High pressure processing and post-high pressure storage induce the change of polyglutamyl folate and total folate from different legumes

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Abstract The aim of this work was to study the effect of high pressure processing (HPP) and post-HPP cold storage on the distribution of polyglutamyl and monoglutamyl folate and the absolute concentration of total folate in green beans, yardlong beans and winged beans using a validated ultra-high performance liquid chromatography-tandem mass spectrometry method. The results showed that HPP led to the deglutamylation of polyglutamyl folate to monoglutamyl folate in all of the investigated beans. The degree of deglutamylation was increased with enhancing processing pressure and extending holding time. During HPP, significant loss of total folate was observed under 600 MPa/10 min treatment. Uniquely 300 MPa/5 min and 450 MPa/5 min could significantly release more folate from yardlong beans and green beans matrix. During the following cold-storage, the deglutamylation keep progressing. For those untreated beans, no significant deglutamylation and total folate loss was observed during cold storage for yardlong beans and green beans while there is slight change for the total folate in winged beans. For those HPP treated beans, total folate loss followed the first order kinetics over the storage. The rate constant of degradation was positively proportional to the applied pressure, holding time and the proportion of monoglutamyl folate. This research provided a reference for understanding the

deglutamylation of polyglutamyl folate and folate loss during HPP treatment and further shelf life.

Keywords High pressure processing · Post-high pressure processing storage · Polyglutamyl folate · Total folate · Legumes

Introduction

Folates belong to B9 group vitamins and folic acid refers to synthetic oxidized forms. Folates are involved in important one-carbon metabolism by serving as the acceptor and donors of one carbon units in a variety of reactions (Hayashi et al. 2007). The biochemical pathways that folates participated in include the methylation and DNA biosynthesis cycle in almost all of the organisms (Forges et al. 2007). Although folate plays a critical role in biological system, only plants and microorganisms are able to synthesize folate *de novo*, human and animals are unable to synthesize folate, and therefore require this vitamin from diet (Basset et al. 2005). Folates are widely distributed in nature. Green leafy vegetables, legumes and some fruits are among the richest sources of folate. Despite of this, epidemiological studies indicate that folate deficiency is one of the most common nutritional deficiencies in the world and can lead to a variety of disorders including neural tube defects such as spina bifida, anencephaly, megaloblastic or macrocytic anemia, occlusive vascular disease and other disease (Kondo et al. 2009). Folate deficiency in China is prevalent especially in northern China because of limited consumption of fresh green leafy vegetables and birth defect rate varied from 4.2 to 10.6‰ (Chen et al. 2009). To decrease the risk of folate deficiency, several countries have launched the policy that cereal based food products

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are mandatory fortified with folic acid to prevent folate-related disorders (De Wals et al. 2007). In addition, transgenic approaches have been used to increase the folate content by biofortification of tomato fruits, potato tubers and rice grains (Zhu et al. 2007). Legumes are important components in traditional diet. Legumes contain high content of protein, minerals and B-vitamins and their consumption is related to several beneficial health effects. Early studies highlights that legumes are good dietary source of folate (Hefni and Witthöft 2014; Rychlik, et al. 2007; Delchier et al. 2013). However legumes are usually processed before consumption which might cause significant loss of folate.

Chemically, folate is composed of a pteridine ring, *p*-aminobenzoate and coupled to polyglutamyl moieties. In subcellular level, folate exists as tetrahydrofolate or dihydrofolate and its derivatives. The derivatives are differentiated by one-carbon substituent at the N 5 and/or N 10 positions (formyl, methyl, methylene, methenyl and formimino), and the length of the γ -glutamyl moieties (Rebeille et al. 2006).

The bioavailability of food folate is usually inferior compared to that of synthetic folic acid and 5-methyltetrahydrofolate (Rebeille et al. 2006). It is recognized that natural food folate which mainly exists in their polyglutamyl forms is absorbed in jejunum as monoglutamyl folates after removal of the polyglutamyl chains by intestinal γ -glutamyl hydrolase (GGH) (Rebeille et al. 2006). There were couples of factors affecting the bioavailability of native folate, for example, hydrolysis of the polyglutamyl chains before absorption by jejunum, food matrix, folate entrapment, GGH inhibitors, folate instability in the digestive system and genetic polymorphism (Rebeille et al. 2006). Early studies have found the polyglutamyl folate was 60 to 80% bioavailable compared with the monoglutamyl form (Gregory 1995). The bioavailability of monoglutamyl folate is significantly greater than that of polyglutamyl forms probably because the latter requires the hydrolysis of the polyglutamyl chains. Relative bioavailability of natural folate was estimated to be only 50% compared with folic acid pills but there were still controversies (Forssen et al. 2000; McNulty and Pentieva 2004).

HPP technology has been gaining popularity applied as a pasteurization method for inactivating vegetative cells of microorganisms and some enzymes, which often resulted in better retention of nutrients without sacrificing shelf life. The effect of high pressure on quality-related plant enzymes was investigated (Terefe et al. 2014).

