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Analysis and characterisation of amino acid contents of *thua nao*, a traditionally fermented soybean food of Northern Thailand

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Abstract: *Thua nao*, a Thai traditional soy-fermented condiment, is extensively consumed in the northern part of Thailand. In this study, an analysis of free amino acid (FAA) profiles was determined using reversed-phase high performance liquid chromatography. For this, *thua nao* samples conventionally produced were collected from six local markets (Mae Wang, Mae Hia, Mae Taeng, Jom Thong, San Patong and San Sai) in Chiang Mai, Thailand and used for the analysis. Results indicate a variation in amino acid compositions and contents among these thua nao products. The quantities of total FAA were found between 11.03 - 61.23 g/kg, as dry basis. Predominant amino acids were Trp, followed by Glu, Cys, Lys and Leu. All essential amino acids (EAA) were present in considerable amounts in which the proportions of EAA_7 and EAA_{10} values were 2.67 - 13.99 and 7.24 - 36.54 g/kg dry basis, respectively. The most abundant taste FAA class was the bitter FAA representing more than 50% of total FAA. This is the first paper to report *thua nao* amino acid profiles with an expectation to further shed light on its nutritive qualities.

Keywords: *Thua nao*, amino acid, fermented soybean, nutritive value

Introduction

Thua nao is a Thai traditional fermented soybean which is popular and widely consumed in the northern part of Thailand. It is protein-rich and thus has long been used as a meat substitute. Traditionally, *thua nao* has been prepared by naturally fermenting the boiled soybeans in a bamboo basket, covered with banana leaves for 2 - 3 days at ambient temperature. After fermentation, raw *thua nao* must be cooked by steaming or roasting in which spices and other ingredients may be added during cooking. Alternatively, raw *thua nao* is crushed and formed into a disc shape, exposed to sunlight resulting in a dried product, the so-called "thua nao *kab*". Other traditionally fermented soybeans similar to thua nao have also been found in Asian countries such as Japanese natto, Indian kinema, Chinese douche, and Korean chungkukjang (Kiuchi and Watanabe, 2004). These products are distinct due to difference in soybean variety, starter culture and fermentation condition. Although mixed cultures of microbes are present, it has been well documented that bacteria in the genus *Bacillus* are responsible for the fermentation of these fermented soybean products. For example, several *Bacillus* species including *B. subtilis, B. pumilus, B. brevis, B. macerans, B. polymyxa*, and *B. licheniformis* can be isolated from *daddawa*, *kinema*, *thua nao* and *chungkukjang* (Sarkar and Tamang, 1995; Omafuvbe *et al.*, 2000; Chantawannakul *et al.*, 2002; Lee *et al.*, 2005; Chukeatirote *et al*., 2006).

It is generally accepted that food composition data are useful and needed to specify association between food and nutritional status, to design regulatory standards, and to help improve product formulation. Although these fermented soybean products are valued for their high protein content, there are only a few in which their food composition data are available. Besides, when considered from the significant role as protein source, the detailed data of amino acid profiles are scarce and restricted to natto (Nikkuni *et al*., 1995; Zarkadas *et al*., 1997), kinema (Sarkar *et al*., 1997) and douchi (Li *et al*., 2007). This criterion is important and can represent as one of the key characteristics of nutritional quality of the food product. Currently, such information is not available for *thua nao* product. This present study was therefore conducted to analyse free amino acid

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components of thua nao samples collected from local markets in Chiang Mai, Thailand.

Materials and Methods

Thua nao samples

Thua nao products used in this study were collected from six different local markets namely Mae Wang (MW), Mae Hia (MH), Mae Taeng (MT), Jom Thong (JT), San Patong (SP) and San Sai (SS), in Chiang Mai, Thailand (Figure 1). The samples once collected were transported to the laboratory in portable coolers and stored at -20°C until analysis.

