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Formation of thiazolidine-4-carboxylic acid (thioproline), an effective nitrite-trapping agent in human body, in *Parkia speciosa* seeds and other edible leguminous seeds in Thailand

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The amounts of thioproline, an effective nitrite-trapping agent in the human body, in various edible leguminous seeds have been determined. Thioproline in uncooked *Parkia speciosa*, the most popular seeds eaten in the south of Thailand, was undetectable but increased markedly to 0.14 ± 0.02 mmol/100 g after boiling. The formation of thioproline was effectively inhibited by the addition of *N*-ethylmaleimide, a thiol-trapping agent. The uncooked *Parkia speciosa* seeds contained substantial amounts of formaldehyde and thiol compounds and the amounts decreased after boiling. The contents of thioproline in other uncooked edible leguminous seeds, *Parkia javanica* and *Archidendron clypearia*, were 0.006 ± 0.002 and 0.31 ± 0.11 mmol/100 g, respectively and increased to 0.54 ± 0.2 and 2.95 ± 0.8 mmol/100 g after boiling. *Archidendron jirringa* and *Leucaena leucocephala*, both uncooked and boiled, showed no detectable amount of thioproline. Copyright © 1996 Elsevier Science Ltd.

INTRODUCTION

Parkia speciosa (Sator) is a tropical leguminous tree in the family of *Leguminosae* found in the northern part of Malaysia as well as in the southern part of Thailand. The seeds are in pods, approximately 30 cm long and 5 cm wide. They are considered to be of high nutritional value. The protein content is approximately 8–9% fresh weight (Suvachittanont & Pothirakit, 1988). Lectin from *Parkia speciosa* seeds has been purified and characterized (Suvachittanont & Peutpaiboon, 1992). *Parkia speciosa* seeds have been eaten as food either cooked or raw in both Malaysia and Thailand. People in southern Thailand believe that the beans have an anti-diabetic effect. Some believe that the beans have a laxative effect. There is some scientific evidence supporting these beliefs (Suvachittanont & Pothirakit, 1988). Sator beans also have distinctive aroma suggesting the presence of sulphur-containing compounds.

The thiol content in the seeds determined previously was high (Suvachittanont *et al.*, 1983). The sulphur-containing compounds in the seeds could be cysteine (cystine) and their derivatives such as glutathione, djenkolic acid and thiazolidine-4-carboxylic acid (TCA) which is often called thioproline. TCA is a cyclic sulphur-containing amino acid, which is a condensation product of formaldehyde and cysteine (Schubert, 1936; Ratner & Clarke, 1937). Endogenous formation of TCA has been considered as a detoxification pathway of formaldehyde (Debey *et al.*, 1958). The potential of TCA as a preventive or curative agent has been recognized, for example, protecting liver against various toxic agents (Peres & Dumas, 1972; Strubelt *et al.*, 1974; Siegers *et al.*, 1978). Some anti-tumour effects of TCA in patients with cancer have been clinically suggested (Brugarolas & Gosalvez, 1980). In addition, Miquel & Economos (1979) and Miquel *et al.* (1982) suggested that TCA had anti-aging effect in both Drosophila and in mice. The *N*-nitrosated derivative of TCA, *N*-nitrosothiazolidine-4-carboxylic acid (nitrosothioproline), is commonly excreted in human urine (Oshima *et al.*, 1983; Tsuda *et al.*, 1983; Tsugane *et al.*, 1992). Furthermore, TCA is an effective nitrite-trapping agent in the human body (Tsuda *et al.*, 1988*a*; Tsuda & Kurashima, 1991), thereby inhibiting the endogenous formation of carcinogenic *N*-nitroso compounds. An inhibitory effect

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of TCA on carcinogenesis by co-administration with *N*-benzyl methylamine and nitrite has also been reported (Tahira *et al.*, 1984, 1988). TCA has been found in various cooked foods such as cod fish (Tsuda *et al.*, 1988*a*), Shiitake mushroom (Kurashima *et al.*, 1990) and various kind of cooked vegetables (Tsuda *et al.*, 1988*b*). Tsuda & Kurashima (1991) have also reported that a remarkable amount of TCA is found in Djenkol beans eaten in Indonesia. This finding led us to investigate whether TCA is present in *Parkia speciosa* seeds and other edible leguminous beans which are popularly eaten in the southern part of Thailand as well as in Malaysia.

