Sensors and Materials, Vol. 36, No. 8 (2024) 3177–3188
MYU Tokyo

S & M 3725

PACKTEST for L-Glutamate Quantification: Development of On-site and High-throughput Analytical Kits Using L-Glutamate Oxidase Mutant

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(Received August 21, 2023; accepted February 27, 2024)

Keywords: L-glutamate, colorimetry, glutamate oxidase, enzyme-based sensing, amino acid analysis

L-glutamate is an umami component found in various foods, and L-glutamate oxidase (LGOX, EC 1.4.3.11) is a FAD-dependent oxidoreductase that catalyzes the oxidative deamination of L-glutamate. A recently reported single-chain LGOX mutant from *Streptomyces* sp. X-119-6 is a potential material for L-glutamate sensors because this mutant is easier to prepare than the wild type. Herein, we report the development of a simple analytical kit using this LGOX mutant for the on-site quantification of L-glutamate. The kit named PACKTEST was developed using a colorimetric method to estimate the L-glutamate concentration in the range of 1–50 mg/L through the visual comparison of the resulting sample color with a standard color sequence. In addition, the PACKTEST kit was combined with a portable photometer for the spectrophotometric quantification of L-glutamate in the range of 0.5–12.0 mg/L. It was used to quantify L-glutamate in commercially available soy sauces, and the results were in good agreement (r = 0.97) with the values obtained by post-column derivatized ion-exchange chromatography. Furthermore, we loaded and dried all the reagents, including enzymes, on a 96-well plate to prepare an enzyme-based sensing device, which showed potential for the high-throughput quantification of L-glutamate.

1. Introduction

L-glutamate is one of the most abundant amino acids in foods, including meat, fish, and vegetables, and imparts a unique taste called *umami*. Umami is known as one of the five basic tastes (sweetness, sourness, bitterness, saltiness, and umami)[1,2] and activates the T1R1/T1R3 heterodimer of the taste receptor type 1 (T1R) family in our body.[3,4] The amount of L-glutamate in food affects not only taste but also process control in food production. In soy sauce, the L-glutamate concentration fluctuates significantly with the fermentation process,[5] and in

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https://doi.org/10.18494/SAM4624

ISSN 0914-4935 © MYU K.K.
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cheese, which requires long-term aging, the concentration of L-glutamate increases as the proteins in the milk break down.[6] These facts suggest that L-glutamate quantification plays an important role not only in food science and physiology but also in industry, such as in the control of food production processes.

Post-column derivatized ion-exchange chromatography[7] and liquid chromatography–mass spectrometry[8] are widely used for the determination of amino acids, including L-glutamate. Although these methods provide comprehensive analysis with high precision and accuracy, they require complex pretreatment and large, expensive analytical equipment, which limits the measurement opportunity. In contrast, analytical techniques using enzymes are generally simple, fast, convenient, and readily available. For these reasons, they have been used to solve the problems of conventional instrumental analytical techniques and to support research on L-glutamate and the control of food production processes.

L-glutamate oxidase (LGOX) from *Streptomyces* sp. X-119-6 has been widely used in enzymatic methods for L-glutamate quantification.[9–11] Wild-type LGOX is translated as a precursor and activated as a mature form by protease cleavage in *Streptomyces* sp. cells.[12,13] Recently, a mutant with activity equivalent to that of the mature form has been created by inserting flexible linkers into the protease cleavage site of the precursor.[14] This LGOX mutant does not require protease cleavage and can be heterologously expressed in *E. coli*. Therefore, highly active LGOX can be easily produced and may be useful as a new material for L-glutamate sensors.

