CHARACTERISTICS OF PHYTASES FROM SOYBEANS AND MICROORGANISMS INVOLVED IN THE TEMPE PRODUCTION

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ABSTRACT

Tempe was prepared from Forest variety of soybeans inoculated with pure culture of the mould Rhizopus oligosporus strain $CT_{11}K_2$. Partially purified phytases from soybeans, mould of the R. oligosporus strain $CT_{11}K_2$, yeasts (Endomycopsis burtonii, Candida diddensiae and Candida tropicalis) and bacteria (Streptococcus faecium and Streptococcus dysgalactiae) involved during tempe production were prepared according to procedure developed by Sutardi (1988). The crude phytases were characterized and phytic acid, inorganic and total phosphorous content of soybeans, intermediate products and tempe, were also determined.

Results show that phytases, produced by microorganisms involved in tempe production, and by endogenous soybean phytase, had significantly different characteristics especially on optimum pH, temperature and substrate, K_m and V_{max} values, inactivation and activation energy and also temperature coefficient. All phytases contribute to the reduction in phytic acid content; and mould phytase, especially the extra-cellular enzyme, and the endogenous soybean phytase, showed the dominant effect on phytic acid degradation compared with other microbial phytases and physical treatments such as soaking, boiling and steaming.

Phytic acid content of soybeans was reduced by more than 50% from about 10.4 to about 4.9 – 5.0 mg/g (DWB) in fresh tempe (after 40 h fermentation at 30°C).

Key words: *Characteristics, pytase, soybeans, microorganisms, tempe.*

INTRODUCTION

Tempe is a popular Indonesian fermented food consisting of tender-cooked soybeans (or occasionally other legumes) bound together into a white cake by a dense cottony mycelia of the mould *Rhizopus oligosporus*. It owes much of its flavour, sliceable meat-like texture, easy digestibility and excellent nutritional properties to the process of fermentation (Shurtleff and Aoyagi, 1979).

Phytase, or myo-inositol hexaphosphate phosphohydrolase (E.C. 3.1.3.8), which can hydrolyze phytic acid to myo-inositol and inorganic phosphate, has been known to be present widely in seeds such as soybeans (Sutardi and Buckle 1986; Viveros et al., 2000), soybeans tempe fermentation (Sutardi, 1992; Sudarmadji, 2000) and many species of microorganisms such as yeast (Turk, et al., 2000; Sutardi, 2003), bacteria (Sutardi, 2003) and mould (Sutardi and Buckle, 1988; Sandberg et al., 1996). The increasing use of soybeans and soybean products as human food and the possible effects of phytate on the bioavailability of minerals in these products have prompted many studies on the reduction and/or elimination of phytates in soybeans and soybean products.

The aims of the present investigation were to study the characteristics of soybean and microbial phytase (s) as an aid to eastablishing whether phytate reduction during tempe production is the result of physical treatments such as soaking, boiling and steaming, or due to enzymic degradation by endogenous phytase (s) or phytase (s) produced by the fermentation mould *R. oligosporus* strain $CT_{11}K_2$, or produced by yeasts and bacteria that proliferate during soaking of the beans. Accordingly, characteristics of phytases from soybeans and microorganisms involved in the tempe production will now be examined.

MATERIALS AND METHODS

Preparation of tempe

Forest variety of soybeans were soaked 24 h in tap water at room temperature, the soaking water was discarded, and the soybeans were boiled in excess water for 5 min. The cooking water was discarded and the soybeans were soaked for a second 24 h. The soaking water was discarded, the soybeans were dehulled by hand, then cooked in steam for 30 min and spread onto a perforated aluminum tray to drain off the excess water and to cool the beans. The beans were inoculated with a

pure culture of *R. oligosporus* strain $CT_{11}K_2$ (Sutardi and Buckle, 1985).

Crude enzyme preparation

Partially purified (crude) enzyme phytase of soybeans, mould, yeast and bacteria that involved during tempe production were prepared by a method modified by Sutardi (1988).

Enzyme assay

Phytase activity was assayed in small glass-stoppered test tubes measuring the rate of increase of P_i using acid method (Watanabe and Olsen, 1965), and protein was determined by the method of Lowry et al., (1951) using bovine serum albumin as a standard.

