Laboratory Exercise

Enrichment of Gamma-Aminobutyric Acid in Bean Sprouts: Exploring Biosynthesis of Plant Metabolite Using Common Household Reagents[S]

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Abstract

The enrichment of plant foods with gamma-aminobutyric acid (GABA) is currently an interesting issue in the field of nutraceuticals and can be used as an experiment for upper-division undergraduate students. Here, an interdisciplinary hands-on experiment to produce GABA-enriched mung bean sprouts using common household reagents is described. Based on the GABA biosynthesis pathways, two key factors, being the availability of L-glutamic acid and the acidification of the germination environment, were chosen for the study of the effects on the enhancement of GABA levels. The activities not only led students to a deeper understanding of biochemistry contents, but also gave the students the opportunity to work with experimental design, analytical chemistry, and statistical data analysis. Furthermore, since mung bean sprouts are familiar foods and the reagents used for germination are easily obtainable and generally recognized as safe, the optimal protocol investigated in the lab could be further applied to the production of bean sprouts with enhanced nutritional values in everyday life, promoting the transfer of knowledge learned in school to practical environments such as home and community.

Keywords: *GABA; mung bean sprouts; biosynthesis*

Introduction

Gamma-aminobutyric acid (GABA) is a nonprotein amino acid found in prokaryotes and eukaryotes. Despite being chemically termed as an amino acid since the molecule contains both an amine and a carboxylic acid, GABA is not an alpha amino acid, meaning the amino group is not attached to the alpha carbon so it is not used for protein synthesis. Instead, GABA acts as an inhibitory neurotransmitter in humans and has been reported in numerous studies for its pharmacological actions including antihypertensive, tranquilizing, anticancer, and immune-enhancing activities [1–3]. In plants, GABA plays several physiological roles, for example, control of carbon/nitrogen balance, regulation of cytoplasmic pH, defense against insects, and protection from oxidative stress [4, 5]. It is primarily synthesized via the decarboxylation of L-glutamic acid in a reaction catalyzed by the cytosolic enzyme, namely glutamic acid decarboxylase (GAD; EC 4.1.1.15) [6] (Fig. 1). Due to the aforementioned health benefits and the occurrence of GABA in many plants, the development and production of GABA-enriched plants as functional foods is currently of interest and actively pursued.

In native plant sources, GABA is usually found at low concentrations [7], however the level is elevated during particular stages such as fruit development and exposure to environmental stress, for example, hypoxia, damage, and drought. In addition, physical and chemical approaches such as heat treatment, cold shock, and fermentation have been practiced to enhance GABA accumulation [8–10]. Among these treatments, sprouting is one of the most

Volume 46, Number 2, March/April 2018, Pages 155–161
[S]Additional Supporting Information may be found in the online version of this article.
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Received 25 September 2017; Revised 5 November 2017; Accepted 7 November 2017
DOI 10.1002/bmb.21100
Published online 7 December 2017 in Wiley Online Library (wileyonlinelibrary.com)

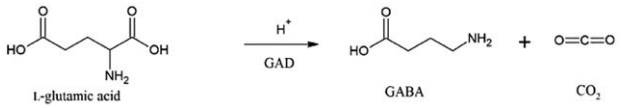

FIG 1 *Decarboxylation of L-glutamic acid into gamma-aminobutyric acid (GABA) by glutamic acid decarboxylase (GAD).*

effective and simple means to stimulate GABA synthesis since the activity of GAD increases during germination [11, 12]. For example, soaking brown rice, wheat or soybeans in water promotes germination and significantly increases the GABA levels [13, 14]. From the point of view of education, it could therefore be interesting for students to apply their knowledge to improve the GABA content in common edible plants by controlling the germination process.

According to literature on the subject, GAD in higher plants catalyzes the synthesis of GABA by using L-glutamate as a substrate at an optimal acidic pH and glutamate decarboxylation is a proton consuming reaction. Based on this fact, the experiment was designed for students to understand the biosynthesis routes of plant metabolites and apply them to control or improve the production of the compound of interest. The main activities were to investigate the effects of external feeding of L-glutamic acid and the acidification of sprouting conditions on the GABA content in bean sprouts by treating seeds with two chemicals, being monosodium glutamate (MSG) flavor enhancer and distilled white vinegar. Besides being relevant to plant biochemistry with respect to source of problem and concept, the students were also required to gain experience with other subjects and skills in order to complete the experiment. These included the extraction of bioactive constituents from plant sources, working with chemical analysis, that is, precolumn derivatization HPLC, as well as the statistical analysis of data and presentation.

