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Increase of "Umami" and "Kokumi" compounds in miso, fermented soybeans, by the addition of bacterial γ-glutamyltranspeptidase

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Abstract

γ-Glutamyltranspeptidase (GGT) hydrolyzes γ-glutamyl compounds and transfers their γ-glutamyl moieties to amino acids and peptides. We previously showed that the "umami" taste of soy sauce could be improved by the addition of salt-tolerant *Bacillus subtilis* GGT to the fermentation mixture, "moromi". Although miso fermentation is a semi-solid fermentation, unlike soy sauce fermentation, this was also the case. When 15 units of purified *B. subtilis* GGT were added to 418 g miso "moromi" (fermentation mixture), the glutamate concentration in "moromi" became 20 mM higher and the "umami" taste became stronger than without the addition of GGT after 2 to 6 months of fermentation. In addition, γ-Glu-Val and γ-Glu-Val-Gly, which are known as "kokumi" peptides, were identified in "tamari", and the concentrations of these γ-glutamyl peptides in "tamari" fermented by the addition of GGT were significantly higher than those of "moromi" without the addition of GGT. These results indicate that *B. subtilis* GGT is able to improve the taste of miso.

Keywords: γ-glutamyltranspeptidase; glutaminase; miso; umami; kokumi; *Bacillus subtilis*

1 Introduction

γ-Glutamyltranspeptidase (GGT; EC 2.3.2.2) consists of one large subunit and one small subunit. GGT catalyzes the transfer of γ-glutamyl moiety from γ-glutamyl compounds to amino acids and peptides, and the hydrolysis of γ-glutamyl compounds (Tate & Meister, 1981). The enzymatic reaction catalyzed by GGT proceeds via a γ-glutamyl enzyme intermediate. The activated oxygen atom of the side chain of the N-terminal threonine residue of the small subunit of GGT attacks the carbonyl carbon of a γ-glutamyl compound to form a γ-glutamyl enzyme intermediate. When this intermediate is subjected to nucleophilic substitution by amino acids or peptides, the reaction is a transpeptidation reaction, producing new γ-glutamyl compounds. When the intermediate is subjected to nucleophilic attack by water, the reaction is a hydrolysis reaction, releasing glutamate. If the original γ-glutamyl compound is glutamine, the hydrolytic reaction is a "glutaminase" reaction (Inoue, Hiratake, Suzuki, Kumagai, & Sakata, 2000).

The quality of soy sauce depends on its glutamate concentration. During its fermentation, soy proteins are cleaved into peptides by proteases from *Aspergillus oryzae* and/or *soyae*, and the

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peptides are cleaved into amino acids by their peptidases. Glutamine liberated from soy proteins by this process is hydrolyzed to glutamate by glutaminase; however, since soy sauce is fermented in the presence of 18% NaCl, glutaminase from these fungi is strongly inhibited. When the activity of glutaminase is insufficient, glutamine is converted spontaneously to tasteless or slightly sour pyroglutamic acid. In the previous study, we showed that by the addition of salt-tolerant *B. subtilis* GGT as glutaminase to the fermentation mixture, "moromi", the glutamate concentration of soy sauce increased and "umami" taste improved (Kijima & Suzuki, 2007).

Miso is a traditional Japanese seasoning widely used in daily meals. It is produced in the presence of about 9% of NaCl from rice or naked barley, soybeans, salt, and koji (steamed rice cultivated with *Aspergillus oryzae* as a source of enzymes). The fate of soy proteins of miso "moromi" is the same as that of soy sauce "moromi". The only difference is that miso fermentation is a semi-solid fermentation, unlike soy sauce fermentation. Whether the addition of salt-tolerant GGT is also effective on semi-solid miso fermentation remains to be elucidated.

Besides the five fundamental tastes, sweet, salty, sour, bitter and "umami", "kokumi" is a taste that is peculiar to Japanese and is used to describe characteristics such as persistence, mouthfulness, and thickness of taste (Ueda, Yonemitsu, Tsubuku, Sakaguchi, & Miyajima, 1997). "Kokumi" substances themselves do not have a "kokumi" taste, but the addition of a small amount of these substances enhances the flavour of food by inducing persistence, depth, and mouth-fullness. It was reported that some γ-glutamyl compounds, such as GSH, are "kokumi" substances (Dunkel, Koester, & Hofmann, 2007; Toelstede, Dunkel, & Hofmann, 2009; Toelstede & Hofmann, 2009). Ohsu et al. (2010) compared the enhancement of "kokumi" by various γ-glutamyl compounds and found that γ-Glu-Val-Gly is the strongest "kokumi" substance. Since GGT can also catalyze the transpeptidation reaction, some γ-glutamyl compounds might be formed from glutamine as a donor, and amino acids and peptides as acceptors when *B. subtilis* GGT is added to "moromi".