Early studies have found polyglutamyl folates were hydrolyzed under HPP conditions in several vegetables (Wang et al. 2011). Different vegetables showed different degrees of deglutamylation under different HPP

condition. In cellular, it is believed that polyglutamyl chains affect folate efficacy and stability (Blancquaert et al. 2014). Early studies indicated that polyglutamyl folate favored binding to folate binding protein (FBP), and polyglutamyl folate was protected from oxidative break down by binding, thus, polyglutamyl folate might be more stable than monoglutamyl form (Blancquaert et al. 2014). Although early studies indicated that polyglutamyl folate in some vegetables could be shortened by HPP, no study continued to investigate the stability of folate during refrigerate storage after HPP treatments (Wang et al. 2011; Verlinde et al. 2008; Melse-Boonstra et al. 2002).

The aim of this study was to evaluate the impact of high pressure–temperature–time combinations; refrigerated storage of post-HPP on the levels of total folate and the deglutamylation of polyglutamyl folate in green beans (*Phaseolus vulgaris*), yardlong beans (*Vigna unguiculata* subsp. *sesquipedalis*) and winged beans (*Psophocarpus tetragonolobus*) by a validated UHPLC–MS/MS method. Three different pressure (300, 450 and 600 MPa) and two holding time (5 and 10 min) were investigated at 30 °C. During the investigation of post-HPP storage, all of beans were stored at 4 °C for 10 days.

Materials and methods

Chemicals and reagents

All of folate standards employed in this study were shown in the followings: (6R,S)-5-methyl-5,6,7,8-tetrahydrofolic acid, folic acid, (6R,S)-5,10-methenyl-5,6,7,8-tetrahydrofolic acid, 10-formylfolic acid, (6R,S)-5-formyl-5,6,7,8-tetrahydrofolic acid and (6R,S)-5,6,7,8-tetrahydrofolic acid were purchased from Schircks Laboratories (Jona, Switzerland). (6S)-5-methyl-5,6,7,8-tetrahydrofolate- $[^{13}\text{C}_5]$ Glu was obtained from Merck Eprova AG (Schaffhausen, Switzerland). LC–MS grade water and acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ). LC–MS Ultra formic acid (99%, purity), 2-mercaptoethanol, sodium borohydride were from Fluka (St. Louis, MO) and acetic acid (99.7%), ammonium acetate, ascorbic acid (99%, crystalline), polypropylene glycol (P 400) were from Sigma (St. Louis, MO). Male rat serum was from Xinpeng (Guangzhou, China), production No. 140824.

Preparation of standard solutions and standard curves

The preparation of stock solutions, working solutions, calibration curves and quantification method followed early report with only minor change (Wang et al. 2010).

The calibration curves were constructed by diluting the standard solution into extraction buffer (100 mM ammonium acetate, 1% ascorbic acid, 0.2% β -mercaptoethanol, pH = 7.89). The y axis of calibration curve was the ratio of peak area of each external standard to internal standard while external standard concentration was x axis. Each standard solution was prepared in four replications to construct the standard curve.

Legumes preparation

Green beans, yardlong beans and winged beans were obtained from test plots in Qingyunshan Farmer's Cooperative in Wengyuan, Shaoguan City, Guangdong Province. The investigated legumes were harvested at 3 months old. The collected legumes were refrigerated and shipped to the laboratory until the next day analysis. Each piece of legumes (ca. 20 g) was packaged and sealed by a vacuum sealer (Model DZ-400A, Chuanka, Shanghai, China) in a clear nylon/EVOH/polyethylene pouch with high barrier properties (Blue Packaging Co., Guangzhou, China). The legumes were treated following the steps as shown in the flow chart (Fig. 1).

High pressure processing

All of the HPP treatments were carried out using a high pressure food processor with a 5 L capacity (model: HPP. L2-600/5, Hua Tai Sen Miao Biotechnology Co., Tianjin, China). Polypropylene glycol was used as the pressure-transmitting fluid. Three pressures at two different holding times were investigated: 300, 450 and 600 MPa at 30 °C for either 5 or 10 min. The pressure vessel was maintained at ambient temperature (25 °C). The HPP system was fully programmable allowing reproducible pressure buildup and decompression times. Pressure come-up time was 120 s, and decompression time was 60 s.