Optimum pH

The effect of pH (mixture adjusted to 3.6, 3.8, 4.2, 4.5, 4.8, 5.0, 5,3, 5.6 and 6.0) on phytase activity was determined in 0.1 M acetate buffer except at pH 6.0 where 0.1 M tris-maleate buffer was used. Incubation was for 30 min at 60°C.

Optimum temperature

Phytase assay mixtures were incubated at pH 4.8 for 30 min over the temperature range 30° – 80°C to determine the optimum temperature for phytase activity.

Optimum substrate

Optimum substrate concentration for phytase activity was determined by incubation for 30 min at 60°C in 0.1 M acetate buffer pH 4.8 in sodium phytate concentration from 0 – 30 mM at 0.5 mM intervals. K_m and V_{max} were determined by the method of Linewaver and Burk (1934).

Rate of denaturation

Enzyme in 0.25 M acetate buffer, pH 4.5, was heated at 55°C and 65°C for 0 – 60 min at interval of 10 min, then cooled, sodium phytate added to a final concentration of 1.0 mM and phytase activity was assayed with the procedure developed by Sutardi and Buckle (1986). The energy and temperature coefficient of inactivation were estimated from Arrhenius equation (Whitaker, 1972).

Effect of incubation time and temperature

Phytase activity was assayed during incubation of the reaction mixture for times from 30 min to 120 min at 37°, 45°, 50°, 55°, 60°, and 65°C. The energy of activation for phytase was estimated from Arrhenius equation (Whitaker, 1972).

Determination of phytic acid

Phytic acid content of each sample was determined by the ion-exchange method described by Harland and Oberleas (1986).

Determination of phosphorus

Inorganic phosphorus (P_i) content of each sample was determined by using the ascorbic acid method described by Watanabe and Olsen (1965), while total phosphorus (total P) content was determined by a method modified by Lopez et al.,(1983).

RESULTS AND DISCUSSION

Soaking

In the first phase of the current study, the phytases of soybeans were extracted, purified and characterized. The results are shown in Table 1. Soybean phytase activity increased during soaking of soybeans (Table 2) and the enzyme (s) hydrolysed phytic acid, consequently the phytic acid level decreased significantly (Table 2). The increase in the activity of soybean phytase was inversely correlated with the phytate content of soybeans during soaking, in agreement with the observation of Sutardi (1992). Soybean phytase was completely destroyed when soybeans were boiled for 5 min (Table 2).

Numerous microorganisms that grow during the soaking of whole soybeans are possibly associated with some of the desirable chemical changes accompanying the early stages of germination. Some of these changes are absent during the soaking of boiled soybeans (Steinkraus et. al.,1961). Under the soaking conditions prevailing in Indonesia, the soybeans undergo a bacterial acid fermentation as well as the early stages of germination. These changes are of nutritional importance since stachyose and raffinose, which are thought to be associated with flatulence, are reduced in oncentration either by enzymic mechanisms within the bean or by the action of microbial enzymes.

To date evidence for hydrolysis of such sugars in the beans caused by microbial enzymes produced during soaking has not been reported.

Although a slight decrease in pH (from pH 7.0 to pH 6.0) was observed in the soaking water, pH and temperature of soak water (ambient temperature) did not prevent some soybean phytase activity during the period of soaking. Three yeasts (***Endomycopsis burtonii, Candida diddensiae*** and ***Candida tropicalis***) and two bacteria (***Streptococcus faecium*** and ***Streptococcus dysgalactiae***) that grow well during the soaking of soybeans have been isolated and identified by Mulyowidarso (1988). In the present study, these microorganisms were examined for their ability to produce phytase; and they produced both extra- and intracellular phytases with the characteristics as shown in Table 1.

