## Chapter 6

## Free Amino Acid Profiles of *Thua Nao* Prepared by *B. subtilis* TN51

### 6.1 Introduction

Traditional *Bacillus*-fermented soybeans are known as a good source of protein and have been produced in different countries such as India (*Kinema*), China (*Douchiba*) and Thailand (*Thua Nao*). *Bacillus subtilis* is the main fermenting bacteria in these products, and during fermentation proteolytic degradation is produced as the major activity in liberating free amino acids (FAA) to the products. This will give rise to highly nutritional fermented soy foods. Therefore, numerous researchers have attempted to verify and improve the profile and quantity of amino acids in these products. For instance, Sarkar *et al*. (1997) tried to improve the qualities of FAA in *Kinema* using various microbial strains and conditions for fermentation. After fermentation, there was a substantial FAA increase as compared to the non-fermented stage, with increased acidic, hydrophobic or apolar amino acids but depleted free basic, hydrophilic and sulfur-containing amino acids. Different profiles and quantity of FAA in fermented soy products *Kinema*, *Dawadawa* and *Chungkukjang* were studied previously with respect to variation of organism strains, as well as temperature and time of fermentation (Sarkar *et al*., 1997; Dakwa *et al*., 2005; Lee *et al*., 2005a). Soy-*Dawadawa* comprised large amount of Leu, Lys, Arg, Pro and Asp (Dakwa *et al*., 2005), while *Kinema* was found to include Glu, Phe and Leu as the major components (Sarkar *et al*., 1997).

In general, free amino acids contribute directly to the taste perception and act as a precursor of flavour enhancement (Qin and Ding, 2007; Yanfang and Wenyi, 2009). For example, increase of Gly, Glu, Asp and Arg in *Chungkukjang* could improve the sweet and savory taste characteristics (Lee *et al*., 2005a), but increase of hydrophobic amino acid groups could lead to a bitter tasting product (Cho *et al*., 2004). Qin and Ding (2007) found abundant bitter tasting free amino acids in *Douchiba* followed by MSG-like and sweet tasting classes. Besides *Bacillus*-

fermented soybean, fungal-soy fermented food was also considered as a nutritive food. Han *et al*. (2004) demonstrated Glu, Leu, Asp, Ala, Phe and Lys as the predominant FAA in Chinese *Sufu*, soybean fermented with pure starter *Actinomucor elegans*. Soy amino acids not only have high nutritive value but also provide several health benefits such as reducing blood cholesterol (Potter, 1995; Gibbs *et al*., 2004), antimutagenicity (Lee *et al*., 2005a), reducing coronary heart disease (Anderson *et al*., 1995) and having antiobesity potential (Allison *et al*., 2003; Fontaine *et al*., 2003). Some amino acids, e.g. Tyr, Met, His, Lys and Trp, are considered to act as antioxidants (Saito *et al*., 2003). In addition, Kim *et al*. (1999) reported that soy glycopeptides, comprising of Asp, Glu, Pro, Gly and Leu, displayed strong cytotoxic activity against cancer cells.

*Thua Nao* is also a basic fermented soybean, popular in the northern part of Thailand. A conventional method of *Thua Nao* production can be started with soaking soybeans over night and boiling for 3 - 4 h. Subsequently, the boiled sample is packed in bamboo baskets, covered with banana leaves, and stored at ambient temperature for 3 days. A sufficiently fermented *Thua Nao* is covered with a slimy substance and brownish in colour, and at this stage an ammonia odour could be detected (Chukeatirote *et al*., 2006). Usually naturally fermented *Thua Nao* has inconsistent quality and is susceptible to contamination with spoilage and/or pathogenic organisms. To improve this product quality and create good manufacturing circumstances, this investigation attempted to use pure starter *Bacillus* strain isolated from local products to produce *Thua Nao*, subsequently analysed for free amino acid contents in comparison with naturally fermented products and the non-fermented soybeans.



**6.2 Materials and methods**

*Bacillus subtilis* TN51 was prepared as starter culture to ferment soybean cultivar TG145 as described in Section 4.2.2 to 4.2.3. Fermented soybeans were collected after 72 h fermentation and smashed before dryness by freeze drier (LABCONCO, FREEZONE PLUS, USA). Free amino acids of lyophilized *Thua Nao* produced by starter culture of *B. subtilis* TN51 (TNB51) and naturally fermented process (TNMX) as well as their cooked non-fermented soybeans (CNF), boiled

(CNF1) and autoclaved (CNF2) soybeans, were extracted with ethanol and then analysed by reversed-phase high performance liquid chromatography (RP-HPLC) after pre-column derivertisation with 9-fluorenylmethyl chloroformate (Fmoc-Cl) as described in Section 3.2.4. Data were expressed as means ± standard deviation of triplicate. The data were also subjected to analysis of variance (ANOVA) and Duncan's multiple range tests. The significant differences between means were defined at $P \leq 0.05$.

