Protein Utilization during Soybean Tempe Fermentation

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The aim was to identify to what extent proteins were utilized during the fermentation of bacteria-free tempe prepared with acidified soybean cotyledons and *Rhizopus oligosporus* NRRL 2710 at 30 °C. Dry matter declined continuously during the fermentation to 980 g/(kg of initial dry cotyledons) at 28 h, 910 g at 46 h (when the tempe was judged mature), and 835 g at 72 h. The decrease in dry matter was due mainly to reduction in crude lipid, amounting to 65 g/(kg of initial dry cotyledons) at 46 h and 135 g/(kg of initial dry cotyledons) at 72 h and representing approximately 70% and 80%, respectively, of the total dry matter loss. Protein oxidation (estimated from ammonia production) was 5 g at 28 h,10 g at 46 h, and 20 g/(kg of initial dry cotyledons) at 72 h. The total amount of soy protein hydrolyzed, including that incorporated into mold biomass, was estimated to be 80 g/(kg of initial dry cotyledons) at 28 h incubation, 95 g at 46 h, and 100 g at 72 h. The hydrolyzed protein at 46 h represented 25% of the initial protein. Of this hydrolyzed protein, it is suggested that approximately 65% remained in the tempe as amino acids and peptides, 25% was assimilated into mold biomass, and 10% was oxidized.

Keywords: *Lipid; protein; Rhizopus oligosporus; soybean; soya bean; tempe; tempeh*

INTRODUCTION

Tempe is a traditional Indonesian product that consists of cooked, dehulled soybeans or other pulses bound into a solid cake by growth of a mold (Nout and Rombouts, 1990; Steinkraus, 1996). The high protein content and pleasant, relatively bland taste has led to it occupying a small but expanding part of the vegetarian market in Japan, the United States, and Europe (Nout and Rombouts, 1990; Liu, 1997).

During the fermentation, there is usually a small increase in crude protein content and a rather larger reduction in crude lipid (Fudiyansyah et al., 1995; van Buren et al., 1972; Murata et al., 1967; van der Riet et al., 1987; Ruiz-Terán and Owens, 1996). Ruiz-Terán and Owens (1996) showed that soybean lipids were utilized from the start of the fermentation and appeared to be a major source of carbon and energy for the mold. Ruiz-Terán and Owens did not study protein metabolism in detail but, on the basis of ammonia production, suggested that less than 5% of the soy protein was oxidized. It is well-established that the growth of the mold is accompanied by the accumulation of amino acids/water-soluble nitrogen compounds in tempe (Murata et al., 1967; Nowak and Szebiotko, 1992; De Reu et al., 1995; Baumann and Bisping, 1995), but the extent to which they are utilized for growth is not clear.

The aim of this study was to identify to what extent proteins/amino acids are utilized during tempe fermentation. In particular, the 'true' hydrolysis of protein during the fermentation was estimated, where the true hydrolysis is equated to proteins in the mold biomass (and presumed to be newly synthesized from monomers) plus any reduction in total protein. This approach gives far more insight into the scale of the metabolic activities of the mold than simple proximate analysis. Changes in proximate composition during fermentation are a consequence of both the degradation of plant tissue and the synthesis of mold tissue, and even negligible changes in proximate composition may mask substantial metabolic activity by the mold. Most studies have measured changes in proximate composition and have not attempted to differentiate between material degraded and material synthesized. Hence, the relationship between the metabolic activities of the mold and changes in the composition of the substrate are still not understood. This lack of understanding hampers the rational selection of mold strains and substrates that is necessary for the development of modified or new tempe-type products.

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MATERIALS AND METHODS

Mold Culture. *Rhizopus oligosporus* NRRL 2710 was grown on potato dextrose agar (PDA; Oxoid CM139) at 30 °C until it sporulated (5 d) and then was stored at 5 °C.

Preparation of Spore Suspension. Spore suspensions were prepared as previously described (Ruiz-Terán and Owens, 1996) except that the fungus was grown on PDA medium instead of Sabouraud dextrose agar and the suspensions were stored at 5 °C until required rather than frozen. Suspensions retained satisfactory viability for at least 1 month.

