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In vitro digestibility of *Bacillus* fermented soya bean

J.L. Kiers*, A.E.A. Van laeken, F.M. Rombouts, M.J.R. Nout

Wageningen University, Agrotechnology and Food Sciences, Laboratory of Food Microbiology, Bomenweg 2, 6700 EV, The Netherlands

Abstract

Bacillus fermented legume products include among others dawadawa and soumbala made from African locust bean, and natto and kinema made from soya bean. *Bacillus subtilis* is the dominant species involved in the fermentation. During *Bacillus* fermentation for 48 h of autoclaved soya bean the quantity of soluble and dialyzable matter increased from 22% and 6% up to 65% and 40%, respectively. Protein and carbohydrate degradation during fermentation of soya bean with several *Bacillus* spp. was investigated and appeared to be substantial during the first 18 h of fermentation resulting in the release of high levels of peptides and oligosaccharides. In vitro digestibility was increased from 29% up to 33–43% after *Bacillus* fermentation for 48 h. It was shown that *Bacillus* spp. were able to degrade soya bean macromolecules to a large extent resulting in water-soluble low molecular weight compounds. In vitro digestion of *Bacillus* fermented soya bean using gastrointestinal enzymes only slightly increased the amount of dialyzable matter, which clearly demonstrated the beneficial effect of *Bacillus* fermentation on food nutrient availability.

Keywords: *Bacillus*; Soya bean; Fermentation; Degradation; Digestibility

1. Introduction

Bacillus subtilis is an important starter culture for Asian and African fermented soya bean foods like Japanese natto, Thai thua-nao, Indian kinema and West African dawadawa (Steinkraus, 1995). These fermentations are characterised by extensive hydrolysis of protein to amino acids, peptides and ammonia, and a rise of the pH. Although most indigenous fermented foods are the result of mixed culture fermentation, it has been shown that for kinema *Bacillus subtilis* is the dominant species (Sarkar et al., 1994).

Dawadawa is generally used as a flavouring agent rather than as a source of dietary protein or calories (Odunfa, 1986), although it is used as a meat substitute by poor families in West Africa (Steinkraus, 1995). However, kinema serves as a major source of protein in the Nepalese diet (Sarkar et al., 1997b). *Bacillus* fermentation of legumes reportedly resulted in improved digestibility (Odunfa, 1986; Sarkar and Tamang, 1995).

In this study we report the effect of fermentation of soya bean using pure cultures of *Bacillus* spp. on the solubilization and degradation of soya bean polymeric substances that may result in increased

*Corresponding author. Tel.: + 31-317-482241; fax: + 31-317-484893.

E-mail address: jeroen.kiers@micro.fdsci.wag-ur.nl (J.L. Kiers).

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digestibility. We used an in vitro digestibility model which has been described and discussed earlier (Kiers et al., 2000).

2. Materials and methods

2.1. Micro-organisms

Five strains of *Bacillus* spp. were selected based on their predominance in kinema and soumbala samples: *Bacillus subtilis* strains B82 and B83 isolated from kinema and B91 isolated from soumbala, *Bacillus badius* strain B97 isolated from soumbala and *Bacillus licheniformis* strain B70 isolated from kinema. All isolates belonged to different genotypical clusters based on randomly amplified polymorphic DNA (RAPD) analyses (data not shown).

Bacillus spp. were maintained on nutrient agar slopes (Oxoid CM3). Before use in an experiment the strains were inoculated into brain heart infusion broth (Difco 0037-17) and incubated for 18 h at 37°C. The culture was diluted in sterile distilled water with 0.85% NaCl and 0.1% peptone (PPS) to approximately 10^5 colony forming units (cfu)/ml^{-1}.

2.2. Fermentation of soya beans

Dehulled yellow-seeded soya beans (*Glycine max*) were soaked overnight in tapwater at 4°C to avoid fermentative acidification. Soakwater was discarded and the beans were cooked in fresh tapwater for 20 min (ratio beans:water of 1:3), cooled and superficially dried at room temperature. Cooked soya beans (100 g) were transferred into glass jars and autoclaved at 121°C for 30 min, cooled and inoculated with 5 ml of diluted culture. After mixing the beans were fermented at 37°C for 24 and 48 h using the five *Bacillus* strains separately. A second fermentation series was carried out with *Bacillus subtilis* B82 and samples were fermented for 6, 12, 18, 24, 36 and 48 h as described above.