During HPP treatment, the temperature of food with high moisture content generally increases about 3 °C for every 100 MPa of compression (Rasanayagam et al. 2003). To achieve similar final process temperature (30 °C) under different pressures, the samples were prechilled at 18, 12 and 4 °C for 300, 450 and 600 MPa treatments, respectively. Samples were prechilled in an incubator (−20–100 °C, Model DC-2006, Boxun, Shanghai, China). The samples were then loaded into a stainless steel cylindrical product holder (1210 × 600 × 2300 mm) insulated with 0.5 cm polytetrafluoroethylene (PTFE). The product holder was then filled with prechilled polypropylene glycol and loaded into the pressure vessel. Time to load and unload the samples into the pressure vessel was standardized to 4 min. The average sample process temperature under pressure was 27–30 °C. Eighteen analytical replicates were performed for each pressure/time condition, and each treatment was conducted in triplicate. The sample pouches were taken out from the pressure chamber and rinsed with water before they were subject to folate analysis and refrigeration storage.

Post-HPP storage

Samples after HPP processing were directly moved to 4 °C refrigerator for storage. The legumes without HPP processing were first vacuum-packed and stored in 4 °C refrigerator as the same as the processed samples. The duration of storage was 10 days. Every 2 days, three replicates with each treatment and non-treatment were drawn from refrigerator for further polyglutamyl folate and total folate analysis.

Folate extraction and determination

The method for folate extraction and determination was according to previous report (Wang et al. 2010). To understand the effect of HPP on the distribution of

Fig. 1 Flow diagram of the green beans, winged beans and yardlong beans treatments

polyglutamyl folates, polyglutamyl folate needed to be stabilized immediately after HPP treatment. Here we boiled the sample right after the HPP treatments because this method was proven to be effective for inactivation of endogenous GGH in previous studies (Wang et al. 2010). To analyze the change of polyglutamyl folate and total folate during HPP processing, three replicates of each treatment were withdrawn from vacuum bags and subject to boiling in extraction buffer (100 mM ammonium acetate, 1% ascorbic acid, 0.2% 2-mercaptoethanol, pH = 7.89) immediately after HPP treatments.

After pre-boiling, the samples were cooled on ice and then homogenized for 2 min to fine puree using a homogenizer (IKA T18 Basic, German) at 4 °C. The homogenates were boiled again for 10 min, cooled and reconstituted to 100 mL. To analyze folate species and total folate, partial of samples was treated with 150 µL rat serum at 37 °C for 6 h to deconjugate the polyglutamyl folate to monoglutamyl folate. Therefore, total folate is the sum of individual folate species concentration with rat serum deconjugation while polyglutamyl folate is the sum of individual folate species concentration without deconjugation. Both deconjugated and undeconjugated samples were centrifuged at 30,000 rpm for 3 min. The supernatant was filtered and injected into the UHPLC–MS/MS. The UHPLC system is consisted with Agilent 1290 Infinity UHPLC, equipped with a binary pump, autosampler, column oven and degasser (Agilent Corp., Santa Clara, CA). The MS/MS system is consisted with an Agilent 6460 triple quadrupole mass spectrometer (Agilent Corp. Santa Clara, CA). During sample preparation, all manipulations were carried out under subdued light as folate is photosensitive. Background folate in the rat serum was subtracted during the quantification of folate.

The distribution of polyglutamyl folate, folate species and total folate were determined with a UHPLC–MS/MS method, which was adapted from early report (Wang et al. 2010).

Data analysis

All results were expressed as the mean $\pm$ standard deviation ($n = 9$). Significant difference was determined by ANOVA with Tukey's *pos hoc* test and one-way *t* test. A significant difference between means was considered to be present when $p < 0.05$. Statistical analysis of data was performed using Minitab 17.0 (State College, PA).

The regression lines were used to show the degradation kinetics of total folate from different legumes during post-HPP storage. The regression lines were plotted as X (storage time: 0, 2, 4, 6, 8, and 10 days) and Y (total folate, µg/100 g) which were generated by Microsoft Office Excel (version 2017, Microsoft Corporation, Seattle, WA, US).

Results and discussion

Influence of HPP on the profile of monoglutamyl and polyglutamyl folate in green beans, yardlong beans and winged beans

The influence of HPP on the distribution of monoglutamyl and polyglutamyl folate in green beans was assessed which was performed at 30 °C at 300, 450, and 600 MPa (5 and 10 min each). An overview of the results was visualized in Fig. 2a–d. The distribution of polyglutamyl and monoglutamyl folate in the untreated green beans consisted of polyglutamyl form (95%) and monoglutamyl folate (5%). Comparing the mildest pressure/time treatment (300 MPa/5 min) to untreated samples, it revealed that significant conversion of polyglutamyl folate to monoglutamyl folate that monoglutamyl folate increased approximately 2.4 fold (Fig. 2b). With prolonging holding time (10 min) under 300 MPa (Fig. 2b), the conversion continued as monoglutamyl increased 5.6 fold and polyglutamyl folate further declined. With increasing pressure (450 and 600 MPa) and extending treatment time, this change became more dramatic with the conversion of the long chain polyglutamyl folate to monoglutamyl form (Fig. 2c, d). Comparison of 600 MPa/10 min (Fig. 2d) to unprocessed green beans (Fig. 2a), results showed monoglutamyl folate increased from 5 to 53%.