In this study, we designed a colorimetric method using this enzyme and developed a simple analytical kit named PACKTEST, which was used to evaluate the performance of the LGOX mutant and quantify L-glutamate in food. PACKTEST is a water quality testing kit consisting of a highly flexible translucent polyethylene tube (10 mm in diameter) containing a powdered mixture of chromogenic reagents that selectively react with analytes [Fig. 1(A)]. A thread-like member inserted at the fusion end of the tube is pulled out to form a through-hole to the outside, and sample water is sucked in like a dropper to react with the chromogenic reagent.[15] The sample water changes color depending on the concentration of the target component, which can

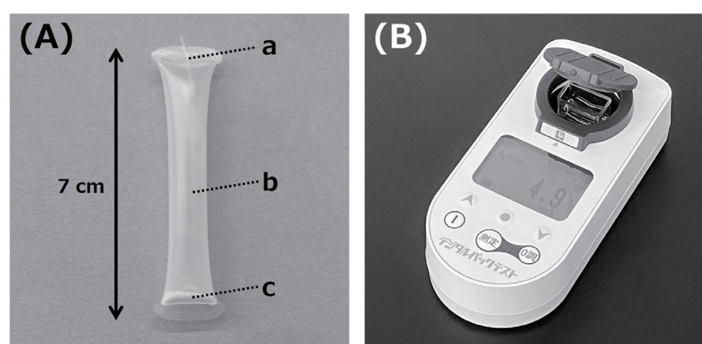


Fig. 1. Appearance of PACKTEST and portable photometer. (A) Components of PACKTEST: (a) thread-like plug, (b) flexible polyethylene tube, and (c) powdered mixture of chromogenic reagents. (B) Portable photometer used in this study for spectrophotometry.

be estimated through the visual comparison of the color tone with the accompanying color chart. The dried powder reagent has excellent long-term stability. For accurate analysis, absorbance spectrophotometric quantification using a portable photometer is also available if necessary.

The Trinder method,[16] a common clinical chemistry method, was used to develop a colorimetric system to analyze L-glutamate in aqueous samples. In the developed reaction system, H_2O_2 is generated from L-glutamate catalyzed by the developed LGOX mutant, and the resulting H_2O_2 is used to couple 4-aminoantipyrine to an aniline derivative (new Trinder reagent) under peroxidase catalysis to produce a blue quinone dye (Fig. 2). To construct a convenient on-site analytical kit, we optimized various parameters, including the composition of the powder reagent, which allowed rapid color reaction and stable long-term storage. The established kit was applied to analyze L-glutamate in food samples. Finally, an enzymatic sensing device was constructed by drying the solution of reagent mixture on a 96-well plate and evaluated for high-throughput quantification.

2. Materials and Methods

2.1 Production of LGOX mutant

The LGOX mutant gene from *Streptomyces* sp. X-119-6 with an N-terminal His-tag was obtained by DNA synthesis and cloned into the pET-16b vector (Merck, Darmstadt, Germany) at the *Nde* I and *Bam* HI sites. The plasmids were transformed into *E. coli* BL21 (DE3) competent cells. BL21 (DE3) cells were grown at 37 °C and induced with 0.1 mM IPTG when OD_{600} reached 1.0. The temperature was lowered to 20 °C, and culturing continued for 16 h. The washed cells, expressing each of the His-tag fusion proteins, were suspended in the lysis buffer (50 mM HEPES, 100 mM NaCl, pH 7.5) and disrupted with an ultrasonic disintegrator (Bioruptor, Cosmo Bio, Tokyo, Japan). The supernatant was applied onto a HisTrap FF crude column (Cytiva, Marlborough, MA, USA), and bound protein was eluted using an imidazole

