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Effect of components in homogeneous extraction suspensions of pea and sweet buckwheat on γ-aminobutyric acid synthesis

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Abstract: The effects of calcium chloride ($CaCl_2$), vitamin B_6 (VB_6), and monosodium glutamate (MSG) on the synthesis of γ-aminobutyric acid (GABA) in the mixed homogeneous suspension of pea and sweet buckwheat (1:1 w/w) were investigated. The composition formula of raw materials in the homogeneous fluid for GABA synthesis was optimized via response surface methodology (RSM). The result showed that the increased content of GABA was dependent on the addition of $CaCl_2$, VB_6, and MSG to the mixed suspension. Box-Behnken design indicated that the optimal added components for GABA synthesis were: $CaCl_2$ at a concentration of 0.85 mmol/l, VB_6 at a concentration of 2.29 mmol/l, and MSG at a concentration of 2.83 mg/ml. Under optimal conditions, a maximal increase of GABA content (51.29 μg/ml) was obtained. Analysis of variance for the regression model suggested that the model can exactly predict GABA synthesis in the mixed homogeneous suspension.

Keywords: pea; synthesis; sweet buckwheat; γ-aminobutyric acid

Gamma-aminobutyric acid (GABA) is a non-protein free amino acid that is widely distributed in bacteria, plants, and vertebrates; it is also a neurotransmitter in the brain and spinal cord of mammals (Manyam *et al*. 1981). GABA was first described as a plant metabolite, then its presence in brain was demonstrated, and it was later described in filamentous fungi suggesting a much broader metabolic role. GABA is primarily produced by the α-decarboxylation reaction of L-glutamic acid (Glu) or its salts, and is catalysed by the enzyme glutamate decarboxylase (GAD), which is dependent on the cofactor pyridoxal-5'-phosphate or vitamin B6 (VB_6) (Mayer *et al*. 1990). GABA shows multiple physiological functions, such as regulation of blood pressure and heart rate, anti-aging, and alleviation of pain and anxiety (Defeudis 1983; Leventhal 2003; Cho & Lim 2016). Medically, GABA was used to treat hyperammonaemia in rats (Paul 2003). In addition, GABA can enhance the flavour of food and has sobering or deodorant functions.

Currently, consumer interest in natural, nutritional, and healthful foods is growing. A plant-based diet, with a main focus on whole coarse cereals, has become one of the most important guidelines to lower the risk of many human diseases (Lawrence & Machlin 1995). Public interest in healthful and nutritious food has

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initiated investigations that use biochemical technology to enhance nutritious components in coarse cereals. Germination has been described as an effective method to increase the content of functional components in coarse cereals and has been used to meet this objective (Ding *et al*. 2018). At present, most of the published studies focus on the biosynthesis of GABA in coarse cereals and its changing regularity to achieve increasing nutritional value during germination (Guo *et al*. 2011; Luo *et al*. 2018). However, when germination conditions are controlled, it remains difficult to ensure protein decomposition to amino acids by endogenous protease, while retaining optimal GAD activity for the further promotion of GABA synthesis in coarse cereals. During germination, lower contents of GABA were synthesized in several coarse cereals due to the increased amount of Glu in combination with a lower GAD activity, or alternatively, a higher GAD activity in combination with a small amount of Glu. Most tests tried to overcome the problem by adding calcium chloride ($CaCl_2$), pyridoxal-5-phosphate (PLP), and Glu to the soaking solution to stimulate GAD activity with limited success (Bai *et al*. 2009). Therefore, novel methods for GABA synthesis need to be explored. The pea is a bean-plant that contains high levels of both proteins and Glu (Ribéreau *et al*. 2018). Sweet buckwheat is a cereal that contains a considerable amount of Glu (Sluková *et al*. 2017). Therefore, both pea and sweet buckwheat offer promising potential for GABA enrichment.

Pea and sweet buckwheat were chosen for this study, and the effects of $CaCl_2$, VB_6, and monosodium glutamate (MSG) on GABA synthesis in the mixed homogeneous suspension of both pea and sweet buckwheat were investigated. Response surface methodology (RSM) was applied to optimize the components in the complex suspension for GABA accumulation. The objective of this work is to provide a new method and scientific basis for the industrialized production of GABA.

MATERIAL AND METHODS

Material. Seeds of a pea (*Pisum sativum* L.) were kindly supplied by the Gansu Academy of Agricultural Sciences (Lanzhou, China) and seeds of sweet buckwheat (*Fagopyrum esculentum esculentum* Moench) were purchased from Dingxi in Gansu Province. All seeds were harvested in 2015, dried naturally, and stored at 4°C prior to use.

GABA (purity > 99%) and phenol were purchased from Sigma-Aldrich (China). Sodium hypochlorite was purchased from Baishi chemical industry Co., Ltd. (China). Other commercial chemicals additives such as $CaCl_2$, VB_6 and MSG were of food grade.