Table 1. Characteristics of phytases from soybeans and microorganisms involved in the tempe production

Phytase source	pH optimum	Temperature optimum (°C)	Substrate optimum (mM)	Km (x 10^{-4} M)	V_{max} (μmole P_i lib./min/mL enzyme)	Inactivation energy (cal/mole)	Temperature coefficient (Q_{10})	Activation energy (cal/mole)
Soybeans	4.8	60	20	24	0,22	47,000	8	11,000
Mould								
R. oligosporus $CT_{11}K_2$								
Extracellular	4,5	55	-*	1,5	0,08	28,300	4	6,100
Intracellular	4,5	55	-	1,7	0,34	33,200	5	9,500
Yeast								
Endomycopsis burtonii								
Extracellular	3,9	70	-	1,4	0,19	76,800	25	7,100
Intracellular	3,2	65	-	0,5	0,09	63,100	15	5,600
Candida diddensiae								
Extracellular	4,2	60	1,0	3,1	0,01	18,600	2	14,100
Intracellular	4,2	55	1,0	0,7	0,004	14,900	2	7,100
Candida tropicalis								
Extracellular	4,8	55	-	1,0	0,02	70,500	25	11,500
Intracellular	4,8	45	-	0,6	0,004	12,600	2	21,700
Bacteria								
Streptococcus faecium								
Extracellular	4,3	45	-	1,2	0,005	13,700	2	3,400
Intracellular	4,3	45	-	2,2	0,007	24,200	3	14,600
Streptococcus dysgalactiae								
Extracellular	4,2	45	-	1,0	0,003	23,800	3	3,600
Intracellular	4,2	40	-	0,5	0,003	12,000	2	7,100

Table 2. Mean (± s.d.)* phytic acid content and phytase activity of soybeans during preparation of tempe prior to fermentation.

Soybean sample	Phytic acid (mg/g DWB)+		Phytase activity (μmole P_i lib./min/mL enzyme)+	
	with antibiotics#	without antibiotic	with antibiotics#	without antibiotic
Whole dry soybeans	10,39 ± 0,04[a]	10,39 ± 0,04[a]	0,04 ± 0,01[a]	0,04 ± 0,01[a]
Soaked beans	10,06 ± 0,09[b]	9,61 ± 0,04[b]	0,06 ± 0,01[b]	0,06 ± 0,01[b]
Boiled beans	9,36 ± 0,04[c]	8,73 ± 0,05[c]	0	0@
Dehulled beans	8,42 ± 0,04[d]	8,46 ± 0,04[d]	0	0
Steamed beans	8,30 ± 0,04[e]	8,38 ± 0,06[e]	0	0
Skin		0,66 ± 0,02		
Soaking water	0,04 ± 0,00[ć]	0,20 ± 0,02[ć]	0,01 ± 0,01	0,03 ± 0,01
Boiling water	0,15 ± 0,02	0,15 ± 0,02		

*Derived from 3 replications

+Differences were determined by t-test

within a column, values with the same superscripts are not significantly different at least at $P \leq 0,05$

#Antibiotic mixture (300 mg/kg each of oxytetracycline, penicillin, streptomycin and cycloheximide) was added to the soak water

@No phytase activity was observed

[ć](mg/mL)

Extracellular phytases produced by yeasts and bacteria during the soaking of soybeans may diffuse into the bean tissue since the hulls become soft and permeable. Subsequently, some enzymic degradation of phytic acid in the beans by yeast and bacterial phytases may be possible. The data in Table 2 shows that soaking water contained phytase activity presumable derived from yeasts and bacteria present in the soaking water, together with any soybean phytase that may have leached from the beans. No phytase activity was observed when an anti-biotics was added to the soaking water to depress any microbial growth. The soaking conditions used would not have inhibited microbial phytases, although they have optimum pH between 3.2 – 4.8 and optimum temperature between 40° – 70°C.

A reduction of phytic acid content (from 10.39 ± 0.04 to 9.61 ± 0.04 mg/g DWB) (Table 2) during the soaking of soybeans was higher than a sample (from 10.39 ± 0.04 to 10.06 ± 0.09 mg/g DWB) in which yeasts and bacteria were inhibited by the presence of antibiotics (Table 3). This tends to indicate that microbial enzymes are more significant in phytate destruction during soaking than is endogenous soybean phytase activity. Decreasing of phytic acid concentration during overnight soaking of whole dry soybeans at ambient temperature was reported by Sutardi and Buckle (1985). Tranggono et al., (1991) demonstrated that for velvet bean (*Mucuna prurienns*), pigeon peas (*Cajanus cajan*), and lima beans (*Phaseolus lunatus*) soaked in water for 24 hours at ambient temperature, the decrease in phytate content were 20; 15 and 40%, respectively.