### 6.3 Results and discussion

Free amino acids are released by hydrolysis of protein with protease enzymes produced by microorganisms during the fermentation. Sarkar *et al.* (1993) reported that *B. subtilis* gave rise to high proteolytic activity in soybean fermentation, thus markedly increased the free-amino acid contents. Furthermore, *Bacillus* strains isolated from Thai traditional *Thua Nao* also produced proteolytic activities (Visessanguan *et al.*, 2005; Chukeatirote *et al.*, 2006; Dajanta *et al.*, 2009). Figure 6.1 and Table 6.1 illustrate typical HPLC chromatograms of FAA profiles, total FAA and essential amino acids (EAA) of fermented and non-fermented soybeans.

The major FAA found in *B. subtilis* TN51 fermented *Thua Nao* (TNB51) were in the order Trp > Glu > Cys ≈ Leu > Phe ≈ His ≈ Lys ≈ Ala > Val ≈ Tyr ≈ Ile ≈ Gly, respectively ($P \leq 0.05$). Whereas, the major FAA in natural product (TNMX) were in the order Trp > Leu ≈ Glu ≈ Cys ≈ Lys ≈ Phe ≈ Tyr > Val ≈ Ala ≈ Ile, respectively ($P \leq 0.05$). These data indicated that these FAA were more susceptible to release by enzymatic hydrolysis during fermentation than by hydrolysis induced by heat for cooked or non-fermented soybeans. For cooked soybeans, the amount of total FAA presented in autoclaved soybean was around 2 times higher than that in boiled soybean, which was probably due to the more severe treatment conditions (121°C) (MacLeod and Ames, 1988). Obviously, the presence of total FAAs in cooked soybeans was relatively low (1 - 2%) when compared to those that occurred in TNB51 and TNMX which were 7 times higher amounts, equivalent to 18.57 and 33.18% respectively of their initial protein content. However, 2 times higher contents of total FAA were also observed in the TNB51 product as compared with the TNMX sample.