Preparation of Tempe. Bacteria-free tempe was prepared with autoclaved, acidified Canadian No. 1 soybeans in Petri dishes according to Ruiz-Terán and Owens (1996). After 0, 28, 46, and 72 h, three dishes were removed from incubation for analysis. Uninoculated cotyledons and tempe cultures were tested for the presence of bacteria with thioglycollate broth (Ruiz-Terán and Owens, 1996).

Chemical and Physical Analyses. Each analysis was done in duplicate on three replicate tempe fermentations with the exception of the determination of fat content, which was determined once only on each tempe fermentation, and glucosamine content, which was determined on four replicate samples.

Dry matter, ash content, and fat content of tempe were determined according to the AOAC (1995). Defatted tempe

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Table 1. Changes in Dry Matter and Crude Lipid during Tempe Fermentation[a]

	dry matter			crude lipid			fungal biomass[b]	
incubation period (h)	concn in tempe at end of period (g /kg wet weight)	amt in tempe at end of period (g/kg of initial dry cotyledons)[c]	net change (g/kg of initial dry cotyledons)[c]	concn in tempe at end of period (g/kg of dry matter)	amt in tempe at end of period (g/kg of initial dry cotyledons)[c]	net change (g/kg of initial dry cotyledons)[c]	concn at end of period (g/kg of dry matter)	amt at end of period[g] (g/kg of initial dry cotyledons)[c]
0−0	372 (1)	1000 (4)	0	265 (4)	265 (5)	0	0	0
0−28	370 (1)	980 (9)	−20	255 (1)	250 (3)	−15	45 (1)	44 (1)
0−46	365 (4)	910 (11)	−90	220 (9)	200 (9)	−65	59 (3)	54 (3)
0−72	350 (6)	835 (15)	−165	160 (10)	130 (8)	−135	56 (4)	52 (4)

[a] Values are means of single (crude lipid), duplicate (dry matter), or quadruplicate (biomass) determinations on triplicate fermentations with standard deviations in parentheses. [b] Glucosamine content ×12. [c] Values are expressed per kg of dry matter present in cotyledons at the start of the fermentation.

flours were used for the determination of fungal biomass, nitrogen content, and filterable nitrogen. Samples were defatted by Soxhlet extraction (Soxtex System HT1043; Tecator Ltd., Bristol, U.K.) with petroleum ether (40−60 °C, Fisher Scientific P/1440/17) at 60 °C for 2 h. The defatted flour was dried at 105 °C for 12 h and then stored in a desiccator until analyzed.

pH was determined by adding a 5-g sample (wet weight) to 20 mL of purified water (Purite RO50 reverse osmosis and ion exchange unit; Purite Ltd., Thame, U.K.), homogenizing and measuring the pH of the suspension with a glass electrode.

Determination of Nitrogen Content. Total nitrogen was determined by combustion (Leco Organic nitrogen analyzer FP 228; Leco Instruments, Stockport, U.K.). Approximately 0.1 g of defatted sample was weighed into each capsule. The capsule was closed and put in the instrument for 3 min. The calibration of the instrument was checked before each batch of samples with five 0.15-g amounts of EDTA for solid samples or five 0.1-mL amounts of $(NH_4)_2SO_4$ solution (containing 1% [w/w] nitrogen) for liquid samples.

Determination of filterable nitrogen was based on Butts et al. (1992). Defatted sample (0.25 g dry weight) was extracted with 1.5 mL of buffered saline (sodium phosphate, 0.1 mol/L; NaCl, 0.145 mol/L; pH 7) by shaking on an orbital shaker at 150 revolutions/min for 30 min. The supernatant was decanted and centrifuged at 15000*g* for 15 min at 3 °C. The supernatant was decanted and retained. The precipitate was washed with 1 mL of buffered saline (shaken at 150 revolutions/min for 15 min), centrifuged again at 15000*g* for 15 min at 3 °C. The second supernatant was added to the first, and the total weight was recorded. The combined supernatant was immediately ultrafiltered through a Vectaspin 3, MWCO 10 000 Da, polysulfone filter (Whatman 6835 1005) by centrifuging at 5000*g* and 5 °C. The ultrafiltrate (low molecular weight fraction, MW <10 000 Da) was weighed and stored frozen until analyzed for total nitrogen. Prior to determination of total nitrogen, the ultrafiltrates were concentrated to half the initial volume by evaporation in an oven at 90 °C for 30−45 min.