Figure 1. Geographic location of *thua nao* sampling sites (Chiang Mai, Thailand). The appearance of the *thua nao* products collected was also showed. MW, Mae Wang; MH, Mae Hia; MT, Mae Taeng; JT, Jom Thong; SP, San Patong; SS, San Sai

Preparation of samples

Two grams of freeze-dried thua nao samples (LABCONCO, FREEZONE PLUS, USA) were the ground to a fine powder, defatted with distilled petroleum ether (Labscan, Dublin, Ireland) in Soxhlet extractors and stored in screw-capped plastic tubes at -20°C until required. Preparation of each sample was carried out in duplicate.

Analysis of free amino acid contents

Defatted samples (100 mg) were extracted with 25 ml of 70% (v/v) ethanol (Merck, Darmstadt, Germany) in an Ultra-Turax (T25 basic IKA-WERKE, Staufen, Germany) at approximately 6500 rpm for 10 sec at room temperature, and then centrifuged (KUBOTA 6930, Japan) at 2000g for 20 min at 4°C. The sample residue from the first extract was subjected to repeat twice more extractions. The solvent was removed from the supernatants by vacuum evaporator (Büchi Rotavapor R-200, Switzerland) at 45°C. The collected suspension was finally dissolved in 10 ml borate buffer and filtered through 0.45 µm filter (Sartorius GmbH, Gottingen, Germany) prior to HPLC analysis.

Free amino acids (FAA) were determined using pre-column derivatisation with 9-fluorenylmethyl chloroformate (Fmoc-Cl) (Sigma-Aldrich Co. St. Louis, MO., USA) followed by reversed-phase high performance liquid chromatography (RP-HPLC) in accordance with the protocol of Sarkar *et al*. (1997). HPLC Shimadzu system was used with following conditions: 5µm Restek C18 column, 250 x 4.6 mm Restek C18 guard column, a column heater, an Fl000 fluorescence detector (263 nm) and emission (313 nm), and a C-R6A Chromatopac (Shimadzu, Tokyo, Japan) integrator. Derivative free amino acids were separated using a binary gradient of Eluent A (20 mM ammonium dihydrogen orthophosphate (Ajax Finechem, New Zealand) and 15% methanol (Fisher Scientific, UK) and Eluent B (90% acetonitrile (Labscan) in water). The flow rate was 1.0 ml/min and the temperature was controlled at 37°C. Amino acids were identified by comparing retention times with a standard mixture of 20 authentic amino acids. The content of each amino acid was calculated on the basis of the standard curve of each authentic amino acid standard. A high linearity of $R^2 > 0.995$ was obtained for each calibration curve.

Amino acid groupings

Amino acids were grouped as basic (Lys + His + Arg), acidic (Asp + Glu + Asn + Gln), total charged (basic + acidic), hydrophilic (total charged + Thr + Ser), hydrophobic (Val + Leu + Ile + Phe + Tyr + Trp + Met) and apolar (hydrophobic – Tyr) (Sarkar et al., 1997). Additionally, taste characteristics as described by Tseng et al. (2005) were also considered and used to categorise amino acids as monosodium glutamate-like (MSG-like) (Asp + Glu), sweet (Ala + Gly + Ser + Thr), bitter (Arg + His + Ile + Leu + Met + Phe + Trp + Val) and tasteless (Cys + Lys + Pro).

Statistical analyses

Data were expressed as means ± standard deviations of duplicate observations. Analysis of variance was also performed by Duncan's multiple range test using the SPSS Version 15.0 software (SPSS Inc., Chicago, IL, USA). The significant differences between means were defined at $P < 0.05$.

