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Protein Hydrolysis during Soybean Tempe Fermentation with *Rhizopus oligosporus*

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Hydrolysis of soybean protein during fermentation in a rotating drum reactor (RDR) was compared with hydrolysis during the traditional stationary tempe fermentation process. An increase of water-soluble nitrogen was observed. After 72 h of fermentation, 18.5, 25.4, 27.1, and 34.0% (w/w) of total N were found for the traditional stationary process at 25, 30, and 37 °C and for the RDR at 36 °C, respectively, compared to 2.0% in cooked soybeans at the start of the fermentation. The fungal activity in the RDR continued longer than in the traditional process. This was achieved by avoiding heat and mass transfer limitations. In the RDR a higher degree of hydrolysis, as well as higher levels of water-soluble and TCA-soluble nitrogen, were found after 72 h of fermentation compared with the stationary process at 37 °C. β -Conglycinin was hydrolyzed more rapidly than glycinin, which is probably related to the chemical structure; β -conglycinin is more sensitive toward protease activity.

Keywords: Soybeans; proteins; *Rhizopus oligosporus*; tempe; glycinin; β -conglycinin

INTRODUCTION

Tempe is a traditional Indonesian fermented food made from dehulled, soaked, and cooked soybeans inoculated with a mold, usually of the genus *Rhizopus*. After fermentation has occurred, the soybeans are bound together into a compact cake by dense cottony mycelium. *Rhizopus* spp. produce a variety of carbohydrases, lipases, proteases, phytases, and other enzymes, which hydrolyze soybean constituents and contribute to the development of the desirable texture, flavor, and aroma of tempe. Enzymatic hydrolysis may also decrease or eliminate antinutritional constituents. Consequently, the nutritional quality of the fermented product may be improved (Nout and Rombouts, 1990; Hachmeister and Fung, 1993).

The effect of fermentation on the total nitrogen content is negligible (Nowak and Szebiotko, 1992), but increases in the concentrations of free amino acids take place. The amino acid pattern, as quantified by the essential amino acid index, is not significantly affected during a 24 h fermentation. Longer fermentation results in losses of lysine (25% lost after 60 h; Winarno and Reddy, 1986; Stillings and Hackler, 1965). Up till now little attention has been paid to the effects of fermentation on the individual subunits of the major soybean proteins.

The major soy proteins are glycinin and β -conglycinin. Glycinin is composed of six subunits (A_n-B_n) which are formed of acidic polypeptides (A_1-A_5) and basic polypeptides (B_1-B_4). These acidic and basic polypeptides are covalently linked to each other by disulfide bonds, to form subunits. Glycinin subunits can be separated into distinct groups: I, $A_{1a}B_2$, $A_{1b}B_{1b}$, and A_2B_{1a} ; and II, A_3B_4 and $A_5A_4B_3$ (Yamauchi *et al.*, 1991).

β -Conglycinin, the other major constituent in soybeans, is less well-defined compared with glycinin.

β -Conglycinin is glycosylated and consists of three subunit combinations of α (57 000–68 000 Da), α' (57 000–68 000 Da), and β (42 000–52 000 Da), giving seven isomers (B_0-B_6) (Yamauchi *et al.*, 1991).

In this study we investigated the influence of incubation temperature and agitation on the proteolysis of soybean proteins during the tempe process. The results obtained with fermentation of *Rhizopus oligosporus* at 25, 30, and 37 °C in the traditional stationary process were compared with those obtained in a previously described rotating drum reactor (RDR) (De Reu *et al.*, 1993). Temperature control during solid-substrate fermentation can be achieved in this laboratory system by either discontinuous or continuous rotation and forced evaporation by aeration. As a result of the rotation a more-or-less granular product is obtained, in contrast to the cakelike product resulting from the traditional process.

MATERIALS AND METHODS

Organism. *Rhizopus microsporus* var. *oligosporus* strain LU 575 (NRRL 5905) was grown and maintained at 30 °C on malt extract agar (Oxoid, CM 59, Basingstoke, Hampshire, England). Sporangiospore suspensions were obtained as previously described by De Reu *et al.* (1994).