The distribution of polyglutamyl and monoglutamyl folate in yardlong beans in untreated sample was 98 and 2%, respectively (Fig. 3a). 300 MPa/5 min treatment demonstrated that significant conversion of polyglutamyl folate to monoglutamyl folate and monoglutamyl folate increased about eightfold compared to untreated yardlong beans (Fig. 3b). Extending high pressure treatment time (from 5 to 10 min) under 300 MPa (Fig. 3b) has a slight conversion of polyglutamyl folate to monoglutamyl folate comparison with 300 MPa/5 min treatment. However, with elevating pressure from 450 to 600 MPa and extending treatment time from 5 to 10 min, the proportion of polyglutamyl folate is decreased from 63 to 44% (Fig. 3c, d). Therefore, by taking HPP at 600 MPa/10 min, the proportion of monoglutamyl folate is increased by 27 fold (from 2 to 56%).

The winged beans followed the similar trend with green beans and yardlong beans (Fig. 4a–d) during storage. Increasing pressure from 300 to 600 MPa and extending treatment time from 5 to 10 min resulted in gradually increasing the proportion of monoglutamyl folate from 12 to 66%. A few investigations have indicated the deglutamylation of long-chain polyglutamyl folate in intact tissue during HPP treatments (Wang et al. 2011; Verlinde et al. 2008; Melse-Boonstra et al. 2002). The possible

mechanisms were speculated here. The conversion of polyglutamyl folates under HPP treatments may be due to the action of vacuolar GGH. GGH (EC 3.4.19.9) is an enzyme that is found ubiquitously in nature including plants, animals, insects and fungi. GGH catalyzes the

Fig. 2 Effect of different pressure, holding time and duration of storage on the distribution of polyglutamyl and monoglutamyl folate of green beans: **a** untreated green beans, **b** 300 MPa, **c** 450 MPa, **d** 600 MPa. Data represented the mean of nine replicates which was acquired from triplicate runs of HPP treatment. For Fig. 2b–d, 1 5 min/0 day, 2 5 min/2 day, 3 5 min/4 day, 4 5 min/6 day, 5 5 min/8 day, 6 5 min/10 day, 7 10 min/0 day, 8 10 min/2 day, 9 10 min/4 day, 10 10 min/6 day, 11 10 min/8 day, 12 10 min/10 day

hydrolysis of polyglutamyl folate into short chain glutamyl folate (Hanson and Gregory 2011). However, if GGH catalyzes the hydrolysis of polyglutamyl folate, enzyme and the substrate have to be brought together. As suggested by Orsomando et al. (2005) polyglutamyl folate and GGH both existed in the vacuole. Polyglutamyl folate bound with FBP to prevent it from hydrolysis by intact GGH (Orsomando et al. 2005). During HPP, FBP was denatured accompanying with the cell permeability which could bring the enzyme and substrate together. The degree of cell permeability has been shown to increase with pressure.

In addition, the larger conversion of polyglutamyl folate with increasing pressure and treatment time might be partially due to promoting the release of membrane-bound enzymes. In addition, GGH might be activated by HPP through the change of secondary and tertiary structure (Terefe et al. 2014). Actually, the activation of plant enzymes induced by HPP has been observed by different researchers. Significant increase was observed in the activity of polyphenol oxidase (PPO) and peroxidase (POD) from apple, banana, tomato, strawberry and raspberry; lipoxygenase (LOX) from green peas and tomato, after treatment at certain pressure (Terefe et al. 2014). In addition about 76% increase in the activity of β -glucosidase was observed after 15 min treatment of strawberries at 400 MPa (Terefe et al. 2014). An increase of pectin methylesterase (PME) activity has been observed in different food matrix (Terefe et al. 2014). Our results showed that the hydrolysis of polyglutamyl folate can be induced during HPP processing. This is important since not the intact polyglutamyl folates, but the monoglutamyl folate has a higher bioavailability. Early study has compared the deglutamylation efficiency under HPP for different vegetables and was observed that carrot had the largest degree of conversion, cauliflower was second, and carrot greens had the least conversion (Wang et al. 2011).

Influence of post-HPP cold storage on the profile of polyglutamyl and monoglutamyl folate in green beans, yardlong beans and winged beans

The influence of post-HPP storage on the polyglutamyl and monoglutamyl profile of folate present in green beans was assessed for storage at 4 °C for 2, 4, 6, 8 and 10 days. An overview of the results was summarized in Fig. 2a–d. For

