Chapter 8

Enhanced Aglycone Production of *Thua Nao* Produced by *B. subtilis* TN51

8.1 Introduction

Soybeans (*Glycine max*) are highly regarded as a healthy food in several Asian countries and are widely consumed as soymilk, *Tofu* and fermented products. In these soybeans and soy-products, several phytochemicals are found and appear to be the active compounds causing many beneficial health effects (Gotoh *et al*., 1998; Potter *et al*., 1998; Park *et al*., 2003; Jung *et al*., 2006). Soy isoflavones in particular are a group of natural heterocyclic phenols comprising of aglycones, β-glucosides, acetylglucosides, and malonylglucosides. These isoflavone compounds are of great importance due to their pharmacological and antioxidant properties. For example, several researchers have shown the beneficial use of isoflavones; these include prevention of mammary cancer (Gotoh *et al*., 1998), reduced risk of cardiovascular diseases (Potter *et al*., 1998; Park *et al*., 2003), improvement of bone health and menopause symptoms (Potter *et al*., 1998; Ishimi *et al*., 2002), antimutagenic effects (Peterson *et al*., 1998; Park et *al*., 2003), and antidiabetic effects (Liu *et al*., 2006). Of these compounds, it appears that the glucoside forms are predominant in soybeans. However, it should be noted that the content and composition of isoflavones are variable depending on many parameters such as soybean variety (Wang and Murphy 1994; Lee *et al*., 2003; 2007b), geographical plantation (Wang and Murphy, 1994; Hoeck *et al*., 2000; Lee *et al*., 2003), storage time (Lee *et al*., 2003), crop year (Wang and Murphy, 1994), and food processing techniques (Jackson *et al*., 2002; Kao *et al*., 2004; Lee *et al*., 2007b).

In Asia, there are several traditional fermented soybean products such as Japanese *Natto*, Korean *Chungkukjang*, Indian *Kinema* and Thai *Thua Nao*. Interestingly, numerous studies have reported that the amount of aglycones is much higher in fermented soybeans compared to that of non-fermented soybeans; such products include *Natto* (Wei *et al*., 2008), *Miso* (Yamabe *et al*., 2007), *Sufu* (Yin *et al*., 2004; 2005), *Douchi* (Wang *et al*., 2007), and *Chungkukjang* (Kwak *et al*., 2007).

At present, the presence of aglycone isoflavones has attracted special interest due to their bioavailability, high rate of absorption in animals and humans (Izumi *et al*., 2000; Murota *et al*., 2002) and high antiproliferative activity on human cancer cells (Peterson *et al*., 1998). Especially for fermented soybeans, several studies have also revealed the role of microbes in soybean fermentation as the active agents to enrich isoflavone aglycones. This conversion of glucoside isoflavones to aglycones during the fermentation is achieved by the activity of microbial β-glucosidase enzymes (Chien *et al*., 2006; Kuo *et al*., 2006; Chun *et al*., 2007; 2008). As part of the programme to improve the nutritional value of *Thua Nao*, we previously screened and identified a strong proteolytic bacterium *Bacillus subtilis* sp. TN51 from commercial *Thua Nao* products (Dajanta *et al*., 2009) and attempted to use such a strain as a pure starter culture in soybean fermentation. This study was therefore undertaken to investigate the content and composition of isoflavone compounds in non-fermented soybean and fermented soybean prepared by *Bacillus subtilis* TN51. The content of isoflavones of *Thua Nao* prepared in a traditional manner was also determined.

8.2 Materials and methods

Bacillus subtilis TN51 was used as starter culture to ferment soybean cultivar TG145 (see Section 4.2.2 and 4.2.3). After 72 h fermentation, isoflavones of fermented soybeans were extracted using methanol and then analysed by HPLC as described in Section 3.2.5. Data were expressed as means ± standard deviation of triplicate observations. The data were also subjected to analysis of variance (ANOVA) and Duncan's multiple range tests. The significant differences between means were defined at $P \leq 0.05$.