Preparation of homogeneous fluid. Intact seeds of pea and sweet buckwheat were picked up, the surface dust was cleaned off with distilled water, seeds were then surface-sterilized with 0.1% (v/v) sodium hypochlorite solution for 30 min, and finally thoroughly rinsed with distilled water. The treated seeds were soaked in distilled water (1:5 w/v) at 30°C for 8 h, then drained the surface water. After that, the soaked seeds were placed in a mortar and ground gently in an ice bath, and removed the separated seed coats with tweezers, and then weighed. Homogeneous fluids were obtained by grinding the seeds under addition of 0.2 mol/l pH 5.7 sodium phosphoric buffer solution (1:1.5 w/v) in an ice bath.

GABA synthesis. A total of 4 g of homogeneous fluid of both pea and sweet buckwheat were mixed in proportion, adding 10 ml of 0.2 mol/l, pH 5.7 sodium phosphate buffer solution containing $CaCl_2$, VB_6, and MSG. Homogeneous complex fluid reacted at 37°C for 2 h at an oscillating speed of 110 rpm. Under these conditions, the maximal amount of GABA was synthesized at the optimal reaction temperature of endogenous protease (optimal enzyme activity at 37°C) and GAD (optimal enzyme activity at 40°C). After the reaction, the mixed suspension was centrifuged at 6578 g for 20 min at 4°C. The supernatant was transferred to clean flask and 10% (w/v) trichloroacetic acid solution (2:1 v/v) was added to remove protein. The supernatant was obtained by centrifuging again at 6578 g for 10 min at 4°C.

GABA determination. The GABA content in the mixed homogeneous suspension of both pea and sweet buckwheat was investigated, following the method of Tsushida and Murai (1987) with modifications. Briefly, 300 µl of the supernatant was collected in test tubes, and 200 µl of 0.1 mol/l sodium tetraborate buffer solution of pH 9.0, 400 µl of 6% (w/v) phenol solution, and 600 µl of 7.5% (v/v) sodium hypochlorite were successively added. The mixture solutions were thoroughly shaken at room temperature and heated in boiling water for 10 min, then immediately placed into an ice bath for 10 min after rapid oscillation. The test tube was continuously shaken until the mixture solution appeared blue-green. Then, 2 ml of 60% (v/v) ethanol were added to the test tubes to stop the reaction. Finally, the absorbance

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of samples at 645 nm was assayed using UV/VIS spectrophotometer.

GABA standard curve. The standard solution of GABA (1 mg/ml) was prepared. 0, 20, 40, 60, 80, and 100 µl of the solution were collected in six test tubes, and distilled water was added to 300 µl. The standard solution of GABA and samples were performed simultaneously. The GABA contents of samples were calculated according to the standard curve.

Determination of endogenous protease activity. The endogenous protease activity was investigated in the mixed homogeneous suspension of pea and sweet buckwheat, following the method of Zhai and Jiao (2009), with modifications. The soaked seeds of both pea and sweet buckwheat were skinned and ground with 0.2 mol/l sodium phosphate buffer solution of pH 5.0. Then, the homogenate was centrifuged at 2570 g for 10 min in a refrigerated high-speed centrifuge at 4°C. The supernatant was the crude endogenous protease fluid. The reaction mixture consisted of 1 ml crude endogenous protease fluid and 2 ml 0.2% (w/v) casein. The reaction solution was incubated at 37°C for 20 min and terminated by adding 2 ml of 10% trichloro acetic acid. The suspension was centrifuged at 6578 g for 10 min at room temperature. The supernatant was collected in a 10 ml measuring flask, distilled water was added to 10 ml, and detected at 274 nm using UV/VIS spectrophotometer. In the blank sample tube, 1 ml crude endogenous protease fluid and 2 ml 10% trichloroacetic acid were added first, followed by addition of 2 ml 0.2% casein. The centrifugation and constant volume steps were identical those of the test tube. The tyrosine content was analysed via standard curve. One unit of enzyme activity was defined as the release of 1 µg tyrosine produced per 1 min at 37°C.

Determination of GAD activity. The GAD activity was investigated in the homogenous extract suspension of pea and sweet buckwheat, following the method of Bai, with modifications (Bai *et al*. 2009). The soaked seeds of both pea and sweet buckwheat were skinned and ground with pH 5.7 phosphate buffer solution. Then, the homogenate was centrifuged at 6578 g for 10 min in a refrigerated high-speed centrifuge at 4°C. The supernatant was crude GAD fluid. The reaction mixture solution consisted of 1 ml crude GAD fluid and 2 ml substrate (0.1% of Glu, pH 5.7). The reaction solution was incubated at 40°C for 2 h and terminated by heating to 90°C for 5 minutes. The suspension was centrifuged at 6578 g for 20 min at room temperature. The GABA content of the supernatant was analysed by the GABA determination method as described above. One unit of enzyme activity was defined as the release of 1 µmol of GABA produced per 30 min at 40°C.