Results and Discussion

The typical HPLC chromatograms of amino acids in thua nao products (together with those from a mixture of standard amino acids) are presented in Figure 2. The chromatogram profiles of standard amino acids and those extracted from soybean products are identical and correlated well as observed by the retention time pattern. Table 1 summarises

profiles of free amino acid (FAA) in thua nao samples collected from six local markets in Chiang Mai, Thailand. Differences in amino acid contents were observed among these samples. According to Table 1, total amounts of FAA ranged from 11.03 (SS sample) to 61.23 g/kg (MT sample). The variation of these data are in agreement with previous studies described the contents of free amino acid in *kinema* (21.21 - 106.11 g/kg) (Sarkar *et al*., 1997) and *chungkukjang* (34.88 - 90.17 g/kg) (Lee *et al*., 2005). The key factors causing such variations are probably due to different types of soybeans, fermenting microbes and process. For example, the studies of Sarkar *et al*. (1997) and Lee *et al*. (2005) reported a large variation of free amino acid contents of *kinema* and *chungkukjang* mainly due to different fermenting microorganisms. The fermenting conditions (i.e., temperature and incubating time) also affected amounts of free amino acids in *kinema* (Sarkar *et al*., 1997) and *dawadawa* products (Dakwa *et al*., 2005).

In general, Trp appears to be the most abundant amino acid, followed by Glu and Cys, respectively. The content of these three amino acids was very high representing more than 39% of total residues (MW, 54.9%; MH, 39.24%; MT, 47.62%; JT, 64.24%; ST, 59.09%; SS, 48.15%). In contrast, previous studies have showed that Glu and Asp are the most two abundant amino acids in *kinema* (Sarkar *et al*., 1997) and *sufu* (Han *et al*., 2004). While the highest content of Leu, Phe, and Glu were presented in *chungkukjang* (Lee *et al*., 2005). In addition, Trp, Cys and Met are described as the major limiting amino acids in kinema (Sarkar *et al*., 1997). The contents of sulfur amino acids (Cys and Met) in soybeans are usually low (Imsande, 2003). Thus high content of Cys in these thua nao samples (except the SS sample) is unexpected; this finding also indicates nutritive value of the thua nao products due to high content of sulfur amino acids.

The profiles of essential amino acids (EAA_7 and EAA_{10}) are also presented in Table 1. Interestingly, all thua nao samples contained sufficient amounts of all EAA. Based on Lee *et al*. (1978), EAA can be classified in groups of either seven or ten amino acids. The EAA profiles of these products were in the range of 20.23 - 35.41% for EAA7 and 59.11 - 65.98% for EAA_{10}, of total FAA with the most abundant of Trp, Leu, and Lys. Compared to *kinema* and *chungkukjang*, all EAAs were also found at considerable amounts ranging between 41 and 53% (Sarkar *et al*., 1997; Lee *et al*., 2005). These data (high content of Cys and EAA) suggest that thua nao products are a good protein source and exhibit favourable amino acid profiles.

Based on amino acid structure and charge (Barrantes, 1975), amino acids can be grouped into hydrophilic, hydrophobic, and apolar classes. Profiles of amino acids are classified based on such criteria are illustrated in Table 2. According to Barrentes (1975), the amino acids can generally be classified depending on their charges; the R values are then calculated as the ratio to indicate the frequencies of occurrence of

Table 1. Compositions and quantities of free amino acid in thua nao products (g/kg dry sample). The results are showed as mean ± SD of the duplicates.