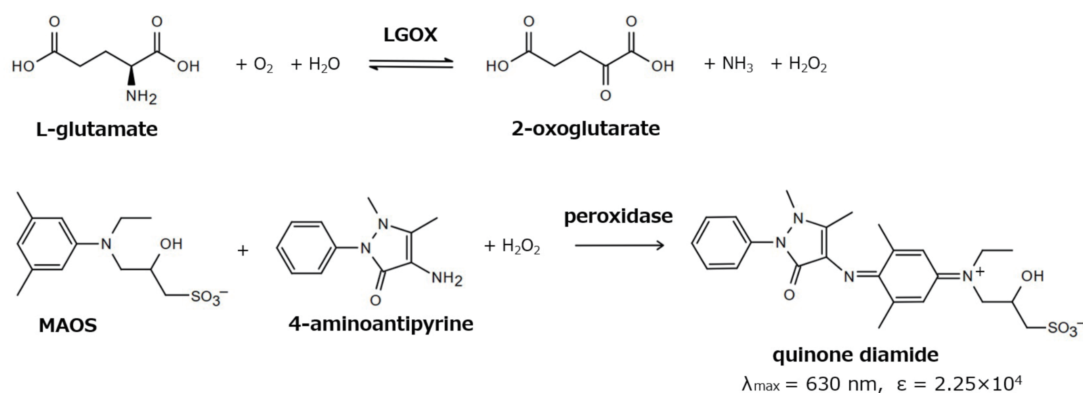


Fig. 2. Detection mechanism of L-glutamate.

gradient (50–500 mM). Fractions containing the target protein were collected, and protein samples were stored in 50 mM phosphate buffer (pH 6.5) or 100 mM NaCl buffer (pH 6.5).

The activity of this mutant was measured by colorimetric assay. The reaction mixture contained 100 mM HEPES (pH 7.5), 10 mM L-glutamate, 15 U/mL peroxidase, 1 mM 4-aminoantipyrine, 3 mM TOOS, and the enzyme solution, which was diluted to 1 μg/mL in a final volume of 200 μL. Absorbance at 555 nm was measured at 37 °C using a microplate reader (Varioskan LUX, ThermoFisher, Waltham, MA, USA). One unit was defined as the amount of enzyme producing 1.0 μmol of 2-oxoglutarate from L-glutamate at pH 7.5 and 37 °C in 1 min. D-sorbitol (1 mg, extra pure grade from FUJIFILM Wako Pure Chemical, Osaka, Japan) was added to 100 U of the LGOX mutant (specific activity ≥ 80 U/mg) and rapidly frozen in liquid nitrogen. The powder was lyophilized.

2.2 Chemical composition and preparation of the mixed reagents in PACKTEST

The composition of the mixed chromogenic reagent used per tube of the established PACKTEST is as follows. The lyophilized enzymes consisted of 5 U of the LGOX mutant and 5.4 U of peroxidase (PEO-302 from Toyobo, specific activity ≥180 U/mg). The pH buffers consisted of 40 mg of pentasodium triphosphate (FUJIFILM Wako Pure Chemical) and 20 mg of dipotassium hydrogen citrate (special grade from Kishida Chemical, Osaka, Japan). To protect the enzyme, 5 mg of D-raffinose pentahydrate (special grade from Nacalai Tesque, Kyoto, Japan) was used. The chromogenic reagents were 0.1 mg of 4-aminoantipyrine (special grade from FUJIFILM Wako Pure Chemical) and 0.20 mg of the sodium salt of *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethylaniline monohydrate (MAOS; Dojindo Laboratories, Kumamoto, Japan). The mixed powder was prepared by weighing 1000 tubes of each reagent base, mixing them uniformly and well in a mortar while keeping them dry, and then dispensing 65 mg of each into polyethylene tubes with the ends welded and sealed for testing. For long-term storage, the tubes were wrapped in aluminum pouches with calcium chloride desiccant and stored at room temperature.

2.3 L-glutamate standard solution

Sodium hydrogen L-glutamate monohydrate (0.318 g, special grade from FUJIFILM Wako Pure Chemical) was dissolved in water (250 mL) and diluted to 1000 mg/L L-glutamate. This standard stock solution was stored, refrigerated, and used within one month. It was diluted with water at the time of use.

2.4 Portable absorbance photometer

A spectrophotometer (DPM-MTSP; KYORITSU CHEMICAL-CHECK Lab., Yokohama, Japan) was used to check and optimize the composition of the reagents for PACKTEST. The instrument is equipped with a white LED light source to measure the absorbance in the visible range of 420 to 670 nm. In this study, we measured the maximum absorption of the chromogenic

products derived from MAOS at 630 nm. For on-site absorbance quantification, we used a commercially available portable photometer (DPM2-ABS, KYORITSU CHEMICAL-CHECK Lab.) [Fig. 1(B)] equipped with three types of LED (470, 525, and 615 nm) in one package as fixed-wavelength light sources and one photodiode as a detector. The photometer registers the standard curve data and calculates the concentrations of targets from the absorbance for screen display. In this study, the absorption of the chromogenic product was measured at 615 nm. In both models, a dedicated cell, made of polystyrene with an optical path length of 2 cm, was used. Since the polystyrene cell attached a 1.5 mL marking line on the wall, it was possible to take a predetermined amount of sample.