Tempe Manufacturing Process. Dehulled yellow-seeded soybeans (*Glycine max*) were soaked, cooked, inoculated, and packed in Petri dishes as described previously by De Reu *et al.* (1994). Petri dishes were incubated at 25, 30, or 37 °C. An autoclaved rotating drum reactor vessel (De Reu *et al.*, 1993) was filled with 1 kg of cooked and inoculated soybeans. The reactor was placed in a temperature-controlled incubator at 30 °C. During the experiments a discontinuous rotating scheme was used to avoid the substrate temperature's exceeding 36 °C. After the substrate temperature reached 36 °C, the vessel was rotated during 1 min according to the following sequence: clockwise (8 s, 8 rpm), hold (2 s), and counterclockwise (8 s, 6 rpm). Due to the rotation, the temperature of the substrate dropped 1.5–2.0 °C. After rotation, the reactor vessel was held until the substrate temperature of 36 °C was reached. Then the rotation cycle was repeated. This procedure was repeated continuously, resulting in a so-called "sawtooth" temperature pattern (De Reu *et al.*, 1993).

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Samples were taken at various times (0, 14, 18, 22, 26, 30, 38, 48, 55, 66, and 72 h) and immediately frozen with liquid nitrogen. The samples were stored at -20°C , lyophilized, and subsequently held at 4°C in the dark until analysis. Although incubation would normally be complete after 30–48 h in a commercial production setting, we continued the fermentation for up to 72 h to observe the effects of ongoing biochemical processes.

Fractionation of Nitrogen-Containing Compounds.

Lyophilized sample (7 g) was extracted with distilled water (35 mL), in a head-over-tail rotator during 30 min at 4°C . The suspension was centrifuged (27000g) for 10 min at 4°C . The supernatant was filtered through a glass filter (Whatman, glass microfiber, catalog no. 1820 037). The supernatant was designated water-soluble solids (WSS). The residue was washed twice with distilled water (50 mL), and the resulting filtrates were discarded. The residue remaining on the filter was added to the water-insoluble residue and was lyophilized and weighed; this fraction was designated water-insoluble solids (WIS).

The WSS fraction was boiled for 15 min to inactivate enzyme activity and was then centrifuged for 10 min at 27000g. The pellet, heat-precipitable water-soluble solids (HPWSS), was washed twice with distilled water and freeze-dried. The remaining supernatant was treated with trichloroacetic acid (TCA) to give a final concentration of 13.6% (w/v) TCA (Adler-Nissen, 1986). After one night of storage at 4°C , the suspension was centrifuged at 4°C for 15 min at 39100g. The TCA pellet was designated TCA-insoluble solids (TIS) and the supernatant as TCA-soluble solids (TSS). The TIS and TSS fractions were lyophilized and weighed.

All fractions were analyzed for their nitrogen content (w/w of dry matter) using the micro-Kjeldahl technique. The standard deviation of the method was determined by analyzing one sample four times. This resulted in a standard deviation of $\text{N} \pm 0.31\%$. On the basis of this result and the total data per fermentation condition, it was decided to analyze the other samples only once.

Electrophoresis. SDS-PAGE was performed on the freeze-dried tempe samples using a Protean II electrophoresis system (Bio-Rad, Richmond, CA) essentially according to the procedure of Laemmli (1970). Homogeneous slab gels [concentration monomers (T) = 12.5 or 15%, cross-linker concentration (C) = 2.2%] were used. The gels were stained with Coomassie Brilliant Blue R-250 according to the instructions of the manufacturer.

The optical density of the bands was measured using a laser densitometer (computing densitometer 300A, Molecular Dynamics, Sunnyvale, CA) equipped with a helium–neon laser (λ 672 nm) using Imagequant 3.15 software (Molecular Dynamics). Although band patterns for all of the obtained samples were available, we elaborated only on the data of samples after 22 and 66 h of incubation, as this method is very laborious. The major peaks were identified following the characterization of Wolf *et al.* (1992); these included peak 1, α' - and α -subunits of β -conglycinin; peak 2, β -subunits of β -conglycinin; peak 3, A_3 -glycinin; peak 4, A_{1a} , A_{1b} , and A_2 -glycinin; and peak 5, B-glycinin.

The peak areas were calculated and expressed as relative intensities. Although equal amounts of protein were used, differences in the absolute recoveries of protein can easily be expected due to possible variation in the (small) amounts of protein brought onto the gels. Therefore, the intensities themselves were not used to compare the various samples.