FAA	*thua nao* samples[1]					
	MW	MH	MT	JT	SP	SS
Ala	2.03±0.03^{b}	1.77±0.09^{b}	4.44±0.30^{a}	0.12±0.01^{d}	1.89±0.09^{b}	0.79±0.04^{c}
Arg	0.34±0.02^{b}	0.24±0.01^{c}	0.52±0.00^{a}	0.10±0.01^{d}	0.52±0.01^{a}	0.12±0.00^{c}
Asn	0.24±0.02^{b}	0.50±0.00^{a}	0.09±0.01^{d}	0.04±0.01^{e}	0.21±0.00^{c}	0.10±0.00^{d}
Asp	1.23±0.04^{b}	1.35±0.08^{ab}	0.27±0.01^{c}	0.22±0.01^{C}	1.53±0.22^{a}	0.26±0.00^{c}
Cys	5.09±0.25^{c}	2.79±0.09^{d}	8.62±0.07^{a}	1.71±0.10^{e}	5.63±0.21^{b}	0.10±0.00^{f}
Glu	4.75±0.04^{b}	4.26±0.39^{b}	4.87±0.18^{b}	1.13±0.07^{c}	6.11±0.47^{a}	1.29±0.02^{c}
Gly	0.66±0.02^{c}	1.07±0.09^{a}	1.06±0.03^{a}	0.15±0.00^{d}	0.93±0.05^{b}	0.25±0.02^{d}
His	1.06±0.02^{c}	1.76±0.18^{b}	3.19±0.19^{a}	0.29±0.00^{d}	1.29±0.13^{c}	0.45±0.03^{d}
OHPro	0.08±0.01^{bc}	0.08±0.02^{bc}	0.10±0.01^{a}	0.06±0.01^{c}	0.09±0.00^{ab}	0.03±0.00^{c}
Ile	1.09±0.04^{c}	1.51±0.03^{b}	1.69±0.09^{a}	0.12±0.00^{e}	1.48±0.07^{b}	0.24±0.02^{d}
Leu	3.50±0.17^{ab}	3.57±0.14^{ab}	3.70±0.19^{a}	0.27±0.00^{c}	3.26±0.10^{b}	0.52±0.02^{c}
Lys	3.43±0.06^{b}	2.46±0.09^{c}	4.40±0.14^{a}	0.35±0.01^{d}	3.29±0.19^{b}	0.50±0.04^{d}
Met	0.46±0.08^{b}	0.34±0.02^{c}	0.44±0.01^{b}	0.13±0.00^{d}	0.63±0.01^{a}	0.13±0.03^{d}
Phe	3.43±0.06^{a}	2.33±0.25^{c}	3.56±0.10^{a}	1.43±0.01^{d}	2.69±0.03^{b}	1.01±0.05^{e}
Pro	0.56±0.00^{d}	1.05±0.01^{b}	3.24±0.12^{a}	0.00±0.00^{f}	0.83±0.00^{c}	0.21±0.00^{e}
Ser	0.23±0.01^{b}	0.70±0.00^{a}	0.68±0.04^{a}	0.05±0.00^{c}	0.21±0.01^{b}	0.05±0.00^{c}
Thr	0.55±0.01^{a}	0.24±0.01^{c}	0.54±0.05^{a}	0.20±0.01^{c}	0.33±0.03^{b}	0.08±0.00^{d}
Trp	17.67±1.08^{b}	6.77±0.39^{c}	15.67±1.95^{b}	5.64±0.10^{c}	21.30±2.04^{a}	3.92±0.43^{c}
Tyr	2.19±0.01^{a}	0.42±0.00^{f}	1.67±0.06^{c}	1.01±0.05^{d}	1.95±0.01^{b}	0.71±0.01^{e}
Val	1.53±0.05^{d}	2.00±0.04^{b}	2.47±0.12^{a}	0.17±0.00^{E}	1.75±0.09^{c}	0.26±0.00^{e}
EAA7[2]	13.99±0.02^{b}	12.47±0.57^{c}	16.80±0.69^{a}	2.67±0.01^{d}	13.43±0.46b^{c}	2.75±0.12^{d}
EAA10[2]	33.05±1.10^{b}	21.23±0.00^{c}	36.19±1.07^{a}	8.71±0.11^{d}	36.54±2.64^{a}	7.24±0.28^{d}
Total	50.11±1.45^{c}	35.22±0.60^{d}	61.23±0.29^{a}	13.20±0.00^{e}	55.92±3.24^{b}	11.03±0.33^{e}

[1]MW = Mae Wang; MH = Mae Hia; MT = Mae Taeng; JT = Jom Thong; SP = San Patong; SS = San Sai.
[2]EAA, essential amino acids were calculated according to the method of Lee *et al*. (1978); EAA_7: Val+Leu+ Ile+Thr+Lys+Phe+Met; EAA_{10}:EAA_7+His+Arg+ Trp.
Data in the same row with different letters were significantly different ($P < 0.05$).