Hydration characteristics during soaking. Hydration characteristics of seeds of both pea and sweet buckwheat were investigated. The seeds (100 g) were soaked with distilled water (1:5 w/v) at 30°C in an incubator. A part of the soaked seeds were removed and weighed at specified times during soaking: 0.5, 2, 4, 8, 12, 16, and 20 hours. The moisture contents at different periods were calculated according to Equation (1):

$$\text{Moisture content (\%)} = (M_1 - M_0)/M_0 \times 100 \quad (1)$$

where: M_1 – represents the weight of seeds soaked at different soaking times; M_0 – represents the weight of untreated seeds.

Effect of soaking time on endogenous protease and GAD activity. Sterilized seeds of pea and buckwheat (50 g/per portion) were soaked with distilled water at 30°C for 4, 8, 12, 16, and 20 h and taken out at specified times as indicated in Table 1 and endogenous protease and GAD activity were measured.

Proportion of homogeneous fluid on GABA synthesis. The homogeneous fluids of pea and sweet buckwheat were mixed at proportions of 1:0, 3:1, 1:1, 1:3, and 0:1 (pea:sweet buckwheat).

Effect of $CaCl_2$, VB_6, and MSG in homogenous extract suspension on GABA synthesis. To determine the proper scope of $CaCl_2$, VB_6, and MSG for GABA synthesis in the homogenous extract suspension of pea and sweet buckwheat, different reaction solutions were prepared. Various concentrations were applied: $CaCl_2$ ranging from 0 to 1 mmol/l, VB_6 ranging from 0 to 5 mmol/l, and MSG ranging from 0 to 4 mg/ml. All solutions were prepared using phosphate buffer solution at pH 5.7.

Optimization of components in mixed homogenous extracts suspension for GABA synthesis.

Table 1. Changes in endogenous protease and GAD activities in response to soaking at 30°C

Soaking time (h)	Endogenous protease activity (U)		GAD activity (U)	
	pea	buckwheat	pea	buckwheat
4	1.88	4.62	0.24	0.31
8	2.18	3.42	0.25	0.39
12	2.36	2.78	0.13	0.41
16	2.92	2.72	0.07	0.43
20	3.20	2.08	0.13	0.34

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Based on a single factor experiment, the components in homogenous extraction mixed suspension, namely $CaCl_2$ (X_1), VB_6 (X_2), and MSG (X_3), for GABA content in the mixed suspension were optimized using RSM (Design-Expert version 7.0.1.0 Trial). The factors and levels were investigated in a Box-Behnken design as shown in Table 2. Fifteen combinations, including three replicates of the centre points were employed to evaluate the combined effects of variables on the resulting GABA content. The second-order polynomial coefficients were calculated and analysed using Design Expert Software. A second-order polynomial model, which includes all interaction terms, was used to predict response as shown in Equation (2):

$$Y = \beta_0 + \sum_{i=1}^{3} B_i X_i + \sum_{i=1}^{3} B_{ii} X_i^2 \sum_{i=1 i<j}^{2} \sum^{3} B_{ij} X_i X_j \quad (2)$$

where: Y – dependent variable (increased content of GABA); β_0 – intercept; B_i, B_{ii}, and B_{ij} – regression coefficients estimated by the model; X_i and X_j – levels of the independent variables.

The model equation represent the linear, quadratic, and interaction regression effects of the X_1, X_2, and X_3 factors on the reaction, respectively. The fitted polynomial equation is then expressed in the form of three-dimensional surface plots to visualize the relationship between the reaction and the experimental levels of each variable and to deduce the optimal reaction conditions. The combination of different optimized variables was determined in an attempt to verify the validity of the model. Subsequently, three additional confirmation experiments were conducted to verify the validity of the obtained statistical experimental strategies.

Calculations and statistical analysis. Average values and standard deviations were computed for all experimental data. Statistical analysis was performed using Fisher's F-test. $P < 0.05$ or 0.01 indicated significance levels. For experiments performed in duplicate, two separate samples were analysed twice. Two values were accepted as replicates only if they differed by not more than 5%. In cases where this did not occur, a third confirmatory analysis was performed and the outlier values were dropped. In triplicate analysis, three samples were analysed.

RESULTS AND DISCUSSION

Hydration characteristics of seeds of pea and sweet buckwheat during soaking. The soaking process of the dry seeds had three distinct phases: physical absorption, sluggish absorption, and growing absorption.

As shown in Figure 1, an obvious difference was found between seeds of pea and sweet buckwheat with regard to the water content during soaking. Under a specific condition of physical absorption

Table 2. Box-Behnken and the responses for increased content of GABA in homogeneous mixed fluids

Trials	$CaCl_2$ (mmol/l)	VB_6 (mmol/l)	MSG (mg/ml)	Increased content of GABA (μg/ml)	
				true value	predicted value
1	–1	–1	0	23.92	23.71
2	1	–1	0	32.92	31.38
3	–1	1	0	35.33	36.88
4	1	1	0	40.45	40.66
5	–1	0	–1	23.26	22.59
6	1	0	–1	41.73	42.39
7	–1	0	1	33.18	32.52
8	1	0	1	23.50	24.17
9	0	–1	–1	22.54	23.42
10	0	1	–1	37.91	37.04
11	0	–1	1	20.79	21.66
12	0	1	1	31.39	30.51
13	0	0	0	49.92	49.91
14	0	0	0	51.01	49.91
15	0	0	0	48.79	49.91