In this study, the effect of the addition of *B. subtilis* GGT to miso "moromi" on its concentrations of glutamate and some γ-glutamyl compounds, and on the taste was compared.

2 Materials and Methods

2.1 Bacterial strain used in this study

Plasmid pCY167 (ColE1 *ori bla*$^{+}$ *lacI*^{q} *rrnBT1* T5*p-ggt*$^{B.subtilis}$) is a derivative of pQE-80L harboring a signal peptide, large and small subunits of *B. subtilis* GGT following the initiation codon, but it does not have a His-tag; that is, the nucleotide sequence within the open reading frame is exactly the same as the wild-type *B. subtilis ggt* gene. *Escherichia coli* K-12 strain SH641 (F^{-} Δ*ggt-2 rpsL recA56 srl300*::Tn*10*) was transformed with this plasmid pCY167 and strain CY168 was obtained (Suzuki et al., 2010).

2.2 Purification of GGT

Strain CY168 was used to purify *B. subtilis* GGT; it was grown at 37^{o}C in 2 L LB Miller medium (Becton Dickinson; Franklin Lakes, NJ) supplemented with 100 μg mL^{-1} ampicillin until $OD_{600} = 0.6$. GGT was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (final concentration, 0.5 mM) and the cells were further incubated at 20^{o}C for 2 days. The periplasmic fraction was prepared as described previously (Suzuki, Kumagai, & Tochikura, 1986a) and GGT was purified with a Gigapite column (Seikagaku Kogyo; Tokyo, Japan) as described previously (Minami, Suzuki, & Kumagai, 2003).

2.3 Determination of GGT activity

GGT activity was measured by the standard assay method described previously (Suzuki, Kumagai, & Tochikura, 1986b). One unit of the enzyme was defined as the amount of enzyme that released 1 µmol of *p*-nitroaniline per minute from γ-glutamyl-*p*-nitroanilide (γ-G*p*NA). The transpeptidation activity was defined as the ac-

tivity enhanced in the presence of an acceptor substrate, glycylglycine.

2.4 Fermentation of miso

Miso was fermented using a “make your own miso kit” (Ikedaya-Jozo; Kumamoto, Japan). “Moromi” (fermentation mixture) was made by mixing 975 g dried rice koji with salt, 1,360 g steamed and dried soybeans, and 80 mL water. “Moromi” was portioned into six plastic Zip-loc bags (418 g each). GGT (15.6 units as hydrolysis activity toward γ- G*p*NA at pH 8.73) dissolved in 20 mM Tris-HCl pH 8.0 was added to three bags (7.44 mL each). To the three control bags, 7.44 mL of the same buffer was added. Excess air was removed from the bags and they were sealed. All six bags of miso “moromi” were incubated at room temperature (20-24^{o}C), and the bags were turned upside down once a week.

2.5 Sampling of “moromi”

Five hundred milligrams of “moromi” were weighed into a sample tube with a medical spoon every 10 days, resuspended thoroughly in 1 mL distilled water, and centrifuged at 12,000 x g for 5 min. The supernatant was collected as the “moromi” sample and stored at -80^{o}C until analysis.

2.6 Sampling of “tamari”

The liquid leaked out from “moromi” during fermentation is called “tamari”, which is thought to be a primitive-type soy sauce. After 6 months of fermentation, “tamari” was collected from the corner of the plastic bags and centrifuged at 14,000 rpm for 5 min. The supernatant was collected and stored at -80^{o}C until analysis.

2.7 Measurement of glutamic acid and glutamine concentration

The concentrations of glutamate and glutamine in “moromi” were measured with an HPLC system (model LC-20; Shimadzu, Kyoto, Japan) equipped with a Shim-pack amino Na column (Shimadzu), with gradient elution at 60^{o}C at a flow rate of 0.6 ml min^{-1}. The gradient of the mobile phase was formed with buffer A (66.6 mM citrate, 1% perchloric acid, 7% ethanol, pH 2.8) and buffer B (200 mM citrate, 200 mM boric acid, 0.12 N NaOH, pH 10). The concentration of buffer B was kept at 0% until 9 min. It was linearly increased to 7% from 9 to 13 min, to 8% from 13 to 17.2 min, and then to 11%. *o*-Phthalaldehyde was used as the detection reagent, and the fluorescence was detected with a fluorescence detector (model RF-10Axl; Shimadzu) as the absorbance at 450 nm, with excitation at 348 nm, as described previously (Suzuki et al., 2003).

