ELSEVIER

PII: S0308-8146(96)00118-5

Food Chemistry, Vol. 59, No. 1, pp. 69–75, 1997

0308-8146/97 $17.00 + 0.00

Amino acid profiles of kinema, a soybean-fermented food

P. K. Sarkar,[a]* L. J. Jones,[a] G. S. Craven,[a] S. M. Somerset[a] & C. Palmer[b]

[a]*Government Chemical Laboratory, Queensland Health, PO Box 594, Archerfield, Brisbane, Q 4108, Australia*
[b]*Amino acid Laboratory, Animal Research Institute, Queensland Department of Primary Industries, Locked Bag Nr 4, Moorooka, Brisbane, Q 4105, Australia*

(Received 3 November 1995; revised version received 16 January 1996; accepted 16 January 1996)

Kinema is an important protein source of traditional Nepalese diets. Total (protein-bound plus free) amino acid profiles of kinema and free amino acid profiles of unfermented and fermented soybeans were determined by reversed-phase high-performance liquid chromatography. Acidic, basic and aromatic amino acids constituted 30.8, 15.1 and 13.0%, respectively, of the total amino acid residues in kinema. The protein component (35.6%, dry weight basis) was rich in all essential amino acids (EAA); EAA_7 and EAA_{10} values (41.7% and 52.6%, respectively, of total protein) were comparable to those of egg and milk proteins. The free amino acid content of unfermented soybeans was only 0.2% of the total dry mass. Processing with *Bacillus subtilis* led to a 60-fold increase in free amino acid content of soybeans. There was a relative increase in free acidic, hydrophobic and apolar amino acids, but depletion of free basic, hydrophilic and sulphur-containing amino acids. However, the sum of free charged amino acids did not change. Although *Enterococcus faecium* had no influence, the presence of yeasts significantly reduced the levels of alanine, isoleucine, serine, aspartic acid, asparagine, arginine, tyrosine, methionine and hydroxyproline, suggesting their preferential uptake by the yeasts. On the other hand, amino acid assimilation by yeasts was indicated by the smaller increase in glycine, leucine, glutamine, lysine, histidine, phenylalanine and proline. The fermentation temperature of 45°C had a significantly adverse effect on free amino acid release.

INTRODUCTION

Kinema is an indispensable dietary component in some parts of the Indian subcontinent, where it has long been used as a meat substitute. To make traditional kinema, soybeans are washed, soaked overnight in water, boiled for about 90 min, crushed to grits, wrapped with fern leaves and sackcloth, and left to ferment for 1–3 days in a warm place. The resulting kinema is fried in oil, then cooked in water with vegetables and spices to prepare a thick curry, and eaten as a side dish with boiled rice (Tamang *et al.*, 1988).

Bacillus subtilis is the predominant microorganism in kinema and is solely responsible for kinema production. *Enterococcus faecium*, *Candida parapsilosis* and *Geotrichum candidum*, occurring in 100%, 50–80% and 40–50%, respectively, of market kinema samples, make up the accompanying flora (Sarkar *et al.*, 1994). Kinema quality can be improved by inoculating sterilized soybeans with *B. subtilis* and incubating at 45°C, leading to a better and faster process (Sarkar & Tamang, 1995).

Since kinema serves as a major source of protein in the Nepalese diet, the distribution of amino acids in kinema is an important nutrition issue. Although kinema contains 48% (dry weight basis) crude protein (Sarkar *et al.*, 1994), there are no published data on its amino acid profile. Therefore, the primary objective of this study was to determine the total (protein-bound plus free) amino acid profile of kinema to provide an insight into its nutritional qualities.

The high pH (approximately 8) of kinema is the result of soy protein hydrolysis and subsequent release of ammonia. During fermentation, there is a significant decrease in protein nitrogen with a coincident increase in water-soluble and trichloroacetic acid-soluble nitrogen content (Sarkar *et al.*, 1993, 1994; Sarkar & Tamang, 1995). Thus, the second aim of this study was to determine the free amino acid profile of kinema produced under various fermentation conditions.