2.5 On-site analysis procedure

First, 1.5 mL of sample water was taken in the polystyrene cell. For visual determination, the sample in the cell was aspirated into a tube containing the reagents and dissolved by shaking approximately 20 times. The color intensity of the solution became constant after 2 min. To estimate the L-glutamate concentration by the visual comparison of the solution color, a color chart (concentration scale: 1, 2, 5, 10, and 20 mg/L) was obtained according to the color tones of the standard solutions. For spectrophotometry, the polystyrene cell containing 1.5 mL of sample was put in a portable photometer to adjust the absorbance to zero. The sample was aspirated into a tube and shaken as above, and then it was transferred into the cell. After 2 min, the absorbance was measured, and the L-glutamate concentration was automatically calculated from the standard curve.

2.6 Food sample

Ten types of soy sauce were purchased from supermarkets in Japan. For PACKTEST measurements, each sample was diluted 2000-fold with water. The sample (1.5 mL) was aspirated into the tube, and the absorbance was measured with the portable photometer. The concentration was calculated from the absorbance using the calibration curve built into the instrument. The same procedure was also used for the additive recovery test. Samples were diluted 2000-fold to a final concentration of 3 mg/L with the L-glutamate standard solution. A post-column derivatized ion-exchange chromatography system (LA8080, Amino SAAYA, Hitachi High-Tech, Tokyo, Japan) composed of a guard column (6.0 × 4.0 mm^2) and an analytical column (60 × 4.6 mm^2) was used to validate the quantitative results. The column temperature was varied in steps from 30 to 73 °C. A 50 µL aliquot of deproteinized sample was injected and citrate buffer was used as the mobile phase.

2.7 Microplate enzyme assay

One tube of PACKTEST reagent was dissolved in 500 µL of water, from which 10 µL was added to each well of a 96-well plate (Thermo, #439454 NN Immunoplate F96 Maxi Soap). The solution was dried at 4 °C. Then, 100 µL of the L-glutamate solution (0–10 mg/L) was added to

each well, and the absorbance at 615 nm was measured every 20 s for 5 min using a microplate reader (VARIOSKAN LUC, ThermoFisher Scientifics). The absorbance at 2 min was used for the standard curve.

3. Results and Discussion

3.1 Optimization of mixed powdered reagents in PACKTEST

Enzymatic activity is generally pH-dependent. Our preliminary investigation confirmed that the optimum pH of the LGOX mutant was in the range of 6.0–8.5. First, pH buffer components as powdered reagents were investigated. The commonly used phosphate pH buffer, comprising potassium dihydrogen phosphate and disodium hydrogen phosphate, was unsuitable for this study because the dissolution rate of the powdered reagent was low, and the absorbance was affected by undissolved reagent residues. Good's buffers, such as HEPES, were also unsuitable owing to the lack of an acceptable counterpart as a powdered alkaline agent. The combination of pentasodium triphosphate and dipotassium hydrogen citrate was adopted because of its good solubility and sufficient pH buffering capacity, which did not affect the chromogenic reaction system. In this method, the reagent composition was adjusted so that pH was 7.5 during the color reaction.

Next, the amount of LGOX mutant, the rate-limiting component in this chromogenic reaction system, was investigated. Increasing the amount of enzyme enabled rapid quantification but increased the cost of reagents. Systematically varied amounts of LGOX were added to 1.5 mL of the L-glutamate standard solution (10.0 mg/L) to observe the absorbance as a function of time (Fig. 3). At 20 ± 2 °C, the absorbance reached the maximum value in 12, 4, and 2 min when the LGOX amounts were 1, 3, and 5 U, respectively. In this study, 5 U of LGOX was used per measurement, and the reaction time was set to 2 min.