Table 2. Contents of free amino acid group based on charge criteria of thua nao products

FAA[1]	*thua nao* samples[2]					
	MW	**MH**	**MT**	**JT**	**SP**	**SS**
Basic	4.82±0.03^{bc}	4.46±0.08^{c}	8.11±0.33^{a}	0.74±0.00^{d}	5.10±0.32^{b}	1.07±0.07^{d}
Acidic	6.22±0.07^{b}	6.11±0.48^{b}	5.23±0.15^{c}	1.40±0.07^{d}	7.84±0.69^{a}	1.66±0.02^{d}
Total charged	11.04±0.05^{b}	10.56±0.39^{b}	13.35±0.48^{a}	2.14±0.07^{c}	12.95±1.01^{a}	2.72±0.05^{c}
Hydrophilic	11.82±0.04^{b}	11.50±0.40^{b}	14.56±0.57^{a}	2.40±0.07^{c}	13.48±1.03^{a}	2.86±0.05^{c}
Hydrophobic	29.88±1.10^{b}	16.95±0.08^{c}	29.20±1.39^{b}	8.76±0.04^{d}	33.06±2.29^{a}	6.80±0.36^{d}
Apolar	27.68±1.11^{b}	16.53±0.08^{c}	27.54±1.45^{b}	7.76±0.10^{d}	31.11±2.29^{a}	6.09±0.35^{d}
R_1	0.40±0.01^{c}	0.68±0.02^{a}	0.50±0.04^{b}	0.27±0.01^{d}	0.41±0.00^{c}	0.42±0.03^{c}
R_2	0.43±0.02^{c}	0.70±0.02^{a}	0.53±0.05^{b}	0.31±0.01^{d}	0.43±0.00^{c}	0.47±0.04b^{c}
R_3	0.37±0.01^{c}	0.62±0.02^{a}	0.46±0.04^{b}	0.24±0.01^{d}	0.39±0.00^{c}	0.40±0.03^{c}
R_4	0.40±0.01^{c}	0.64±0.02^{a}	0.49±0.04^{b}	0.28±0.01^{d}	0.42±0.00^{c}	0.45±0.03^{bc}

[1]Calculated according to Barrantes (1975). Ratio 1 (R1), hydrophilic/hydrophobic; ratio 2 (R2), hydrophilic/apolar; ratio 3 (R3), total charged/hydrophobic; ratio 4 (R4), total charged/apolar.
[2]MW = Mae Wang; MH = Mae Hia; MT = Mae Taeng; JT = Jom Thong; SP = San Patong; SS = San Sai.
Data in the same row with different letters were significantly different *(P < 0.05)*.

Table 3. Contents of free amino acid class based on taste characteristics of thua nao products.

FAA[1]	*thua nao* samples[2]					
	MW	**MH**	**MT**	**JT**	**SP**	**SS**
MSG-like taste	5.98±0.09^{b}	5.61±0.48^{b}	5.14±0.17^{b}	1.36±0.07^{c}	7.63±0.69^{a}	1.55±0.02^{c}
Sweet taste	3.46±0.05^{b}	3.79±0.00^{b}	6.71±0.42^{a}	0.52±0.00^{d}	3.36±0.15^{b}	1.17±0.03^{c}
Bitter taste	31.27±1.14^{b}	18.94±0.09^{c}	32.91±1.20^{ab}	9.16±0.04^{d}	34.87±2.42^{a}	7.36±0.33^{d}
Tasteless	9.07±0.18^{c}	6.31±0.19^{d}	16.27±0.32^{a}	2.06±0.10^{e}	9.75±0.02^{b}	0.81±0.05^{f}

[1]Calculated according to Tseng et al. (2005): MSG-like = Asp + Glu; sweet = Ala + Gly + Ser + Thr; bitter = Arg + His + Ile + Leu + Met + Phe + Trp + Tyr + Val; tasteless = Cys + Lys + Pro.
[2]MW = Mae Wang; MH = Mae Hia; MT = Mae Taeng; JT = Jom Thong; SP = San Patong; SS = San Sai.
Data in the same row with different letters were significantly different *(P < 0.05)*.