The enzymic degradation of phytic acid in soak water does not contribute much to the overall reduction in the level of phytate from soybeans. During soaking 0.04 – 0.20 mg/ml phytic acid is leached into the soaking water (Table 2 and Table 3), a result perhaps supported by the observation of Sutardi and Buckle (1985).

Soybean, yeast and bacterial phytases are capable of hydrolyzing phytic acid during soaking, treatments such as boiling, however, destroy most vegetative microorganisms and endogenous phytases.

The corresponding effects of soaking on phytate-P, P_i and total P are presented in Table 3. P_i increases during soaking as the result of hydrolysis of phytic acid by phytase derived from either soybeans and/or yeasts and/or bacteria. The decrease in P_i during soaking was not specifically related to the activity of soybean and microbial phytase (Table 3), as liberated P_i was presumably reused by microorganisms or utilized by beans in the early stages of germination for plant growth and development. Table 3 shows that the soak water contained 0.21 mg/ml P_i, while the "control" sample (treated with antibiotics) contained 0.22 mg/ml P_i. Analogues with the phytic acid reduction, total P of soybeans decreased during soaking as the result of leaching of water-soluble phosphorus compounds; 0.40 mg/ml total P was observed in the soak water (Table 3).

Table 3. Mean (± s.d.)* phytic acid$^{\pi}$, inorganic P and total P of soybeans during preparation of tempe prior to fermentation.

Soybean sample	Phytic acid (mg/g DWB)+			Inorganic P (mg/g DWB)+			Total P (mg/g DWB)+		
	with antibiotic#	without antibiotic	level of significance	with antibiotic#	without antibiotic	level of significance	with antibiotic#	without antibiotic	level of significance
Whole dry soybeans	10,39± 0,04^{a}	10,39± 0,04^{a}	NS	0,53 ± 0,02^{a}	0,53 ± 0,02^{a}	NS	4,70± 0,02^{a}	4,70±0,02^{a}	NS
Soaked beans	10,06± 0,09^{b}	9,61± 0,04^{b}	0,0005	0,43 ± 0,02^{b}	0,44 ± 0,01^{b}	0,0005	4,67±0 ,01^{b}	4,66± 0,04^{b}	NS
Boiled beans	9,36 ± 0,04^{c}	8,73 ± 0,05^{c}	0,0005	0,29 ± 0,01^{c}	0,31 ± 0,01^{c}	0,10	4,63± 0,02^{c}	4,63± 0,02^{c}	NS
Dehulled beans	8,42 ± 0,04^{d}	8,46 ± 0,04^{d}	NS	0,14 ± 0,01^{d}	0,14 ± 0,01^{d}	NS	4,60± 0,01^{c}	4,54± 0,04^{d}	0,05
Steamed beans	8,30 ± 0,04^{e}	8,38 ± 0,06^{e}	NS	0,12 ± 0,01^{d}	0,13 ± 0,02^{d}	0,10	4,58± 0,02^{c}	4,53± 0,03^{d}	0,000
Skin		0,66 ± 0,02		0,04 ± 0,01	0,04 ± 0,02		0,85± ,02	0,79± 0,02	
Soaking water	0,04± 0,00$^{¢}$	0,20 ± 0,02$^{¢}$		0,12 ± 0,02$^{¢}$	0,11 ± 0,01$^{¢}$		0,40±0,01$^{¢}$	0,40±0,02$^{¢}$	
Boiling water	0,15± 0,02$^{¢}$	0,15 ± 0,02		0,30 ± 0,02	0,30 ± 0,02		0,45 ± 0,02	0,45 ± 0,04	

*Derived from 3 replications

$^{\pi}$Phytic acid (mg/g DWB) x 28,2/100 ≡ Phytate-P

+Differences were determined by t-test

within a column, of data, values with the same superscripts are not significantly different (at least at P ≤ 0,05); within a row, actual significance levels are shown

#Antibiotic mixture (300 mg/kg each of oxytetracycline, penicillin, streptomycin and cycloheximide) was added to the soak water

¢(mg/m

Boiling

Boiling of soaked soybeans facilitates dehulling as well as partially cooking the beans, and destroys most vegetative microorganisms. Boiling for 5 min caused the phytic acid level of soybeans to decrease significantly (Table 3). Boiling is more likely to extract soybean components, particularly water-soluble ones including phytate, than it is to cause chemical destruction of phytate. It was observed that the boiling water was both turbid and dark in colour, indicating extensive extraction of water-soluble components. The corresponding effect of boiling on P_i and total P levels is shown in Table 3. Both P_i and total P decreased significantly.