Nonfilterable-N (high molecular weight fraction, MW >10 000) was calculated as the difference between total-N and filterable-N. Filterable-N other than ammonia was obtained by subtracting ammonia-N from filterable-N.

Soybean proteins, with the exception of some trypsin inhibitors, have molecular masses greater than 10 000 Da (Wolf 1972); hence, the nonfilterable N-fraction is considered to comprise proteins, nucleic acids, chitin, etc. On the assumption that this fraction was predominantly proteins, protein content was estimated by multiplying nonfilterable-N by 5.7 (Bender and Bender, 1997). The filterable-N fraction (MW <10 000 Da) is considered to comprise peptides, amino acids, ammonium, and other low molecular weight nitrogenous compounds.

Determination of Ammonia Nitrogen. Fresh tempe (2.5 g wet weight) was homogenized with 10 mL of 1 mol/L perchloric acid. Ten milliliters of purified water was added to the homogenate, the pH value was adjusted to 7 with 10 and 1 mol/L KOH solutions, and the volume was made up to 50 mL with purified water. The mixture was cooled in a refrigerator for about 30 min and then centrifuged at 6900*g* at ambient temperature. The supernatant was frozen, thawed, and centrifuged, and the supernatant was assayed for ammonia enzymically (Boehringer 1112 732; Boehringer-Mannheim, Mannheim, Germany).

Table 2. Changes in Nitrogen Compounds during Tempe Fermentation

	nitrogen compounds[a] (g of N/(kg of initial dry cotyledons))				
incubation period (h)	total	non-filterable[b]	filterable[c]	filterable other than ammonia[d]	ammonia
0	72 (1)	70.5 (1)	1.6 (0.4)	1.6 (0.4)	0.04 (0.01)
28	74 (1)	61.5 (2)	12.5 (0.8)	11.5 (0.8)	0.8 (0.03)
46	76 (3)	62 (3)	14 (1)	12 (1)	1.8 (0.1)
72	75 (5)	60 (5)	15 (1)	11.3 (1)	3.7 (0.2)

[a] Values are means of duplicate determinations on triplicate fermentations with standard deviations in parenthese. Values are expressed per kg of dry matter present in the cotyledons at the start of the fermentation. [b] Total-N minus filterable-N. [c] Separated by ultrafiltration with MWCO of 10 000 Da. [d] Filterable-N minus ammonia-N.

Estimation of Protein Oxidation. The amount of protein oxidized to carbon dioxide during tempe fermentation was estimated by multiplying the ammonia-N produced by 5.7 (Bender and Bender, 1997) and assuming that the N remained in the tempe while the CO_2 was lost to the atmosphere.

Estimation of True Protein Hydrolysis. The true extent of protein hydrolysis was estimated as the increase in filterable-N × 5.7 plus protein present in mold biomass.

Determination of Mold Biomass. Mold biomass in defatted samples was estimated from glucosamine content as previously described (Ruiz-Terán and Owens, 1996), but the final precipitate was resuspended in 25 mL of purified water instead of 5 mL. A conversion factor of 12 g of dry fungal biomass/g of glucosamine was used (Sparringa and Owens, 1999). Protein content of biomass was estimated by assuming that it contained 80 g of N/kg of dry matter (Stanbury et al., 1995) and multiplying the nitrogen content by 5.7.