8.3 Results and discussion

A typical HPLC chromatogram of the isoflavone standards, cooked non-fermented soybeans (CNF), and fermented soybean products are presented in Figure 8.1. Due to their different chemical structures, these isoflavone compounds were successfully separated by the HPLC system established in this study. The chromatogram of isoflavones extracted from CNF and fermented soybeans were

similar to that of the pure isoflavone standards (Figure 8.1). The reliability of the extraction method was also assessed by addition of a known concentration of glycitein standard into non-fermented soybean before extraction of isoflavones because slight content of this component has been verified in soybean. Recovery percentages for glycitein were calculated as 98% (n = 5). By this means, the correlation coefficients (R^2) of all standard curves of isoflavone standards were over 0.99 (data not shown). Besides, the HPLC chromatograms also showed several peaks of unidentified compounds (X1 – X6). Based on previous reports (Lee *et al*., 2004; Kim *et al*., 2007), these unknown compounds were possibly malonyldaidzin, malonylglycitin, malonylgenistin, acetyldaidzin, acetylglycitin, and acetylgenistin. These compounds are also isoflavone derivatives and their content appeared to be much higher in non-fermented soybean (Figure 8.1B). These glucosides-based compounds have been reported in small amounts and thus not taken into account in our study. It is also evident that these compounds are abundant in soybeans but not fermented soybeans.

The concentration and composition of isoflavones in cooked non-fermented soybeans (boiled and autoclaved) are shown in Table 8.1. The total isoflavones are expressed as the sum of glucosides (daidzin + glycitin + genistin) and aglycones (daidzein + glycitein + genistein). According to Table 8.1, the data clearly show that cooking method also affects the isoflavone contents. For non-fermented soybeans, autoclaving (CNF2) appears to promote the concentration of total isoflavones especially the glucoside forms. Boiled soybean (CNF1) showed a lower level of glucoside isoflavones probably due to heat damage (boiling in water for 4 h). This finding is in agreement with data previously described by Jackson *et al*. (2002) and Kao *et al*. (2004) where the process of soaking and heating soybeans in water led to a decrease of glucosides. Total glucosides were the largest proportion in both non-fermented soybeans samples and were accounted for 78 and 84% of the total isoflavones, respectively. This was slightly lower than the 88% proportion as reported by Wei *et al*. (2008) for autoclaved non-fermented soybean. This minor discrepancy was probably due to the difference in the variety of soybean and isoflavones extraction method (Achouri *et al*., 2005; Yamabe *et al*., 2007).