MATERIALS AND METHODS

Chemicals and samples

L-Cysteine hydrochloride and *p*-toluenesulfonyl-*N*-methyl-*N*-nitrosoamide, a reagent for diazomethane generation, and *N*-ethylmaleimide (NEM) were purchased from Tokyo Kasei Co. (Tokyo, Japan). L-Thiazolidine-4-carboxylic acid (L-thioproline) was obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Acetylacetone for the Nash test (Nash, 1953) was obtained from Wako Pure Chemical Industries (Osaka, Japan). 5,5′-Dithiobis-2-nitrobenzoic acid (DTNB) was purchased from BDH, UK. All other chemicals were standard commercial products.

Fresh seeds of *Parkia speciosa* (locally known as Sator) and other edible leguminous seeds, *Parkia javanica* (Riang), *Archidendron clypearia* (Niang Nok), *Leucaena leucocephala* (Kratin) and *Archidendron jirringa*, djenkol bean (Niang) were purchased from a local market in Hat Yai, Songkla, Thailand, and kept frozen. The weight of dried beans was determined after being air dried and weighed until constant weight was obtained. Dried weights of these beans were found to be 23, 26, 29, 44 and 52% of fresh weight for *Parkia speciosa*, *Parkia javanica*, *Leuceaena leucocephala*, *Archidendron jirringa* and *Archidendron clypearia*, respectively.

Preparation of supernatant of the uncooked and cooked beans

Five grammes (fresh weight) of each of the uncooked beans were homogenized with 50 ml of distilled water, or homogenized after boiling for 5 min with 50 ml of distilled water. The homogenates were centrifuged twice

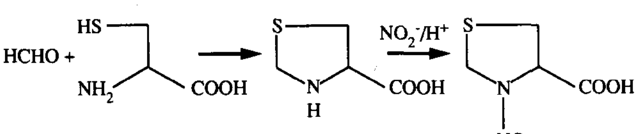

Fig. 1. Formation of thiazolidine-4-carboxylic acid (TCA) from formaldehyde and cysteine and derivatization to *N*-nitrosothiazolidine-4-carboxylic acid (NTCA).

at 3000 rpm for 10 min and the combined supernatants were used immediately for formaldehyde and thiol determination as well as for nitrite treatment.

Determination of formaldehyde and thiol content in the uncooked and cooked beans

Formaldehyde in each sample was determined based on the formation of colour compound (λ_{max} = 420 nm) with acetylacetone in ammonium acetate and acetic acid, which is a modification of the method of Nash (1953). The colour compound was extracted with isoamyl alcohol (Iwami *et al.*, 1974). Thiol contents in the supernatants of these beans were determined by the reaction of thiol group with DTNB according to Ellman's method (Ellman, 1959). Although a direct method to measure cysteine content via formation of colour compound is available (Gaitonde, 1967), the method was not applicable for samples with high formaldehyde content.

Determination of TCA in uncooked and cooked beans

For determinations of TCA content in Sator and others leguminous seeds, the supernatants were treated with excess amount of nitrite under acidic condition to convert TCA to the corresponding *N*-nitrosothiazolidine-4-carboxylic acid (NTCA, *N*-nitrosothioproline) as described by Kurashima *et al.* (1990) (Fig. 1). The NTCA obtained after nitrite treatment was extracted with ethylacetate containing 10% (v/v) methanol and was converted to methylated NTCA by treatment with diazomethane. Thus methylated NTCA was then determined by gas chromatography–thermal energy analyzer (GC-TEA) as described by Tsuda *et al.*, (1986) using the same conditions as described by Kurashima *et al.* (1990).

A GC-9A gas chromatograph (Shimadzu Corp., Kyoto, Japan) was used with argon carrier gas (40 ml/min) and was connected to a thermal energy analyszer (TEA, Model 543; Thermoelectron Corp., Waltham, Massachusetts, USA). A glass column (2.6 mm i.d. × 3.6 m), packed with 5% OV-1 on Gas Chrom Q (80–100 mesh), was programmed from 90 to 200°C at 4°C/min. The GC-chromatogram was recorded with a chromatopac CR3-A (Shimadzu). The retention times of *N*-nitrosoazetidine carboxylic acid (NAZCA) used as internal standard and of methylated NTCA were 15.3 and 21.9 min, respectively. In the case of endogenous TCA determination, addition of *N*-ethylmaleimide (NEM) to the bean sample prior to homogenization and preparation of supernatant was necessary to block TCA formation during the analysis.

Addition of *N*-ethylmaleimide

Since TCA is synthesized from cysteine and formaldehyde at room temperature (Ratner & Clarke, 1937), NEM was added when necessary to trap cysteine and to avoid artificial formation of TCA during analysis. The amount of NEM needed in trapping the thiol group in each sample (50 × thiol group) was predetermined. Thus, the endogenous TCA in the bean sample was the amount of TCA obtained from this analysis with the addition of NEM. The amounts of TCA in the uncooked and NEM added samples were practically the same as without NEM (Kurashima *et al.*, 1990). In this study a similar result was obtained using Thai leguminous beans. On the other hand, if NEM was added to the sample prior to boiling, NEM blocked the thiol group in the sample and inhibited TCA formation.