Degree of Hydrolysis. Twenty-five milliliters of a hot solution (80°C) of sodium dodecyl sulfate (10 g/L) and 1,4-dithiothreitol (3 g/L) was added to ± 2.0 g of sample (approximately 1 g of protein). The mixture was placed in a water bath (100°C) for 1 h. The solution was then cooled to room temperature, with continuous stirring. The solution was adjusted to pH 6, before excess (4.5 mL) formaldehyde (37%, Merck) was added. Finally, the mixture was titrated with 0.1 M sodium hydroxide to pH 9.5. The required volumes for both the fermented sample and the cooked soybeans determined the degree of hydrolysis (Adler-Nissen, 1986).

Table 1. Partition of Nitrogen (% w/w of Total N) in the Different Fractions of Soybeans

	WIS ^a (%)	WSS ^a (%)	HPWSS ^a (%)	TIS ^a (%)	TSS ^a (%)
raw beans	54.3	45.7	29.6	12.36	2.9
soaked beans	92.6	7.4	1.0	6.10	6.1
cooked beans	113.6	2.0	0.0	2.54	2.5

^a WIS, water-insoluble solids; WSS, water-soluble solids; HPWSS, heat-precipitable water-soluble solids; TIS, TCA-insoluble solids; TSS, TCA-soluble solids.

Table 2. Total Nitrogen and pH during the Fermentation of Soybeans with *R. oligosporus* at 25, 30, and 37 °C in Petri Dishes and at 36 °C in the RDR after 65 h

sample	total (N \times 6.25) content (% w/w of dry matter)	pH
raw beans	44.1	6.7
soaked beans	46.1	4.4
cooked beans	45.0	4.5
RDR, 65 h	49.5	5.9
25 °C, 65 h	51.7	5.8
30 °C, 65 h	52.4	5.8
37 °C, 65 h	53.8	6.1

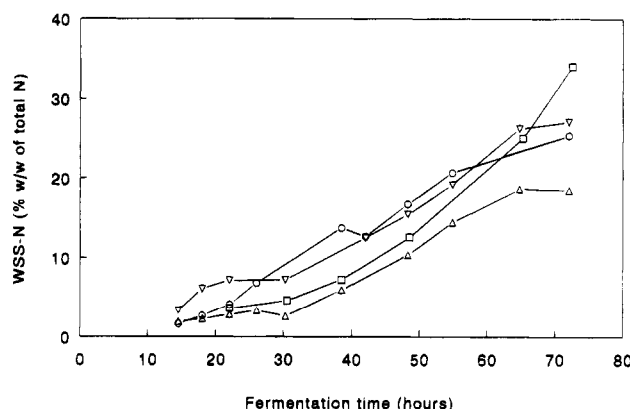


Figure 1. Water-soluble nitrogen (WSS-N) content in the fermentation of soybeans with *R. oligosporus* during the traditional process at 25 (Δ), 30 (○), and 37 °C (▽) and with the RDR at 36 °C (□).

RESULTS

Initially 45.7% (w/w of total N) of the nitrogen was water-soluble in the raw beans as shown in Table 1. During soaking and acidification the water-soluble nitrogen proportion decreased to 7.4% (w/w of total N). Cooking decreased the water-soluble nitrogen proportion further to 2.0% (w/w of total N).

During fermentation we observed increases in protein content (based on solids content) and pH of the freeze-dried material as shown in Table 2. During soaking the pH of the beans decreased from 6.7 to 4.4. Cooking resulted in a slight increase to 4.5, while during fermentation the pH increased to approximately 6.0.

The proportion of the water-soluble nitrogen (WSS-N) for the different fermentations is shown in Figure 1. The WSS-N proportion increased during fermentation initially in this order: 37 °C, 30 °C, RDR, and 25 °C. At 25 °C the increase in the proportion of WSS-N started slowly but still reached 18.7% (w/w of total N) after 72 h. Incubation at 30 and 37 °C increased the proportion of WSS-N to 25.4 and 27.1% (w/w of total N), respectively. In the RDR the proportion of WSS-N increased at the same rate as the traditional process at 25 °C up to 48 h. However, during the third day of the process the increase was more pronounced compared to any of the nonagitated fermentations and resulted in a final