2.3. pH measurements and microbial analysis

Samples (10 g) were homogenised with 90 ml of sterile water. The pH was measured in this suspension using a glass electrode. Prior to pH measurement decimal dilution series in PPS were prepared and 1 ml of the dilutions was mixed with molten (45°C) nutrient agar and poured into plates. After solidification a covering layer of nutrient agar was applied and the plates were incubated at 37°C for 24 h.

2.4. Solubility, absorbability and digestibility

Samples were freeze-dried and ground to a fine flour (Fritsch, type Pulverisette 14, Germany). All samples were defatted by extraction with petroleum-ether (40:60) in Soxhlet extractors, as this was required for use in the in vitro digestibility model (Kiers et al., 2000). The solvent was evaporated, and quantification of the total crude lipid content (% of dry weight) was carried out gravimetrically.

Solubility, absorbability and digestibility were determined as described earlier (Kiers et al., 2000). In short, residues obtained after defatting 5 g of sample were suspended in 30 ml distilled water and incubated for 30 min at 37°C. After centrifugation, the dry matter content of the supernatant and pellet was determined and solubility was calculated, representing the percentage of a defatted sample that is water-soluble. Supernatants were dialysed (Medicell Int Ltd, Visking, size 8, cut-off 12–14 kDa) against running tapwater for 15 h at 4°C. Retentates were quantitatively collected and their dry matter content was determined. The percentage of a defatted sample that was able to pass the dialysis membrane was defined as absorbability. Similarly, after solubilization samples were treated with α-amylase, pepsin, lipase and pancreatin. Digestibility was defined as the percentage of a defatted sample that was able to pass the dialysis membrane after enzymatic digestion.

2.5. Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using a Pharmacia PhastSystem according to the instructions of the manufacturer. To reduce the protein, in order to obtain subunits, disulfide bonds were cleaved by β-mercaptoethanol. Reduction and solubilization of protein was obtained by head over tail mixing for 90 min and treatment in an ultrasonification bath at 60°C for 15 min twice (Marsman et al., 1997).

Finally the sample was boiled for 15 min. Runs were performed in Gradient 8-25 Phastgels. Gels were fixed and stained with Coomassie Brilliant Blue R-250.

Since the samples based on autoclaved soya bean could not be separated adequately using this method, we decided to carry out an additional fermentation experiment. In this experiment we did not autoclave the soya beans after cooking as was done for the two other fermentation series. We inoculated the cooked soybeans with *Bacillus subtilis* B83 as described before and fermented the soya beans at 37°C for 24, 48 and 72 h.

2.6. Gel permeation chromatography (GPC)

Molecular weight distribution of the water-soluble dry matter was performed on a LC-10Ai HPLC (Shimadzu) equipped with a Superdex Peptide column (Pharmacia Biotech 17-5003-01) and elution at 30°C with 0.1% (v/v) trifluoroacetic acid and 30% (v/v) acetonitrile at 0.5 ml/min. Calibration was performed using a range of proteins and peptides ranging from 7000 Da to 200 Da. The eluate was monitored using a UV detector at 200 nm. On the basis of the calibration curve the molecular weight corresponding to various segments of the chromatogram was calculated. The area under the curve was determined in three segments (7000–5200 Da, 5200–1100 Da and 1100–200 Da) and was expressed as the percentage of the total area of the three segments together.

The supernatant obtained after determining the solubility (as described before) of the samples obtained from the second fermentation series was subjected to analysis.

2.7. High performance size exclusion chromatography (HPSEC)

High-performance size-exclusion chromatography (HPSEC) was performed on a SP8800 HPLC (Spectra Physics) equipped with three columns (each 300×7.5 mm) of Bio-Gel TSK in series (40XL, 30XL and 20XL; Bio-rad Labs) in combination with a TSK guard column (40×6 mm) and elution at 30°C with 0.2 M $NaNO_3$ at 0.8 ml/min. Calibration was performed using dextrans ranging from 500 kDa to 180 Da. The eluate was monitored using a refractive index detector.