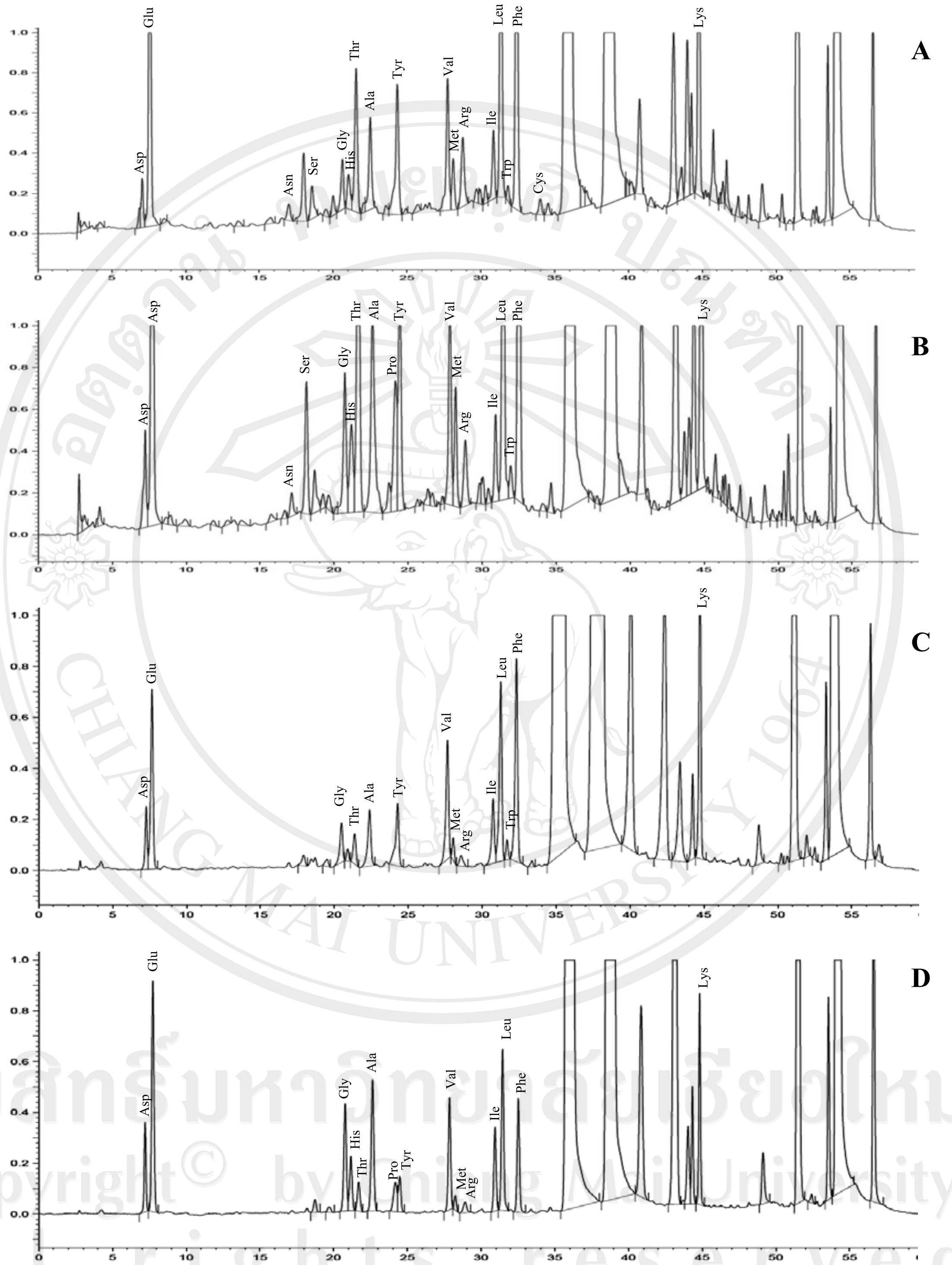


Figure 6.1 Reverse-phase HPLC patterns of free amino acids: A, boiled non-fermented soybeans (CNF1); B, autoclaved non-fermented soybeans (CNF2); C naturally fermented *Thua Nao* (TNMX) measured at concentration of 4%; D, *B. subtilis* TN51 fermented *Thua Nao* (TNB51) measured at concentration of 2%. Peaks are labeled with three letter abbreviations for amino acids.