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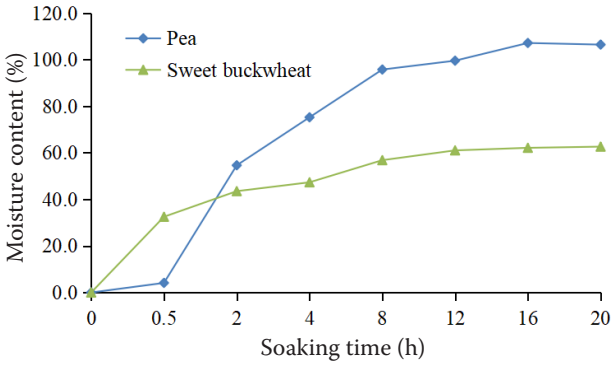

Figure 1. Hydration characteristics of seeds of pea and sweet buckwheat in response to soaking at 30°C

before 8 h of soaking, the moisture content of peas first exceeded 95% and then changed rarely after 8 hours. Because the volume swell of seeds was limited by the hull, the absorption velocity slowed gradually and even reached a balance of 108% after 16 to 20 h of soaking. In contrast, the moisture content of sweet buckwheat seeds reached 57% for 8 h of soaking, changed rarely after 8 to 12 h of soaking, and retained sluggish absorption with 62%.

Effect of soaking time on endogenous protease and GAD activity. Physiological and biochemical reactions occurred after soaking of seeds, showing vigorous life activity and synthesis as well as decomposition metabolic functions.

The activities of both endogenous protease and GAD in seeds changed during soaking (Table 1). Endogenous protease activity in pea increased by 1.88 to 3.20 from 4 to 20 h, respectively. Endogenous protease activity in sweet buckwheat was the highest during soaking for 4 h and then gradually decreased from 4.62 to 2.08. The highest GAD activity in pea was obtained during soaking for 8 h, however, in sweet buckwheat for 12 hours. GAD activity of both pea and sweet buckwheat first increased and then decreased. Therefore, the optimal soaking time was 4 h, the turning point from the physical absorption phase to sluggish absorption. At this point, both endogenous protease and GAD activity were vigorous.

Published studies have reported that plant seeds dramatically change during soaking. Amino acids stored in seeds as storage proteins are decomposed under the action of endogenous protease activated by water absorption and change into transportable amides (Sharma *et al*. 2018). GAD is activated upon water absorption, and Glu is converted to GABA (Komatsuzaki *et al*. 2007).

Effect of proportion of homogeneous fluid on GABA synthesis. Proteins in seeds were decomposed to amino acids by endogenous protease. L-Glu can be converted to GABA by GAD. When the homogeneous fluids of pea and sweet buckwheat were mixed in specific proportion, the content of synthesized GABA was higher than that of individual pea or sweet buckwheat (Figure 2). The highest GABA content was obtained when pea and sweet buckwheat (1:1 w/w) were mixed. Thus, the mixture of both pea and sweet buckwheat was more conducive to GABA synthesis.

Effect of $CaCl_2$ in homogeneous extraction suspension on GABA synthesis. There is a calmodulin (CaM) binding domain at the C-terminus of GAD in plant (Akama *et al*. 2007) and GAD activity is regulated by calcium ion (Ca^{2+}) or CaM. Recent studies have indicated that increasing cytosolic Ca^{2+} concentration stimulates GAD activity and promotes GABA synthesis, which also confirms that GAD is regulated by the Ca^{2+}/CaM signal transduction pathway (Wang *et al*. 2010). The effect of increasing exogenous $CaCl_2$ concentration on the ncreased content of GABA is presented in Figure 3.

The produced content of GABA increased first and then decreased. When $CaCl_2$ concentration was

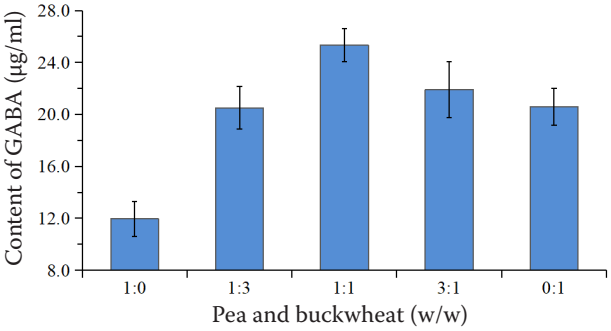

Figure 2. Effect of the proportion of homogeneous extract suspension on GABA synthesis