The direct contact of the enzyme powder with the pH buffering reagents could affect the stability of PACKTEST during storage owing to the partial inactivation of the enzyme. To avoid this problem, the enzyme was mixed with a protective agent in the powder form before it was

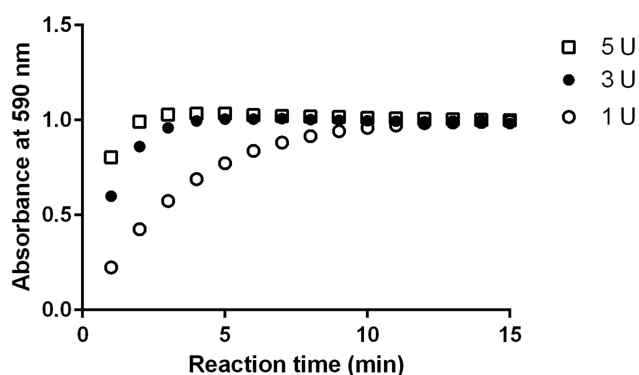


Fig. 3. Reaction rate depending on the units of LGOX mutant per tube: 1, 3, and 5 U.

added to other reagents. Several types of protective agent were tested, and trisaccharide raffinose was selected because it did not interfere with the chromogenic reaction system and gave good results with high storage stability.

In a chromogenic system using oxidase, such as in this study, 4-aminoantipyrine and aniline derivatives (new Trinder reagents) are commonly used as chromogenic substrates. Several types of aniline derivative have been proposed,[17] and the color tone and sensitivity of their chromogenic products vary according to the type of functional group. Usually, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS; λ_{max} of its product: 555 nm, ε: 3.92×10^4)[17] is considered suitable because of its high sensitivity. In addition, food samples are often contaminated with yellow to brown coexisting components derived from foreign substances. Therefore, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3,5-dimethylaniline (MAOS; λ_{max} of its product: 630 nm, ε: 2.25×10^4)[17] was adopted in this study because of its intense blue color.

3.2 Determination range for L-glutamate

When the sample solution was aspirated into the tube containing the chromogenic reagent, a distinct blue color was observed after a reaction time of 2 min. Because this color depended on the L-glutamate concentration, a standard color sequence was prepared using the standard solution (Fig. 4), which allowed the rapid estimation of the L-glutamate concentration in the range of 1–50 mg/L through visual comparison. In addition, a calibration curve was prepared by measuring the absorbance of a chromogenic solution obtained from 0 to 13 mg/L of the standard solution using a portable photometer (DPM2-ABS). As a result, linearity was confirmed, with $R^2 \geq 0.9999$ (Fig. 5). The determination limit governed by the blank values (10σ; $n = 15$) was calculated to be 0.1 mg/L. In this study, we adopted 0.5 mg/L as the practical determination limit, which is 5 times the 10σ value considering individual and environmental differences in an actual on-site analysis. According to the upper limit of absorbance measurable with the photometer, the determination range with the portable photometer was 0.5–12.0 mg/L. Photometric determination was repeatable, with $CV \leq 4\%$ (Table 1; *CV*: coefficient of variation). Although the determination range of DPM2-ABS was narrower than that of the visual colorimetric method, the concentration of L-glutamate was determined with high repeatability using DPM2-ABS, suggesting that DPM2-ABS can be used in applications requiring high accuracy.

Fig. 4. (Color online) Visual comparison of the reacted PACKTEST with standard colors.