Approximately 160 mg of defatted samples obtained from the second fermentation series was added to 10 ml 10% TCA, mixed, placed for 20 h at 4°C and centrifuged to remove protein and peptides. The supernatant was subjected to analysis.

3. Results

3.1. Growth and pH changes

The growth of the *Bacillus* spp. was rapid during the first 18–24 h (Tables 1 and 2) and exceeded 10^9

Table 1
Colony count, pH, solubility, absorbability and digestibility of *Bacillus* fermented soya bean[a]

Strain	Code	Time (h)	Colony count (log cfu g^{-1})	pH	Solubility (% defatted dry matter)	Absorbability (% defatted dry matter)	Digestibility (% defatted dry matter)
		0	3.0*	6.9*	22.3*	6.4*	28.9**
B. licheniformis	B70	24	9.4	6.5	52.1	30.7	ND
		48	9.4	7.6	54.6	32.6	ND
B. subtilis	B82	24	9.5	7.3	59.0	31.8	ND
		48	9.7	7.7	61.1	35.2	ND
B. subtilis	B83	24	9.6	7.2	52.8	29.0	34.4
		48	9.9	8.2	60.4	37.0	42.8
B. subtilis	B91	24	9.8	6.7	51.2	25.5	32.6
		48	9.3	7.8	61.9	35.9	37.9
B. badius	B97	24	8.8	7.4	51.5	26.9	30.9
		48	9.1	8.4	64.8	39.9	34.9

[a] Data represent the average of duplicate determinations. * Average of five time duplicate determinations; ** average of three time duplicate determinations; ND not determined.

Table 2
Colony count, pH, solubility, absorbability and digestibility of *Bacillus subtilis* (B82) fermented soya bean[a]

Time (h)	Colony count (log cfu g^{-1})	pH	Solubility (% defatted dry matter)	Absorbability (% defatted dry matter)	Digestibility (% defatted dry matter)
0	2.9	6.9	21.1	6.5	29.5
6	6.6	7.0	20.8	6.7	27.8
12	ND	ND	43.3	20.3	33.0
18	9.2	6.8	48.0	24.7	33.2
24	9.3	7.8	52.7	25.8	29.0
36	ND	ND	56.5	29.1	30.8
48	9.7	8.3	58.5	31.4	32.6

[a] Data represent the average of duplicate determinations. ND not determined.

cfu g^{-1} wet weight after 48 h fermentation. Although two of the five strains studied, B70 and B91, showed a small decrease in pH after 24 h of fermentation compared to time zero, a pH increase from 6.9 for the cooked and autoclaved beans to 7.6–8.4 after 48 h of fermentation was observed.

3.2. Changes in solubility and absorbability

During fermentation the crude lipid content increased from 28.8% to 32.7–37.2% of total dry matter after fermentation for 48 h (data not shown).

Water-soluble dry matter increased markedly after fermentation for 24 h and exceeded 50% of defatted dry matter (Table 1). The highest increase was observed between 6 and 12 h of fermentation for B82, whereas solubility increased during prolonged fermentation up to about 60% of total dry matter for all strains studied (Tables 1 and 2). Similarly, the quantity of dialyzable dry matter (absorbability) increased during fermentation. After fermentation 30–40% of the total defatted dry matter was dialyzable (Tables 1 and 2).

3.3. Protein degradation

SDS–PAGE analysis carried out on samples which had been cooked and subsequently autoclaved prior to fermentation resulted in a very poor separation of the soya bean protein subunits. The majority of the protein from the sample obtained at time zero could not diffuse into the gel, although samples obtained after 24 h of fermentation showed hardly any protein in the top of the gel anymore (data not shown). In Fig. 1 the SDS–PAGE profile is shown for cooked and subsequently fermented soya bean with *Bacillus subtilis* B83 for 24, 48 and 72 h (lanes 2–5). The major protein subunits can be clearly identified in the cooked soya bean (lane 2). Already after fermentation for 24 h (lane 3) all protein subunits are degraded to a large extent, and after 72 h of fermentation virtually all proteins have disappeared (lane 5).

