant and heat stable glutaminase has been receiving much attention for applications in both the food and pharmaceutical industries (Yoshimune et al., 2004).

As a part of our study on glutaminase, we have investigated the properties of glutaminases of food-grade *Lactobacillus* spp. Traditional dairy strains of *Lactobacillus* spp. are commonly used in the food fermentation industry for production of cheeses, buttermilk, sauerkraut and yogurt (Salminen et al., 1998). They play beneficial roles in maintaining health, which makes them promising probiotic organisms (Weingand-Ziadé et al., 2003). As many lactobacilli do, *Lactobacillus reuteri* also produces an antimicrobial substance (reuterin) which can inhibit pathogens in the gastrointestinal tract (Shornikova et al., 1997). In addition, many strains of *L. reuteri* have been isolated from meat or dairy products and are widely used in the food industry. Therefore, *L. reuteri* is a good candidate to be applied as a flavor enhancer in natural food sources with quality assurance.

In our previous studies, we isolated various *lactobacilli*

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Abbreviations: MSG; Monosodium glutamate, **KGA;** Kidney (K-type) glutaminase**, LGA**; Liver (L-type) isoenzyme.

from Korean adults (Choi et al., 2002). Among them, we have been interested in two strains for development of functional probiotics: one is *Lactobacillus plantarum* KLB213 which exhibits a high salt-tolerance, and the other is *Lactobacillus paracasei* KLB58 which produces a high amount of exopolysaccharide (Lee et al., 2008). In this study, we attempted to investigate the enzymatic properties of glutaminase from *Lactobacillus reuteri* KCTC3594. In addition, the glutaminase gene cloned from *L. reuteri* KCTC3594 was introduced into *L. plantarum* KLB213 and *L. paracasei* KLB58 in expectation of increasing glutaminase activity.

MATERIALS AND METHODS

Bacterial strains and media

All of the *Lactobacillus* spp. were grown anaerobically in MRS medium (Difco, West Molesey, United Kingdom) at 37℃. *E. coli* DH5α used for replication of plasmids was grown in LB medium (Bacto tryptone 10 g/L, Bacto Yeast extract 5 g/L, Sodium chloride 10 g/L) at 37℃ with shaking. Erythromycin for transformants of *Lactobacillus* spp. and ampicillin for *E. coli* harboring vectors were added to the media at final concentrations of 5 and 50 μg/mL respectively.

Glutaminase assay

Cultivated cells were harvested by centrifugation for 10 min at 8,000 xg. To remove remnant ingredients of media, cell pellets were washed twice with 1 ml of TE buffer (50 mM Tris - HCl and 5 mM EDTA; pH 7.5). The cells were resuspended with 1 ml of TE buffer, and were subsequently homogenized by sonication for 1 min. The concentration of total protein was measured by the Bradford method (Bradford, 1976) using crystalline bovine serum albumin as the standard. The initiation solution (0.1 ml of fresh 2% L-glutamine solution and 0.2 ml of 100 mM Tris - HCl buffer; pH 7.5) was equilibrated at 37℃ for 5 min. The reaction was started immediately where 0.1 ml of cell extract was then added to the initiation solution. The sample was then incubated at 37℃ for 10 min. To stop the reaction, the sample was boiled for 3 min and centrifuged at 8,000 xg for 5 min. An aliquot of 50 μl of the supernatant was added to first reaction mixture containing 1 ml of hydroxylamine buffer (0.25 M hydroxylamine and 20 mM EDTA, pH 8.0), 0.5 ml of 10 mM NAD^+ solution, 1 ml of distilled water and 20 U of glutamate dehydrogenase (GLDH). After incubation for 30 min at 37℃, the glutaminase activity was determined by measuring optical density at 340 nm in a spectrophotometer. One unit (U) of glutaminase activity was defined as the amount of enzyme which was consumed to produce 1 μmol of NADH per min at 37℃. To find the optimum temperature, pH and salt concentration, the parameters in the initial equilibration steps were varied in the following ranges: 20 - 60℃, pH 3 - 11 and 0 - 20% NaCl.