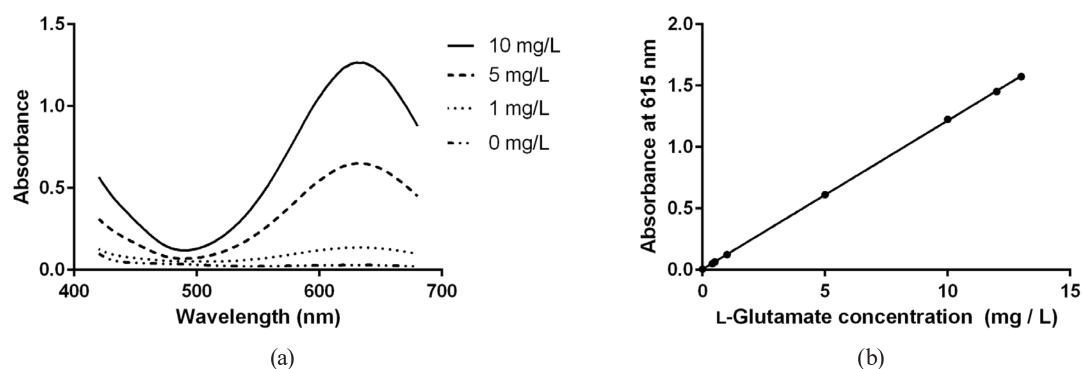


Fig. 5. Absorption spectrum and standard curve. (a) Absorption spectra of the reacted solutions. (b) Absorbance at 615 nm of each L-glutamate solution measured with a portable photometer (DPM2-ABS) (n = 3, mean ± S.D.). The linear regression equation was $y = 0.1208\,x + 0.008$ ($R^2 \geq 0.9999$).

Table 1
Repeatability of PACKTEST for L-glutamate quantification. Absorbance at 615 nm of each L-glutamate solution measured using a portable photometer (DPM2-ABS).

Glutamate	Absorbance			
(mg/L)	Mean	S.D.	*CV* (%)	*n*
0	0.008	0.001	13.9	15
0.4	0.051	0.001	1.7	3
0.5	0.064	0.002	3.2	3
1	0.125	0.001	0.9	3
5	0.613	0.003	0.5	3
10	1.226	0.005	0.4	3
12	1.452	0.007	0.4	3
13	1.574	0.010	0.6	3

3.3 Selectivity

Coexisting components in food samples can interfere with L-glutamate measurements. First, using the developed PACKTEST, the reactivity of L-glutamate was compared with those of 19 other amino acids generally present in large amounts in foods. The results are shown in Table 2. The values obtained for L-glutamate were in agreement with the amount of L-glutamate added. In contrast, when glutamine, aspartate, and alanine were added in excess (1000 mg/L), the obtained values were 0.7, 1.7, and 0.5 mg/L, respectively. In addition, the concentrations of the other amino acids were below the determination limit of the portable photometer (DPM2-ABS). These results indicated that the developed PACKTEST was highly selective for L-glutamate.

Next, the effects of coexisting components assumed to be present in food samples were investigated. Ascorbic acid at the concentration of 2 mg/L interfered with the determination negatively, suggesting that H_2O_2, an intermediate product of the chromogenic reaction, was consumed. Because ascorbic acid is assumed to be present in food samples, such as fruits and vegetables, pretreatment such as dilution or decomposition is necessary to improve the accuracy of quantification. In contrast, inosinic acid, guanylic acid, citric acid, tartaric acid, glucose,

Table 2
Selectivity for L-glutamate in the presence of other proteinogenic amino acids.

Amino acids	Added (mg/L)	Found[1)] (mg/L)	Relative selectivity (%)
L-glutamate	10	10.0	100.0
L-alanine	1000	0.5	0.05
L-aspartate	1000	1.7	0.17
L-glutamine	1000	0.7	0.07
L-arginine, L-asparagine, L-cystine, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine, L-valine	1000	< 0.5[2)]	< 0.05

[1)] Determined by spectrophotometry with the portable photometer, DPM2-ABS.
[2)] Below the determination limit.

sucrose, and starch did not interfere with the determination of L-glutamate even when they were present at 1000 mg/L. Sodium chloride, a typical edible salt component assumed to be present in seasonings, did not interfere even when it was present (at least 10000 mg/L).