Participants and Teaching Strategies

In order to stimulate teamwork and fit in the multi-task workload, the experiment was assigned to groups of 5 upper-division undergraduate students as a collaborative activity. Prior enrollment in basic courses such as biochemistry and analytical chemistry was recommended so that the students would already have the necessary fundamental knowledge and be familiar with laboratory techniques. In terms of the size of class, the experiment could either be implemented for small classes or adapted for labs with a large number of students as long as the facilities, for example, HPLC instruments are available.

As earlier described, this experiment is interdisciplinary and therefore provides students with opportunities to become exposed to a variety of knowledge types as well as the practice of laboratory techniques in several fields. As shown in many reports, this type of learning helps to demonstrate to students the connections between concepts and ideas across different disciplinary boundaries, thus enhancing the students' problem solving skills in real life [15–19]. To implement the experiment, the instructor may simply follow the protocol described here as a laboratory handbook. This strategy illustrates to the students the significance and applications of the plant biochemistry which they have learned from lectures. However, to enhance the learning outcomes, the experiment could be redesigned as an inquiry-based teaching method, in which students are expected to be capable of developing solutions for complex questions—a life skill which enables students to be successful in the 21st century. In this inquiry learning system, the teacher introduces the problems to the students (without providing a laboratory manual) and allows them to actively find the solutions themselves by formulation of hypotheses, design, and conduction of experiments, collection and analysis of data, and final conclusions. During the course of the process, the teacher interacts with the students by providing them with necessary information as well as supporting and motivating them positively. From this prototype experiment, the problems may be diversified and used for further inquiry, for example, by asking the students how to increase GABA in other types of seeds or grains which are locally available, or if other factors, for example, the addition of nonglutamate amino acids or plant elicitors (i.e., extrinsic substances which trigger and enhance the synthesis of metabolites) such as chitosan, affect the yield. This is therefore a relatively simple but effective and adaptable experiment.

Experimental Procedure

Materials and Overview

In the experiment described here, a common and inexpensive bean for sprouting, namely mung bean (*Vigna radiata*), was chosen because the seeds rapidly germinated within a few days and its sprouts could be eaten raw or cooked. Furthermore, the reagents used for the germination in the study were easily available household items, rendering the protocols not only suitable for implementation as an experiment in the classroom, but also transferrable to practical environments such as home and community for the production of mung bean sprouts with enhanced nutritional values in everyday life.

The experiment required several days, however the laboratory schedules could be arranged discontinuously as appropriate since the products from each part could be kept frozen until used in the next part. Firstly, mung bean seeds were germinated under different treatment conditions and the sprouts were collected for drying. This step took about 4–5 days. Subsequently, GABA was isolated from the plant materials. Based on its water soluble properties, GABA could be easily extracted by using water with the aid of heat and this process could be finished in one