**Table 6.1** Free amino acids in cooked non-fermented soybeans and *Thua Nao* products

| FAA | Cooked non-fermented soybean | | Fermented soybean (*Thua Nao*) | |
|---|---|---|---|---|
| | **CNF1** | **CNF2** | **TNB51** | **TNMX** |
| Ala | 0.13±0.00$^{c}$ | 0.42±0.13$^{c}$ | 7.17±0.16$^{a}$ | 1.39±0.79$^{b}$ |
| Arg | 0.21±0.05$^{b}$ | 0.16±0.01$^{c}$ | 1.40±0.16$^{a}$ | 0.25±0.31$^{b}$ |
| Asn | 0.04±0.00$^{c}$ | 0.06±0.01$^{c}$ | 0.19±0.02$^{a}$ | 0.18±0.02$^{b}$ |
| Asp | 0.09±0.02$^{c}$ | 0.16±0.01$^{c}$ | 4.90±0.26$^{a}$ | 1.07±0.26$^{b}$ |
| Cys | 2.31±0.01$^{c}$ | 1.67±0.43$^{c}$ | 10.59±0.11$^{a}$ | 4.97±0.76$^{b}$ |
| Glu | 0.65±0.03$^{c}$ | 1.82±0.43$^{c}$ | 15.86±0.80$^{a}$ | 3.84±1.24$^{b}$ |
| Gly | 0.05±0.00$^{c}$ | 0.16±0.03$^{c}$ | 4.20±0.17$^{a}$ | 0.44±0.11$^{b}$ |
| His | 0.14±0.01$^{c}$ | 0.40±0.04$^{b}$ | 7.45±0.20$^{a}$ | 0.60±0.05$^{b}$ |
| OHPro | 0.04±0.00$^{d}$ | 0.05±0.00$^{c}$ | 0.12±0.00$^{a}$ | 0.09±0.01$^{b}$ |
| Ile | 0.09±0.01$^{c}$ | 0.13±0.03$^{c}$ | 4.66±0.34$^{a}$ | 1.22±0.46$^{b}$ |
| Leu | 0.69±0.10$^{c}$ | 0.86±0.10$^{c}$ | 10.58±0.21$^{a}$ | 3.73±1.25$^{b}$ |
| Lys | 0.86±0.03$^{d}$ | 1.53±0.01$^{c}$ | 7.41±0.19$^{a}$ | 4.91±1.30$^{b}$ |
| Met | 0.13±0.01$^{d}$ | 0.28±0.01$^{c}$ | 1.24±0.05$^{a}$ | 0.67±0.36$^{b}$ |
| Phe | 0.80±0.02$^{c}$ | 1.48±0.23$^{c}$ | 7.81±0.08$^{a}$ | 3.87±0.82$^{b}$ |
| Pro | 0.05±0.00$^{b}$ | 0.47±0.07$^{b}$ | 3.86±0.66$^{a}$ | 0.72±0.03$^{b}$ |
| Ser | 0.03±0.01$^{b}$ | 0.13±0.01$^{b}$ | 0.56±0.06$^{a}$ | 0.10±0.01$^{b}$ |
| Thr | 0.17±0.00$^{d}$ | 0.47±0.05$^{b}$ | 1.43±0.02$^{a}$ | 0.38±0.05$^{c}$ |
| Trp | 3.21±0.06$^{d}$ | 8.84±1.12$^{c}$ | 51.26±2.90$^{a}$ | 42.38±5.48$^{b}$ |
| Tyr | 0.43±0.03$^{c}$ | 0.94±0.23$^{c}$ | 5.05±0.41$^{a}$ | 2.79±0.18$^{b}$ |
| Val | 0.19±0.02$^{c}$ | 0.28±0.05$^{c}$ | 5.39±0.03$^{a}$ | 2.10±0.86$^{b}$ |
| EAA$_7$ | 2.93±0.15$^{c}$ | 5.04±0.48$^{c}$ | 38.51±0.44$^{a}$ | 16.99±5.09$^{b}$ |
| EAA$_{10}$ | 6.49±0.28$^{d}$ | 14.44±1.64$^{c}$ | 98.63±2.11$^{a}$ | 60.21±10.88$^{b}$ |
| **Total FAA** | **10.28±0.34$^{d}$** | **20.32±2.97$^{c}$** | **151.12±2.62$^{a}$** | **75.79±13.89$^{b}$** |

Data are mean ± standard deviation (n = 3) and expressed in the unit of g/kg dry basis. Means within a row varying letter are significantly different ($P \leq 0.05$). CNF1, CNF2 = Cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, TNB51 = *Thua Nao* fermented by naturally occurring bacteria and *B. subtilis* TN51, respectively. FAA, free amino acids; EAA, essential amino acids were calculated according to the method of Sarkar *et al.* (1997); EAA$_7$: Val+Leu+Ile+Thr+Lys+Phe+Met; EAA$_{10}$: EAA$_7$+His+Arg+ Trp.

When comparing the total FAA found in TNB51 *Thua Nao* and in *Kinema*, which is also fermented by a pure culture starter (Sarkar *et al.*, 1997), TNB51 *Thua Nao* exhibited higher concentration (151.12 g/kg dry basis) than that in *Kinema* (106.11 g/kg dry basis). After fermentation, EAA also increased extensively (5 - 8 times) with respect to their original amounts in non-fermented soybeans (Table 6.1). Pure *Bacillus*-fermented *Thua Nao* contained EAA at significantly higher levels than present in the naturally fermented sample ($P \leq 0.05$). Similar results were reported by Sarkar *et al.* (1997), who used a pure starter culture to develop Indian *Kinema*. However, in comparison with *Kinema*, TNB51 in this study displayed $EAA_7$ lower quantity (38.51 g/kg) than those in *Kinema* (48.76 g/kg), whereas $EAA_{10}$ in TNB51 *Thua Nao* showed higher quantity (98.63 g/kg) than those in *Kinema* (53.50 g/kg) (Sarkar *et al.*, 1997) as a result of the presence of high level of His and Trp. This means that *Thua Nao* produced by pure starter *Bacillus* is a good source of essential amino acids. The amino acids indicated as essential nutrients for infant growth (FAO/WHO/UNU, 2007) such as His, Arg, Cys and Tyr were found 12, 6, 2 and 2 times higher for pure *Bacillus*-fermented *Thua Nao* than for the naturally fermented product, respectively. In addition, Fuller and Garlick (1994) stated that sulphur-containing amino acids (Met and Cys) were the most critical essential amino acids because they were easily lost from the body; thus their supplementation into food is greatly required. In this study Met and Arg were detected in both pure *Bacillus*-fermented *Thua Nao* and naturally fermented samples at relatively low amounts. However these quantities were in accord with Sarkar *et al.* (1997) and Han *et al.* (2004), who investigated essential amino acids in fermented *Kinema* (3.15 g/kg for Met and 0.22 g/kg for Arg) as well as *Sufu* (1.5 g/kg for Met and 1.2 g/kg for Arg) respectively.