No phytase activity was observed in boiled beans until the prepared soybeans were fermented into tempe.

Dehulling

Dehulling was performed after boiling of the beans by rubbing the beans together and then washing. The phytic acid content decreased from 8.73 $\pm$ 0.05 to 8.46 $\pm$ 0.04 mg/g (DWB) which was significantly different (Table 2). The loss of phytic acid during dehulling and washing was probably due to leaching into water. The level of P_i and total P decreased significantly although total P for sample added with antibiotics (as control sample) did not change significantly (Table 3).

Steaming and cooling

Although steaming for 30 min in an atmospheric pressure cooker would destroy most microorganisms present in the beans, draining and cooling in the open air before fermentation allows microorganisms to contaminate the beans. The level of contamination is rarely sufficient to interfere with the fermentation, although the contaminating organisms also may produce phytases that could contribute to the reduction of phytic acid during tempe fermentation. Although phytic acid in soybeans is known to be a heat-labile anti-nutritional factor (Liener, 1981), steaming without pressure, however, is unlikely to result in significant phytic acid degradadtion. Table 2 and Table 3 show that the phytic acid level decreased from 8.46 $\pm$ 0.04 to 8.38 $\pm$ 0.06 mg/g (DWB), which was statistically significance different ($p \leq 0.05$). Previous investigation (Sutardi, 1981) showed that treatments such as boiling, dehulling, steaming, draining and cooling decreased slightly the phytic acid content of soaked soybeans although the reduction was statistically not significant ($p \geq 0.01$).

Fermentation

The mould *R. oligosporus* or other *Rhizopus spp.* produce the greatest effect on phytate during tempe fermentation, while at the same time they bind steamed, dehulled soybeans into a compact, cake-like mass as edible tempe. *R. oligosporus* strain $CT_{11}K_2$, among the hundreds of strains isolated and purified from commercial tempe in Indonesia, has been examined previously and shown to produce good quality tempe compared with another five strains of *R. oligosporus* and the traditional inoculum (*usar*) (Sutardi and Buckle, 1985).

R. oligosporus strain $CT_{11}K_2$ produced both extra- and intracellular phytases which appear to play an important role in phytic acid degradation during tempe fermentation. Mould phytases are likely to be important phytases since the mycelia of the mould penetrate deeply into the beans (Sudarmadji, 2000) enabling phytase to breakdown phytic acid in the beans more easily.

In the current study, phytases of the mould were characterized (Table 1) and its significant effects on phytic acid reduction observed. Table 4 shows about 40% of the phytic acid present in soybeans at the commercement of fermentation was reduced after 40 h fermentation (fresh tempe). The reduction of phytic acid level was not significantly different between tempe produced with *R. oligosporus* strain $CT_{11}K_2$. Van der Riet et al., (1987) reported that in tempe made from two different soybean cultivars, the reduction of phytic acid content after 48 h fermentation was between 75 and 88%.

Table 4 shows that beans inoculated with *R. oligosporus* strain $CT_{11}K_2$ contained phytase activity, while a control that was not inoculated had no phytase activity. Any yeasts or bacteria, probably present only in low numbers during fermentation, would not grow to any extent since by this stage the conditions are more conducive for mould growth. It is possible, however, that some of the contaminating yeasts and bacteria may produce some phytase activity.

Phytase activity increased with increase in fermentation time and paralleled the decrease in phytic acid level (Table 4). The highest reduction of phytic acid level occurred during the first 12 h fermentation; with a further increase in incubation time and the rate of reduction of phytic acid level decreased. *R. oligosporus* strain $CT_{11}K_2$ did not produce a significantly different level of phytic acid after 40 h fermentation.

Phytase activity was affected by both the time and temperature of fermentation. Van der Riet *et al.* (1987) reported a reduction of phytic acid content during tempe fermentation that was significantly higher than that of the current study, possibly due to the longer incubation time and higher incubation temperature (72 h at 31° – 35°C). On the other hand, Turk et al., (2000) reported that *Saccharomyces cerevisiae* (baker's strain) with a high expression of phytase during fermentation, could be one way of achieving extensive phytate degradation during bread-making. The condition of fermentation, such as temperature, can influence significantly phytase activity, although mould phytases have an optimum temperature of about 55°C, rather higher than that of the incubation temperature (30°C).