During fermentation of soya bean with *Bacillus subtilis* B82 the amount of peptides (200–1100 Da)

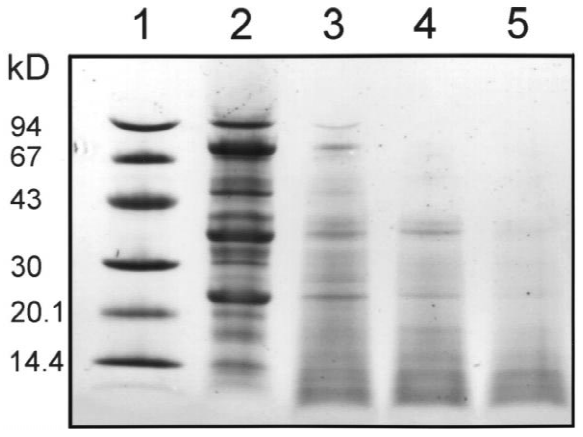

Fig. 1. SDS–PAGE profile of fermented soya bean. In lane 1 markers of several molecular weights are shown. Cooked soya bean fermented with *Bacillus subtilis* (B83) for 0, 24, 48 and 72 h is shown in lanes 2–5.

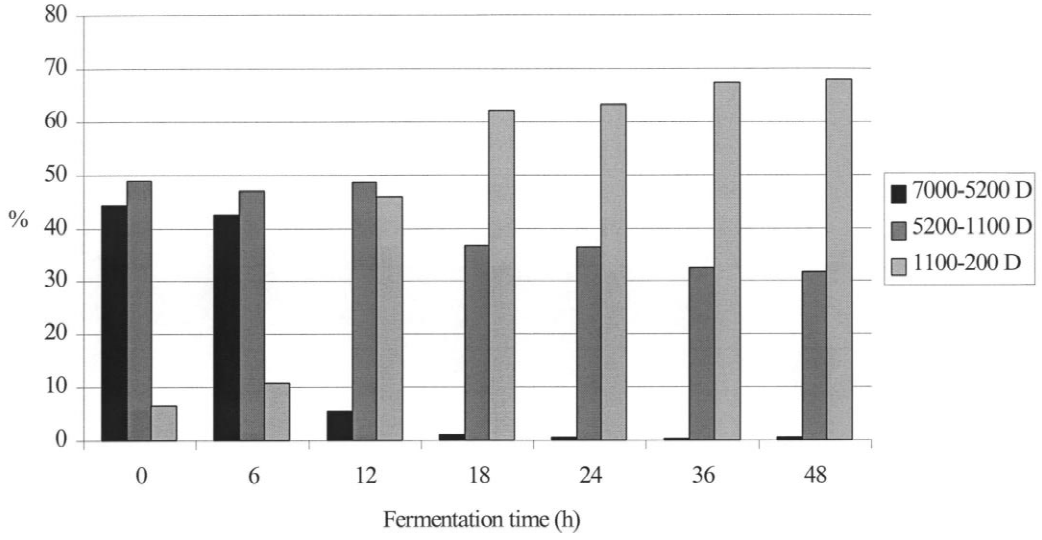

Fig. 2. Molecular weight distribution of water-soluble protein and peptides during *Bacillus subtilis* (B82) fermentation of soya bean. Bars indicate the percentage of the total (7000–200 Da).

increased markedly at the expense of the protein fraction (5200–7000 Da) (Fig. 2). After 18 h of fermentation only small changes were observed.

3.4. Degradation of polysaccharides

From the dextran standards used it was shown that fermentation of soya bean with *Bacillus subtilis* B82 resulted in a shift from polymeric to oligomeric matter of <5 kDa (Fig. 3). Fermentation times over 24 h resulted in an increased level of intermediate polymeric material from about 500–10 kDa.

3.5. Digestibility

From Tables 1 and 2 it can be seen that digestibility increased from 29% of defatted dry matter in cooked and subsequently autoclaved soya beans to 30–40% in soya beans fermented for 48 h. Compared to the values obtained for absorbability (quantity of dialyzable material without enzymatic degradation) these values are only slightly higher, except for time zero (Table 1) and 0–18 h (Table 2).