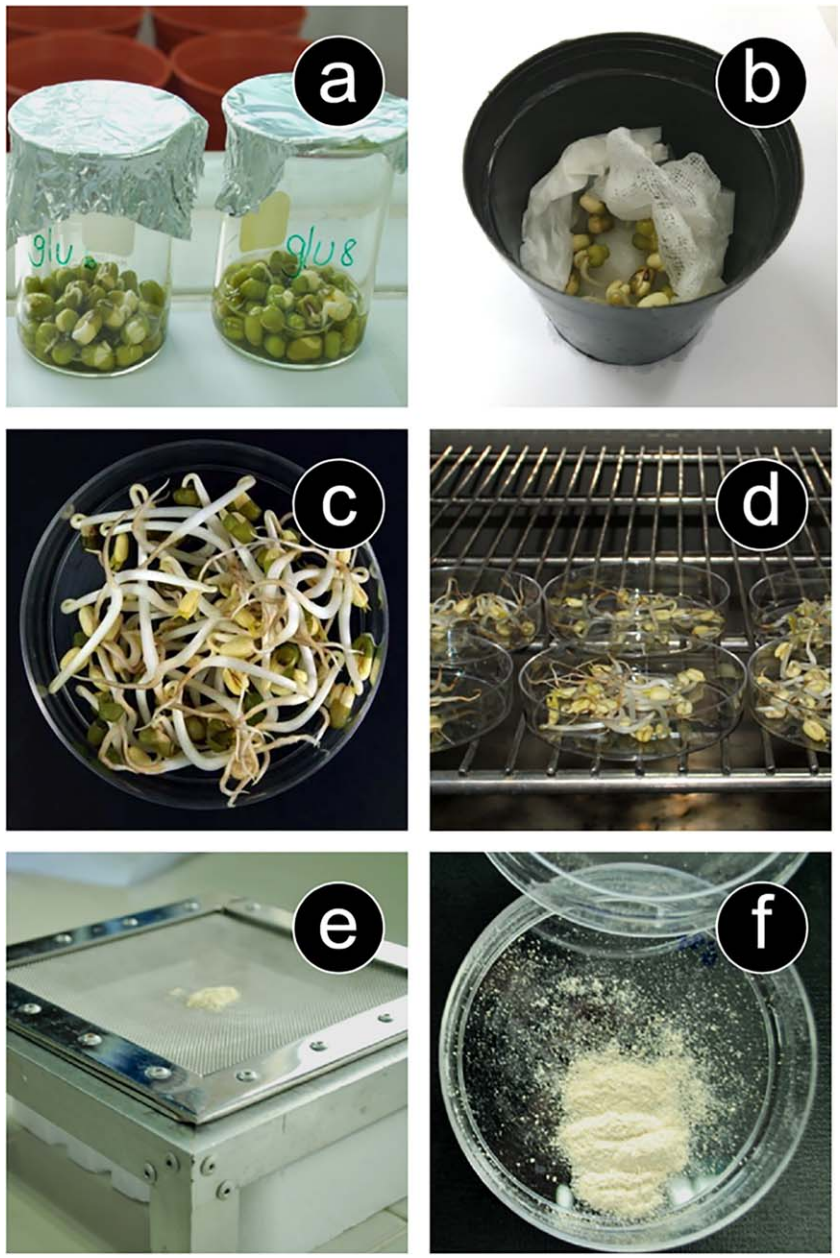

FIG 2 *Germination of mung bean seeds and collection of sprouts: (a) soak seeds in treatment solution and leave overnight (b) move into container with damp cloth and continue soaking twice a day for 3 days (c) collect sprouts and wash (d) dry in hot air oven (e) grind and seive (f) powder of bean sprouts. [Color figure can be viewed at wileyonlinelibrary.com]*

day. Since GABA itself is generally a weak chromophore (does not absorb UV light), the last part which required 2 days was dedicated to the derivatization of GABA with phenylisothiocyanate (PITC) to form the UV-absorbing phenylthiocarbamyl derivatives, that is, PITC-GABA, followed by the quantitation by reversed phase HPLC using a UV detector and data analysis.

Preparation of Treatment Solutions

Treatment solutions used for soaking seeds were prepared to study the effects of two factors, being the external supplementation of L-glutamic acid and the acidity of the germination environment. For this purpose, MSG solutions at different concentrations (2, 4, 6 and 8 mM) were prepared in distilled water (1 mM = 0.17 g MSG/L) and the pH of all solutions was adjusted to 7 by slight additions of 1 M sodium hydroxide solution. The pH effect of the soaking solutions was investigated by using water added to distilled white vinegar or 1 M sodium hydroxide solution to produce various levels of acidity (pH 4, 5, 6, 7 and 8). In addition, the combined effect of these two factors was studied by treating seeds with 6 mM MSG solution having a pH of 5.