*To whom correspondence should be addressed (Present address: Microbiology Laboratory, Department of Botany, University of North Bengal, Siliguri 734430, India).

MATERIALS AND METHODS

Organisms

Bacillus subtilis DK-W1 (MTCC[1] 1747), *Enterococcus faecium* DK-C1, *Candida parapsilosis* DK-Sm1 (MTCC 1744) and *Geotrichum candidum* DK-Ch1 (MTCC 1735), used in this investigation, were representative of isolates from commercial kinema (Sarkar *et al.*, 1994).

Preparation of kinema

Soybean seeds (*Glycine max* (L.) Merrill cultivar 'local yellow') were purchased from a market in Gangtok town (Sikkim, India) and stored in a polythene bag for about 1 year at room temperature (10–30°C) before use. The seeds were washed and soaked in deionized water (water:beans, 5:1, w/w) for 16 h at 22–25°C. After decanting the water, fresh water was added (water:beans, 2:1, w/w), autoclaved at 121°C for 15 min, and cooled to about 50°C. The cooked beans were drained, transferred to a sterile polythene bag and pestled from outside the bag so that about two-thirds of the beans were dehulled and the cotyledons separated and crushed to grits.

The bacillus, enterococcus and yeast inocula were prepared by introducing 5 ml of sterile water onto slant cultures of 18 h-old (incubated at 37°C) plate-count agar (Difco Laboratories, Detroit, MI, USA), 24 h-old (incubated at 37°C) all-purpose Tween agar (Difco) and 24 h-old (incubated at 28°C) malt-extract agar (Oxoid, Basingstoke, UK), respectively. Colonies were scraped off into tubes and agitated for 30 s on a Vortex mixer (Scientific Instruments, Springfield, MA, USA). Cell numbers were determined using a Neubauer counting chamber and a phase-contrast microscope. The suspensions were used as inocula with concentrations of bacillus, enterococcus and yeast cells of 10^{6-7}, 10^{5-6} and 10^{2-3}, respectively, per gram of sterilized beans. After mixing with inoculum, the beans were distributed in approximately 50 g (wet weight) amounts in sterile 250 ml Erlenmeyer flasks plugged with cotton wool, and incubated at 95% relative humidity in an environmental chamber. Processing variables included: beans unfermented and fermented in the presence of *B. subtilis*, *B. subtilis* plus *E. faecium*, and a mixture of all four microorganisms, and under fermentation temperatures of 37°C and 45°C.

Preparation of samples

Unfermented (soaked and cooked) and fermented beans (kinema) were blended for 1 min to smooth pastes using a Bamix (Switzerland) Model 122 blender. They were frozen at −20°C, freeze-dried (Dynavac freeze-drier Model FD12), ground to a fine powder, defatted with distilled petroleum ether (Recochem, Lytton, Australia) in Soxhlet extractors and stored in screw-capped plastic tubes at −20°C until use. The ether extract was evaporated under a stream of nitrogen and quantified-gravimetrically.

Extraction of amino acids

The procedure followed for protein hydrolysis was based on that of Finlayson (1964). Defatted samples (200 mg) were hydrolysed with 20 ml of 6 N HCl (Ajax Chemicals, Sydney, Australia) in sealed Pyrex tubes under nitrogen atmosphere for 18 h. Each hydrolysate was washed into a volumetric flask and made to an appropriate volume with glass-distilled water. Amino acid standard H (Pierce Chemical Co., Rockford, IL, USA) was made to an appropriate volume with 0.1 N HCl. An aliquot was spiked with DL-norleucine (Sigma Chemical Co., St Louis, MO, USA) as an internal standard, and dried under vacuum on a rotary evaporator (Buchi Laboratoriums-Technik, Flawil, Switzerland). The dried mass was washed twice with distilled water, evaporated to dryness between washings, and made to an appropriate volume with 0.1 N HCl. The sample solution was passed through a 0.45 μm cellulose acetate filter prior to analysis.