Free amino acids can be grouped based on either chemical structure or taste characteristics. Sarkar *et al.* (1997) had grouped the amino acids based on their chemical structure into total charged, hydrophilic, hydrophobic and apolar. While Tseng *et al*. (2005) had classified FAA by their taste attributes into MSG-like, sweet tasting, bitter tasting and tasteless. In general, apolar amino acids that contribute hydrophobic interaction were the major group of FAA in soybeans as compared to hydrophilic FAA group as shown in Table 6.2. For TNB51 fermented soybean, acidic amino acid groups, which related to MSG-like amino acids including Asp and Glu, displayed 9 times increase from the original autoclaved sample. In naturally fermented *Thua Nao*, the corresponding increase was 6 times from the original boiled stage. Similar effects were shown for MSG-like amino acids with increasing concentration 9 and 6 times in TNB51 and TNMX, respectively, suggesting that TNB51 gave rise to significantly higher concentration of MSG-like free amino acids as compared to the TNMX sample. Relationship between acidic and MSG-like tasting amino acids also reported previously in several *Bacilli*-fermented soybeans (see Table 6.3) (Nikkuni *et al*., 1995; Sarkar *et al*., 1997; Dakwa *et al*., 2005; Lee *et al*., 2005a). The other groups of FAA such as basic amino acids (His and Arg) as well as hydrophobic amino acids (Val, Leu, Ile, Phe, Tyr, Trp, and Met) contributed to bitter taste (Tseng *et al.*, 2005). In this study, basic amino acids after fermentation increased 7 times for TNB51, compared to those in natural fermented *Thua Nao* which increased 4 times after fermentation. Furthermore, the hydrophobic group in TNB51 and TNMX also increased 6 and 9 times, respectively. The increase in both of these two groups were comparable to the increase of bitter substances in Table 6.2, indicating that alkaline-fermented soybean brings about bitter flavour which is a unique characteristic of these products. The most abundant bitter tasting amino acids which related to high contents of hydrophobic and basic amino acids also reported in commercial *Thua Nao* and other alkaline-fermented soybeans including *Natto*, *Kinema*, *Dawadawa*, and *Chungkukjang* (Table 6.3) (Nikkuni *et al*., 1995; Sarkar *et al*., 1997; Dakwa *et al*., 2005; Lee *et al*., 2005a). In previous study for organoleptical evaluation of *Douchiba*, a moderate umami taste and only a slight bitterness were detected by panelists although high content of bitter amino acid than MSG-like amino acids assisted in the fermented product. The balance and interaction between different taste components were also described by Qin and Ding (2007).

The sweet tasting amino acids in TNB51 increased 11 times while those in the TNMX samples increased 5 times from their initial non-fermented stage, which apparently related to the increase in hydrophilic FAA in both TNB51 and TNMX products. However, the quantities of sweet FAA displayed in Table 6.2 were relatively low, which hardly contributed to product attributes. Therefore, the sweet perception of this product might come from the presence of free sugars which were detected with high concentration 11.61% in autoclaved soybean and 4.32% in boiled sample (see Table 4.3). Overall, the tasting FAA of *Thua Nao* improved after fermentation process; as a consequence, pure starter culture could liberate more palatable FAA.