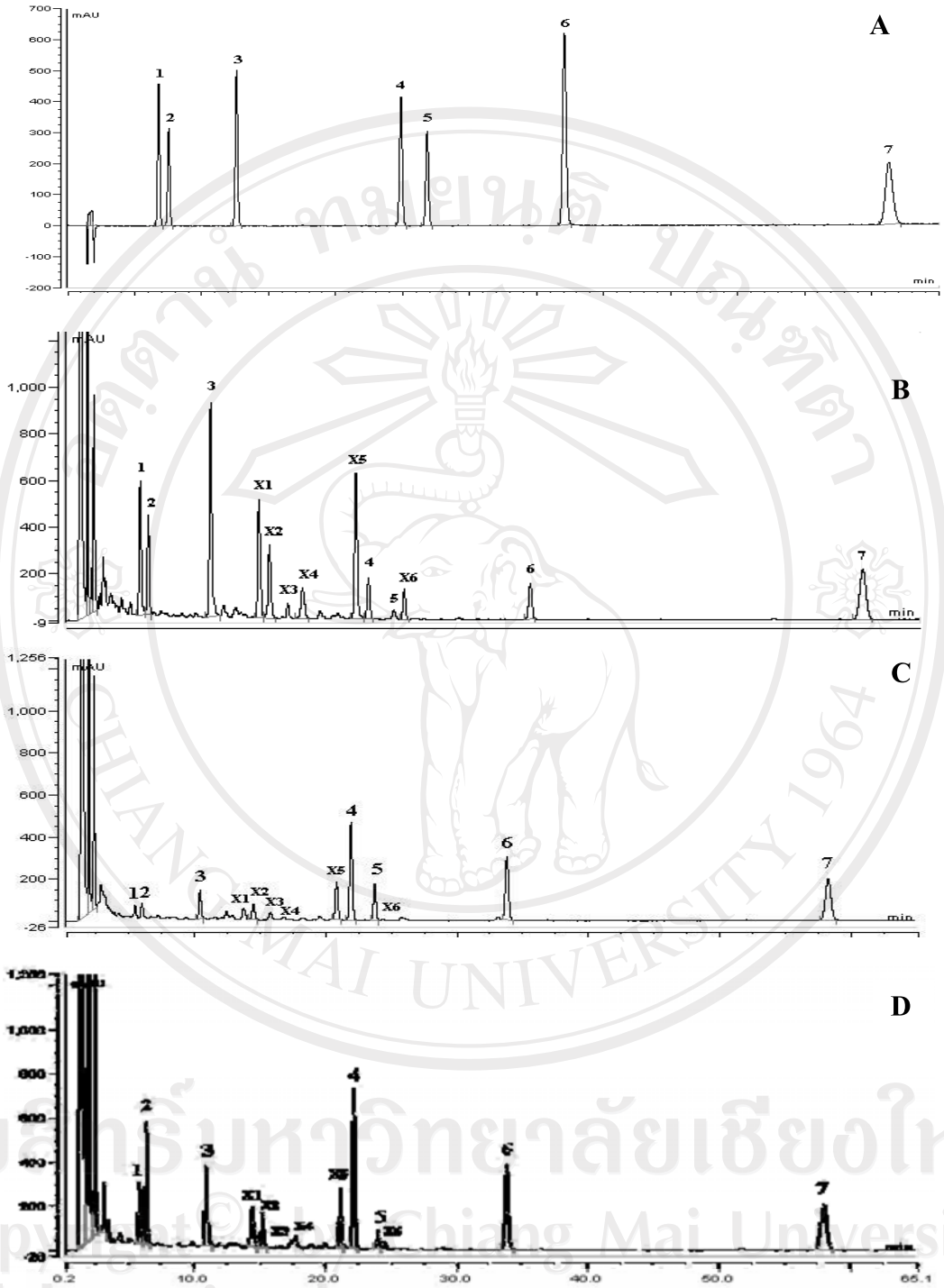

Figure 8.1 HPLC chromatograms of isoflavone compounds: A, isoflavones standard; B, autoclaved soybean (CNF2); C, naturally fermented *Thua Nao* (TNMX); D, *Thua Nao* produced by *B. subtilis* TN51 (TNB51); 1, daidzin; 2, glycitin; 3, genistin; 4, daidzein; 5, glycitein; 6, genistein; 7, flavone (internal standard). X1 - X6 were unidentified compounds, possibly malonylglucosides and acetylglucosides (Lee *et al*., 2004; Kim and Chung, 2007).

Table 8.1 Concentrations of isoflavone components in cooked non-fermented soybeans and *Thua Nao* products

Isoflavone	**Cooked non-fermented soybean**		**Fermented soybean (*Thua Nao*)**	
	CNF1	**CNF2**	**TNB51**	**TNMX**
Glucosides	255 ± 18^{b} (78)	375 ± 42^{a} (84)	343 ± 27^{a} (54)	60 ± 14^{c} (19)
Daidzin	72 ± 5^{b} (22^{B})	100 ± 19^{a} (22^{B})	68 ± 8^{b} (11^{C})	11 ± 2^{c} (3^{C})
Glycitin	68 ± 5^{c} (21^{B})	108 ± 15^{b} (24^{B})	191 ± 12^{a} (30^{A})	24 ± 6^{d} (7^{C})
Genistin	116 ± 7^{b} (35^{A})	167 ± 12^{a} (37^{A})	84 ± 8^{c} (13^{B})	26 ± 7^{d} (8^{C})
Aglycones	73 ± 6^{c} (22)	71 ± 4^{c} (16)	297 ± 14^{a} (46)	260 ± 22^{b} (81)
Daidzein	37 ± 3^{c} (11^{C})	36 ± 2^{c} (8^{C})	195 ± 9^{a} (31^{A})	135 ± 14^{b} (42^{A})
Glycitein	10 ± 1^{c} (3^{E})	12 ± 1^{c} (3^{D})	32 ± 3^{b} (5^{D})	63 ± 10^{a} (20^{B})
Genistein	25 ± 2^{c} (8^{D})	23 ± 1^{c} (5^{CD})	70 ± 4^{a} (11^{BC})	61 ± 7^{b} (19^{B})
Total Isoflavones	328 ± 24^{c} (100)	447 ± 46^{b} (100)	640 ± 31^{a} (100)	320 ± 32^{c} (100)

Data are mean ± standard deviation (n = 3) with the unit of μg/g of dry sample and number in parentheses are percentage of each isoflavone relative to total isoflavones. Means in the same row with different small letters are significantly different in type of soybean products ($P \leq 0.05$) and that in the same column with different capital letters are significantly different in each of isoflavone compounds ($P \leq 0.05$). CNF1, CNF2 = Cooked non-fermented soybeans prepared by boiling and autoclaving, respectively; TNB51, TNMX = *Thua Nao* fermented by naturally occurring bacteria and *B. subtilis* TN51, respectively.