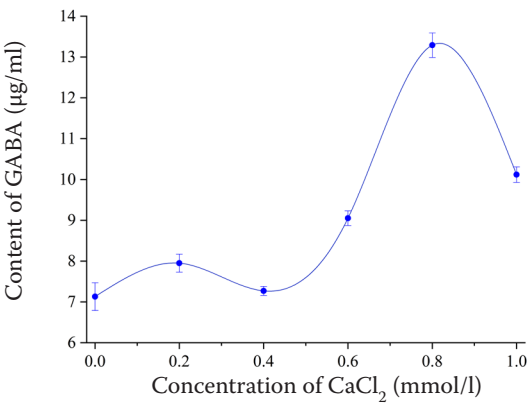

Figure 3. Effect of $CaCl_2$ in homogeneous extract suspension on GABA synthesis

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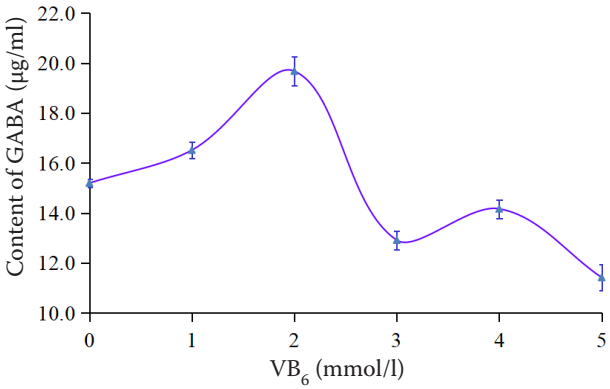

Figure 4. Effect of VB_6 in homogeneous extract suspension on GABA synthesis

in the range of 0–0.8 mmol/l, the GABA content increased with increasing $CaCl_2$ concentration and the maximal content reached 13.16 μg/ml at a $CaCl_2$ concentration of 0.8 mmol/l. $CaCl_2$ concentrations decrease steeply at 0.8–1 mmol/l. Therefore, the optimum added amount of $CaCl_2$ is 0.8 mmol/l. By adding exogenous Ca^{2+} in germinated millet, the maximal GABA yield of 37.9 mg/100 g *FW* appeared at Ca^{2+} concentration of 2.5 mM, and the maximum of GAD activity was stimulated via that was 1.0 mM (Bai *et al.* 2009). The findings of the present study show that increasing Ca^{2+} levels promoted GABA synthesis due to GAD activity, which stimulated homogeneous extraction suspension.

Effect of VB_6 in the homogeneous extraction suspension on GABA synthesis. Pyridoxal 5-phosphate (PLP) is a coenzyme of GAD and plays an important role as a cofactor in stimulating GAD activity (Tong *et al.* 2002). It has been reported that GAD is specific to the substrate Glu and requires PLP through qualitative analysis *in vitro* to crude extract fluid of GAD in plants tissue. Pyridoxamine (VB_6) is a cheap analogue of PLP, which is often used to promote the synthesis and accumulation of GABA.

Within the concentration range of 0–5 mmol/l of VB_6, the GABA content first increased and then decreased (Figure 4). There was a gradual increase in GABA content with increasing VB_6 concentration of 0–2 mmol/l, but a sharp decrease occurred when the VB_6 concentration exceeded 3 mmol/l, following little change with VB_6 concentration of 3–5 mmol/l. Therefore, the optimal concentration of VB_6 was 2 mmol/l.

The increase of the PLP concentration favoured GABA-transaminase activity, which was dependent on the PLP concentration and converted GABA to succinate semialdehyde (Narayan & Nair 1990). The data obtained in the present study show that addition of VB_6 into the mixture suspension enhanced GABA synthesis.

Effect of MSG in the homogeneous extraction suspension on GABA synthesis. Glu or its salts were the reaction substrate during GABA synthesis. Glu addition increased the substrate concentration in the vicinity of the cytosolic GAD, and further regulation of GAD activity and GABA content (Komatsuzaki *et al.* 2005). In this study, MSG was chosen instead of Glu due to its solubility in distilled water. The effect of MSG addition, in homogeneous extraction suspension of pea and sweet buckwheat, on increased content of GABA is shown in Figure 5. GABA synthesis increased continuously with increasing MSG. The maximal content of GABA synthesis was 334.82 μmol/l under the condition of optimal concentration of 17.74 mmol/l; however, it rapidly decreased at an MSG concentration of 17.74–23.65 mmol/l. These data indicated that higher concentration of MSG was not conducive to GABA synthesis.

Recently, the regulation of GAD activity by glutamate availability has been investigated *in situ* (Scott-Taggart *et al.* 1999). Elevated glutamate levels stimulated GABA synthesis in isolated *Asparagus* mesophyll cells (Cholewa *et al.* 1997). Increases of GABA levels were likely caused by an increase of the substrate in the vicinity of the cytosolic GAD, which suggests that both GAD activity and GABA content were regulated by glutamate addition (Komatsuzaki *et al.* 2005).