4. Discussion

4.1. Changes in pH

The pH increase observed during fermentation presumably resulted from proteolysis and the release of ammonia due to utilisation of amino acids for growth. The decrease in pH upon fermentation of soya beans during the first 24 h with two of the *Bacillus* spp. used could be due to the ability of these strains to initially use sugars as substrates for growth as shown before (Sarkar et al., 1993).

4.2. Increase in soluble and dialyzable material

We ascribe the ability of the *Bacillus* spp. to solubilize over 50% of the defatted dry matter to their high enzymatic activity. An increase in water-soluble compounds has been shown earlier for tempe fermentation where solubility increased from 7.0% for cooked soya bean to maximum of 27.3% after *Rhizopus oryzae* fermentation for 24 h (Kiers et al., 2000).

The increase in absorbability was very likely due to the breakdown of macromolecular substances into

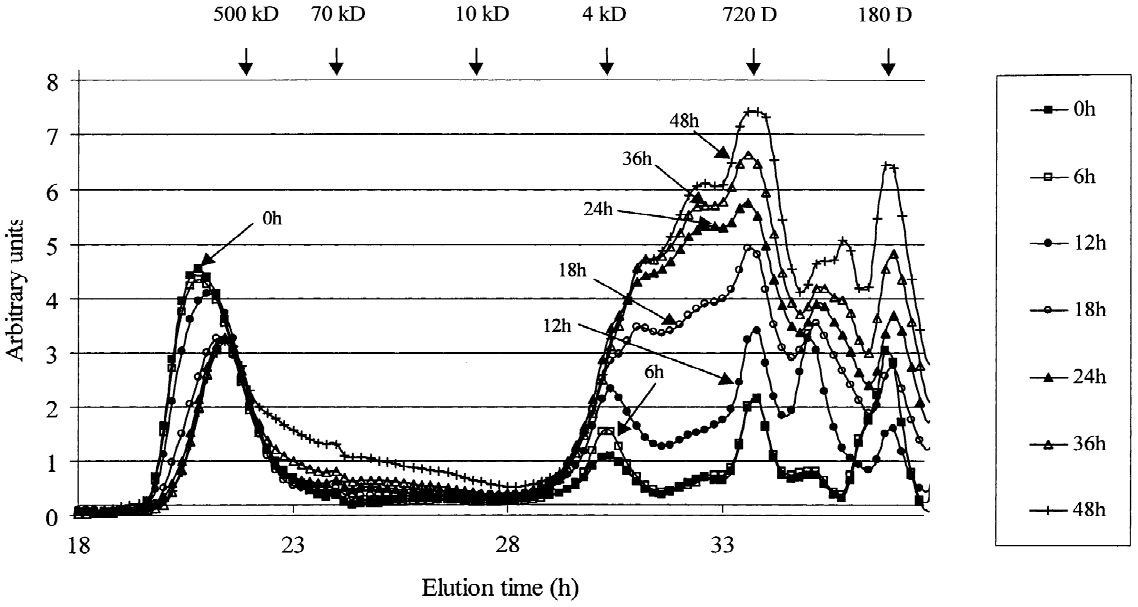

Fig. 3. HPSEC elution pattern of water-soluble carbohydrates during *Bacillus subtilis* (B82) fermentation of soya bean. Molecular weight of several dextran standards are shown at the top of the figure.

water-soluble low-molecular weight molecules, being small enough to pass the dialysis membrane.

4.3. Degradation of protein

Soya beans contain two main storage proteins, β-conglycinin and glycinin, which are composed of several subunits. β-conglycinin consists of three components, the α, α′ and β subunit, whereas glycinin consists of an acidic (A) and a basic (B) polypeptide. More or less complete breakdown of all three subunits from β-conglycinin and both polypeptides from glycinin was observed after *Bacillus subtilis* (B83) fermentation of cooked soya bean resulting in an accumulation of low molecular weight compounds as shown in Fig. 1. These observations reflect the considerable proteolytic activity reported for *Bacillus* spp. (Sarkar et al., 1993; Sarkar and Tamang, 1995). Maximum proteolytic activity is reached after 12–24 h and it remains fairly constant during prolonged fermentation (Allagheny et al., 1996; Sarkar et al., 1993). Processing of soya bean with *Bacillus subtilis* led to a 60-fold increase in free amino acids which accounted for approximately 26% of the total amino acid content (Sarkar et al., 1997b).