Cloning of glutaminase gene and transformation from *L. reuteri* KCTC3594

Based on the genome sequence of *L. reuteri* F275 (NC009513 in NCBI), the specific primers for amplification of glutaminase gene were designed as follows: Glu-for (5' - AGT CAC GAG TGA CTT CTG – 3') and Glu-rev (5' – AGC GGT GGC AAA GTA GTC – 3'). PCR was carried out using the genomic DNA of *L. reuteri* KCTC3594 as templates. The sequencing analysis of the PCR product was performed at SolGent, Co. (Daejeon, Republic of Korea). The sequence homology was analyzed using BLAST program on the NCBI web site (http://www.ncbi.nlm.nih.gov/BLAST/). The amplified glutaminase gene was inserted to pGEM T easy vector (Promega). The product of insertion was named pL3594. To construct a transformation vector, the 1.2 kb fragment of *Eco*RI-digested pL3594 and *Eco*RI-digested pTRKL2, *E. coli* – *Lactobacillus* shuttle vector (Danie and Todd, 1993) were ligated. The 7.5 kb circular plasmid constructed after ligation was named pGlu-16 and was introduced into *L. plantarum* and *L. paracasei* by electroporation method (Hashiba et al., 1990; Chung et al., 2008). The transformants were then selected on MRS agar containing 5 μg/mL of erythromycin. To confirm transformation, PCR was conducted using two PCR primer sets for amplification of the erythromycin resistance gene (forward, 5'- GAA ATT GGA ACA GGT AAA GG-3', reverse , 5' - GAT ATT CTC TGA CCC AT-3') and glutaminase gene (Glu-for and Glu-rev).

RESULTS AND DISCUSSION

Glutaminase activity of *Lactobacillus spp*

When glutaminase activity was compared among *L. reuteri* KCTC3594, *L. plantarum* KLB213 and *L. paracasei* KLB58, *L. reuteri* KCTC3594 showed the highest activity of 0.516 U/mg. It was 2.4-fold higher than that of *L. plantarum* KLB213. On the other hand, *L. paracasei* KLB58 showed no glutaminase activity (Figure 1).

To determine the hydrolyzing ability of glutaminase, a glutaminase assay is commonly used. In the assay, the glutaminase activity is indirectly determined by measuring the amount of NADH which is produced during conversion of L-glutamate to 2-oxoglutarate by glutamate dehydrogenase (Masuo et al., 2005; Brown et al., 2008). There are many enzymes involved in glutamate metabolism. Among them, two enzymes participate in direct and reversible pathways between L-glutamine and L-glutamate (Guillamon et al., 2001). One is glutaminase which we are interested in, and another is glutamine synthetase (Figure 2). However, in the cases of both of them, it is too difficult to determine the activity of the enzymes directly. Thus, we also used the common indirect method of performing a glutaminase assay as described in Materials and Methods. The three species of *Lactobacillus* used in this study showed different levels of glutaminase activities.

Regarding glutamate metabolism, there are two major enzymes involved, glutaminase (EC 3.5.1.2) and glutamine synthetase (EC 6.3.1.2), between L-glutamine and L-glutamate (16, 18). Based on the previous genomic data, *L. plantarum* has only glutamine synthetase while *L. reuteri* has both enzymes. There has been no genomic data until now for *L. paracasei* on glutamate metabolism (http://www.ncbi.nlm.nih.gov/BLAST/). This genomic data was supported by the fact that the glutaminase gene was amplified in only *L. reuteri* KCTC3594 (data not shown). These studies suggest that glutaminase participates mainly in glutamate metabolism from glutamine to glutamate by EC 3.5.1.2 (Figure 2). On the other hand, there are also the two enzymes on the genomic data of *Lacto* -