Fig. 3 Effect of different pressure, holding time and duration of storage on the distribution of polyglutamyl and monoglutamyl folate of yardlong beans: **a** untreated yardlong beans, **b** 300 MPa, **c** 450 MPa, **d** 600 MPa. Data represented the mean of nine replicates which was acquired from triplicate runs of HPP treatment. For Fig. 3b–d, 1 5 min/0 day, 2 5 min/2 day, 3 5 min/4 day, 4 5 min/6 day, 5 5 min/8 day, 6 5 min/10 day, 7 10 min/0 day, 8 10 min/2 day, 9 10 min/4 day, 10 10 min/6 day, 11 10 min/8 day, 12 10 min/10 day

untreated green beans, the profile of polyglutamyl and monoglutamyl folate almost remained no change with a slight trend of deglutamylation upon storage for 4 days. However, for high pressure treated samples, the proportion of monoglutamyl folate kept increasing during storage. For treatments with 300 MPa/5 min, 300 MPa/10 min and 450 MPa/5 min, significant conversion had progressed where polyglutamyl folate kept decreasing with monoglutamyl folate increasing upon storage and the proportion of monoglutamyl folate was increased about 1.9, 1.2 and 1.5 fold from 0 day to the 10th day storage. For samples after treatment with 450 MPa/10 min, 600 MPa/5 min and 600 MPa/10 min, the deglutamylation phenomenon is more dramatic during storage and the proportion of monoglutamyl folate reached to 100% after storage for 10 days.

The influence of post-HPP cold storage on the distribution of monoglutamyl and polyglutamyl folate in yardlong beans was assessed. An overview of the results was visualized in Fig. 3a–d. The distribution of monoglutamyl folate increased significantly from 2 to 22% during 10 days storage in the untreated yardlong beans. However, for those HPP treated samples, the distribution of polyglutamyl and monoglutamyl folate was altered dramatically during storage. For example, the proportion of monoglutamyl folate in 300 MPa (5 or 10 min treatment) and 450 MPa/5 min was increased to 52, 75 and 80% after storage for 10 days. The polyglutamyly folate in yardlong beans treated with 450 MPa/10 min, 600 MPa/5 min and 600 MPa/10 min was almost completely converted to monoglutamyl folate after 10 days storage.

The distribution of monoglutamyl and polyglutamyl folate in winged beans followed the similar trend with green beans and yardlong beans during storage after HPP treatments as shown in Fig. 4a–d. The proportion of monoglutamyl folate almost retained no change during storage for untreated sample while polyglutamyl folate converted partially to monoglutamyl folate in those samples after treatment under 300 MPa/5 min, 300 MPa/10 min and 450 MPa/5 min. Also the polyglutamyl folate in winged beans treated with 450 MPa/10 min, 600 MPa/5 min and 600 MPa/10 min was almost completely converted to monoglutamyl folate after 10 days storage.

Therefore, for those HPP treatment samples, the conversion of polyglutamyl folate into monoglutamyl folate kept progressing during storage. The reasons given to explain the results are the action of GGH. It appeared that HPP might activate GGH by converting it into more active form or release more GGH from membrane-bound matrix. It has been recognized that high pressure may cause inactivation or activation of enzymes depending on the applied pressure and the type of enzyme. Application of pressure less than 100 MPa has been observed to activate some

Fig. 4 Effect of different pressure, holding time and duration of storage on the distribution of polyglutamyl and monoglutamyl folate of winged beans: **a** untreated winged beans, **b** 300 MPa, **c** 450 MPa, **d** 600 MPa. Data represented the mean of nine replicates which was acquired from triplicate runs of HPP treatment. For Fig. 4b–d, 1 5 min/0 day, 2 5 min/2 day, 3 5 min/4 day, 4 5 min/6 day, 5 5 min/8 day, 6 5 min/10 day, 7 10 min/0 day, 8 10 min/2 day, 9 10 min/4 day, 10 10 min/6 day, 11 10 min/8 day, 12 10 min/10 day

disruption (Terefe et al. 2014). Higher pressure (>450 MPa) usually induced irreversible or reversible inactivation. GGH has been well characterized and cloned in soy, tomato, and *Arabidopsis* (Orsomando et al. 2005; Akhtar et al. 2008; Huangpu et al. 1996). The GGH from different plants presented different optimum pH and substrate specificity (Orsomando et al. 2005; Huangpu et al. 1996; Tamura et al. 1972). Apparently, GGH remained its activity even after 600 MPa/10 min treatment that indicated GGH might be very resistant to the pressure or be protected by the presence of other components in the vegetables. Further study will be needed to investigate the stability of GGH under HPP to make better use of this enzyme in food industry.

Effect of high pressure processing on the total folate in green beans, yardlong beans and winged beans

In this study, green beans, yardlong beans and winged beans were selected in order to study the effect of HPP on the stability of total folate (sum of folate species including 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, tetrahydrofolate, 10-formylfolic acid and 5, 10-methenyl-tetrahydrofolate). Since previous study indicated folate loss during HPP treatments was mainly caused by leaching (Melse-Boonstra et al. 2002), a closed system was used in this investigation.