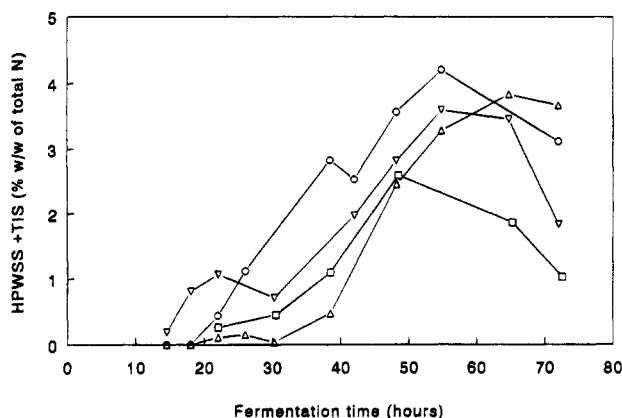


Figure 2. Sum of the residue after boiling the water-soluble fraction (HPWSS) and the TCA-insoluble fraction (TIS) in the fermentation of soybeans with *R. oligosporus* during the traditional process at 25 °C (Δ), 30 °C (\circ), and 37 °C (∇) and with the rotating drum reactor at 36 °C (\square).

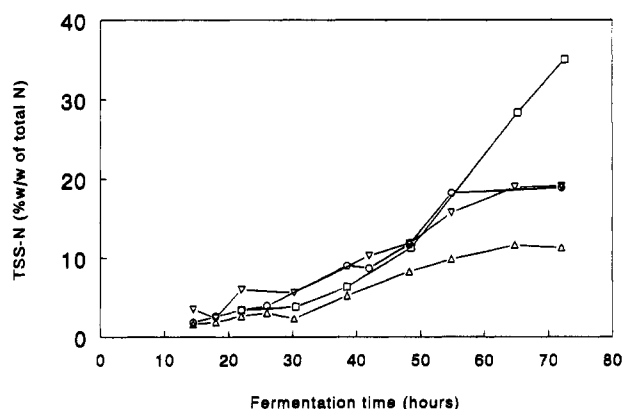


Figure 3. TCA-soluble nitrogen (TSS-N) content in the fermentation of soybeans with *R. oligosporus* during the traditional process at 25 °C (Δ), 30 °C (\circ), and 37 °C (∇) and with the RDR at 36 °C (\square).

WSS-N proportion of 34.0% (w/w of total N) after 72 h of fermentation. As expected, the WIS nitrogen proportion showed an inverse pattern to the increasing proportion of WSS-N (data not shown). The nitrogen proportions of the two fractions should add up to give 100%. In this study (40 samples), an average value of $95.1 \pm 4.6\%$ was obtained.

The sums of nitrogen present in the fractions HPWSS and TIS expressed as the proportion of total N are shown in Figure 2. They formed a minority of the WSS fraction, as a maximum of 4% (w/w of total N) was found in those fractions. The maxima indicate that after 50–60 h of fermentation, the fractions were hydrolyzed further to smaller peptides or amino acids which were soluble in 13.6% TCA. For the RDR the maximum is reached after 48 h and for the traditional process at 25, 30, and 37 °C after 65, 55, and 55 h, respectively.

The proportions of TCA-soluble nitrogen (TSS-N) for the different fermentations are shown in Figure 3. The level of TSS-N increased less at 25 °C than at higher temperatures. The differences between 30 and 37 °C were small, while after 50 h, the level in the RDR increased rapidly to 34% (w/w of total N).

The degree of hydrolysis (DH) of the different fermented samples is shown in Figure 4. The hydrolysis at 25 °C started later and increased less than at 30 and 37 °C. Until 48 h the differences between 30 and 37 °C and the RDR were small, but beyond 48 h the DH increased rapidly to a level of 46% in the RDR.

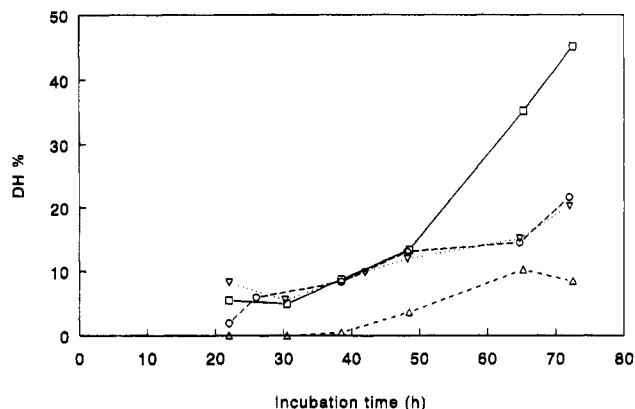


Figure 4. Degree of hydrolysis (DH) in the fermentation of soybeans with *R. oligosporus* during the traditional process at 25 °C (Δ), 30 °C (\circ), and 37 °C (∇) and with the RDR at 36 °C (\square).