Expression of Amounts of Chemical Components Relative to the Amount Present in the Initial Dry Cotyledons. The amount of ash was assumed to remain constant during the fermentation (Ruiz-Terán and Owens, 1996), and amounts of chemical components are expressed relative to the amount of ash present in the initial dry cotyledons (23.8 ± 0.4 [standard deviation] g/kg of dry matter) as gram of material/kilogram of dry matter present in the initial dry cotyledons.

RESULTS AND DISCUSSION

Growth of *Rhizopus oligosporus* and Substrate Utilization. Turbidity was not observed in any tubes of thioglycollate medium inoculated with uninoculated cotyledons, with incubated cotyledons, or with tempe samples, confirming the absence of bacteria from the

Table 3. Protein Utilization during Tempe Fermentation[a]

	amino acid and peptides					protein			
incubation period (h)	concn at end of period (g/kg of dry matter)	amt at end of period[b] (g/kg of initial dry cotyledons)[i]	net change (g/kg of initial dry cotyledons)[i]	protein oxidized to NH_3[c] (g/kg of initial dry cotyledons)[i]	protein in fungal biomass[d] (g/kg of initial dry cotyledons)[i]	concn at end of period[e] (g/kg of dry matter)	amt at end of period[f] (g/kg of initial dry cotyledons)[i]	net change[g] (g/kg of initial dry cotyledons)[i]	"true" protein hydrolysis[h] (g/kg of initial dry cotyledons)[i]
0−0	9.1 (2)	9.1 (2)	0	0	0	400 (7)	400 (9)	0	0
0−28	68 (4)	67 (4)	+58	5 (0.2)	20	360 (9)	340 (9)	−60	80
0−46	76 (6)	69 (6)	+60	10 (0.8)	25	390 (20)	330 (10)	−70	95
0−72	77 (4)	64 (4)	+55	20 (1)	24	410 (30)	325 (10)	−75	100

[a] Values are calculated from data in Tables 1 and 2. [b] Filterable-N other than $NH_4^+ \times 5.7$. [c] NH_3-N production × 5.7. [d] Estimated by assuming biomass contained 80 g of N/kg of dry matter. [e] Nonfilterable-N × 5.7. [f] (Initial total-N − filterable-N) × 5.7. [g] Increase in filterable-N × 5.7. [h] Net change plus protein in biomass. [i] Values are expressed per kg of dry matter present in cotyledons at the start of the fermentation.

fermentations. Hence, changes in chemical components during the fermentation can unequivocally be attributed to activities of the mold.

Growth of the mold was largely complete after 46 h incubation when mold biomass in the mature tempe was 59 g of dry biomass/kg of dry tempe, equating to 54 g of dry biomass/kg of initial dry cotyledons (Table 1). The pH of the initial acidified, cooked soybean cotyledons was 4.6; this rose during the fermentation to 5.6 at 28 h, 6.6 at 46 h, and 7.1 after 72 h incubation.

Dry matter declined continuously during the fermentation, being 980 g/kg of initial dry cotyledons at 28 h, 910 g at 46 h (when the tempe was judged mature with the cotyledons bound into a compact cake by mold mycelium), and 835 g at 72 h (Table 1). The overall weight loss of 90 g/kg of initial dry cotyledons observed here in mature tempe is similar to values reported previously (Ruiz-Terán and Owens, 1996; Steinkraus 1996; van der Riet et al., 1987).

The decrease in dry matter (Table 1) was due mainly to reduction in crude lipid. This reduction in crude lipid amounted to 65 g/kg of initial dry cotyledons at 46 h and 135 g/kg of initial dry cotyledons at 72 h, representing approximately 70% and 80%, respectively, of the total dry matter loss. Net protein loss (estimated as ammonia production × 5.7; Table 2) was 5 g at 28 h,10 g at 46 h, and 20 g/kg of initial dry cotyledons at the end of the fermentation.

Lipid Utilization. The concentration of crude lipid in tempe decreased by about 15% during the first 46 h of fermentation, but when values are expressed per kilogram of dry matter present in the cotyledons at the start of the fermentation, it is seen that nearly 25% of initial lipid had been utilized (Table 1). The utilization of lipids continued in overincubated tempe, and at 72 h, 50% of the initial crude lipid had been used.