Untreated (0.1 MPa) yardlong beans, green beans, and winged beans contained about 106, 63, and 67 $\mu\text{g}/100\text{ g}$ total folate. These findings were in accordance with early reports (Delchier et al. 2012, 2013; Rychlik et al. 2007).

When comparing total folate in untreated samples with that in HPP treatment samples, significant difference was observed and p values from one way t -test were shown in Table 1. For yardlong and green beans, there is significant increase of total folate after treatment under 300 and 450 MPa/5 min although there is slightly decrease under 300 MPa/5 min for winged beans ($p = 0.0378$). The increase of total folate is in the range of 11–20%. The increase of total folate content under certain HPP treatments could be ascribed to the increasing extractability of folate from food matrix under high pressure treatment. This phenomenon has been observed for other bioactive compounds that HPP could denature the food matrix and increase the extraction of bioactive compounds from

enzymes, especially monomeric enzymes (Terefe et al. 2014). Moderate high pressure (300–450 MPa) can be used to enhance beneficial enzyme reactions in plant tissue as it allowed to increase enzyme-substrate contact due to tissue

Table 1 *p*-value of one-way *t*-test of total folate between untreated and HPP samples

Treatments	Yardlong beans	Green beans	Winged beans
300 MPa/5 min	↑0.0030*	↑0.0002*	↓0.0378*
300 MPa/10 min	↓0.0029*	0.2394	0.0584
450 MPa/5 min	↑0.0014*	↑0.0018*	0.1390
450 MPa/10 min	↓0.0012*	0.1126	↑0.0110*
600 MPa/5 min	0.2962	0.3127	0.0572
600 MPa/10 min	↓0.00002*	↓0.0091*	↓0.0483*

* Designates significance at $p < 0.05$. ↑ represents the increase of total folate comparison with untreated beans and ↓ represents the decrease of total folate comparison with untreated beans

Table 2 The ANOVA *P*-value considering two factors (pressure and time) with interaction term on total folate content from three legumes

<i>P</i> value	Yardlong beans	Green beans	Winged beans
Pressure (<i>P</i>)	0.000*	0.125	0.010*
Treatment time (<i>T</i>)	0.000*	0.002*	0.670
<i>P</i> * <i>T</i> interaction	0.001*	0.831	0.022*

* Designates significance at $p < 0.05$. Data was analyzed by two factors variance analysis (Minitab 17.0)

product matrix after processing (Gupta et al. 2011). Treatments with 450 and 600 MPa/10 min caused significant loss of total folate for yardlong beans in the value of 26 and 35%, respectively. For green beans and winged beans, there is 18 and 6% loss of total loss under 600 MPa/10 min respectively. Folate loss during HPP has been

reported by several investigators. Melse-Boonstra et al. (2002) investigated folate in cauliflower at 200 MPa for 5 min and found 43% loss of total folate due to leaching into the medium because the investigators did not pack the vegetables in vacuum bags. Verlinde et al. (2008) found 48–78% folate loss in vacuum-packed broccoli at 600 MPa/30 min upon different processing temperature. However, Arroqui et al. (2004) found 5-methyltetrahydrofolate was fairly stable at 500 MPa and 60 °C for 5–100 min in vacuum-packed asparagus and a 49% loss of total 5-methyltetrahydrofolate in carrot juice was found under the same treatment. Wang et al. (2011) found losses up to 30% of total 5-methyltetrahydrofolate for carrot and cauliflower during HPP. Verlinde et al. (2009) reported significant losses of 5-methyltetrahydrofolate in a water system under 200–800 MPa which was induced through oxidation. The author also identified several degradation compounds including *s*-triazine, 5-methyldihydrofolic acid, and *p*-aminobenzoyl-L-glutamate.

The effect of high pressure treatment on the content of total folate from different legumes depends on the applied pressure and treatment time. To test which factor has a strong effect on the folate retention, interaction model was fitted in this study by application of ANOVA to test the significance of the interaction term. The resulting ANOVA table of interaction model is shown in Table 2 and interaction figures are shown in Fig. 5. We could find the *P*, *T* and their interaction term are statistically significant at an alpha level of 0.05 for yardlong beans. For winged beans, pressure and *P*, *T* interaction term have a significant impact

Fig. 5 Effect of different pressure and holding time on total folate of three beans: **a** yardlong beans, **b** green beans, **c** winged beans. Values are the mean (SD) of nine replicates. Significant difference was determined by ANOVA with Tukey's *post hoc* test ($p < 0.05$) for yardlong beans, green beans and winged beans, respectively. Values without sharing a same letter indicate that the treatment resulted in a significant difference on the concentration of total folate

on the total folate while only treatment time has a significant impact on the total folate from green beans.

Effect of post-HPP storage on the total folate in green beans, yardlong beans and winged beans

The total folate levels in green beans, yardlong beans and winged beans as a function of storage time after HPP treatments were given in Table 3. During subsequent storage for 10 days, the loss of total folate varied upon different HPP treatments and storage duration.