2.8 Evaluation of “umami” and “kokumi” taste of miso and “tamari”

Evaluation of “umami” and “kokumi” taste of miso and “tamari” samples were performed as described previously (Kijima & Suzuki, 2007) with slight modifications.

For this research, we made commercial-style miso from soybeans and rice koji. Commercially, miso made from soybeans and rice koji is usually shipped out to the market after 6 months of fermentation. Therefore, to evaluate “umami” taste, 10 grams of miso samples were collected after 6 months of fermentation and dissolved in 100 mL hot water. Twenty panel members were trained with 0.1% monosodium glutamate as the standard “umami” taste solution. Then, they tasted one teaspoonful of each miso soup and compared the “umami” taste. The panel members were told about the purpose of the test, but were not told what each sample was. The test was done in the regular laboratory and they were asked which miso soup had a stronger “umami” taste. The evaluation was performed on a five-point scale: sample A (without GGT) has much stronger “umami” taste; sample A (without GGT) has stronger “umami” taste; cannot distinguish “umami” taste of sample A and B; sample B (with GGT) has stronger ”umami” taste; sample B (with GGT) has much stronger ”umami” taste.

Similarly, “tamari” sampled after 6 months of fermentation was evaluated by 14 panel mem-

bers. They were asked to evaluate which sample had stronger "kokumi" taste. They licked teaspoons with "tamari" samples made with and without the addition of GGT, and evaluated "kokumi" taste by their thickness, continuity, and mouthfulness. The evaluation was performed on a five-point scale: sample A (without GGT) has much stronger "kokumi" taste; sample A (without GGT) has stronger "kokumi" taste; cannot distinguish "kokumi" taste of sample A and B; sample B (with GGT) has stronger "kokumi" taste; sample B (with GGT) has much stronger "kokumi" taste. As we described in the introduction, "kokumi" substances themselves do not have a "kokumi" taste, but the present of small amount of these substances enhances the flavour of food. Therefore, we cannot use a pure chemical as a standard.

2.9 Identification and measurement of kokumi substances in "tamari"

Glutathione, γ-Glu-Val, and γ-Glu-Val-Gly were analyzed by Agilent CE-TOFMS system (Agilent Technologies; Waldbronn, Germany) equipped with Agilent 6210 time of flight mass spectrometer, Agilent 1100 isocratic HPLC pump, Agilent G1603A CE-MS adapter kit, and Agilent G1607A CE-ESI-MS sprayer kit, as described previously (Sugimoto et al., 2010). The system was controlled by Agilent G2201AA ChemStation software version B.03.01 for CE. Metabolites of interest were analyzed with a fused silica capillary (50 μm inner diameter × 80 cm total length), with commercial cation electrophoresis buffer (Solution ID: H3301-1001; Human Metabolome Technologies, Tsuruoka, Japan) as the electrolyte. The samples were diluted 10-fold with distilled water and injected at a pressure of 50 mbar for 10 sec (approximately 10 nL). The applied voltage was set at 27 kV. Electrospray ionization-mass spectrometry (ESI-MS) was conducted in positive ion mode and the capillary voltage was set at 4,000 V. The spectrometer was scanned from 50 to 1,000 $m\ z^{-1}$.
Raw data obtained by CE-TOFMS were processed with a software, "Master Hands", which was developed by Institute for Advanced Biosciences, Keio University (Tsuruoka, Yamagata, Japan). Signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites were excluded, and all signal peaks potentially corresponding to authentic compounds were extracted, and then their migration time (MT) was normalized using those of the internal standards. Thereafter, peaks were aligned according to the $m\ z^{-1}$ values and normalized MT values. Finally, peak areas were normalized against that of the internal standard, MetSul. The resultant relative area values were further normalized by the sample amount.