As cyst(e)ine and methionine are partially destroyed by acid hydrolysis, a separate analysis for these was required. They were oxidized with performic acid (Sigma) to cysteic acid and methionine sulphone, respectively, prior to hydrolysis, according to the procedure of Moore (1963). Cysteic acid and methionine sulphone standards were obtained from Sigma.

Tryptophan was determined by the method of Allred and MacDonald (1988). Defatted samples (300 mg) were hydrolysed with 10 ml of 4.2 N NaOH (Ajax) in a sealed Pyrex tube in a nitrogen atmosphere at 110°C for 20 h. The hydrolysate and a tryptophan standard (BDH, Poole, UK) were adjusted to pH 4.25 with 32% (v/v) HCl and made up to 50 ml with 0.2 N sodium citrate (pH 4.25) (Ajax). Aliquots (approximately 10 ml) were centrifuged (Centra-8R; International Equipment Co., Needham Heights, MA, USA) at 1800*g* for 10 min. Approximately 2 ml of the supernatant was transferred to an Eppendorf tube and spun for 20 min in a microfuge (Clements Orbital 100; Phoenix Scientific Industries, North Hyde, NSW, Australia) at maximum speed. The supernatant was poured into an autosampler vial for analysis.

Free amino acids were estimated using the procedure of Minero-Amador *et al.* (1992). Defatted samples (100 mg) were homogenized with 25 ml of 70% (v/v) ethanol (Recochem) in an Ultra-Turax (Janke and Kunkel, Staufen, Germany) Model TP18/10 for 10 s (approximately 1600 rpm) at 2°C. The homogenates were centrifuged at 1700*g* for 15 min at 2°C, and the supernatants decanted. The extraction procedure was repeated twice more. The supernatants were combined

[1]Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India.

and dried under vacuum on a rotary evaporator at 45°C, resuspended in 10 ml of 0.1 N HCl and filtered through a 0.22 μm filter (Millipore, Milford, USA).

Determination of amino acids

Amino acids were determined by reversed-phase high-performance liquid chromatography (RP-HPLC). Samples were analysed using a Waters system (Millipore) equipped with an interface module, Waters Model 510 pumps, a temperature control module, a Waters 80002 ion-exchange column, a post-column reaction system and a UV detector. Equipment controls, peak identification and integration were performed by computer using Waters Millennium 2010 Chromatography Manager Version 2. The buffering system, used as a mobile phase, contained buffer A [pH 3.08; 19.6 g litre^{-1} trisodium citrate (Ajax), 10 ml conc. HNO_3 (Ajax)] and buffer B [19.6 g litre^{-1} trisodium citrate, 58.4 g litre^{-1} NaCl (Ajax), adding HNO_3 dropwise to pH 6.45]. Ninhydrin (Fluka, Buchs, Switzerland) was used for the post-column derivatization of amino acids, and absorption was measured at 546 nm. Proline was simultaneously detected at 436 nm. The column was held at 60°C and the post-column reaction coil at 110°C. For tryptophan, a Waters μ-Bondapak C_{18} (10 μm silica particle size) column (300 mm×3.9 mm) was used with an 8.5 mM (pH 4.0) sodium acetate (Ajax) mobile phase and UV detection at 280 nm, and the column was held at 45°C. Amino acids were eluted by a gradient control from 100% A to 100% B at 0.5 ml min^{-1}. A 20 μl sample was applied to the column. Ninhydrin was pumped at a constant flow rate of 0.3 ml min^{-1}. Amino acids were quantified by comparing areas under the curve with those of the internal standard (norleucine).

