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Journal of food safety

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https://doi.org/10.1111/j.1745-4565.1993.tb00114.x

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EFFECT OF PROCESSING CONDITIONS ON THE FORMATION OF BIOGENIC AMINES AND ETHYL CARBAMATE IN SOYBEAN TEMPE

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Received for Publication May 25, 1993
Accepted for Publication August 19, 1993

ABSTRACT

Tempe is a fermented soybean product originating from Indonesia. The effects of manufacturing conditions, i.e., soaking, boiling, fermentation and home cooking by stewing or frying, and the effect of pure cultures of microorganisms commonly occurring in tempe towards their production of toxicants like biogenic amines and ethyl carbamate were investigated. The level of biogenic amines in soaked soybeans was rather low (total < 280 ppm), and not significantly affected by boiling, but considerably increased by fermentation. The functional fungus Rhizopus oligosporus *mainly produced tyramine and some putrescine (total biogenic amines approximately 1800 ppm). With added inoculation of* Klebsiella pneumoniae *and* Trichosporon beigelii, *the total amount of biogenic amines increased slightly (2000, resp. 2100 ppm) with a shift towards cadaverine. With added* Lactobacillus plantarum, *a reduction of tyramine levels resulted in a considerably lower total level of biogenic amines (approx. 1000 ppm). Storage at 5C did not affect the level of biogenic amines, whereas at 25C, increased levels of putrescine were observed. Home cooking by stewing had little effect, but frying in oil resulted in significant decreases of both*

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putrescine and tyramine. Preventive measures to keep biogenic amines at low levels in tempe are recommended. They include inoculation with selected lactic acid bacteria which cannot produce but can degrade biogenic amines, and frying instead of stewing of tempe. Ethyl carbamate levels were negligible (<11 ppb) in all treatments; this was attributed to the absence of significant concentrations of ethanol in the product.

INTRODUCTION

Biogenic amines and ethyl carbamate are toxic substances which can be generated in foods, mainly by microbial enzymatic activity. Biogenic amines of toxicological relevance include histamine, tyramine, β-phenylethylamine, tryptamine, putrescine and cadaverine (Stratton *et al.* 1991). The major pathway of formation in foods is by decarboxylation of free amino acids. Various lactic acid bacteria and Enterobacteriaceae possess the required decarboxylase activity (Stratton *et al.* 1991). No data are available on the production of decarboxylases by fungi.

Ethyl carbamate (urethane) has mutagenic and carcinogenic properties (Mirvish 1986), and can be formed from reaction with ethanol and naturally occurring carbamyl phosphate during the fermentation process. Both compounds mainly result from yeast metabolism; ethyl carbamate can also be formed by the reaction of ethanol and urea produced naturally from amino acids like arginine and citrulline (Ough 1976). The latter compound was found to accumulate in raw soy sauce and act as a major precursor for ethyl carbamate formation during the pasteurization operation (Matsudo *et al.* 1993).

Tempe is a fermented food of Indonesian origin obtained by fungal solid-substrate fermentation of soaked and cooked soybeans, legumes, cereals or other suitable raw materials (Ko and Hesseltine 1979; Nout and Rombouts 1990). The manufacturing process of soybean tempe includes soaking of the dehulled beans in water overnight, followed by boiling or steaming. After discarding the water, the beans are allowed to air-dry and cool, and accordingly inoculated with spore preparations of *Rhizopus* spp., usually *R.oligosporus*. Fermentation of the inoculated beans takes place in perforated containers.

In addition to filamentous fungi, considerable levels of bacteria and yeasts occur in tempe as a result of cross-contamination from equipment, use of impure inoculum, etc. Lactic acid bacteria, e.g., *Lactobacillus plantarum* prevail in tempe when lactic fermentation takes place during the soaking stage (Nout *et al.* 1987). Enterobacteriaceae are also common in tempe and some of them, e.g., *Klebsiella pneumoniae* have been associated with the production of vitamin B12 in tempe (Okada *et al.* 1985). Yeasts, e.g., *Trichosporon beigelii* occur in varying quantities (Samson *et al.* 1987). Due to the continuing microbial and

enzymic activity, freshly harvested tempe has a limited shelf-life and should preferably be prepared for consumption the same day or be kept refrigerated. Home preparation prior to consumption consists of cooking, e.g., by boiling in stew or by frying in oil.