Genistin was shown to be the most predominant in both types of cooked soybeans followed by daidzin and glycitin as amount 116 - 167, 72-100, and 68 - 108 μg/g, respectively. For the aglycones group, daidzein (36 - 37 μg/g) was found at significantly higher level than genistein (23 - 25 μg/g) and glycitein (10 - 12 μg/g) ($P \leq 0.05$).

Conversion of glucoside into aglycone isoflavones during soaking and cooking has been reported previously. Kao *et al*. (2004) demonstrated that soaking temperature and time affect the content and conformation of isoflavone compounds in soybean. After soaking soybean at 25, 35, and 45 °C for 12 h, the concentration of aglycone isoflavones (daidzein, glycitein, and genistein) clearly increased, conversely

there was a decrease of conjugated (malonylglucosides and acetylglucosides) and β-glucoside isoflavones. This suggests that malonylglucoside can be converted to acetylglucoside and further converted to glucoside or aglycone isoflavones during the soaking process. Chein *et al*. (2005) also indicated that the conformation of glucosides could be changed during moist heating, with the highest rate of conversion of malonylgenistin to genistin, followed by malonylgenistin to acetylgenistin, and acetylgenistin to genistin. In this study, soybean seeds were soaked in water at 25°C for 16 h and boiled in boiling water for 4 h or autoclaved at 121°C for 40 min; therefore, genistin predominates in all of the cooked soybeans.

Similar results were presented in this study for genistin and daidzein, which are the major glucoside and aglycone forms in non-fermented soybean (Kim and Chung, 2007) although Lee *et al*. (2007) showed higher amounts of genistein than daidzein in Ohio soybeans. A number of investigators reported that malonylgenistin is most predominant in soybeans (Lee *et al*., 2004; Kim and Chung, 2007). However, this study has not calculated the concentration of malonylglucoside and acetylglucoside isoflavones in soybeans.

Apart from physical factors, it is interesting to note that microbial fermentation also plays a key role in isoflavone content variation. In this study, fermentation of bacterial pure starter culture (TNB51) tends to promote the increase of total isoflavones (Table 8.1); 43% of total isoflavones enhanced from initial soybean. Although the similar concentration of total isoflavones in traditionally fermented soybeans (TNMX) and cooked non-fermented soybeans (CNF1) was observed, but differences in the form of isoflavones present. Besides, traditionally fermented soybeans contained higher concentration of aglycone isoflavones, including daidzein (135 μg/g), glycitein (63 μg/g), and genistein (61 μg/g), than cooked non-fermented soybeans (36 - 37, 10 - 12, and 23 - 25 μg/g, respectively). Therefore, *B. subtilis* fermented soybeans appear to be a better source of bioavailable soy isoflavones as it has been reported that aglycone isoflavones are absorbed faster and in higher amounts than their glucosides in humans (Izumi *et al*., 2000; Murota *et al*., 2002).

Despite the high content of total isoflavones present in TNB51 *Thua Nao* (2 times higher), the diverse structural conformations of the compounds were also found in these products. Glucosides appear to be abundant in *Thua Nao* fermented by *B. subtilis* TN51 (54% of total isoflavones) with the most abundant being glycitin (30%). Although the lower content of the aglycone form was present in this product, a high amount of daidzein aglycone (31%) was found, which was in similar proportion as glucoside glycitin. Previously investigators also reported a major component of glucoside isoflavone forms in *Tempeh* and *Natto* (Nakajima *et al.*, 2005; Wei *et al.*, 2008). In naturally fermented *Thua Nao*, aglycone isoflavones (81%) clearly presented the greatest amount with the highest content being of daidzein (42%).