Optimization of components for GABA synthesis

Analysis of Box-Behnken experiments. The Box-Behnken design and the corresponding experimen-

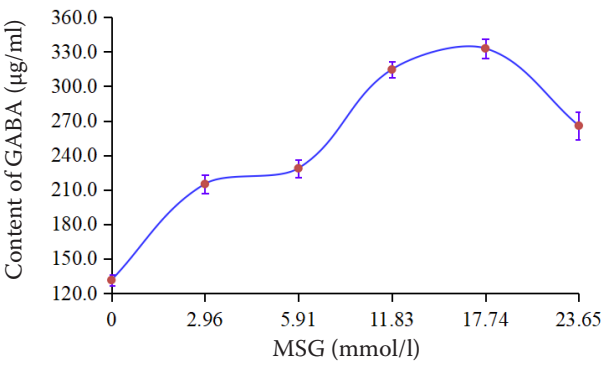

Figure 5. Effect of MSG in homogeneous extract suspension on GABA synthesis

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tal data are shown in Table 2. Multiple regression analysis of the data demonstrated that the RSM design model was consistent with the second-order polynomial Equation (1). The second-order polynomial model, describing the correlation between GABA content and the three variables in this study is shown in Equation (3):

$$Y = 49.91 + 2.86X_1 + 5.61X_2 - 2.07X_3 - 0.97X_1X_2 - 7.04X_1X_3 - 1.19\ X_2X_3 - 7.25X_1^2 - 9.51X_2^2 - 12.24X_3^2 \quad (3)$$

where: X_1, X_2, and X_3 – codes of $CaCl_2$, VB_6, and MSG concentration, respectively.

Statistical analysis (Table 3) indicated that the proposed model was adequate and achieved a satisfactory R^2 value of 0.9920. The 'Pred R^2' of 0.8939 was in reasonable agreement with the 'Adj R^2' of 0.9775. The F test reflected the effective value of regression model, containing lack of fit test and significance test of regression equation. An F of 68.64 was obtained by ANOVA, which implied that the model was very significant ($P < 0.01$), and an adequate precision of 22.150 showed an adequate signal. The 'Lack of Fit F' of 2.63 implies that the Lack of Fit is not significant relative to the pure error. These results proved the validity of the experimental model.

X_2, X_1X_3, X_1^2, X_2^2, and X_3^2 were very significant ($P < 0.01$), X_1 and X_3 were significant ($P < 0.05$), X_1X_2 and X_2X_3 were not significant ($P > 0.05$), see Table 3. Non-significant terms were not deleted for convenience of analysing and discussing the model.

Interaction analysis between two factors. Response surfaces plots were used to illustrate the interactive effects of $CaCl_2$, VB_6, and MSG concentrations on GABA synthesis in Figures 6A, B and C.

Figure 6A shows the effect of $CaCl_2$ and VB_6 on GABA content at an MSG concentration of 2.83 mg/ml. Both $CaCl_2$ and VB_6 had most significantly effects on GABA accumulation, but $CaCl_2$ and VB_6 did not interact significantly ($P > 0.05$) (Table 3). At a fixed $CaCl_2$ concentration, the GABA content first increased sharply with addition of VB_6, then decreased for a VB_6 concentration above 2.29 mmol/l. When the VB_6 addition was set, $CaCl_2$ led to a gradual increase of GABA synthesis at an optimal concentration of 0.85 mmol/l.

Figure 6B shows the effect of $CaCl_2$ and MSG on the GABA content at a constant VB_6 concentration of 2.29 mmol/l. There was a significant interaction ($P = 0.0003$) between $CaCl_2$ and MSG, which also influenced GABA synthesis. At a fixed MSG concentration, the GABA content increased sharply and decreased later. At fixed $CaCl_2$ addition, GABA accumulation constantly increased with MSG addition at the beginning, but later decreased at an MSG concentration of 2.83 mg/ml. The maximal GABA content was obtained at a $CaCl_2$ concentration of 0.85 mmol/l and a MSG concentration of 2.29 mg/ml.

Table 3. Analysis of variance (ANOVA) for the response surface regression model

Source	Sum of squares/10^{-3}	*df*	Mean square/10^{-3}	*F*	*P*	Significance level
Model	1506.50	9	167.39	68.64	0.0001	***
X_1	65.61	1	65.61	26.90	0.0035	**
X_2	252.11	1	252.11	103.38	0.0002	***
X_3	34.36	1	34.36	14.09	0.0132	**
X_1X_2	3.76	1	3.76	1.54	0.2692	*
X_1X_3	198.11	1	198.11	81.23	0.0003	***
X_2X_3	5.69	1	5.69	2.33	0.1872	*
X_1^2	193.85	1	193.85	79.49	0.0003	***
X_2^2	333.64	1	333.64	136.81	< 0.0001	***
X_3^2	553.47	1	553.47	226.96	< 0.0001	***
Residual	12.19	5	2938.53	–	–	–
Lack of Fit	9.73	3	6155.34	–	–	–
Pure error	2.46	2	525.92	2.63	0.2873	*
Cor total	1518.69	14	–	–	–	–

*not significant at $P > 0.05$; **significant at $P < 0.05$; ***significant at $P < 0.01$; $R^2 = 0.9920$; adj $R^2 = 0.9775$; pred $R^2 = 0.8939$; adequate precision = 22.150

