

# DETERMINATION OF FOLATE RETENTION DURING TOFU PREPARATION USING TRIENZYME TREATMENT AND MICROBIOLOGICAL ASSAY

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## ABSTRACT

Megaloblastic anaemia, neural tube defects, coronary heart disease, and some forms of cancer have been linked to an inadequate dietary intake of folate. Legumes are good sources of folate, however, processing may affect folate availability in legume foods. This study investigated the folate content and retention during the preparation of tofu through the trienzyme treatment (protease,  $\alpha$ -amylase, and human plasma) and microbiological assay using *Lactobacillus casei*. The results showed that the total folate content was considerably reduced during tofu preparation, from 273.4  $\mu\text{g}$  100 g<sup>-1</sup> dwb in soybean seed to 108.4  $\mu\text{g}$  100 g<sup>-1</sup> dwb in tofu, yielding a folate retention of 40%. Folate losses were mainly due to leaching into the water during soaking and pressing as well as heat destruction during milling and boiling. High folate loss (51%) was particularly found in the whey. A shorter soaking time (8 hours), optimum extraction, and pressing methods were suggested with respect to minimizing folate losses during tofu preparation. This study suggests that the consumption of 100 g of deep-fried tofu may satisfy 21% of the recommended dietary intake (RDI).

[**Keywords:** Folate, soyfoods, enzyme activity, microbiological assay]

## INTRODUCTION

The interest in the study of folate, a B group vitamin has considerably increased in recent years due to the growing evidence linking folate deficiency to neural tube defects in the newborn and coronary heart disease (Bower 1996; Butterworth and Bendich 1996) as well as some forms of cancer (Christopherson 1996). Another deficiency condition is megaloblastic anaemia, which commonly occurs in pregnant women and young infants (Hawkes and Villota 1989). The prevalence was about 3-5% in developed countries, while it was much higher (22-66%) in developing countries (Rain *et al.* 1989).

Most of the folate deficiencies are related to inadequate dietary intake of folate (Hawkes and Villota 1989). This suggests that measurement of folate content in diverse foods becomes important. However, determination of naturally occurring folates is

difficult due to a large number of chemical forms, which differ in stability and bioavailability as well as their existence in relatively low concentrations. Nearly 80% of folates in foods exist as polyglutamates with the most common forms of tetrahydrofolate (H<sub>4</sub> folate), 5-methyl-H<sub>4</sub> folate and 5-formyl-H<sub>4</sub> folate (Gregory 1989).

Microbiological assay (MA) is the most widely used method for folate analysis in foods (Tamura 1998). This method includes the extraction of folate from the sample matrix, deconjugation of polyglutamates to monoglutamates and detection of the concentration through the turbidimetric bacterial growth of *Lactobacillus casei* (ATCC 7469). The frequent uses of chicken pancreas and hog kidney as folate conjugase sources have been reported (Gregory 1984; Holt *et al.* 1988; Engelhardt and Gregory 1990; Aiso and Tamura 1998; Shrestha *et al.* 2000), while only few studies have been published regarding the use of human plasma despite its advantages over other conjugase sources. Human plasma is easily available, cheaper, used in small quantity, produces monoglutamates as the end products, and contains low endogenous folate content (Lakshmaiah and Ramasastri 1975a; Goli and Vanderslice 1992), hence it was chosen as a conjugase source in this study. Furthermore, the trienzyme treatment (protease,  $\alpha$ -amylase and conjugase) was reported to be effective in achieving a maximum value for folate in particular foods that are rich in starch and protein (Tamura 1998). Martin *et al.* (1990) observed an average increase of 19% of total folate content using this procedure during extraction of 12 food items compared to the traditional method using folate conjugase alone. Similar findings were also reported by DeSouza and Eitenmiller (1990); Pfeiffer *et al.* (1997); Rader *et al.* (1998); Aiso and Tamura (1998) and Shrestha *et al.* (2000), suggesting the importance of trienzyme treatment in folate analysis.

Legumes are important sources of protein in the human diet, particularly in developing countries.