Aglycone isoflavones in fermented soybeans (TNMX and TNB51) produced around 4 times higher levels than those in their cooked non-fermented soybeans (CNF1 and CNF2) ($P \leq 0.05$). Content of aglycone isoflavones in fermented soybeans varied with the starter culture evidence from *Thua Nao* prepared from starter *B. subtilis* TN51 showed the significant greater abundance of total aglycone isoflavones than those in natural fermented *Thua Nao*. It has been reported that the increase of aglycone occurs during soybean fermentation when using *Bacillus* strains and it has been suggested that β-glucosidase is a key enzyme for the conversion of isoflavone forms in soybean fermented foods *via* deglycosylation reaction (Figure 8.2).

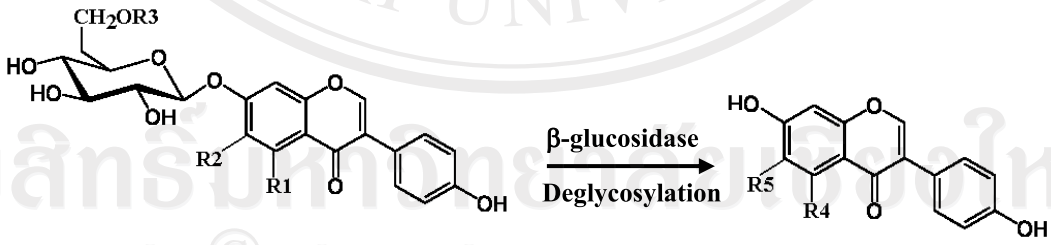

Figure 8.2 Biotransformation of glucosides to aglycones via β-glucosidase.

Wei *et al*. (2008) reported that aglycone isoflavone concentration in *B*. *subtilis* BCRC14718 fermented soybeans increased significantly. Likewise, Ibe *et al*. (2001) reported that isoflavone glucosides in soybean were hydrolysed by β-glucosidase which was produced by *B*. *subtilis* IF9916, and Kuo *et al*. (2006) indicated that daidzin and genistin glucoside were converted into aglycone isoflavone, daidzein and genistein by means of deglycosylation by β-glucosidase produced by *B*. *subtilis* NTU-18 during fermentation of black soybean. Hydrolysis of glucoside isoflavones started at 8 h after inoculating with *Bacillus* culture. Also, other isoflavone conjugates that are acetylglucoside and malonylglucoside isoflavones contained in soybean might be deglycosylated into aglycone forms; therefore, in this study decreased β-glucosides, malonylglucosides, and acetylglucosides peak areas were observed in fermented soybeans HPLC chromatograms (Figure 8.1C-D).

It is not only *B*. *subtilis* that can produce β-glucosidase during soybean fermentation; other microorganisms used for fermented soybeans such as lactic acid bacteria and bifidobacteria with soymilk (Chun *et al*., 2007; 2008), *Actinomucor elegans* with *Sufu* (Yin *et al*., 2004; 2005), *Rhizopus* with *Tempeh* (Miura *et al*., 2002), and *Aspergillus* with *Miso* (Yamabe *et al*., 2007) were reported in previous literature. Chien *et al*. (2006) suggested that β-glucosidase deglycosylation caused a significant decrease of malonylglucoside and acetylglucoside along with a significant increase of aglycone isoflavone during fermentation of soymilk with lactic acid bacteria and bifidobacteria. Malonylglucoside isoflavones are easily converted to glucosides owing to breakdown of weak bonds between sugar and malonyl group caused by heat; moreover, the effect of β-glucosidase, which hydrolyses β-glucosidic linkages of oligosaccharides and other glucosides conjugated compounds to form isoflavone aglycones.

Compared with non-fermented soybean, TNMX revealed 3.6 times increasing of total aglycones reversion of 4.2 times decreasing of glucoside, while the difference trend was found in starter fermented product with 4.2 times raising of aglycone isoflavones despite of changeable of glucoside compounds. These results confirmed in principal role of microbial fermentation in generating the high content of aglycone compounds. Interestingly, regarding individual compounds, daidzin (85%) and

glycitein (530%) were the greatest reduced and increased concentrations from their initial value in naturally fermented *Thua Nao*, while in starter fermented product a similar situation was found for genistin and daidzein, respectively.