RESULTS

Amount of formaldehyde in the uncooked and cooked beans

The amount of formaldehyde in these beans varied, depending on the type of beans analyzed. Uncooked Sator seeds were found to have the highest formaldehyde content (0.77 ± 0.07 mmol/100 g) while Kratin and Thai Djenkol beans had a low level of formaldehyde (less than 0.03 ± 0.02 mmol/100 g) as shown in Table 1. The levels of formaldehyde decreased upon boiling for those with a high formaldehyde content. A similar observation has been reported for Shiitake mushroom (Kurashima *et al.*, 1990). However, for beans which contained small amounts of formaldehyde, boiling increased the formaldehyde content (Table 1). The decrease in formaldehyde content in Sator, Riang and Niang Nok after boiling could be owing to volatilization of the formaldehyde as well as the formation of TCA in these samples.

Content in the uncooked and cooked beans

Thiol contents in these beans also varied even though all of them have distinctive sulphur aroma, the thiol content was high in four samples analyzed. Boiling decreased thiol content in two specimens, Sator and Riang, but Niang Nok and Djenkol beans increased their thiol contents after boiling (Table 1). Only the amount of thiol in Kratin remained constant after boiling (Table 1). The decrease in thiol content after boiling, in Sator and Riang, may result from the formation of TCA. However, the increase in TCA in Niang Nok, was accompanied by an increase in thiol content.

Amount of TCA in uncooked and cooked beans

Uncooked Sator, Kratin, and Thai djenkol bean (Niang) did not have any detectable amount of TCA, while Riang had some TCA (5.67 ± 2.00 μmol/100 g) and Niang Nok had quite high endogenous TCA (0.31 ± 0.11 mmol/100 g). TCA present in these beans was not formed during the analytical process. The level of TCA in the sample was practically the same whether the uncooked sample was homogenized with or without NEM before the preparation of supernatant. Upon boiling the beans for 5 min, the TCA content increased significantly in three kinds of beans, Sator, Riang and Niang Nok (Table 1). The TCA formation was effectively inhibited if NEM was added to the beans before boiling.

It is interesting to note that, in beans with higher formaldehyde and thiol content, the TCA contents after boiling were generally high though the stoichiometric relationships were not so good. This is in good agreement with the fact that TCA is a condensation product of formaldehyde and cysteine. When these beans were boiled with varying amount of formaldehyde, (0.5 and 50 mmol/100 g fresh beans), TCA content in four kinds of beans, Sator, Riang, Niang Nok and Indonesian Djenkol beans, increased with increasing amount of formaldehyde whereas beans with undetectable TCA (in both uncooked and boiled Thai Djenkol beans and Kratin) still showed no TCA after boiling with the addition of formaldehyde (Table 2). These results suggested that the thiol molecules (components) in these two samples may not be in the form of cysteine.

Table 1. Amounts of formaldehyde, thiol and thiazolidine-4-carboxylic acid in leguminous beans eaten in Thailand

Leguminous beans	mmol/100 g dry beans*					
	Formaldehyde		Thiol		TCA	
	uncooked	boiled	uncooked	boiled	uncooked	boiled
Parkia speciosa (Sator)	0.77 ± 0.07	0.25 ± 0.18	4.4 ± 0.6	2.5 ± 0.16	< 0.001**	0.14 ± 0.02
Parkia javanica (Riang)	0.21 ± 0.15	0.15 ± 0.11	1.0 ± 0.01	0.6 ± 0.01	0.006 ± 0.002	0.54 ± 0.0.20
Archidendron clypearia (Niang Nok)	0.25 ± 0.13	0.13 ± 0.12	1.8 ± 0.01	5.1 ± 0.01	0.31 ± 0.11	2.95 ± 1.06
Leuceana leucocephala (Kratin)	0.03 ± 0.02	0.11 ± 0.02	1.5 ± 0.02	1.5 ± 0.01	< 0.001	< 0.001
Archidendron jirringa (Djenkol bean, Niang)						
Southern Thai beans	0.01 ± 0.01	0.03 ± 0.03	0.10 ± 0.01	0.90 ± 0.01	< 0.001	< 0.001
Indonesian beans	0.05 ± 0.01	0.21 ± 0.03	0.30 ± 0.01	0.80 ± 0.01	0.03 ± 0.01	0.44 ± 0.06

*Mean of duplicated assay.
**Detection limit.