Table 4. Mean (± s.d.)* phytic, phytate-P content and phytase activity of tempe during fermentation at 30°C.

Time of incubation (h)	Phytic acid (mg/g DWB)+		Phytate-P (mg/g DWB)		Phytase activity ((μmole P_i lib./min/mL enzyme)	
	with antibiotics#	without antibiotic	with antibiotics	without antibiotic	with antibiotics	without antibiotic
0	8,30 ± 0,04[a]	8,38 ± 0,06[a]	2,34 ± 0,01[a]	2,36 ± 0,02[a]	0@	0[a]
12	8,27 ± 0,05[a]	6,12 ± 0,02[b]	2,33 ± 0,02[a]	1,73 ± 0,01[b]	0	0,01 ± 0,00[a]
24	7,97 ± 0,02[b]	5,50 ± 0,04[c]	2,25 ± 0,01[b]	1,55 ± 0,01[c]	0	0,04 ± 0,01[b]
36	7,86 ± 0,06[c]	5,12 ± 0,06[d]	2,23 ± 0,02[c]	1,44 ± 0,01[d]	0	0,09 ± 0,01[c]
40	7,80 ± 0,04[c]	4,89 ± 0,04[e]	2,20 ± 0,01[d]	1,38 ± 0,01[e]	0	0,14 ± 0,01[d]
	P ≤ 0,05	P ≤ 0,0005	P ≤ 0,05	P ≤ 0,0005		P ≤ 0,0005

*Derived from 3 replications
+Differences were determined by t-test
within a column, values with the same superscripts are not significantly different at least at P ≤ 0,05
#Antibiotic mixture (300 mg/kg each of oxytetracycline, penicillin, streptomycin and cycloheximide) was added to the soak water
@No phytase activity was observed

During fermentation, the temperature in the bean cake may rise by 10° – 16°C. Above that of the incubation temperature (Steinkraus et al., 1960; Martinelli and Hesseltine, 1964), providing ideal conditions for mould phytases. The slightly highly optimum temperature (55°C) of mould phytase was in agreement with the study conducted by Affrifah et al., (2005), and they reported that phytase of cowpea flour with moisture content adjusted to 10, 25 and 35% and heated in sealed retort pouches at 70° to 95°C for periods of 2 to 32 min showed a high thermal resistance with residual activity ranging between 50% and 95%.

CONCLUSSION

Good quality and low phytic acid level of tempe was produced by a modified Indonesian traditional method, with soaking, boiling, dehulling and steaming of soybean cotyledons inoculated with the mould of *R. oligosporus* strain $CT_{11}K_2$. Tempe preparation decreased by about 50% the phytic acid content of soybean.

Phytases produced by endogenous soybean phytase and microorganisms involved in tempe production, all contribute to the reduction in phytic acid content; the endogenous soybean phytase and mould phytases, especially the extracellular enzyme, showed the dominant effect on phytic acid degradation compared with other microbial phytases (yeasts and bacteria phytases) and physical treatments such as soaking, boiling and steaming.

All of the identified phytases such as soybean phytase, mould phytases, yeast phytases and bacterial phytases that active during tempe production had different characteristics in pH, temperature and substrate optimum, as well as K_m and V_{max} values, inactivation and activation energy, and also temperature coefficient.

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REFERENCES

Affrifah, N.S., Chinnan, M.S. and Phillips, R.D. 2005. Heat-moisture treatments of cowpea flour and their effects on phytase inactivation. J. Food Sci. 70 (2): E98 – E103.

Harland, B.F. and Oberleas, D.A.1986. A modified method for phytase analysis using ion-exchange procedure: Application to textured vegetable protein. Cereal Chem. 54: 827 – 832.

Liener, I.E. 1981. Factors affecting the nutrional quality of soya products. J. Am. Oil. Chem. Soc. 58: 406 - 415.

Lineweaver, H. and Burk, D. 1934. The determination of enzyme dissociation constant. J. Am. Oil Chem. Soc. 56: 658 – 666.