3.4 Storage stability

To assess the practicality of PACKTEST developed in this study, its storage stability was investigated. The stability of PACKTEST stored at room temperature (20 ± 10 °C) is shown in Fig. 6. Here, the absorbance of the L-glutamate standard solution at three concentrations was measured over a long period of time using PACKTEST, which was stored at room temperature under light shielding. The results showed that PACKTEST had the same reactivity after at least 600 days as on the first day of production, indicating high storage stability. Because the reagents were prepared in the dry, powder form, they were likely resistant to degradation from oxidation. Notably, because PACKTEST was highly stable during storage at room temperature, we considered it suitable for on-site measurements even in locations without refrigeration facilities.

3.5 Quantification of L-glutamate in food

To investigate the applicability of the developed PACKTEST to food analysis, 10 commercially available soy sauces were diluted 2000-fold and the concentration of L-glutamic acid was determined. The results of PACKTEST analysis were compared with those of instrumental analysis, giving a correlation coefficient (r) of 0.97 (Fig. 7). When the L-glutamate standard was added at a final concentration of 3 mg/L, the recovery rate was 93.3–123.3%, with an average of 101.0%. These results indicated that the determination of the L-glutamate concentration was highly accurate and unaffected by impurities in the soy sauce samples. One of the reasons for this may be that soy sauce contains a high concentration of L-glutamate, and the effect of other components is reduced by dilution.

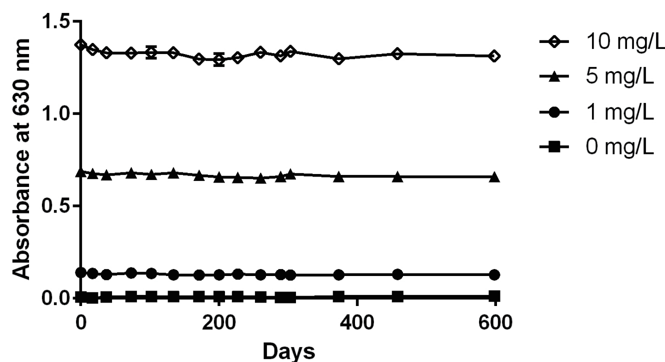


Fig. 6. Storage stability of PACKTEST for L-glutamate quantification. Absorbance of the reacted solution from standard at different concentrations (n = 3, mean ± S.D.).

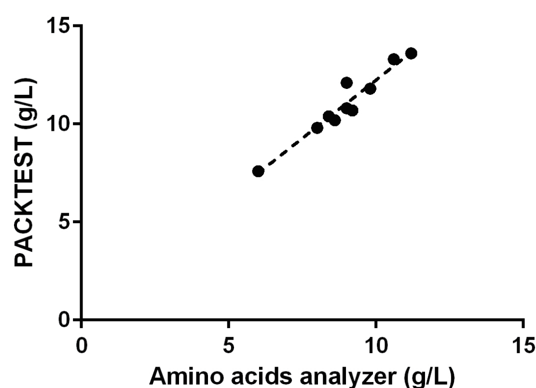
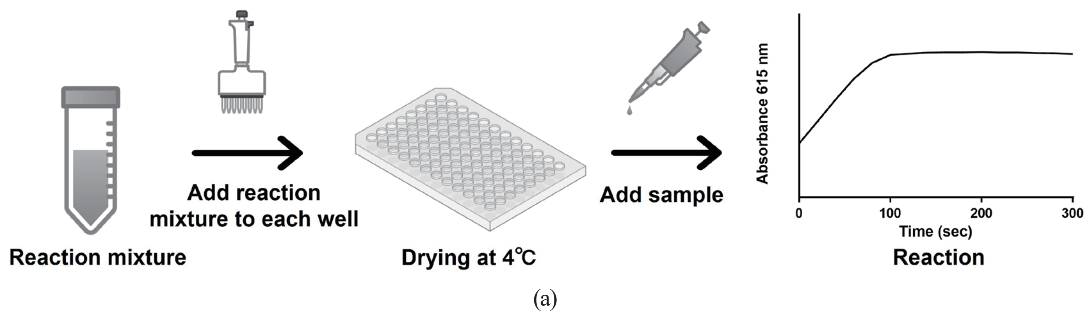