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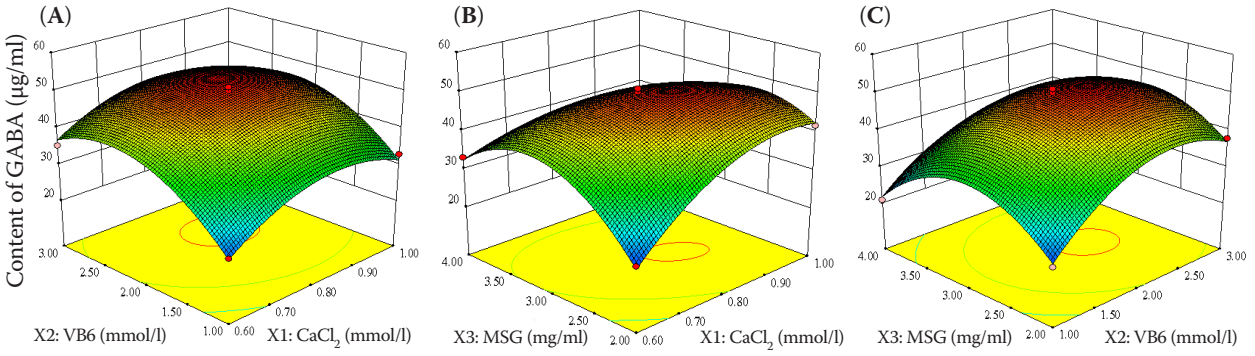

Figure 6. Response surface plots showing effects of: (**A**) $CaCl_2$ and VB_6, (**B**) $CaCl_2$ and MSG, (**C**) VB_6 and MSG on increased content of GABA in homogeneous extract suspension

The effect of VB_6 and MSG, at a constant $CaCl_2$ concentration of 0.85 mmol/l, on GABA synthesis is shown in Figure 6C. The interaction between two variables, in the model, on GABA synthesis was not significant ($P > 0.05$) (Table 3). The GABA content increased when VB_6 addition increased from 0 to 2.29 mmol/l, but decreased above 2.29 mmol/l at a fixed MSG concentration. When the VB_6 concentration was set, GABA synthesis sharply increased with the addition of MSG and the optimal concentration was 2.83 mg/ml.

Component optimization and model verification. According to the RSM test results, the optional components in the homogeneous extraction suspension of pea and sweet buckwheat for GABA synthesis were 0.85 mmol/l of $CaCl_2$, 2.29 mmol/l VB_6, and 2.83 mg/ml MSG. Under optimal conditions, the maximal increased content of GABA was 51.29 μg/ml. Verification of the model in Equation (2) was performed under the optimum conditions for GABA synthesis. The observed increased content of GABA under optimal conditions was 49.91 μg/ml, which agreed with the predicted value of 51.29 μg/ml in the model. The experimental results proved that the model was valid.

Verifying test. Under the conditions of different added contents obtained by RSM test results and other treatments, the resulting GABA contents are presented in Table 4.

Table 4. Verifying test

Verification	Test conditions			GABA (μg/ml)
	$CaCl_2$ (mmol/l)	VB_6 (mmol/l)	MSG (mg/ml)	
No-addition	0	0	0	25.21
RSM test	0.85	2.29	2.83	51.29
Optimal single factor	0.80	2.00	3.00	48.92
No-VB6	0.85	0	2.83	40.76

CONCLUSIONS

When seeds of pea and sweet buckwheat were steeped for 4 h, endogenous protease and GAD activities were stimulated. When the homogeneous fluids of both pea and sweet buckwheat were mixed, GABA synthesis dramatically increased compared to single pea or sweet buckwheat fluids. The effect of $CaCl_2$, VB_6, and MSG on γ-aminobutyric acid synthesis in the homogeneous extraction suspension of pea and sweet buckwheat were studied. The results showed that addition of $CaCl_2$, VB_6, and MSG dramatically increased GABA content. ANOVA analysis in RSM demonstrated that the designed model was valid. $CaCl_2$, VB_6, and MSG addition markedly influenced GABA synthesis; however, the interaction between $CaCl_2$ and MSG was most significant on GABA synthesis. The optimal combination in the homogeneous complex suspension was 0.85 mmol/l $CaCl_2$, 2.29 mmol/l VB_6, and 2.83 mg/ml MSG. Under optimized conditions, the highest GABA content of 51.29 μg/ml was obtained. All these results proved the mixture of pea and sweet buckwheat for GABA synthesis as an effective method.

References

Akama K., Takaiwa F. (2007): C-terminal extension of rice glutamate decarboxylase (OsGAD2) functions as an autoinhibitory domain and overexpression of a truncated mutant results in the accumulation of extremely high levels of GABA in plant cells. Journal of Experimental Botany, 58: 2699.