The presence of varying levels of biogenic amines in cheese, sausage, wine, beer, soy sauce and miso (Stratton *et al.* 1991), and of ethyl carbamate in distilled spirits and wines, and fermented foods such as bread, soy sauce, miso and yeast spread (Diachenko *et al.* 1992) have been reported. In the absence of data concerning tempe, the present investigation was carried out to evaluate the influence of processing, and of representative microorganisms on the possible formation of biogenic amines and ethyl carbamate in tempe prepared from soybeans.

MATERIALS AND METHODS

Microorganisms

Inocula used included *Rhizopus oligosporus* (NRRL5905 = LUW575) grown on Malt Extract Agar (MEA; Oxoid CM59) at 30C for 1 week, *Klebsiella pneumoniae* (isolated from commercial tempe) grown on Plate Count Agar (Oxoid CM325) at 30C for 24 h, *Trichosporon beigelii* (isolated from commercial tempe; LUW692) grown on MEA at 30C for 3 days, and *Lactobacillus plantarum* (Pioneer RG1) grown on de Man, Rogosa & Sharpe (MRS) agar (Merck 10661 with 1.2% technical agar Oxoid L13) at 30C for 24 h. Microbiological analyses for total mesophilic aerobic bacteria, Enterobacteriacaea, yeasts and lactic acid bacteria were carried out in freshly prepared samples in duplicate, according to Nout *et al.* (1987a).

Tempe Manufacture

Tempe was manufactured from dry abrasion-dehulled yellow variety soybeans at a laboratory scale (Nout *et al.* 1987a) with different soaking conditions and inoculations with starter organisms. Soaking conditions included (1) 4C overnight to allow hydration while avoiding microbial activity; (2) 30C overnight to allow spontaneous fermentation (Nout *et al.* 1987b); and (3) inoculation with 10% v/v of fermented soaking water from a previous batch and incubation at 30C overnight to achieve accelerated natural fermentation (Nout et al. 1987b). After the usual boiling treatment, the soybeans were sterilized by dispensing them in 100 g portions in screw-capped glass jars followed by autoclave treatment at 121C for 30 min. Sterilization is not a normal part of the tempe production process, but it was incorporated to enable an assessment of the single and combined effects of added pure cultures of tempe-associated microorganisms. In all treatments, *Rhizopus oligosporus* sporangiospores were

added (10^9 cfu/Kg cooked beans) as the functional starter. Additional inoculation with *Klebsiella pneumoniae*, *Trichosporon beigelii* and *Lactobacillus plantarum* was at rather high levels (approximately 10^8 cfu/Kg cooked beans) to ensure maximum effect. In trade practice, perforated PVC lunch boxes are used to incubate the inoculated beans. As these cannot be sterilized, we incubated the inoculated beans at 30C for 44 h in the glass jars in which they had been autoclaved, and a perforated polyethylene film was used to cover the jars.

Commercial Tempe

Commercial tempe of good quality was purchased from a local retailer in Wageningen. It was used for boiling and frying on the day of purchase, and duplicate samples were stored at 25C for 1 week, and at 5C for 2 weeks in their original polyethylene wrapping. In case of boiling, cubes of 1 cm^3 were immersed in boiling water during 10 min; for frying, slices of 5 mm thickness were immersed in sunflower oil at 170C until they were golden-brown (3-5 min) and excess oil was removed using absorbent paper.

Samples for chemical analysis were prepared by grinding 100 g of composite sample from duplicate treatments, followed by frozen storage at -20C until analysis. Chemical analyses were carried out in duplicate.