They are also rich in folate, which may range from 100 to 600  $\mu\text{g } 100 \text{ g}^{-1}$  (Witthoft *et al.* 1999). Legumes are usually processed into foods prior to consumption in which losses of folate may occur, as they are water soluble and sensitive to light, air, heat, and pH. During soaking, boiling, and cooking of legumes, losses of folate ranged from 34% to 81% (Sutrisno *et al.* 1982; Hoppner and Lampi 1993; Dang *et al.* 2000). However, information on folate retention during tofu preparation is hardly available.

Tofu is one of the soybean products, which is commonly consumed in Asian countries, such as China, Japan, and Indonesia. About 40% of soybean available for consumption in Indonesia was used for tofu preparation (Silitonga and Djanuardi 1996). In addition, about 19% and 45% of the households consumed tofu every day and 2-5 times per week, respectively (ICBS 1999), reflecting the important role of tofu in the Indonesian diet. This study aimed to determine folate retention during tofu preparation through trienzyme treatment and MA as well as to estimate the potential of tofu as a dietary source of folate.

## MATERIALS AND METHODS

Tofu was prepared from a domestic variety of soybean (*Glycine max* (L.) Merr. var. Wilis) according to the method presented in Fig. 1. Prior to analysis, samples taken from each step of tofu preparation were freeze-dried and stored at  $-20^{\circ}\text{C}$ , while samples of soak water and whey were directly stored at  $-20^{\circ}\text{C}$ . The preparation of tofu was performed in the Indonesian Legume and Tuber Crops Research Institute (ILETRI) Malang, Indonesia, while folate analysis was done in the Department of Food Science and Technology, UNSW, Sydney during the year of 2000-2001.

### Sample Extraction

Sample extraction was performed under protection from direct daylight and followed the procedure introduced by Ruggeri *et al.* (1999) with a minor modification to the trienzyme treatment. This included the sequence of the enzymes added and the use of human plasma as a conjugase source instead of hog kidney. About 5 g of ground samples or 15 g of soak water were suspended in 40 mL 0.05 M CHES-HEPES buffer pH 7.85, containing 2% (w/v) sodium ascorbate and 0.01 M 2-mercaptoethanol, flushed with nitrogen, covered/capped and mixed well using a

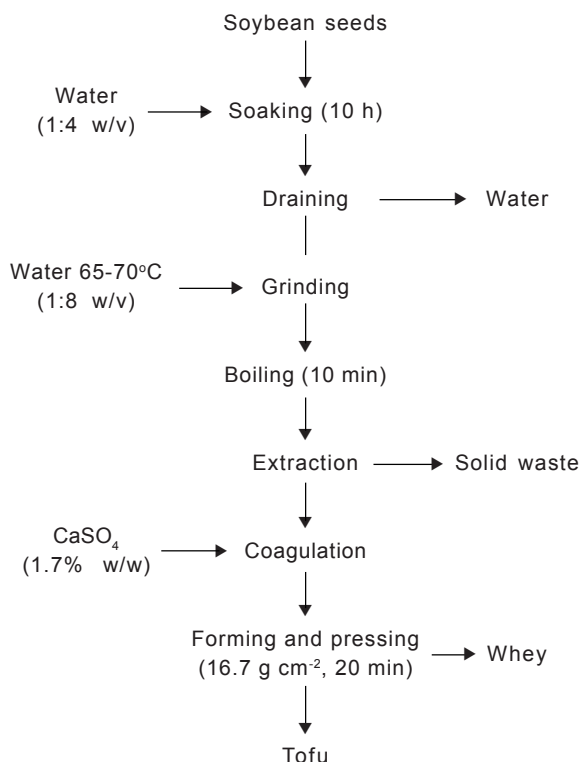


Fig. 1. Flow chart for tofu preparation (Indrasari and Damardjati 1991; Utomo and Ginting 1999).