2.10 pH profile of *B. subtilis* GGT using glutamine as a γ-glutamyl donor in the presence of NaCl

Transpeptidation and hydrolysis activity of *B. subtilis* were compared at various pHs in the presence of 9% of NaCl; 10 mM glutamine was used as the donor substrate and 25 mM Val-Gly was used as the acceptor substrate. After incubation for 1 h at 37°C, the reaction was terminated by the addition of TCA (final concentration, 10%). The concentration of γ-Glu-Val and γ-Glu-Val-Gly was quantified as described above.

3 Results and Discussion

3.1 Effect of the addition of GGT to miso "moromi" on glutamate and glutamine concentrations in "moromi"

B. subtilis GGT was purified from the periplasmic fraction of strain CY168 with a Gigapite column and added to miso "moromi". Glutamate and glutamine concentrations of "moromi" during fermentation were measured (Figure 1). Glutamate concentration in "moromi" fermented by the addition of GGT gradually increased, peaked after two months and remained nearly unchanged until 6 months, and it was nearly 20 mM higher than that without GGT (Figure 1a). On the other hand, the concentration of glutamine in "moromi" fermented by the addition of GGT also

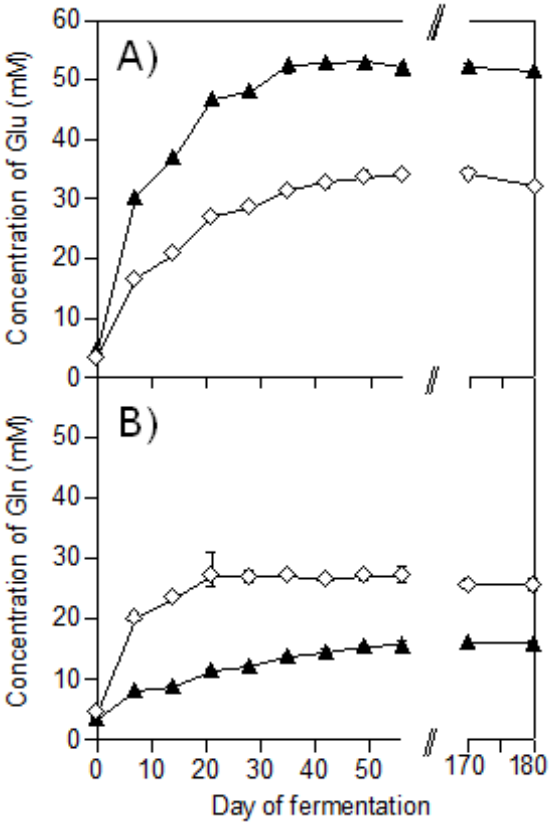

Figure 1: Concentrations of glutamate (A) and glutamine (B) in miso "moromi" with (filled triangles) and without (open diamonds) the addition of *B. subtilis* GGT. The average of three bags was plotted and deviations are shown by error bars.

increased, but it was about 10 mM less than that without GGT (Figure 1b). The results indicated that the addition of *B. subtilis* GGT accelerated the conversion of glutamine to glutamate in "moromi". The sum of glutamate and glutamine was about 10 mM higher in "moromi" with the addition of GGT than that without GGT. This indicated that more glutamine may have been lost as pyroglutamate without the addition of GGT, although the concentration of pyroglutamate was not measured.

3.2 Comparison of the taste of miso

The "umami" of miso fermented with and without the addition of GGT was evaluated. Eighteen out of 20 panel members recognized a difference between the soup made of miso with and without GGT and indicated that the soup made of miso with GGT had a stronger "umami" taste than that without GGT; two of them could not distinguish the difference (Figure 2a).
"Kokumi" of "tamari" fermented with and without the addition of GGT was evaluated. Twelve out of 14 panel members recognized a difference between "tamari" fermented with and without GGT and commented that "tamari" made with GGT had a stronger "umami" taste than that without GGT; two of them could not distinguish the difference (Figure 2b).