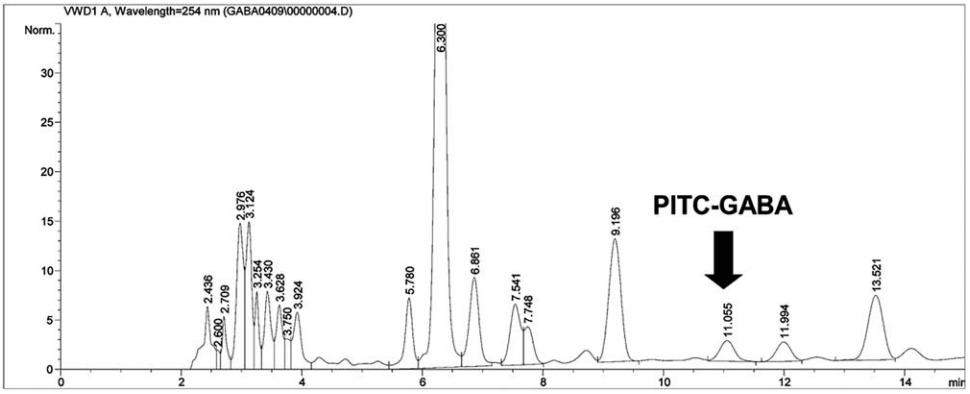

FIG 3 *Chromatogram corresponding to precolumn derivatization HPLC assay of GABA in dried mung beans sprouts.*

For comparison, sprouts soaked with distilled water (no provision of glutamate or acid environment) and ground nongerminated seeds were used as controls.

Germination of Mung Bean Seeds and Collection of Sprouts

Mung bean seeds were purchased from local grocery stores. The flawless seeds were rinsed with tap water and soaked in the treatment solutions previously described at room temperature (30°C) to imbibe (Fig. 2). After 12 h, they were removed from the solutions and incubated in containers by placing them between thick layers of moist cloth and stored in darkness. The seeds were allowed to germinate for 3 days while being watered with the treatment solutions and drained off twice a day. Throughout the sprouting process, germination behavior was observed. For sample collection, the sprouts were washed with tap water and then dried in a hot air oven at 60°C for 24 h. The dried plant materials were then ground into powder, sieved through 40-mesh, and stored in a freezer until subjected to analysis for GABA content.

Extraction and Quantitative Analysis of GABA

0.02 g of sprout powder was accurately weighed and extracted with 1 mL of deionized water at 70°C in an incubator shaker for 30 min. The mixture was then centrifuged at 10,000 rpm for 10 min and the clear supernatant was collected. A 200 μL aliquot of the supernatant was dried under vacuum. The residue was dissolved in 20 μL of ethanol-water-triethylamine mixture (2:2:1 v/v) and evaporated to dryness under vacuum. A 30 μL of ethanol-water-triethylamine-PITC (7:1:1:1 v/v/v) mixture was then added to the residue and allowed to react for 20 min at room temperature to form PITC-GABA derivatives. The excess reagent was then removed under vacuum. The dry residue containing PITC-GABA was dissolved in 100 μL of the mobile phase, consisting of a mixture of 92% Solution A (aqueous solution of 8.205 g sodium acetate and 0.5 mL triethylamine in 1000 mL, adjusted to pH 5.8 with acetic acid) and 8% acetonitrile. To construct the calibration curve, 200 μL of 0.2, 0.4, 0.6, 0.8 and 1.0 mM standard GABA solutions were used instead of the supernatant and subjected to derivatization with PITC following the same procedures.

An isocratic HPLC analysis was conducted using a C-18 column (250 $\times$ 4.6 mm I.D., particle size 5 μm), with a flow rate of 1 mL/min, column temperature of 50°C and injection volume of 20 μL. The UV detector was set at 254 nm. As shown in Figure 3, PITC-GABA was eluted from the column at about 11 min. The standard curve of GABA concentration in mM (X) versus peak area (Y) was linear over the concentration range of 0.2–1.0 mM, yielding a regression equation $Y = 209.32X + 5.16$ with a coefficient of correlation of 0.998.

Statistical Analysis

All data are reported as means $\pm$ standard deviation (S.D.) for 5 replicates. The means of two treatments, for example, nongerminated seeds versus those germinated by soaking with distilled water, were compared using a student's *t*-test in the Data Analysis ToolPak of Microsoft Excel. A one–way ANOVA with post hoc test was used to analyze significant differences between the means among more than 2 treatments, for example, the effect of MSG concentrations (0, 2, 4, 6 and 8 mM). This test was done via the free online statistic calculator available at http://statistica.mooo.com. $p < 0.05$ was considered as statistically significant.