Table 2. Effects of boling formaldehyde, on thiazolidine-4-carboxylic acid content in leguminous beans eaten in Thailand

Leguminous beans	TCA mmol/100 g dry beans*		
	Boiled alone	With HCHO 0.5 mmol/110 g	With HCHO 5 mmol/110 g
Parkia speciosa	0.14 ± 0.02	0.36 ± 0.01	0.76 ± 0.04
Parkia javanica	0.54 ± 0.20	0.78 ± 0.28	1.15 ± 0.34
Archidendron clypearia	2.95 ± 1.06	3.79 ± 0.72	4.46 ± 0.40
Leuceana leucocephala	< 0.001**	< 0.001	< 0.001
Archidendron jirringa			
Southern Thai beans	< 0.001	< 0.001	< 0.001
Indonesian beans	0.44 ± 0.06	0.51 ± 0.11	0.92 ± 0.07

*Mean of duplicated assay.
**Detection limit.

Among the southern Thai beans analyzed, Niang Nok had the highest TCA content. Riang and Sator also had a relatively high TCA, whereas Kratin and Thai Djenkol beans did not have any TCA. However, dried Djenkol beans, obtained from Indonesia had a high level of TCA, and the amount increased upon boiling (Tsuda & Kurashima, 1991).

DISCUSSION

Formaldehyde shows genotoxicity in various *in vitro* assay systems (Takahashi *et al.*, 1985). Although formaldehyde has been proved to be carcinogenic to the nasal cavity of rodents when administered by inhalation (Kerns *et al.*, 1983), there is no evidence that formaldehyde is carcinogenic when administered orally to the experimental animals. A mechanism for detoxifying formaldehyde by the formation of TCA has been suggested (Cavallini *et al.*, 1956). The level of formaldehyde in Thai food has not been previously determined. In this study, the fomaldehyde content in Thai leguminous beans was determined to find whether there was any relationship between formaldehyde and TCA content. It is interesting to note that, in beans with higher formaldehyde and thiol contents, the TCA content after boiling is generally high. This suggests that the higher thiol content in the beans also reflects high level of cysteine.

The amount of TCA in Niang Nok was very high, the beans gave a good smell (like that of Japanese Shiitake mushroom) when cooked. Although all of the five beans had some sulphur aroma, they showed varying capacities for TCA formation. Frozen Djenkol beans from southern Thailand, either cooked or raw, did not show any detectable amount of TCA. However, dried Djenkol beans obtained from Indonesia had high levels of TCA and the amount markedly increased upon boiling (Tsuda & Kurashima, 1991). The difference of TCA formation capacity between frozen Thai Djenkol beans and the dried Indonesian beans may be due to the drying process, in addition to the storage conditions which affected the available contents of cysteine and formaldehyde, the precursors of TCA. For instance, raw Shiitake mushrooms did not have any detectable TCA whereas dried mushrooms had high TCA contents (Kurashima *et al.*, 1990). This discrepancy of TCA formation between Thai and Indonesian Djenkol beans should be clarified in further investigation. Generally, TCA has been found at low levels in fish, shell fish and vegetables. The level in vegetables was less than 0.5 ppm or 0.375 μmole/100 g (Tsuda *et al.*, 1987)

High levels of TCA, an effective nitrite-trapping agent *in vivo*, present in popular edible leguminous beans, may lead to blocking of the endogenous formation of carcinogenic *N*-nitroso compounds in the human body as proposed by Tsuda *et al.* (1988*b*) and Tahira *et al.* (1988). The amount of these leguminous seeds eaten per person per day is approximately 100–200 g fresh weight. TCA from the beans would be 10–400 mg/day/person if 200 g of fresh beans were cooked and eaten. This amount of TCA consumed could possibly prevent formation of carcinogenic *N*-nitroso compounds (Tsuda & Kurashima, 1991). Recently, Srivatanakul *et al.* (1991) reported higher urinary excretion of NTCA and *N*-nitroso-2-methylthiazolidine-4-carboxylic acid (NMTCA) in the southern Thai population than the northern population, indicating a higher intake of TCA in the southern population. Stomach cancer incidence in the south (Songkla) was also lower than that in the north (Chiang Mai), i.e. 3.2 vs 10.7 for male and 1.0 vs 6.1/100 000 for female, respectively (Vatanasapt *et al.*, 1993). Stomach cancer incidence in the southern province of Thailand (Songkla), was considerably lower than the national incidence in both males (3.2 vs 5.8/100 000) and females (1.0 vs 2.9/100 000) (Vatanasapt *et al.*, 1993). It is possible that these leguminous beans, commonly eaten in the southern part of Thailand, contribute to this observation.

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