vortex stirrer. They were heated for 10 minutes in a boiling water bath and swirled occasionally, which then cooled immediately in an ice bath. A volume of 2 mL protease (2 mg mL<sup>-1</sup>) was added and incubated in a shaking water bath at 37°C for 2 hours. The enzyme was deactivated by heating the samples for 3 minutes in a boiling water bath and cooled on ice. The pH was adjusted to 6.0 with 4 M HCl, followed by addition of 5 mL of crude human plasma conjugase and 1 mL of  $\alpha$ -amylase (20 mg mL<sup>-1</sup>). The samples were incubated for 4 hours in a shaking water bath at 37°C. Finally, the samples were heated again in a boiling water bath for 5 minutes, cooled on ice and centrifuged for 15 minutes at 10,000 rpm, 4°C. The residue was suspended in 10 mL extraction buffer and recentrifuged for 15 minutes. The supernatants were combined, made up to exact volume (50 mL) with the extraction buffer and filtered with a Minisart CE syringe filter (0.45  $\mu\text{m}$  pore size; 25 mm I.D.; No. 17598, Sartorius). The filtrates were flushed with nitrogen and stored at  $-20^{\circ}\text{C}$  until analysis. All samples were analyzed in triplicate. An enzyme blank (without sample) was also included to examine folate content in the enzymes used. This value was subtracted from the folate content in the samples to give actual folate concentrations.

### Folate Analysis

Microbial assay was used to determine the total folate content. The sample filtrates were diluted with dilution buffer (0.1M phosphate buffer, 1% ascorbic acid, pH 6.1) prior to performing the MA. The dilution factor for each sample depended upon the estimated folate content in the sample to fall within the concentration range of folic acid standards (0.2-1.0 ng mL<sup>-1</sup>). The culture of *L. casei* ATCC 7469 was obtained from The School of Microbiology and Immunology, UNSW, Sydney, Australia and the preparation of the glycerol-cryoprotected culture of *L. casei* was done according to Wilson and Horne (1982). The use of folic acid (F-7876, Sigma Chemical Co., St. Louis, MO) as a standard, the preparation of standard solutions and sample tubes as well as incubation (37°C, 16-18 hours), and the absorbance measurement using a spectrophotometer (540 nm) were performed following the procedure introduced by Keagy (1985) and Shrestha *et al.* (2000).

### Quality Control

Recovery studies were evaluated by adding or spiking a known concentration of folic acid standard solution to one selected sample in every batch of the assay and calculated using the formula given by Rader *et al.* (1998).

## RESULTS AND DISCUSSION

### Initial Total Folate Content in Soybean Seed

The total folate content obtained in soybean seed (273.4 µg 100 g<sup>-1</sup> dwb) varied widely with other studies, which ranged from 100 µg 100 g<sup>-1</sup> (Lakshmaiah and Ramasastri 1975b), 168 µg 100 g<sup>-1</sup> (Sutrisno *et al.* 1982) to 250-320 µg 100 g<sup>-1</sup> (Shin *et al.* 1975). These disparities may be due to differences in soybean varieties, growing condition, and postharvest handling as well as analytical methods (extraction procedure, antioxidant used, conjugase sources, deconjugation procedure and pH of the assay) as also stated by Keagy (1985) and Tamura (1998). In particular, most of above studies used conjugase as a single enzyme, while trienzyme treatment was performed in this study. Using human plasma alone, Lakshmaiah and Ramasastri (1975b) reported much lower amount of folate in soybean (100 µg 100 g<sup>-1</sup>) relative to the value obtained in this study, suggesting the advantage of trienzyme treatment over the single enzyme.

However, Wong (2000) noted a higher value of total folate content in soybean (400-450 µg 100 g<sup>-1</sup> dwb) when trienzyme treatment was used. A different variety of soybean and source of conjugase (chicken pancreas) as well as a longer deconjugation time (16 hours) employed in the latter study may be attributed to such differences.