3.3 γ-Glu-Val and γ-Glu-Val-Gly concentrations in "tamari"

Many "kokumi" compounds are γ-glutamyl compounds; for example, glutathione, γ-Glu-Val and γ-Glu-Val-Gly are recognized as "kokumi" compounds. Since "tamari" from miso fermented with GGT had a stronger "kokumi" taste than without GGT, it was supposed that more γ-glutamyl compounds were formed in miso fermented by the addition of GGT, and this was confirmed. According to Ohsu et al. (2010), the intensity of the enhancement of "kokumi" by γ-Glu-Val and γ-Glu-Val-Gly were 0.61 times and 12.8 times relative to glutathione, respectively. The concentrations of reduced and oxidized glutathione, γ-Glu-Val, and γ-Glu-Val-Gly in "tamari" obtained after 6 months of fermentation with and without the addition of *B. subtilis* GGT were measured by CE-TOF-MS. As shown in Table 1, neither reduced nor oxidized glutathione was found. However, the concentrations of γ-Glu-Val and γ-Glu-Val-Gly in "tamari" fermented with the addition of GGT were significantly higher than those without GGT. The result showed that *B. subtilis* GGT catalyzed the transpeptidation reaction in "moromi", although its pH was acidic. On the other hand, since cysteine residues are quite reactive, it may be dif-

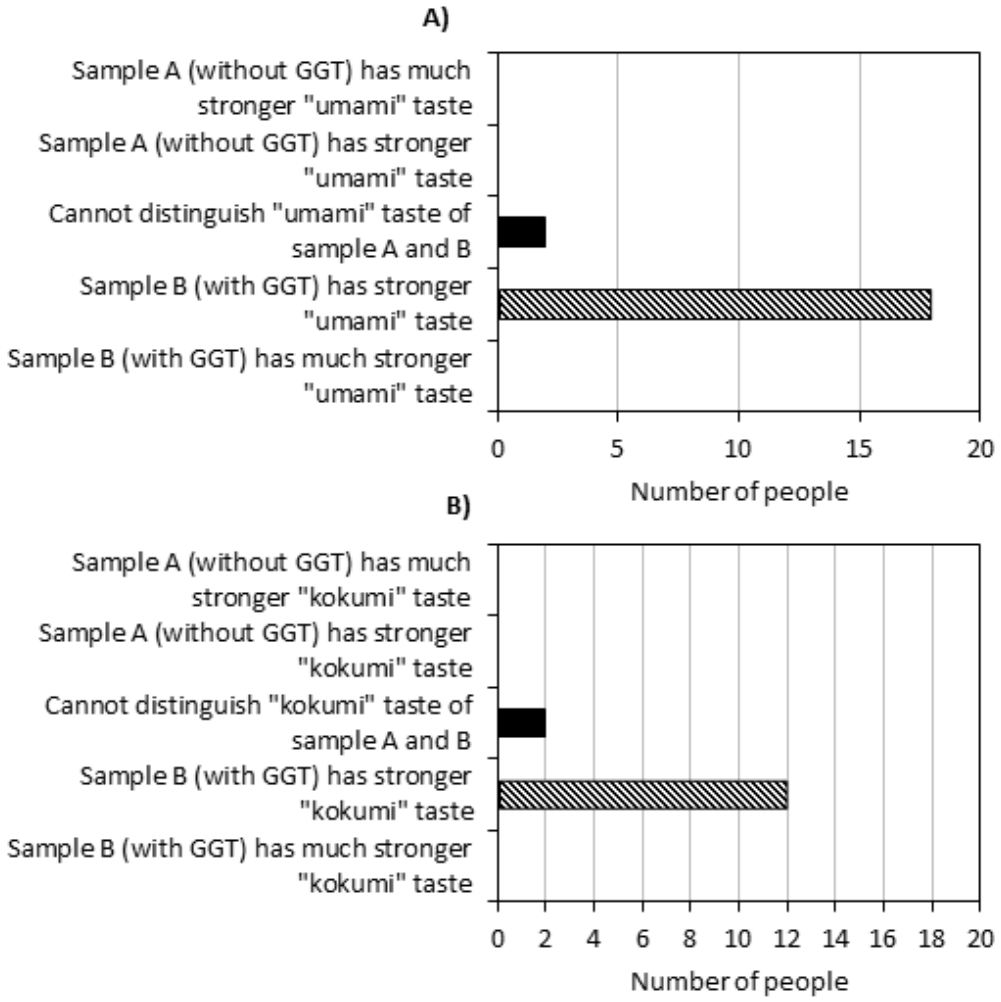

Figure 2: Comparison of "umami" taste of miso "moromi" (A) and "kokumi" taste of miso "tamari" (B) between miso fermented with and without the addition of *B. subtilis* GGT. Sample A: without GGT, sample B: with GGT.