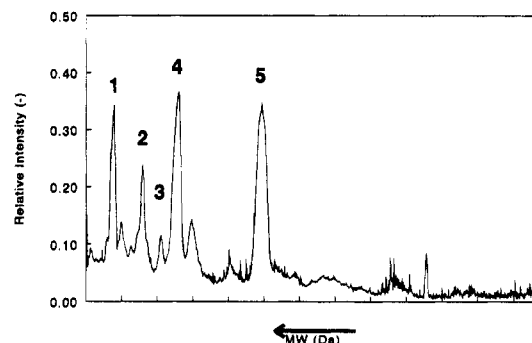


Figure 5. Intensities of bands on a representative SDS-PAGE gel of freeze-dried tempe obtained after 22 h of fermentation at 25 °C. Peak 1, α' - and α -subunits of β -conglycinin; peak 2, β -subunits of β -conglycinin; peak 3, A₃-glycinin; peak 4, A_{1a}-, A_{1b}-, and A₂-glycinin; peak 5, B-glycinin.

Table 3. Ratios of β -Conglycinin:Glycinin, α - and α' -Subunits of β -Conglycinin: β -Subunits of β -Conglycinin, and A-Glycinin:B-Glycinin in Soybeans during the Traditional Stationary Process at 25, 30, and 37 °C during the Agitated Process in the RDR at 36 °C

	β -conglycinin: glycinin		α - and α' -subunits: β -subunits of β -conglycinin		A-glycinin: B-glycinin	
	22 h	65 h	22 h	65 h	22 h	65 h
raw	0.52		3.77		1.54	
soaked	0.42		2.86		1.21	
cooked	0.43		1.65		1.33	
RDR	0.32	0.28	1.28	1.11	1.10	0.72
25 °C	0.35	0.23	1.77	1.24	1.03	0.94
30 °C	0.42	0.30	1.44	1.12	1.10	1.11
37 °C	0.39	0.31	1.68	0.77	1.35	1.20

The freeze-dried samples were analyzed by SDS-PAGE to characterize the hydrolysis pattern in tempe. A typical pattern is shown in Figure 5 after 22 h of fermentation at 25 °C. This pattern was determined by measuring the intensities of the different bands on the gel.

In Table 3 the changes in the ratio between β -conglycinin (peaks 1 and 2) and glycinin (peaks 3 + 4 + 5) as well as the ratio of α - and α' -subunits of β -conglycinin (peak 1) to β -subunits of β -conglycinin (peak 2) and the ratio of A-polypeptides of glycinin (peaks 3 + 4) to B-polypeptides of glycinin (peak 5) are shown during the process. During preprocessing and fermentation conglycinin appears to be hydrolyzed to a higher extent than glycinin, as the ratio decreased from 0.52 in the raw beans to a minimum of 0.23 after 65 h of fermenta-

tion at 25 °C. The α - and α' -subunits of β -conglycinin are hydrolyzed to a larger extent than the β -subunits of β -conglycinin, while the ratio of A-polypeptides of glycinin to B-polypeptides of glycinin decreased more rapidly in the RDR than in the nonagitated fermentations.

DISCUSSION

During fermentation a slight apparent increase in total protein content was observed. Several investigators reported on the total protein content during tempe fermentation. Van Buren *et al.* (1972) observed a slight decrease in total protein from 43.2 to 41.6% (w/w of dry matter). Slight increases were observed by Nowak and Szebiotko (1992) from 45.1 to 48.8% (w/w of dry matter), by Van der Riet *et al.* (1987) from 40.1 to 49.3 (w/w of dry matter), and by Murata *et al.* (1967) from 45.7 to 50.6% (w/w of dry matter). In addition, Baumann *et al.* (1990) concluded that there were no differences in nitrogen content during fermentation. The most likely explanation of an apparent increase in total protein content is that there is a shift in the composition of the dry matter during fermentation. There is a loss of 10% dry matter during fermentation (Van der Riet *et al.*, 1987). During fermentation carbohydrates and lipids (De Reu *et al.*, 1994) were used to form biomass, carbon dioxide, and water. This would result in a relative increase in protein content. Increases in total protein content are small but seem to be related to the growth rate of *R. oligosporus*. The optimum temperature for growth is about 37–42 °C; the lower the incubation temperature, the smaller the observed increases in total protein content.