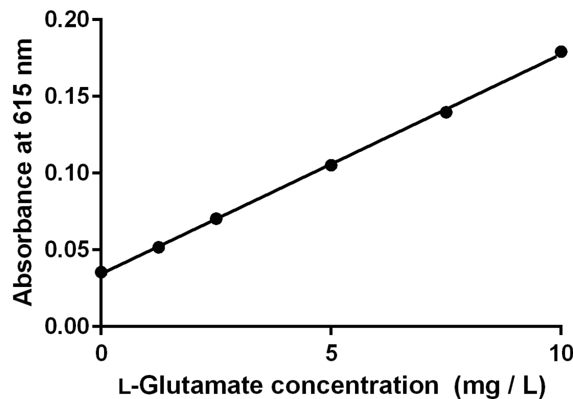
Fig. 7. Correlation between PACKTEST and instrumental analyses. The concentration values (g/L) were obtained by converting each measurement value (mg/L) by the portable photometer to the concentration in the undiluted solution. LA8080 (Amino SAAYA) was used as an amino acid analyzer.

3.6 Application of developed PACKTEST to high-throughput enzyme-based sensing

As mentioned above, the evaluation results suggested that the LGOX mutants were useful for L-glutamate quantification. In addition, the possibility of the LGOX mutants for high-throughput enzyme-based sensors was investigated. An enzyme-based sensing device was prepared by dropping an aqueous solution of the reagents in PACKTEST onto a 96-well plate for ELIZA, and the solution was dried at 4 °C. When 0–10 mg/L of L-glutamate solution was added, the reaction terminated in 100 s [Fig. 8(a)]. These results indicated that the enzyme and other reagents were stable after drying the solution. A standard curve was constructed using the absorbance at 2 min after the start of the reaction, and a linear relationship between the concentration and the absorbance was confirmed [Fig. 8(b)]. The detection limit and the lower limit of quantitation were 0.12 and 0.21 mg/L, respectively. Therefore, this sensing device can be used to quantify L-glutamate for high-throughput analysis.



(a)



(b)

Fig. 8. Application of developed PACKTEST to a microplate biosensing. (a) The reaction mixture in PACKTEST for L-glutamate quantification was dried on the surface of a 96-well plate. Each component was dissolved after the sample solution was added to the well. Images of the experimental materials and equipment are from TogoTV (© 2016 DBCLS TogoTV, CC-BY-4.0. https://creativecommons.org/licenses/by/4.0/). (b) Standard curve for L-glutamate quantification. The linear regression equation was $y = 0.0143\ x + 0.0344$. R^2 was more than 0.999 and CV was less than 2% ($n = 3$, mean ± S.D.).

4. Conclusions

In this study, we developed a practical analytical kit named PACKTEST for the colorimetric quantification of L-glutamate using a novel LGOX mutant from *Streptomyces* sp. X-119-6. By optimizing the amount of enzyme and reagent components, we succeeded in developing PACKTEST to quantify L-glutamate within 2 min of the measurement. This PACKTEST was stable during storage at room temperature for over 600 days. The standard curve for L-glutamate with a portable photometer was highly linear, and the *CV* of the analysis was less than 4%. The correlation coefficient of the relationship between the results of PACKTEST and instrumental analyses was $r = 0.97$, and the recovery rate ranged from 93.3 to 123.3%. In addition, the solution of reagent mixture was dried on a 96-well plate for constructing an enzyme-based sensing device. The successful results showed that the proposed device can be used to quantify L-glutamate in food samples for high-throughput analysis.

Acknowledgments

We deeply thank Dr. Taro Toyota (The University of Tokyo), Dr. Atsushi Shoji, and Dr. Yukiko Moriiwa (Tokyo University of Pharmacy and Life Sciences) for supporting our collaboration. We are grateful to Dr. Chinatsu Kasamatsu (Ochanomizu University) for supporting and encouraging this research. We wish to thank Mr. Kazuki Noguchi and Ms. Yixin Wang for their technical assistance. We also thank Dr. Hisayuki Uneyama and Ms. Taeko Inamura (Ajinomoto Co., Inc.) for their helpful discussions. We thank Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

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