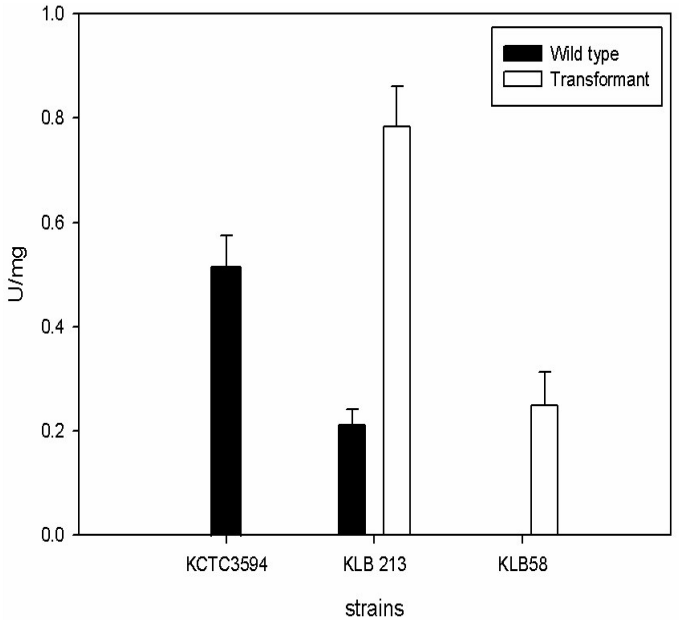

Figure 1. Comparison of glutaminase activity among different *Lactobacillus* spp. KCTC3594, KLB213 and KLB58 refer to *L. reuteri* KCTC3594, *L. plantarum* KLB213 and *L. paracasei* KLB58, respectively. The activity of each enzyme was measured in triplicate.

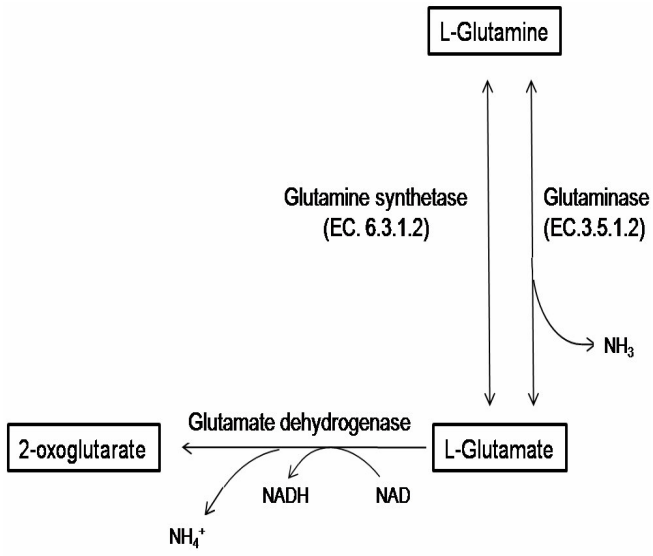

Figure 2. Scheme of reactions related to the glutaminase assay. The main reaction of glutaminase is known as conversion of glutamine to glutamate and that of glutamine synthetase is anti-oriented although both of the two enzymes are reversible.

bacillus gasseri LGAS0507 and *Lactobacillus johnsonii* NCC533. Based on the data, we assayed glutaminase activity in *L. gasseri* and *L. johnsonii* isolated from Korean adults. The activities of both species were higher than *L. plantarum* although lower than *L. reuteri* (data not shown).

Table 1. Properties of glutaminase from *L. reuteri* KCTC3594.

Property	Glutaminase
Substrate specificity	L-glutamine
Optimal temperature	40℃
Thermostability	60℃ (30 min)
Optimal pH	7.0 - 8.0
Stable pH	7.0-11.0
Optimal NaCl	5%
Salt tolerance [a]	50%
Km (L-glutamine) [b]	4.5 ± 1.7
Vmax	101.6 ± 1.05

[a] Relative activity in 20% NaCl; the activities without NaCl were considered to be 100%.
[b] The Km for ammonia was calculated from Michaelis-Menten equation.

Properties of glutaminase activity of *L. reuteri* KCTC3594

We examined the effect of pH, temperature and salt on the glutaminase activity of *L. reuteri* KCTC3594, as summarized in Table 1. The glutaminase worked optimally at pH 7.5. The optimum temperature was 40℃ and 78% activity still remained after incubation at 60℃ for 10 min. To determine the salt dependence of glutaminase activity, the activity was assayed in a reaction mixture of 0 - 20% (w/v) NaCl. It was shown that *L. reuteri* KCTC3594 was salt-tolerant as the relative activity displayed 110 % in the condition of 5% NaCl and remained over 50% in the presence of 20% NaCl. The kinetic parameters of glutaminase were calculated by non-linear Michaelis-Menten equation. As shown in Table 1, the Km values of the *L. reuteri* KCTC3594 for glutamine were similar to *Lactobacillus rhamnosus* and *Anabaena* sp. (Weingand-Ziadé et al., 2003; Zhou et al., 2008).