4.4. Degradation of carbohydrates

During the first 24 h of fermentation substantial quantities of polymeric matter were degraded into compounds <5 kDa, whereas during prolonged fermentation also intermediate compounds appeared. Levels of oligosaccharides like raffinose and stachyose responsible for flatus formation resulting from soya bean ingestion are reduced by soaking, cooking and *Bacillus* fermentation (Sarkar et al., 1997a). No identification of the oligosaccharides liberated during *Bacillus* fermentation was done in this study.

4.5. Digestibility

Bacillus fermentation of legumes has been associated with improvements in digestibility (Odunfa, 1986; Sarkar and Tamang, 1995). Digestibility of fermented soya beans was only slightly increased compared to the cooked and autoclaved beans, whereas absorbability was much higher for fermented soya beans. The same phenomenon was seen during tempe fermentation of cooked soya beans (Kiers et al., 2000), although the values found for absorbability and digestibility were higher in case of *Bacillus* fermentation. In case of tempe fermentation,

it was shown from the ratio between absorbability and digestibility that mould fermentation attributed up to 50–60% of the digestibility (Kiers et al., 2000). In case of *Bacillus* fermentation this ratio was higher than 80% after fermentation for 24 h increasing up to 95% after fermentation for 48 h. This points to a considerable predigestion during *Bacillus* fermentation, leading to a product in which luminal gastro-intestinal enzymes hardly can increase nutrient bioavailability.

5. Conclusion

Fermentation of soya bean using several *Bacillus* spp. resulted in major biochemical changes in the substrate leading to an increase in soluble and dialyzable material. Investigations at protein and carbohydrate level revealed major breakdown of polymers into water-soluble low molecular weight peptides, oligosaccharides and monosaccharides. From the results obtained it is concluded that after 18–24 h of fermentation considerable substrate modification had occurred leading to a product with high nutrient availability in which the need for degradation of nutrients by gastro-intestinal enzymes is minimal.

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References

Allagheny, N., Obanu, Z.A., Campbell Platt, G., Owens, J.D., 1996. Control of ammonia formation during *Bacillus subtilis* fermentation of legumes. Int. J. Food. Microbiol. 29, 321–333.

Kiers, J.L., Nout, M.J.R., Rombouts, F.M., 2000. In vitro digestibility of processed and fermented soya bean, cowpea and maize. J. Sci. Food. Agric. 80, 1325–1331.

Marsman, G.J.P., Gruppen, H., Mul, A.J., Voragen, A.G.J., 1997. In vitro accessibility of untreated, toasted, and extruded soybean meals for proteases and carbohydrases. J. Agric. Food. Chem. 45, 4088–4095.

Odunfa, S.A., 1986. Dawadawa. In: Reddy, N.R., Pierson, M.D., Salunkhe, D.K. (Eds.), Legume-based Fermented Foods. CRC Press, Boca Raton, FL, USA, pp. 173–189.

Sarkar, P.K., Tamang, J.P., 1995. Changes in the microbial profile and proximate composition during natural and controlled fermentations of soybeans to produce kinema. Food Microbiol. 12, 317–325.

Sarkar, P.K., Cook, P.E., Owens, J.D., 1993. *Bacillus* fermentation of soybeans. World J. Microbiol. Biotechnol. 9, 295–299.

Sarkar, P.K., Tamang, J.P., Cook, P.E., Owens, J.D., 1994. Kinema – a traditional soybean fermented food: proximate composition and microflora. Food Microbiol. 11, 47–55.

Sarkar, P.K., Jones, L.J., Craven, G.S., Somerset, S.M., 1997a. Oligosaccharide profiles of soybeans during kinema production. Lett. Appl. Microbiol. 24, 337–339.

Sarkar, P.K., Jones, L.J., Craven, G.S., Somerset, S.M., Palmer, C., 1997b. Amino acid profiles of kinema, a soybean-fermented food. Food Chem. 59, 69–75.

Steinkraus, K.H., 1995. Indigenous fermented foods involving an alkaline fermentation. In: Steinkraus, K.H. (Ed.), Handbook of Indigenous Fermented Foods. Marcel Dekker, New York, USA, pp. 349–362.