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Bai Q., Chai M., Gu Z., Cao X., Li Y., Liu K. (2009): Effect of components in culture medium on glutamate decarboxylase activity and γ-aminobutyric acid accumulation in foxtail millet. Food Chemistry, 116: 152–157.

Cho D.H., Lim S.T. (2016): Germinated brown rice and its bio-functional compounds. Food Chemistry, 196: 259–271.

Cholewa E., Cholewinski A.J., Shelp B.J., Snedden W.A. (1997): Cold-shock-stimulated γ-aminobutyric acid synthesis is mediated by an increase in cystolic Ca^{2+}, not by an increase in cytosolic H^{+}. Canadian Journal of Botany, 75: 375–382.

Defeudis F.V. (1983): γ-aminobutyric acid and cardiovascular function. Experientia, 39: 845–849.

Ding J., Ulanov A.V., Dong M., Yang T., Nemzer B.V., Xiong S., Zhao S., Feng H. (2018): Enhancement of gama-aminobutyric acid (GABA) and other health-related metabolites in germinated red rice (*Oryza sativa* L.) by ultrasonication. Ultrasonics Sonochemistry, 40: 791–797.

Guo Y., Hui C., Yu S., Gu Z. (2011): Effects of soaking and aeration treatment on γ-aminobutyric acid accumulation in germinated soybean (*Glycine max* L.). European Food Research & Technology, 232: 787–795.

Komatsuzaki N., Shima J., Kawamoto S., Kimura T., Momose H. (2005): Production of γ-aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. Food Microbiology, 22: 497–504.

Komatsuzaki N., Tsukahara K., Toyoshima H., Suzuki T., Shimizu N., Kimura T. (2007): Effect of soaking and gaseous treatment on GABA content in germinated brown rice. Journal of Food Engineering, 78: 556–560.

Lawrence J.M., Machlin L.J. (1995): Critical assessment of the epidemiological data concerning the impact of antioxidant nutrients on cancer and cardiovascular disease. Critical Reviews in Food Science and Nutrition,35: 41–49.

Leventhal A.G., Wang Y., Pu M., Zhou Y., Ma Y. (2003): GABA and its agonists improved visual cortical function in senescent monkeys. Science, 300: 812–815.

Luo X., Wang Y., Li Q., Wang D., Xing C., Zhang L., Xu T., Fang F., Wang F. (2018): Accumulating mechanism of γ-aminobutyric acid in soybean (*Glycine max* L.) during germination. International Journal of Food Science & Technology, 53: 106–111.

Manyam B.V., Katz L., Hare T.A., Kanifefski K., Tremblay R.D. (1981): Isoniazid-induced elevation of cerebrospinal fluid (CSF) GABA levels and effects on chorea in Hunitington's disease. Annals of Neurology, 10: 35–37.

Mayer R., Cherry J., Rhodes D. (1990): Effects of heat shock on amino acid metabolism of cowpea cells. Plant Physiology, 94: 796–810.

Narayan V.S., Nair P.M. (1990): Metabolism, enzymology and possible roles of 4-aminobutyrate in higher plants. Phytochemistry, 29: 367–375.

Paul V. (2003): Inhibition of acute hyperammonemia-induced convulsions by systemically administered gamma aminobutyric acid in rats. Pharmacology Biochemistry & Behavior, 74: 523–528.

Ribéreau S., Aryee A.N.A., Tanvier S., Han J., Boye J.I. (2018): Composition, digestibility, and functional properties of yellow pea as affected by processing. Journal of Food Processing & Preservation, 42: e13375. doi: 10.1111/jfpp.13375

Scott-Taggart C.P., Cauwenberghe O.R.V., Mclean M.D., Shelp B.J. (1999): Regulation of γ-aminobutyric acid synthesis in situ by glutamate availability. Physiologia Plantarum, 106: 363–369.

Sharma S., Saxena D.C., Riar C.S. (2018): Changes in the GABA and polyphenols contents of foxtail millet on germination and their relationship with *in vitro* antioxidant activity. Food Chemistry, 245: 863–870.

Sluková M., Levková J., Michalcová A., Horáčková Š., Skřivan P. (2017): Effect of the dough mixing process on the quality of wheat and buckwheat proteins. Czech Journal of Food Sciences, 35: 522–531.

Tong J.C., Mackay I.R., Chin J., Law R.H., Fayad K., Rowley M.J. (2002): Enzymatic characterization of a recombinant isoform hybrid of glutamic acid decarboxylase (rGAD67/65) expressed in yeast. Journal of Biotechnology, 97: 183–190.

Tsushida T., Murai T. (1987): Conversion of glutamic acid to γ-aminobutyric acid in tea leaves under anaerobic condition. Argicultural and Biological Chemistry, 51: 2865–2871.

Wang L., Xu D.X., Lv Y.G., Zhang H. (2010): Purification and biochemical characterisation of a novel glutamate decarboxylase from rice bran. Journal of the Science of Food & Agriculture, 90(6): 1027–1033.

Zhai W., Jiao Y. (2009): Sprouting condition optimization based on protease activity and GABA of black soybean. Food science, 30: 51–54.

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