### Folate Retention during Tofu Preparation

Currently, no specific study has reported folate retention during tofu preparation using MA. Wong (2000) performed a similar study on soymilk preparation, however, the processing steps were slightly different as no coagulation process was done. During the preparation of tofu, differences in total folate content were noted, which were followed by different levels of folate retention (Table 1). Total folate content in soaked seed (10 hours) was substantially lower (81%) than the initial total folate content in soybean seed. According to Hoppner and Lampi (1993) and Dang *et al.* (2000), leaching into the soak water was the major cause of folate loss during soaking. This was supported by the loss of folate in the soak water of approximately 10%, based on the amount of water added. Similar folate retention (79%) was also reported in overnight soaked soybean with 15% of folate loss in the soak water (Wong 2000). This showed that amounts of water added as well as soaking time were attributed to folate losses during soaking. In fact, the ratio of soybean to water used in this study was sufficient for soaking purposes (Indrasari and Damardjati 1991). In addition, soaking of 8-12 hours is commonly performed during tofu preparation (Shurtleff and Aoyagi 1979) and 10 hours

**Table 1. Folate content, retention and losses in samples collected during tofu preparation.**

Sample	Total folate (µg 100 g <sup>-1</sup> dwb) <sup>1)</sup>	Folate retention (%)
Soybean seed		
var. Wilis	273.4 + 14.9	100
Soaked seed	220.4 + 19.3	81
Milled seed	135.5 + 16.8	50
Boiled slurry	106.6 + 6.4	39
Soymilk	214.1 + 25.7	78
Tofu	108.4 + 11.7	40
Water sample	Total folate (µg 100 g <sup>-1</sup> ww) <sup>1)</sup>	Folate loss (%)
Soak water	4.5 + 0.5	10
Whey	31.9 + 2.6	51

<sup>1)</sup>Values are means ± SD of triplicate analysis; dwb = dry weight basis; ww = wet weight basis

was used in this study. Therefore, a shorter soaking time (8 hours) can be suggested with respect to reducing folate losses during soaking.

Milling of the soaked seed in a large amount of hot water (1:8 w/v; 65-70°C) considerably decreased the amount of folate in the slurry (Table 1), yielding a folate retention as low as 50%. The use of this amount of water was recommended as it would give optimum yield recovery as well as good texture of tofu (Watanabe 1962). Additionally, milling can be done using either hot or cool water. In this study, hot water was used as it may remove the beany flavor of soybean (Schroder and Johnson 1972). Nevertheless, this treatment may also cause loss of folate as it is sensitive to heat.

During boiling of the slurry (10 minutes), folate retention slightly decreased to as low as 39%. This may be due to quick heating, causing only a small destruction of folate. A considerable increase of total folate content, which was equal to 78% folate retention was observed in soymilk (Table 1). Removing the solid part of the slurry (*okara*) would clearly result in a lower weight of soymilk than that of the slurry, thus increasing the amount of folate in soymilk by at least two-fold. In fact, loss of folate may occur along with removal of the *okara*, reflecting that not all folate can be extracted into the soymilk. This is possible since the extraction process was performed manually, suggesting that small amounts of water/soymilk may be retained in the *okara* mass. Hence, optimum extraction methods, such as using a hydraulic or mechanical press is essential to obtain maximum amounts of soymilk.

No comparable data on folate content in soymilk produced during tofu preparation is available. Wong (2000) stated that total folate found in ultra high temperature (UHT) treated soymilk was about 276  $\mu\text{g } 100 \text{ g}^{-1}$  dwb, while Souci *et al.* (1994) reported much lower total folate content in soymilk (9  $\mu\text{g } 100 \text{ g}^{-1}$  dwb). Different processing conditions used to produce soymilk may contribute to such differences in folate content. Additionally, in the present study as well as described by Wong (2000), trienzyme treatment was used for folate extraction. Therefore, it is understandable if the results obtained from these studies were much higher compared to that of Souci *et al.* (1994) who used single enzyme (conjugase) for the assay.

The total folate content in tofu was significantly lower than that in soymilk, with the folate retention decreasing from 78% to 40%. However, folate loss into the whey, a liquid released after pressing the tofu mass, was estimated at about 51%. This sug-

gested that more folate was found in the whey rather than in the tofu itself. Precipitation of protein may lead to the release of folate into the whey as folate is soluble in water and naturally bound to protein. Total folate found in tofu was about 108.4  $\mu\text{g } 100 \text{ g}^{-1}$  dwb. Souci *et al.* (1994) reported that total folate in tofu was 15  $\mu\text{g } 100 \text{ g}^{-1}$  at 84.6% moisture content or about 97.4  $\mu\text{g } 100 \text{ g}^{-1}$  if converted to dry weight basis, slightly lower relative to the value obtained in this study. It was expected that total folate content found in the present study would be much higher than that of Souci *et al.* (1994) as the trienzyme treatment was performed, while single enzyme was used in their study.