For untreated green beans and yardlong beans, there is no significant loss of total folate over post-HPP storage as shown in Table 3. A minor change of total folate in untreated winged beans was observed during cold storage. However, for those samples subject to HPP treatments, total folate was degraded over the storage. Comparison with untreated yardlong beans, the retention of total folate is about 59, 33, 48, 6.4, 30 and 0% after 10 days storage when treatment with 300 MPa/5 min, 300 MPa/10 min, 450 MPa/5 min, 450 MPa/10 min, 600 MPa/5 min and 600 MPa/10 min, respectively. As shown in Table 3, comparison with the untreated green beans, the retention of total folate is about 64, 45, 56, 30, 40 and 17% after 10 days storage when treatment with 300 MPa/5 min, 300 MPa/10 min, 450 MPa/5 min, 450 MPa/10 min, 600 MPa/5 min and 600 MPa/10 min, respectively. Comparison with the untreated winged beans, the retention of total folate is 57, 37, 33, 30, 24 and 3% after 10 days storage when treatment with 300 MPa/5 min, 300 MPa/10 min, 450 MPa/5 min, 450 MPa/10 min, 600 MPa/5 min and 600 MPa/10 min, respectively. As shown in Table 4, total folate concentration for three beans decreased following the first order kinetics over the storage time (their linear correlation coefficients were in the range of 0.9104–0.9975). The rate constant of degradation was positively proportional to increasing of pressure and extending of holding time for most cases as shown in Table 4. In addition, we found the rate constant of degradation was positively correlated with the proportion of monoglutamyl folate in the beans. In other words, higher content of monoglutamyl folate, folate is more susceptible to degrade. Folate binding protein was a folate receptor which bound folates with high affinity and 1:1 molar stoichiometry (Salter et al. 1981). Folate binding protein did not preserve enzyme activity and played a critical role in binding folate. Folate binding protein can protect folate from oxidation and modulate folate metabolism. During HPP treatment, FBP may have been denatured. Thus, loss of binding may cause a decrease of total folate intake because folate was very susceptible to oxidative degradation without binding with FBP. Different folate species showed different binding ability with FBP that

Table 3 Total folate retention ($\mu\text{g}/100\text{ g}$) in different beans subjected to different HPP treatments and the duration of cold-storage (days)

Treatments	Storage (day)	Yardlong beans ($\mu\text{g}/100\text{ g}$)	Green beans ($\mu\text{g}/100\text{ g}$)	Winged beans ($\mu\text{g}/100\text{ g}$)
0.1 MPa	0	^a 106 (1)	^a 63 (9)	^{ab} 67 (2)
	2	^a 114 (7)	^a 74 (9)	^{bc} 63 (1)
	4	^a 110 (7)	^a 72 (2)	^{bc} 63 (6)
	6	^a 109 (2)	^a 68 (7)	^a 72 (2)
	8	^a 100 (3)	^a 62 (2)	^{bc} 62 (2)
	10	^a 108 (6)	^a 62 (2)	^c 58 (1)
300 MPa/5 min	0	^a 118 (3)	^a 72 (9)	^a 63 (2)
	2	^{ab} 103 (11)	^{ab} 68 (1)	^{ab} 58 (2)
	4	^b 96 (9)	^{bc} 58 (2)	^{abc} 52 (9)
	6	^{bc} 87 (6)	^{cd} 52 (1)	^{bcd} 47 (1)
	8	^{cd} 76 (1)	^{cd} 45 (1)	^{cd} 44 (4)
	10	^d 63 (3)	^d 40 (7)	^d 38 (2)
300 MPa/10 min	0	^a 86 (2)	^a 62 (7)	^a 69 (2)
	2	^{ab} 73 (6)	^{ab} 57 (5)	^b 54 (6)
	4	^{bc} 60 (7)	^{bc} 48 (1)	^b 50 (6)
	6	^{cd} 51 (10)	^{cd} 42 (3)	^{bc} 46 (4)
	8	^{de} 43 (3)	^{de} 35 (2)	^c 37 (2)
	10	^e 35 (2)	^e 28 (1)	^d 25 (1)
450 MPa/5 min	0	^a 127 (2)	^a 72 (10)	^a 71 (7)
	2	^b 109 (4)	^{ab} 65 (10)	^b 53 (2)
	4	^c 92 (4)	^{abc} 58 (7)	^{bc} 45 (5)
	6	^c 81 (8)	^{bcd} 49 (2)	^{cd} 38 (1)
	8	^d 66 (7)	^{cd} 41 (1)	^{de} 30 (1)
	10	^e 51 (2)	^d 35 (1)	^e 22 (3)
450 MPa/10 min	0	^a 78 (3)	^a 58 (4)	^a 73 (2)
	2	^b 62 (3)	^{ab} 49 (4)	^b 59 (2)
	4	^c 49 (5)	^{bc} 41 (6)	^c 41 (4)
	6	^d 31 (9)	^{cd} 35 (1)	^d 30 (6)
	8	^e 17 (3)	^{de} 28 (2)	^{de} 25 (3)
	10	^e 7 (2)	^e 19 (2)	^e 20 (1)
600 MPa/5 min	0	^a 107 (5)	^a 65 (2)	^a 69 (2)
	2	^b 89 (4)	^b 53 (1)	^b 52 (2)
	4	^b 75 (5)	^b 48 (3)	^c 45 (2)
	6	^c 57 (6)	^c 38 (2)	^d 38 (2)
	8	^{cd} 41 (10)	^d 30 (1)	^e 29 (2)
	10	^d 32 (3)	^d 25 (5)	^f 16 (4)
600 MPa/10 min	0	^a 69 (1)	^a 52 (6)	^a 63 (3)
	2	^b 47 (2)	^{ab} 45 (2)	^b 3 (2)
	4	^c 22 (5)	^b 40 (1)	^c 34 (2)
	6	^d 12 (1)	^c 28 (2)	^d 20 (2)
	8	^e 1 (6)	^c 21 (1)	^e 10 (2)
	10	^e 0 (4)	^d 11 (3)	^f 2 (4)