Lopez, Y., Gordon, D.T. and Fields, M.L. 1983. Release of phosphorus from phytate by natural lactic acid fermentation. J. Food Sci. 48: 953 – 954.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randel, R.J. 1951. Protein estimation with folin phenol reagent. J. Bio. Chem. 193: 265 – 275.

Martinelli, A.F. and Hesseltine, C.W. 1964. Tempeh fermentation: Package and tray fermentation. J. Food Technol. 18: 167 – 171.

Mulyowidarso, R.B.K. 1988. The microbiology and biochemistry of soybean soaking for tempe fermentation. The University of New South Wales. Sydney. PhD. Thesis.

Sandberg, A.S., Rossander, R. and Turk, M. Dietary Aspergillus niger phytase increases iron absorption in human. J. Nutr. 126: 476 – 480.

Shurtleff, W. and Aoyagi, A. 1979. The book of Tempeh. New York: Harper and Row.

Sudarmadji, S. 2000. Determination of relationship between enzyme activity of phytase and fermentation on soybean tempe. Research Report. Institute of Research. Gadjah Mada University. Yogyakart. Contract No. 3129/J.01/KL.04/2000.

Sutardi. 1981. Changes in phytic acid levels in soybeans during tempeh production. The University of New South Wales. Sydney. MAppSc. Thesis.

Sutardi and Buckle, K.A. 1985. Phytic acid changes in soybeans fermented by traditional inoculum and six strains of *Rhizopus oligisporus*. J. Appl. Bacteriol. 38: 539 – 543.

Sutardi and Buckle, K.A. 1986. The characteristics of soybean phytase. J. Food Biochem. 10: 197 – 216.

Sutardi. 1988. Phytase activity during tempe production. The University of New South Wales. Sydney. PhD. Thesis.

Sutardi and Buckle, K.A. 1988. Characteristics of extra- and intracellular phytases from *Rhizopus oligosporus* used in tempeh production. Int. J. of Food Microbiol. 6: 67 – 79.

Sutardi. 1992. Changes of phytic acid content and phytase activity during production, storage and cooking of tempe. Agritech. 12 (1): 2 – 15.

Sutardi. 2003. Characterization of extra- and intracellular phytases from proliverating yeast during tempeh production. Agritech 23 (2): 57 – 66.

Sutardi. 2003. Characterization of extra- and intracellular phytases from bacteria proliverating during tempe production. Indonesian Food and Nutrition Progress 10 (2): 96-105.

Steinkraus, K.H., Hwa, Y.B. and van Buren, J.P. 1960. Studies on tempeh – An Indonesian fermented soybean food. Food Res. 25: 777 – 788.

Steinkraus, K.H., Hand, D.B., van Buren, J.P. and Hackler, L.R. 1961. Pilot plan studies on tempeh. Proc. Conf. Soybean products for protein in human foods. Peoria, Ill., USDA-ARS 71 – 22; 78 – 84.

Tranggono, Sutardi and Meta Mahendradatta. 1991. Phytase activity during tempe production from velvet beans (*Mucuna pruriens*), pigeon peas (*Cajanus cajan*) and lima beans (*Phaseolus lunatus*) using traditional inoculum (usar). Agritech. 11 (4): 2 – 10.

Turk, M., Sandberg, A.S., Carlsson, N.G. and Andlid, T. 2000. Inositol hexaphosphate hydrolysis by baker's yeast, capacity, kinetics and degradation products. J. Agric. Food Chem. 48: 100 – 104.

Van der Riet, W.B., Weight, A., Cilliers, J.J.L. and Datel, J.M. 1987. Food chemical analysis of te,peh prepared from South African-grown soybeans. Food Chem. 25: 197 – 206.

Viveros, A., Centeno, C., Brenes, A., Canales, R. and Lozano, A. 2000. Phytase and acid phosphatase activities in plant feedstuffs. J. Agric. Food Chem. 48: 4009 – 4013.

Watanabe, E.S. and Olsen, S.R. 1965. Test of an ascorbic acid method for determining phosphorus in water and $NaHCO_3$ extracts from soil. Soil Sci. Soc. Am. Proc. 29: 677 – 678.

Whitaker, J.R. 1972. Principles of enzymology for the food science. New York. Marcel Dekker.