Differences in processing steps, particularly pressing process would significantly influence the final total folate in tofu. Pressing would dictate the firmness of tofu as well as amounts of the whey released. Pressing with a pressure of 5-15  $\text{g cm}^{-2}$  for 10-15 minutes is commonly used for preparing soft tofu, while a pressure of 20-100  $\text{g cm}^{-2}$  is used for firm tofu (Shurtleff and Aoyagi 1979). Additionally, the longer time needed for pressing, the firmer tofu obtained as well as the more whey released. In this study, moderate firm tofu was prepared using a pressure of 16.7  $\text{kg cm}^{-2}$  for 20 minutes. This type of tofu is mostly preferred in Indonesia, while soft or silken tofu is popular in Japan and China. In the preparation of silken tofu with a little pressure and quick pressing (Kusbiantoro 1993) or without pressing (Antarlina *et al.* 2002), more folate can be expected in the tofu mass. Therefore, pressing should be performed optimally, depending upon the types of tofu prepared with respect to minimizing folate loss in the whey.

### Quality Control

On average, the recovery of added folic acid was 92 + 8% ( $n = 3$ ), suggesting that the assay used in this study, including the test microorganism was effective and accurate for folate analysis. According to the AOAC Peer-Verified Methods Program in Konings (1999), the recovery of 80-110% was determined to be acceptable.

### Potential of Tofu as a Dietary Source of Folate

Based on the amount of folate found in tofu (108.4  $\mu\text{g } 100 \text{ g}^{-1}$  dwb), it is grouped as a rich source of folate (Department of Health of Scottish Office in Witthoft *et al.* 1999). For estimation of folate availability in tofu for consumption, a deep-fried tofu is chosen as a



representative food derived from tofu. Using similar folate retention (79%) for deep-fried *tempe* (Wong 2000), it was estimated that total folate in deep-fried tofu was about 42  $\mu\text{g}$  100  $\text{g}^{-1}$  (at 51% moisture content). Hence, the consumption of 100 g of deep-fried tofu per serving would satisfy 21% of the recommended dietary intake (RDI) at a level of 200  $\mu\text{g}$  per day for normal adults (NHRMC 1991). It is reported that 19% and 45% of Indonesian households consumed tofu everyday and 2-5 times a week, respectively (ICBS 1999), reflecting a considerable contribution of tofu to folate intake. Currently, no systematic study on the occurrence of NTDs has been reported in Indonesia due to folate deficiency. However, megaloblastic anaemia may be associated with iron deficiency anaemia (Muhilal *et al.* 1996), hence increased consumption of foods that are rich in folate, such as tofu, would help to reduce folate deficiency.

## CONCLUSION

Significant reduction in the folate content and retention were observed during tofu preparation. Losses of folate were noted during soaking, milling, boiling, and pressing. High folate loss (51%) was particularly found in the whey. In terms of reducing folate losses during tofu preparation, a shorter soaking time (8 hours), optimum extraction and pressing methods are suggested. The trienzyme treatment employed in this study gave higher value of total folate in soybean seed compared to other studies, which only used single enzyme, reflecting the essential use of trienzyme treatment in folate analysis. Total folate content in tofu was 108.4  $\mu\text{g}$  100  $\text{g}^{-1}$  dwb, suggesting that tofu is a rich source of dietary folate.

## ACKNOWLEDGEMENTS

The authors thanked Suprpto, Kus Setiarjo, Elok, Yuni (Indonesian Legume and Tuber Crops Research Institute, Malang, Indonesia) as well as Ashok, Eileen and Camillo (Department of Food Science and Technology, UNSW, Sydney) for their technical assistance during sample preparation and folate analysis.

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