Hazard

MSG is not considered a dangerous substance, however unnecessary contact should be avoided. Distilled white vinegar, sodium hydroxide, and triethylamine are acidic or alkali chemicals that can cause irritation of the skin or mucous membranes. Acetonitrile is flammable and toxic when swallowed or inhaled, or upon contact with the skin. Despite the small amounts used in the experiment which are unlikely to cause severe or immediate health effects,

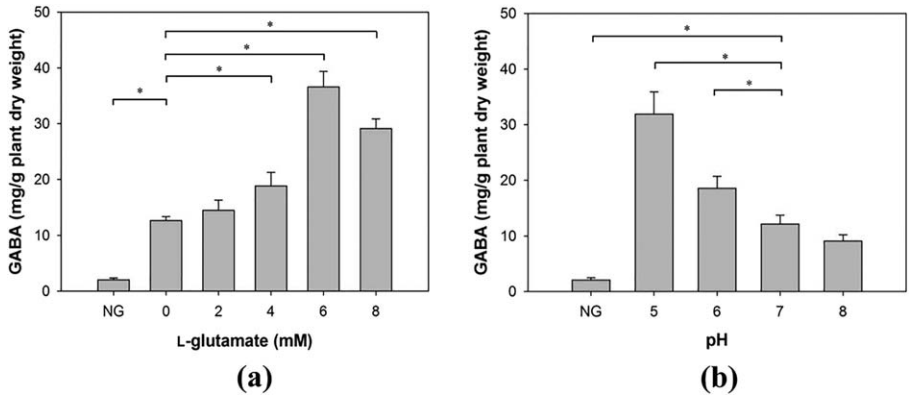

FIG 4 *Effects of MSG concentration (a) and pH of germination (b) on the GABA content in mung bean sprouts. NG is nongerminated beans. Bars represent the mean ± S.D., the asterisks indicate the significant difference at* $p < 0.05$.

PITC is harmful if inhaled or swallowed and causes burns to the skin and upper respiratory tract. The derivatization steps should therefore be performed in fume hoods. Adequate personal protective equipment should always be worn in a laboratory.

Results and Discussion

From the germination experiment, it was found that mung bean sprouts soaked with distilled water at pH 7 for 3 days contained a significantly higher level of GABA (12.2 mg/g dry weight), than nongerminated seeds (2.1 mg/g dry weight). This finding therefore supported the theory that the production and accumulation of GABA in mung bean seeds, similar to other plants, was actively stimulated during germination. To further improve the GABA levels, L-glutamate and acidic conditions were independently created during germination and their effects were studied. Clearly, the GABA content in the bean sprouts was enhanced by feeding seeds with MSG as a precursor for GABA biosynthesis at concentrations of up to 6 mM where GABA reached a peak level (Fig. 4a). Beyond this point, although the bean sprouts grew normally, the GABA content began to decline slightly. In a similar way, the GABA content was dramatically elevated with an increase in acidity (lowering of pH) of the germination environment (Fig. 4b) and the accumulation was highest in the sprouts treated with water, pH 5. This result was in agreement with the fact that protons are required for glutamate decarboxylation and most GAD enzymes in higher plants are favorably active under acidic conditions ranging from pH 5–6. Furthermore, the treatment of seeds with water which had a $pH \leq 4$ adversely retarded or inhibited the germination. From both studies, students would realize the importance of the optimal levels of treatment that should be adequate to produce desirable effects without detrimental consequences.

After the optimal concentration of MSG in the soaking solution and pH of germination were established from the separate experiments, the combined effect of both factors was studied. Interestingly, the use of 6 mM MSG solution of which the pH was deliberately adjusted to 5 further increased the GABA content in bean sprouts, giving the highest GABA level among all the treatments tested (Fig. 5). These results therefore suggested that enrichment of mung bean sprouts with GABA could be achieved by simple regulation of the sprouting conditions, that is, supplementation of L-glutamate or acidification of germination conditions. In addition, when these two factors were concurrently applied, an additive effect was obtained.