Determination of Biogenic Amines

A portion of 15 g dry matter was homogenized with 50 ml 5% trichloroacetic acid at 70C in a 100 ml glass beaker using a Waring Blendor. After cooling to room temperature, the mixture was transferred quantitatively into a 100 ml volumetric flask and made up to the mark with 5% trichloroacetic acid. After mixing, a 50 ml portion was transferred into a centrifuge tube and centrifuged at 2500 g for 10 min. The supernatant was filtered through filter paper (Schleicher and Schull 595 1/2, nr.311645). A 25 ml aliquot of filtrate was transferred into a 100 ml volumetric flask and diluted to the mark with distilled water. The diluted filtrate was filtered again through a 0.45 μm pore size filter (Millipore filter, type HVLP 04700). Biogenic amines were separated by ion exchange liquid chromatography (LC) and detected with a fluorescence detector after post-column derivatization with o-phthalaldehyde adapted from Walters (1984). The detection limit was 1 mg/Kg, and data have a coefficient of variation of 10%. The LC conditions were as follows: column: stationary phase Zorbax 300 SCX strong cation exchange resin (DuPont nr. 28768); mobile phase (eluent): 70 vol. parts of 0.1 M phosphate buffer pH 6.1 and 30 vol. parts of methanol; elution rate: 1.0 ml/min. Post-column derivatization was carried out at 45C in a 900 $\times$ 0.5 mm i.d. Teflon reactor spiral. The reagent for post-column derivatization was added at 0.8 ml/min and consisted of freshly mixed solutions of A and B. Solution A: 12.5 g boric acid (Merck art.165) in

475 ml distilled water adjusted with KOH to pH 10.4. Solution B: 300 mg o-phthalaldehyde (Merck art. 11452) in 5 ml ethanol 96% (Merck art. 983) to which 100 μl 2-mercaptoethanol (Merck art. 805740) was added. Detection was carried out with a fluorescence detector (Waters type 420 with lamp 78245) with $\lambda_{excitation} = 338$ nm and $\lambda_{emission} > 420$ nm.

Detection of Ethyl Carbamate

Ten grams of sample were homogenized and extracted with methylene chloride according to the procedure for bread (Canas *et al.* 1989). Concentrated extracts were analyzed by gas liquid chromatography (GLC) according to Kesselmans *et al.* (1986), applying the following conditions: column: CP-Wax-52 CB, WCOT fused silica length 50 m, inner diameter 0.32 mm; carrier N_2 (120 kPa), make-up He (115 kPa), detector H_2 (67 kPa), air (102 kPa); injection volume 1.0 μl on-column; temperature program: 20C for 20 s, 20-87C in 40 s, 87-180C at a rate of 8C per min, 180-190C at a rate of 10C/min, 190C for 20 min. The recoveries for ethyl carbamate (EC) in spiked tempe samples varied from 45 to 50%. The detection limit of EC in extract by GLC was 10 μg/L, corresponding with 11 μg/Kg (11 ppb) in the original sample. Data have a coefficient of variation of 5.5% at 200 ppb level.

RESULTS AND DISCUSSION

Table 1 shows the effect of preparatory soaking and boiling treatments involved in the tempe manufacturing process, and the levels of cadaverine, histamine, putrescine, tryptamine and tyramine expressed as mg/Kg sample dry weight.

When soaking took place at 4C (treatment A), no bacterial fermentation occurred, as indicated by the high pH 6.5 which is close to the original pH of soybeans. At 30C (treatment B), spontaneous bacterial fermentation resulted in a modest decrease to pH 5.2, while an active acidification till pH 4.4 took place after inoculation (treatment C). During the soaking stage at 4C a noticeable accumulation of tyramine takes place. This can be explained by the action of endogenous decarboxylases on free amino acids (tyrosine) in the soybeans (Devi and Prasad 1992). When actively growing microorganisms are present (treatments B and C), some of the free amino acids are not converted into biogenic amines. In addition, the decreased pH in the beans soaked at 30C may inhibit endogenous decarboxylases, as histidine decarboxylase has an optimum pH of approximately 6.5 (Janssen 1988). Spontaneously soaked beans usually contain a mixed microbial flora in which Enterobacteriaceae predominate

whereas inoculated soaks contain mainly lactobacilli (Nout *et al.* 1987b). The levels of biogenic amines of soaked beans also differed with respect to putrescine and tyramine. However, the data do not allow conclusions to be made about the role of particular groups of microorganisms.