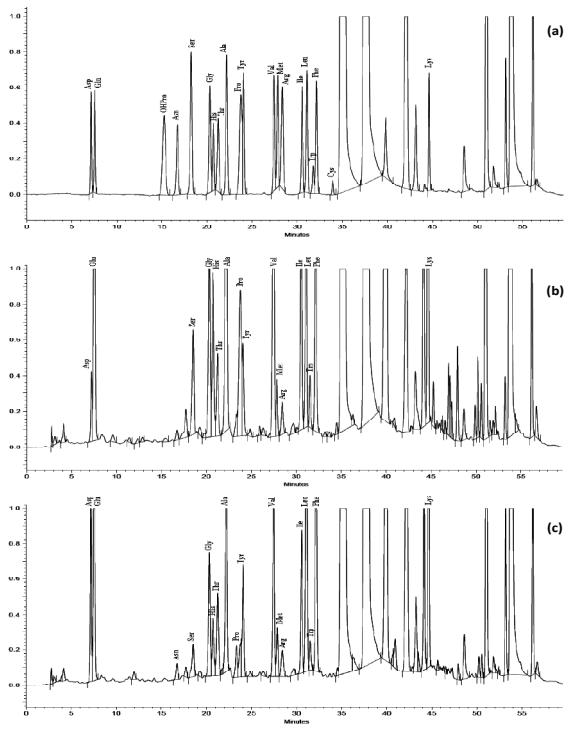

Figure 2. Reverse-phase HPLC patterns of authentic amino acids (a) and those extracted from *thua nao* products of Mae Taeng (b) and Mae Wang (c). Peaks are labelled with three-letter abbreviations for amino acids

these groups. This present study shows that there appeared to be a preferential accumulation of some certain amino acids and thus representing their abundance in a particular group. Our results clearly indicated the high content of amino acids with hydrophobic rather than hydrophilic property. In addition, the apolar amino acids were also a major group in these thua nao products. This finding is in agreement with numerous studies describing a preferential accumulation of hydrophobic amino acids possibly due to microbial fermentation (Nikkuni *et al*., 1995; Sarkar *et al*., 1997; Dakwa *et al*., 2005; Lee *et al*., 2005).

Moreover, when considered from taste characteristics, FAA can also be differentiated into four major groups: MSG-like, sweet, bitter and tasteless (Tseng *et al*., 2005). As shown in Table 3, the most abundant tasty FAA class of the *thua nao* samples was the bitter FAA representing 53.75 - 69.39% of total FAA. High concentrations of the bitter FAA class were also described in Chinese douchiba especially at ripening state (Qin and Ding, 2007). It has been proposed that hydrophobic amino acid content may cause a bitter flavour of the food product (Cho *et al*., 2004). Such a correlation (high content of hydrophobic and bitter FAA) was also observed in this study.

Conclusions

Food composition data are necessary considered from nutritionist's viewpoint. It provides valuable information of nutritive value of the food products. In addition, these data can be used as nutritional standard or recommendation on Government's health policy. This is required for the benefit of the nation and its own people. The development of the product can also be improved with an expectation that the nutritional quality would be better. *Thua nao* products in this study appear to be a good protein source based on the amino acid profiles. This study provides useful information relevant to amino acid contents of *thua*

nao. Further work on development of *thua nao* nutritive quality using pure starter culture is being undertaken and the availability of these data is thus important as standard values.

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