These observations are in agreement with previous reports of reductions in total lipid during tempe fermentation (Murata et al., 1967; van Buren et al., 1972; Fudiyansyah et al., 1995; Ruiz-Terán and Owens, 1996). From van der Riet et al.'s (1987) data, it is possible to calculate that utilization of crude lipid accounted for about 32% of total dry matter loss in Prima variety soybean tempe at 48 h incubation and 50% after 72 h. Ruiz-Terán and Owens (1996) reported that lipid utilization accounted for 30% of total dry matter loss in mature tempe and 70% in greatly overincubated tempe. In the fermentations observed here, lipid loss represented about 70% of total dry matter loss at 46 h and 80% at 72 h. It is evident that there are very wide differences between the values reported by different authors. It is not known why the variation is so great, but it is possible that some of it is due to measurements being made on tempes at different stages of maturity.

The determination of crude lipid includes acylglycerols and free fatty acids; therefore, the decrease in crude lipid indicates an actual loss during the fermentation, presumably as result of oxidation by the mold. Thus, the utilization of lipids was substantially greater than the utilization (i.e., oxidation to carbon dioxide and ammonia) of proteins.

Nitrogen Metabolism. Total nitrogen, within the errors of measurement, remained constant during the fermentation, indicating that ammonia was not lost from the tempe/Petri dish to the air (Table 2). Filterable nitrogen other than ammonia (presumed to represent amino acids and small peptides) increased during the early growth phase, up to 28 h incubation, and then remained constant. Ammonia nitrogen was continuously produced, reaching 3.7 g/kg of initial dry cotyledons by the end of the fermentation (Table 2).

The ammonia production observed here is similar to levels reported previously (Murata et al., 1967; van Buren et al., 1972; Ruiz-Terán and Owens, 1996). If it is accepted that ammonia production is a consequence of protein/amino acid oxidation, then in mature tempe (46 h) about 2.5% of the initial protein had been oxidized. However, other metabolic reactions could also contribute to the release of ammonia.

A substantial proportion of the total nitrogen was converted to filterable nonammonia nitrogenous-materials, presumably amino acids and small peptides (Table 2). This nonammonia filterable-nitrogen fraction represented about 15% (w/w) of total nitrogen at 46 h. Increases in free amino acids during tempe fermentation have been reported previously (Murata et al., 1967; Nowak and Szebiotko, 1992; Baumann and Bisping, 1995).

The nonfilterable-nitrogen fraction, representing proteins, nucleic acids and chitin, etc., includes nitrogen from soybean nitrogenous compounds that have been assimilated into fungal biomass. Hence, the proportion of soybean nitrogenous compounds (presumably mainly proteins) hydrolyzed is much greater than is suggested by net changes in protein (Table 3). The true hydrolysis of soybean protein can be estimated by combining the net decrease in total protein in the tempe with protein present in the mold mycelium (on the assumption that this is synthesized de novo). This suggests that nearly 25% (w/w) of the initial protein present in the cotyledons had been hydrolyzed in mature tempe at 46 h incubation (Table 3). Of this hydrolyzed protein, approximately 65%

remained in the tempe as amino acids and peptides, 25% was assimilated into mold biomass, and 10% was oxidized to ammonia.

The estimation of the true extent of protein hydrolysis as done in Table 3 is inherently rather imprecise, not least because it involves the calculation of differences and the use of multipliers of uncertain accuracy, such as N to protein and glucosamine to biomass conversion factors. Nevertheless, such calculations are useful to convey the scale of metabolic activities by the mold in the fermentation in a way that is not provided by simple proximate analysis.

It is clear that, during tempe fermentation of soybean, while lipids serve as the major energy source for the mould, proteins are also very substantially hydrolyzed. This study shows that the extent of the protein hydrolysis is substantially greater than has previously been suggested and in mature tempe may amount to 25% of the initial soybean protein.

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