In previous studies on glutaminases from *Micrococcus luteus* K-3 type *maltophilia*, it has been reported that the activity of glutaminase decreased when salt concentration increased (Yoshimune et al., 2004). In the case of glutaminase of *L. rhamnosus*, however, the relative activity was 1.8 fold higher in the presence of 2.5% NaCl which exhibited the highest activity than the control (no salt) (Weingand-Ziadé et al., 2003). *L. reuteri* KCTC3594, in this study, also showed similar osmotolerance as *L. rhamnosus*. The above results indicate that glutaminase from lactobacilli can probably be more tolerant to salt than other species reported. In addition, the enzymes from *L. rhamnosus* (Weingand-Ziadé et al., 2003) and *L. reuteri* KCTC3594 used in this study were also stable in broad ranges of pH and temperature (Table 1). The glutaminase activity was detected at a pH range from 5.0 - 11.0 which was broader than 7.5 - 9 for *Cyanobacterium Anabaena* sp. PCC7120 (Zhou et al., 2008), or 6.0 - 9.0 for *L. rhamnosus* (Weingand-Ziadé et al., 2003). These results support that *L. reuteri* KCTC3594 can be used to

increase the amount of glutamic acid by hydrolysis of glutamine in the stressful conditions of elevated salt concentration or temperature.

Cloning of the glutaminase gene

The 1.2 kb PCR product harboring the glutaminase gene was obtained from *L. reuteri* KCTC3594 by PCR using the specific primers. The nucleotides of the glutaminase gene showed 48 and 46% identities with *Lactobacillus gasseri* and *Lactobacillus johnsonii*, respectively. When the nucleotide sequence was converted into an amino acid sequence, the similarities with *L. gasseri* and *L. johnsonii* were 69 and 68% respectively (data not shown). On the other hand, we obtained no PCR products from *L. plantarum* KLB213 and *L. paracasei* KLB58 using the same primers.

Elevation of the enzyme activity by transformation

To ascertain whether the glutaminase gene was applicable to various species, we attempted to introduce the glutaminase gene cloned from *L. reuteri* KCTC3594 into other *Lactobacillus* species, *L. plantarum* KLB213 and *L. paracasei* KLB58, in which the glutaminase gene was not amplified. All the transformed strains with the exogenous plasmids harboring the glutaminase gene showed substantially increased glutaminase activity (Figure 1). Especially, the transformants of *L. plantarum* KLB213 displayed an approximately 4–fold higher activity than the wild type. Additionally, the activity of the transformants of *L. plantarum* KLB213 showed a 1.5 fold higher glutaminase activity than the wild type of *L. reuteri* KCTC3594. Although the wild type *L. paracasei* KLB58 showed no activity, the glutaminase activity of the transformants increased to half of the activity of *L. reuteri* KCTC3594. The increase of the glutaminase activities of *L. plantarum* KLB213 and *L. paracasei* KLB58 after transformation indicates that the glutaminase gene from *L. reuteri* KCTC3594 is able to elevate the glutaminase activities of other species. Therefore, it is expected that the glutaminase gene can be used to develop a variety of functional lactic acid bacteria. When the glutaminase gene is introduced into a thermotolerant *Lactobacillus* sp., for example, the manipulated species may be able to become a good candidate for probiotic food additives with flavor enhancing effects.

The introduction of heterologous genes being offered to different species opens new possibilities for improving their functionality. However it also creates ethical and regulatory issues related to GMOs that have to be dealt with (Guillamon et al., 2001). Therefore, we are now currently in the process of developing food-grade functional *Lactobacillus* strains with high glutaminase activity for flavor enhancing effects. In addition, we will attempt to investigate the deletion of an antibiotic marker gene inserted for selection of transformants because the use of an antibiotic resistance gene as a selectable marker gene is inappropriate for the food industry (Renault et al., 2002). It is also necessary to establish practical conditions for high yield production of glutaminase.

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