The decrease in the water-soluble nitrogen during soaking was probably due to leaching into the soak water and more importantly to the drop in pH to 4.2 in the soak water. Lah and Cheryan (1980) showed that pH has a strong effect on the water solubility of soybean proteins. During cooking the remaining water-soluble protein became insoluble due to denaturation. In the traditional process the fractions TSS-N in WSS-N were 0.61, 0.74, and 0.70 after 72 h of incubation at 25, 30, and 37 °C, respectively, while in the RDR all WSS-N had turned into TSS-N after the same incubation time. On the basis of the higher values for the DH and the fact that almost all of the WSS-N was soluble in TCA, it can be concluded that in the RDR an exoprotease was active and that the product was hydrolyzed to smaller fractions (e.g. amino acids).

In the literature little attention has been paid to protease production by *R. oligosporus*. Wang *et al.* (1974) characterized the extracellular proteases as aspartic proteases, having endopeptidase activities. In the RDR the gradients of oxygen, carbon dioxide, and temperature are less than in the traditional system. Therefore, the activity of exoproteases might be related to the greater availability of oxygen, which enhances growth, and hence greater production of protease or to shear forces during rotation or to temperature.

Protease activity in *R. oligosporus* was measured intracellularly (2 units/mg of protein after 48 h of incubation), extracellularly (4.1 units/mg after 48 h of incubation), and in the debris (2 units/mg of protein) by Baumann (1992). It was not determined whether the proteases had exo or endo activity. Due to disruption of the hyphae, both the extracellular and intracellular enzyme activities as well as some of the activity in the debris might be pooled, and the resulting in-

creased level of extracellular activity might be an explanation for the increased degree of hydrolysis in the RDR.

In previous studies we have shown that it was possible to control the temperature during the fermentation of soybeans to tempe (De Reu *et al.*, 1993). In this study it is shown that there are no significant differences of proteolysis between the traditional stationary process and the rotating drum reactor during the first 48 h of fermentations. However, the fungal activity lasted longer in the RDR than in the traditional process, as shown by the higher degree of hydrolysis, the higher content of WSS-N, and the differences in TSS nitrogen. Figure 3 shows that during nonagitated fermentations (at 25, 30, and 37 °C) the TSS-N leveled off after 48 h while the level still increased in the RDR. This might be due to the limited heat and mass transfer in the nonagitated systems.

It is shown by De Reu *et al.* (1995) that the lagphase is reduced at higher incubation temperatures. Therefore, we were not surprised that in Figure 4 the rate of hydrolysis in the stationary process at 37 °C is higher than at 30 and at 25 °C.

During fermentation we observed a shift in the ratio of β -conglycinin to glycinin from 0.52 of raw beans to a minimum of 0.23 after 65 h of fermentation at 25 °C. This implies that β -conglycinin was decomposed more rapidly than glycinin. This phenomenon was reported earlier by Kim *et al.* (1990) for different microbial enzymes and also by Romagnola *et al.* (1990) in the rumen of Holstein cows. The resistance to degradation of glycinin compared to β -conglycinin is probably associated with its chemical and physical structure.

The shift in the ratio of α - and α' -subunits of β -conglycinin to β -subunits of β -conglycinin from 3.77 in the raw beans to 0.77 in the fermented product after 65 h of incubation at 37 °C implies that the α' - and α -subunits are more rapidly degraded during fermentation. Qi *et al.* (1992) observed similar patterns due to endogenous soybean protease activity (protease C₁) during seed imbibition. It is expected that during the soaking of the soybeans endogenous enzymes could be active; however, they will be inactivated during cooking. During the fungal fermentation stage the degradation of the α - and α' -subunits of β -conglycinin is prolonged. This is probably caused by fungal proteases instead of endogenous protease activity.

The shift in the ratio of A-polypeptides of glycinin to B-polypeptides of glycinin from 1.54 in the raw beans to 0.72 in the RDR after 65 h of incubation implies that the A-polypeptides are decomposed more rapidly than the B-polypeptides of glycinin; Romagnola *et al.* (1990) observed a similar pattern for proteolysis in the rumen of Holstein cows.

As tempe manufacture fermentation is usually stopped after 30–48 h, the differences in protein hydrolysis of the traditional product and the RDR product are small. Protein hydrolysis as an important factor affecting the taste of the final product is the subject of further study.

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