In general, daidzein aglycone isoflavone was found as a larger proportion than genistein and glycitein in fermented soybean. Moreover, *B. subtilis* TN51 fermented soybeans contained significantly higher daidzein and genistein isoflavones than other strains ($P \leq 0.05$), while mixed natural microorganisms fermented soybean showed the highest content of glycitein. Wei *et al*. (2008) indicated that the concentration of daidzein in soybean fermented with *B. subtilis* BCRC14718 was higher than genistein throughout 48 h of fermentation time. Kuo *et al*. (2006) demonstrated faster rate of deglycosylation in daidzin (100%) than genistin (75%) by β-glucosidase which was produced from *B. subtilis* NTU-18.

Compared with other fermented soybean products, Thai soybean cultivar TG145 fermented with *B. subtilis* TN51 showed lower content of total isoflavones than *Chungkukjang* which was produced from Korean and Chinese soybean, but exhibited higher amount of aglycone forms. Furthermore, *B. subtilis* TN51 fermented soybean also produced larger amounts of all glucoside and aglycone isoflavone compounds than *Doenjang* produced from Korean and Chinese soybeans (Lee *et al*., 2007). This is probably due to the different soybean cultivar, fermentation process, strain of microbial fermentation, and isoflavone extraction method (Yin *et al*., 2004; 2005; Achouri *et al*., 2005; Yamabe *et al*., 2007; Wei *et al*., 2008).

8.4 Conclusion

This is the first study to describe the content and composition of isoflavone compounds in *Thua Nao*. The results as described in this Chapter showed that the content of total isoflavones in *Thua Nao* prepared by *B. subtilis* TN51 greatly increased (43%) compared to that of the CNF2 sample. In addition, a dramatic increase of aglycones was also observed (318%) as illustrated in Figure 8.3. Aglycones are of great interest due to their beneficial properties on human health. Further work on fermentation improvement using *B. subtilis* TN51 as starter culture would help develop a novel functional food rich in aglycone compounds.

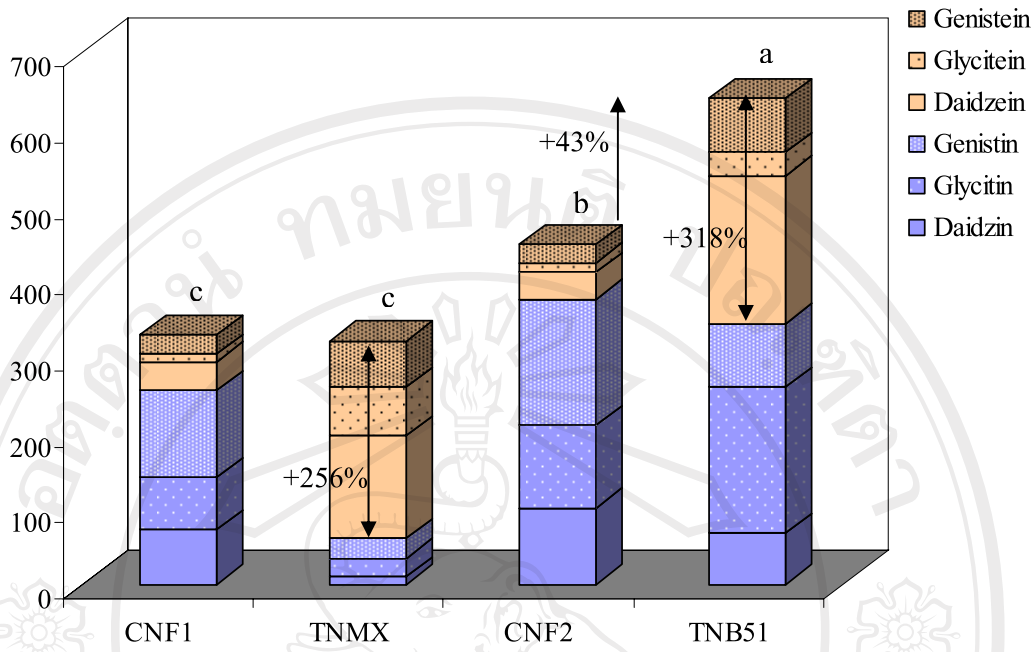

Figure 8.3 Improvement of isoflavones in *Thua Nao* after fermentation. CNF1, boiled non-fermented soybeans; TNMX, naturally fermented *Thua Nao*; CNF2, autoclaved non-fermented soybeans; TNB51, *B. subtilis* fermented *Thua Nao*. Each value represents mean (n = 3). Means (bar value) with different letters are significantly different ($P \leq 0.05$).