Values are the mean (SD) of nine replicates. Significant difference of each treatment was determined by ANOVA with Tukey's *post hoc* test ($p < 0.05$) for yardlong beans, green beans and winged beans, respectively. Values without sharing a same letter indicate that the treatment resulted in a significant difference on the concentration of total folate

Table 4 Equation of regression lines and regression coefficients of total folate during cold storage after different HPP treatments

Treatments	Yardlong beans	R ²	Green beans	R ²	Winged beans	R ²
300 MPa-5 min	$Y = -5.2X + 116.6$	0.9905	$Y = -3.4X + 72.6$	0.9901	$Y = -2.6X + 62.6$	0.9936
300 MPa-10 min	$Y = -5.1X + 83.2$	0.9858	$Y = -3.5X + 62.6$	0.9968	$Y = -3.9X + 66.5$	0.9588
450 MPa-5 min	$Y = -7.4X + 124.8$	0.9958	$Y = -3.8X + 72.3$	0.9975	$Y = -4.6X + 66.1$	0.9666
450 MPa-10 min	$Y = -7.3X + 77.0$	0.9950	$Y = -3.8X + 57.2$	0.9963	$Y = -5.4X + 68.3$	0.9427
600 MPa-5 min	$Y = -7.7X + 105.2$	0.9929	$Y = -4.0X + 63.1$	0.9868	$Y = -4.9X + 65.9$	0.9786
600 MPa-10 min	$Y = -7.0X + 60.4$	0.9140	$Y = -4.1X + 53.5$	0.9887	$Y = -6.0X + 58.5$	0.9799

X: storage time (0, 2, 4, 6, 8 and 10 days), Y: total folate content (μg/100 g)

monoglutamyl folate species had less affinity for FBP, they might also be more vulnerable to degradation in HPP-processed vegetables (Blancquaert et al. 2014). These could explain why the rate constant of degradation is positively correlated with the proportion of monoglutamyl folate.

Conclusion

High pressure processing of intact green beans, winged beans and yardlong beans led to the deglutamylation of polyglutamyl folate to monoglutamyl folate. The degree of deglutamylation was increased with enhancing processing pressure and extending holding time. Under extreme processing condition in this study (600 MPa/10 min), the monoglutamyl folate was increased by 9.6, 27 and 4.5 fold for green beans, yardlong beans and winged beans. High pressure processing at 600 MPa/10 min caused a significant loss of total folate. Uniquely 300 MPa/5 min and 450 MPa/5 min could significantly release more folate from yardlong beans and green beans matrix. During the following cold storage after HPP, the deglutamylation of polyglutamyl folate to monoglutamyl folate kept progressing. For those samples under mild or moderate pressure and short processing time (300 MPa/5 min, 300 MPa/10 min, 450 MPa/5 min), partial deglutamylation was induced upon 10 days storage. Under higher pressure and longer processing time (450 MPa/10 min, 600 MPa/5 min and 600 MPa/10 min), only monoglutamyl folate existed after 10 days storage. For those untreated beans, deglutamylation and total folate loss was not significantly observed or minor changed during cold storage. For those HPP treatment samples, the loss of total folate in three beans followed the first order kinetics over the storage time. The rate constant of degradation was positively proportional to increasing of pressure and extending of holding time and was positively correlated with the proportion of monoglutamyl folate in the beans. This research provided a reference for understanding the deglutamylation

of polyglutamyl folate and folate loss during HPP treatment and further shelf life. Further study needs to elucidate possible mechanisms to understand the significant loss during storage and find a way to prevent the loss.

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Auhtor contribution Shuangyan Luo and Hanying Duan contribute equally to this paper.

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