Table 6.2 Group of free amino acids based on chemical structure[1] and taste characteristics[2] in cooked non-fermented soybeans and *Thua Nao* products

| **Free amino acids** | **Cooked non-fermented soybean** | | **Fermented soybeans (*Thua Nao*)** | |
|---|---|---|---|---|
| | **CNF1** | **CNF2** | **TNB51** | **TNMX** |
| Basic | 1.22±0.10$^{d}$ | 2.10±0.05$^{c}$ | 16.26±0.17$^{a}$ | 5.76±1.61$^{b}$ |
| Acidic | 0.77±0.05$^{c}$ | 2.04±0.43$^{c}$ | 20.95±0.53$^{a}$ | 5.08±1.53$^{b}$ |
| Total charged | 1.99±0.15$^{d}$ | 4.14±0.47$^{c}$ | 37.20±0.36$^{a}$ | 10.84±3.14$^{b}$ |
| Hydrophilic | 2.19±0.14$^{d}$ | 4.74±0.53$^{c}$ | 39.20±0.32$^{a}$ | 11.32±3.20$^{b}$ |
| Hydrophobic | 5.53±0.21$^{d}$ | 12.81±1.77$^{c}$ | 85.98±1.85$^{a}$ | 56.87±9.03$^{b}$ |
| Apolar | 5.11±0.18$^{d}$ | 11.88±1.54$^{c}$ | 80.94±2.26$^{a}$ | 54.08±9.21$^{b}$ |
| MSG-like FAA | 0.74±0.05$^{c}$ | 1.98±0.42$^{c}$ | 20.75±0.55$^{a}$ | 4.90±1.50$^{b}$ |
| Sweet FAA | 0.37±0.01$^{c}$ | 1.09±0.08$^{c}$ | 13.36±0.37$^{a}$ | 2.31±0.96$^{b}$ |
| Bitter FAA | 5.89±0.28$^{d}$ | 13.37±1.21$^{c}$ | 94.84±1.49$^{a}$ | 57.71±9.34$^{b}$ |
| Tasteless FAA | 3.21±0.03$^{c}$ | 3.66±0.35$^{c}$ | 21.85±0.96$^{a}$ | 10.60±2.05$^{b}$ |

Data are mean ± standard deviation (n = 3) and expressed in the unit of g/kg dry basis. Means within a row varying letter are significantly different ($P \leq 0.05$). CNF1, CNF2 = Cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNMX, TNB51 = *Thua Nao* fermented by naturally occurring bacteria and *B. subtilis* TN51, respectively.

[1]Calculated according to the method of Sarkar *et al.* (1997). Basic: Lys+His+Arg; Acidic: Asp+Glu+Asn; Total charged: basic+acidic; Hydrophilic: total charged+Thr+Ser; Hydrophobic: Val+Leu+Ile+Phe+Tyr+Trp+Met and Apolar: hydrophobic-Tyr.

[2] Calculated according to the method of Tseng *et al.* (2005). MSG-like FAA: Asp+ Glu; Sweet FAA: Ala + Gly + Ser + Thr; Bitter FAA: Arg + His + Ile + Leu + Met + Phe + Trp + Tyr + Val; Tasteless: Cys + Lys + Pro.

Table 6.3 Free amino acids based on chemical structure and taste characteristics in *Thua Nao* and related products

| FAA | *Thua Nao* | | *Natto*[1] | *Kinema*[2] | *Chungkukjang*[3] | *Dawadawa*[4] |
|---|---|---|---|---|---|---|
| | **TNCM** | **TNB51** | | | | |
| Basic | 0.74 – 8.11 | 16.26 | 5.11 | 12.7 | 3.39 | 0.4 |
| Acidic | 1.40 – 7.84 | 20.95 | 4.96 | 27.31 | 5.08 | 0.27 |
| Hydrophobic | 6.8 – 33.06 | 85.98 | 19.12 | 42.61 | 20.4 | 0.76 |
| Apolar | 6.09 – 31.11 | 80.94 | 15.35 | 37.75 | 18.52 | 0.67 |
| MSG-like | 1.36 – 7.63 | 20.75 | 4.96 | 27.31 | 5.08 | 0.27 |
| Bitter | 7.36 – 34.87 | 94.84 | 17.22 | 42.22 | 20.61 | 0.89 |

Data are expressed in the unit of g/kg dry sample. [1]Nikkuni *et al*. (1995). [2]Sarkar *et al*. (1997). [3]Lee *et al*. (2005a). [4]Dakwa *et al*. (2005).

## 6.4 Conclusion

Total FAA presented in TNB51 was found at significantly higher concentration than those determined in TNMX ($P \leq 0.05$). Both fermented *Thua Nao* exhibited much higher quantity of FAA than their corresponding non-fermented samples. The amounts of EAA detected in TNB51 sample were significantly higher than those in TNMX. Typical bitter FAA of *Thua Nao* predominantly arose from hydrophobic FAA plus basic FAA. Umami FAA mainly came from acidic FAA.