Students' Assessment and Feedbacks

The students were monitored and evaluated in several ways. Prelaboratory quizzes were asked to ensure that

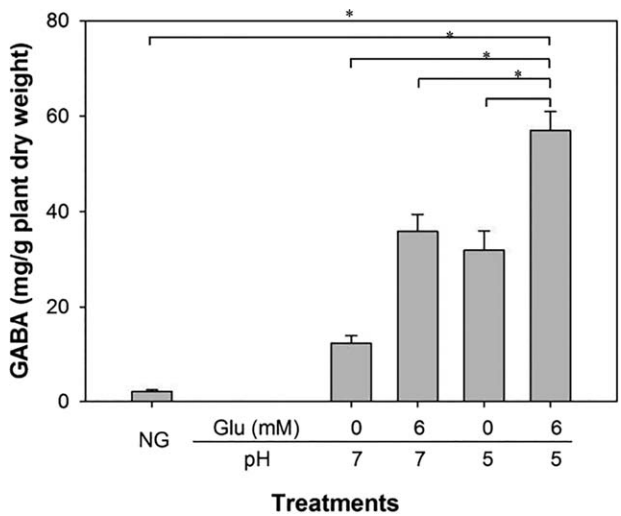

FIG 5 *Combined effect of the optimal MSG concentration and pH of germination on the GABA content in mung bean sprouts. NG is nongerminated beans. Bars represent the mean ± S.D., the asterisks indicate the significant difference at* $p < 0.05$.

students had prepared themselves and understood the principles and methods addressed in the laboratory procedures given or the protocol they had developed. The quiz results also well reflected their ability and attempts at the literary search, for instance, biology students might need to look for chemistry references in order to fulfill their knowledge of instrumental analysis. In terms of working performance, points were awarded for participation, teamwork, problem-solving ability, attention and responsibility by observation during the experiment and group discussion. For the skills of numeric analysis and information technology, the students had to be able to perform statistical tests correctly using Microsoft Excel or open-source statistical calculators available on the internet. Finally, students were evaluated by their end-of-laboratory presentation based on the contents of the presentation and their communication skills. Lastly, the students' knowledge and understanding was scored on their ability to answer questions on their work and discuss it proficiently with the audience.

From observation and interviews, students responded positively to the experiment and were particularly intrigued by the use of simple household materials. Many of them stated that they now had a better appreciation of the fact that biochemistry is not just in a classroom, but is in fact all around us. Compared with other typical experiments dealing with the extraction and quantitation of bioactive compounds from natural products, this experiment was unique in that students had a chance to develop the plant materials themselves, beginning from seeds. These activities actively engaged the students' interests, so that they looked forward to seeing which treatment of germination would give the highest yield of GABA. From a teamwork aspect, this multi-task experiment helped to strengthen students' skills in this area since they had to learn how to work effectively in a group, for example, by sharing ideas, planning schedules and deciding on a responsible person for each task. Finally, at the end of the course, students were asked to present their work orally to other students who had worked on different projects. Obviously, they were proud of showing off their innovative and practicable work to the public. In the same way, the feedback from the audience indicated that this experiment was interesting. Moreover, the experiment sparked the enthusiasm in the minds of some of the students, as well as members of the audience, to further conduct the experiment with other plants, implying that this experiment was well-received and could effectively establish enjoyable and successful laboratory experiences for students in the future.

Conclusions

Described here is a simple, straightforward and illustrative hands-on experiment that provides students with opportunities to learn about and apply knowledge of plant biochemistry to enhance the biosynthesis of the compounds of interest. Since the experiment is interdisciplinary, it allows students to learn by looking across subject boundaries and it is suitable for undergraduate students in many different fields such as biochemistry, chemistry, pharmaceutical sciences and agriculture. For instructors, the protocol can be directly implemented as a laboratory handbook or further modified into an inquiry based learning activity. Beyond just learning in the lab, the optimal protocol successfully developed can be further applied to growing bean sprouts with enhanced nutritional values in everyday life, enhancing the transfer of knowledge in school to practical environments such as home and community in a real world context.

Acknowledgments

We are grateful for financial support from Faculty of Pharmacy, Silpakorn University. Also, we gratefully acknowledge Henry Erskine-Hill for his valuable help in editing and proofreading the manuscript. Disclosure: The authors have no conflicts of interest in this work.

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