TABLE 1.
ACCUMULATION OF BIOGENIC AMINES (MG/KG DRY MATTER) DURING LABORATORY PREPARATION OF SOYBEAN TEMPE. EFFECT OF SOAKING AND BOILING.

Treatment code:	A	B	C
Beans soaked at:	4 C	30 C	30 C inoculated[1]
Cadaverine	<17	<17	<17
Histamine	<3	<3	<3
Putrescine	<8	67	8
Tryptamine	<3	<3	<3
Tyramine	250	75	167
After boiling (30 min., 100 C):			
Cadaverine	<25	<25	<25
Histamine	<5	<5	<5
Putrescine	<13	88	<13
Tryptamine	<5	<5	<5
Tyramine	200	113	113

1: inoculated with 10% v/v of soaking water from previous batch.

Boiling had no significant effect on the levels of biogenic amines in the variously treated beans. Likewise, autoclaving only had a slight but insignificant decreasing effect (data not shown) on the level of tyramine in beans of treatment A.

TABLE 2.
ACCUMULATION OF BIOGENIC AMINES (MG/KG DRY MATTER) DURING LABORATORY PREPARATION OF SOYBEAN TEMPE. EFFECT OF FUNCTIONAL AND ASSOCIATIVE MICROORGANISMS

Treatment code:	A1	A2	A3	A4	C1
Soaked at:	4 C	4 C	4 C	4 C	30 C inoculated[1]
After fermentation with:					
(45 h, 30 C)	R.oli.[2]	R.oli. + K.p.[3]	R.oli. + T.b.[4]	R.oli. + L.p.[5]	R.oli. + L.p.
Cadaverine	<25	950	750	<25	<25
Histamine	<5	<5	<5	<5	<5
Putrescine	163	125	150	88	225
Tryptamine	<5	<5	<5	<5	<5
Tyramine	1575	900	1175	925	725

1: inoculated with 10% v/v of soaking water from previous batch; 2: R.oli.: *Rhizopus oligosporus*; 3: K.p.: *Klebsiella pneumoniae*; 4: T.b.: *Trichosporon beigelii*; 5: L.p.: *Lactobacillus plantarum*.

In Table 2, the effect of incubation with pure culture inocula of functional and associative microorganisms is shown. Treatment A was chosen to serve as a versatile substrate for all microorganisms tested (A1-A4) as the neutral pH of the sterilized beans would enable their growth. In contrast, treatment C was also autoclaved but only inoculated with *R.oligosporus* + *L.plantarum* (C1). Microbiological analyses using selective media (data not shown) for Enterobacteriaceae, yeasts and filamentous fungi, and lactic acid bacteria confirmed that the tempe treatments A1, A2, A3, A4 and C1 did not contain detectable levels of contaminating microorganisms other than the inoculated strains. Fermentation treatments caused a significant increase in levels of cadaverine, putrescine and tyramine. Comparing fresh tempe of treatments A1, A2, A3 and A4 it is clear that *R.oligosporus* itself can produce significant levels of putrescine and tyramine. In the presence of either *K.pneumoniae* or *T.beigelii*, reduced tyramine levels were found but due to a strong production of cadaverine, total levels of biogenic amines were even somewhat higher than with only *R.oligosporus*. With added *L.plantarum*, no such cadaverine accumulation was observed. Both *L.plantarum* inoculated treatments (A4 and C1) resulted in similar patterns of biogenic amines, i.e., mainly tyramine with some putrescine, with total biogenic amines well below that of treatment A1 (only *R.oligosporus*). These limited data suggest that the initial pH of the soaked soybeans did not play an important role in the formation of biogenic amines by *L.plantarum*.