Table 1: Concentrations of reduced and oxidized gltathione, γ-Glu-Val and γ-Glu-Val-Gly in "miso tamari"

Kokumi substances	Concentartion (μM)					
	without GGT			with GGT		
	#1	#2	#3	#1	#2	#3
Glutathione (reduced)	ND	ND	ND	ND	ND	ND
Glutathione (oxidized)	ND	ND	ND	ND	ND	ND
γ-Glu-Va	38	40	37	70	66	76
γ-Glu-Val-Gly	10	9.8	10	16	16	18

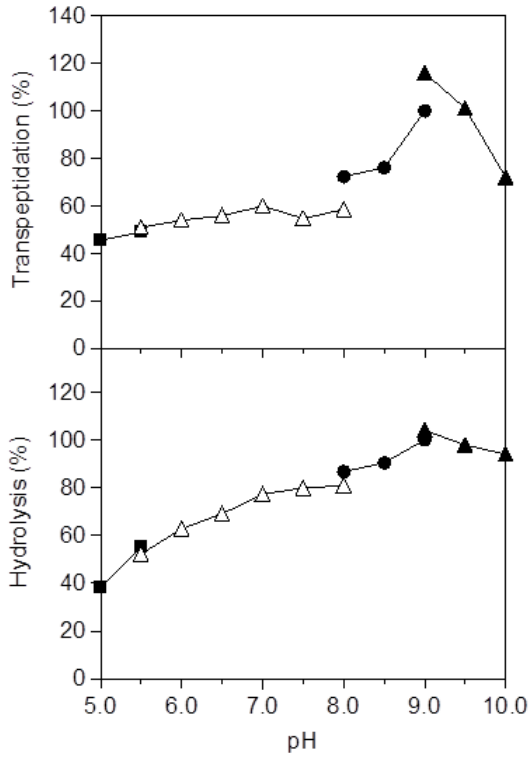

Figure 3: The pH profile of relative transpeptidation (A) and hydrolysis (B) activities of *B. subtilis* GGT in the presence of 9% NaCl. Transpeptidase activity was measured with 10 mM glutamine and 25 mM Val-Gly as substrates. Hydrolysis activity was measured using 10 mM glutamine as a substrate. The buffers used in reactions were 50 mM Na-acetate buffer (pH 5-5.5) (filled squares); 50 mM potassium phosphate buffer (pH 5.5-8.0) (open triangles); 50 mM Tris-HCl buffer (pH 8.0-9.0) (filled circles) and 50 mM imidazole-HCl buffer (pH 9.0-10) (filled triangles). The amount of purified enzyme used was 50.1 mU mL^{-1} (A) and 150 mU mL^{-1} (B) as transpeptidation activity measured under standard conditions, respectively.

ficult for glutathione to persist after a long fermentation period.

3.4 pH profile of the enzyme activity

The pH of miso "moromi" is around 5.5 and the optimum pH of the transpeptidation reaction of *B. subtilis* GGT is pH 9.5 using L-glutamine and Gly-Gly as substrates in the presence of 18% NaCl (Minami et al., 2003); therefore, it was surprising to find that γ-Glu-Val and γ-Glu-Val-Gly were formed in "moromi". We obtained the pH profiles of the transpeptidation reaction using L-glutamine as a γ-glutamyl donor substrate and Val-Gly as a γ-glutamyl acceptor substrate in the presence of 9% NaCl (Figure 3a) and that of the hydrolysis reaction using L-glutamine as a substrate (Figure 3b). Although the pH optima for both reactions were around pH 9, both reactions proceeded even at acidic pH in the presence of 9% NaCl.

4 Conclusions

Traditional fermentation of miso is carried out in the open-air and cannot prevent contamination from the environment; therefore, "moromi" for miso was fermented in the presence of 9% NaCl to inhibit the growth of contaminated microorganisms. At such a high salt concentration, however, enzymes from *Aspergillus oryzae* and *soyae*, which are important for fermentation, are strongly inhibited (Kijima & Suzuki, 2007). Glutaminase is needed to generate glutamate, which is in turn important for the "umami" taste. GGT has glutaminase activity and GGT from *B. subtilis* is very salt-tolerant (Minami et al., 2003). The addition of *B. subtilis* GGT to the fermentation mixture, "moromi", of miso increased not only the concentration of glutamate in miso, but also those of γ-Glu-Val and γ-Glu-Val-Gly. The result indicated that both hydrolysis and transpeptidation reactions of *B. subtilis* GGT occur in miso "moromi" even though it was a semi-solid fermentation and the quality of miso can be improved.

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