Table 3 shows that the levels of biogenic amines in different samples of commercial tempe can be quite variable. Major factors causing such differences would include the microbiological composition and conditions and duration of storage.

Storage for 1 week at room temperature resulted in a significant increase of putrescine levels. Histamine and tyramine levels increased in one sample. On the other hand, storage for 2 weeks in the refrigerator did not result in increased levels of biogenic amines. In some cases there even was a slight decrease.

Home preparation by stewing caused some reduction of putrescine level whereas frying had stronger reducing effects on putrescine in both samples, and on histamine and tyramine in one sample.

Considering the total levels of biogenic amines of 1000-4000 mg/kg dry weight in samples of laboratory-made and commercial tempe, and an estimated amount of 50 g dry weight of tempe consumed per person per meal (Nout and Rombouts 1990), this corresponds to an intake of 50-200 mg total biogenic amines per meal. An intake of >40 mg biogenic amines per meal has been considered potentially toxic (Ayres *et al.* 1980). However, not all amines are equally toxic. Especially histamine and tyramine are of concern. Histamine was found at low levels, but tyramine was present in significant quantities. Consequently, all possible measures should be taken to reduce the level of

biogenic amines when tempe is produced. Options are inoculation with actively growing lactic acid bacteria, selected for their inability to produce biogenic amines combined with an ability to degrade them (Beutling 1992). Frying in oil may also be expected to give a safer product compared to spontaneously fermented and stewed tempe.

TABLE 3.
EFFECT OF STORAGE AND PREPARATION ON LEVELS OF BIOGENIC AMINES (MG/KG DRY MATTER) IN COMMERCIAL SOY TEMPE.

	Fresh tempe		Storage 1 week, 25 C		Storage 2 weeks, 5 C	
Sample:	I	II	I	II	I	II
Cadaverine	<5	225	<5	200	<22	100
Histamine	<5	100	<4	225	<4	<25
Putrescine	475	3200	1510	4500	225	777
Tryptamine	<5		<4		<4	
Tyramine	500	575	500	821	500	733
			Stewed[1]		Fried[2]	
Fresh tempe sample:			I		I	II
Cadaverine			<25		<11	
Histamine			<5		<2	<10
Putrescine			250		90	218
Tryptamine			<5		<2	
Tyramine			500		200	632

1: in excess water 10 min at 100C; 2: in sunflower oil 3-5 min at 170C

With regard to ethyl carbamate, all samples contained less than 11 ppb (the detection limit). This appears to correspond with the reported absence of ethyl carbamate in a range of fermented foods in which yeasts were not involved as major population (Diachenko *et al.* 1992). Levels up to 84 ppb (soy sauce) were found in fermented foods in which some alcoholic fermentation had taken place and an average of approximately 200 ppb was reported for alcoholic beverages. Also, citrulline and arginine were reported to act as precursors for ethyl carbamate formation (Matsudo *et al.* 1993). The tolerance level of ethyl carbamate as applied in Canada varies from 30 ppb in wines to 400 ppb in distilled spirits (Diachenko *et al.* 1992). This implies that ethyl carbamate is not a factor of public health relevance in soybean tempe. Most likely, the absence of ethyl carbamate is due to inadequate levels of precursors or ethanol in the product.

ACKNOWLEDGMENT

We gratefully acknowledge the chromatographic assistance for biogenic amines by Mrs. D.P. Venema and Ir. P.C.H. Hollman of the State Institute for Quality Control of Agricultural Products RIKILT-DLO, Wageningen. We are also indebted to Mr. T.M.M. Rondags and Ir. L. Terwel of the Inspectorate for Health Protection, Food Inspection Services at Maastricht and Rotterdam, The Netherlands, respectively, for carrying out the chromatographic analyses for ethyl carbamate.

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