Free amino acid levels were determined also by pre-column derivatization (Haynes *et al.*, 1991). For this analysis, the extracted amino acids were resuspended in borate buffer [200 mM boric acid (BDH, UK), pH 8.5 with 5 M NaOH (Fisons, Homebush, NSW, Australia) solution] instead of 0.1 N HCl as previously used. The amino acid solution (200 μl) was combined with 200 μl of 9-fluorenylmethylchloroformate [Fmoc-Cl (Sigma) dissolved in acetonitrile (EM Science, NJ, USA) as a 4.16 mg ml^{-1} solution] in a tube which was agitated and allowed to stand for 90 s. A 120 μl volume of cleavage reagent [prepared in 1 ml batches by mixing 680 μl of 850 mM NaOH solution with 300 μl of 500 mM hydroxylamine hydrochloride (BDH, Kilsyth, Australia) solution and 20 μl of 2-(methylthio)ethanol (Sigma)] was added and the solution mixed and allowed to stand. After 3.5 min, 200 μl of quenching reagent (acetonitrile–water–acetic acid, 20:3:2) was added, and an aliquot of the resulting mixture was analysed by HPLC. Amino acids were identified by comparing retention times with a standard mixture of Fmoc derivatives of 20 protein amino acids and 4-hydroxyproline (Sigma), and quantified by measuring peak heights.

For analysis of Fmoc derivatives of amino acids, the chromatographic system consisted of System Gold pumps (Beckman Instruments, San Ramon, CA, USA), a 5 μm Alltima C_{18} column, a Waters Nova-Pak C_{18} guard column, a column heater, a F1000 fluorescence detector (Hitachi Instruments, Danbury, CT, USA) with excitation wavelength of 263 nm and emission wavelength of 313 nm, and a C-R6A Chromatopac (Shimadzu, Tokyo, Japan) integrator. Derivatives were separated using a binary gradient. Eluent A contained 20 mM ammonium dihydrogen orthophosphate (BDH, Australia) [a 2.67 M stock solution was adjusted to pH 6.5 with NH_4OH (Ajax)] and 15% (v/v) methanol (Biolab Scientific, Clayton, Australia). Eluent B was 90% (v/v) acetonitrile in water. The flow rate was constant at 1.0 ml min^{-1} and the column was maintained at 37°C. A sample volume of 5 μl was introduced onto the column with a Waters WISP 710B automatic sample injector. The gradient profile was as follows: 0–3 min, 18% B; 3–10 min, 23% B; 10–36 min, 48% B; 36–37 min, 55% B; 37–50 min, 100% B; 50–55 min, 18% B.

Amino acid groupings

Amino acids were grouped (Barrantes, 1973, 1975) as basic (lysine + histidine + arginine), acidic (aspartic acid + glutamic acid + asparagine + glutamine), total charged (basic + acidic), hydrophilic (total charged + threonine + serine), hydrophobic (valine + leucine + isoleucine + phenylalanine + tyrosine + tryptophan + methionine) and apolar (hydrophobic−tyrosine).

Protein determination

Protein mass in kinema was determined as described by Horstmann (1979). A mean residue weight (WE, μg nmol^{-1}) was calculated using the formula:

$$\mathrm{WE} = \sum_{i=1}^{19} (a_i b_i)$$

where a is the mole fraction of amino acid i found in the analysed aliquot and b is the molecular weight of amino acid residue i (μg). A conversion factor (CF), which is the apparent average residue molecular weight increase in proportion to the missing tryptophan, cyst(e)ine and methionine values and which is characteristic for each protein, was calculated from the following equation:

$$\mathrm{CF} = \mathrm{WE}/[1 - (a_{\mathrm{Trp}} + a_{\mathrm{Cys}} + a_{\mathrm{Met}})]$$

A conversion factor CF′ (μg nmol^{-1}) was also calculated using the above equation for determining protein mass in the absence of tryptophan, cyst(e)ine, methionine and 4-hydroxyproline. The amount of protein P (mg) in each hydrolysate was then calculated as:

$$P = \mathrm{CF} \sum_{i=1}^{16} x_i$$

where x_i represents the quantity (nM) of each amino acid *i* found in the analysed aliquot.

Statistical analyses

Data were analysed by determining standard error of measurements, coefficient of variation and analysis of variance (Snedecor & Cochran, 1989).

RESULTS AND DISCUSSION

The total (protein-bound plus free) amino acid profile of kinema (Fig. 1(a)) and free amino acid profiles of unfermented and fermented soybeans are summarized in Table 1. Amino acids were determined by ion-exchange chromatography followed by post-column derivatization with ninhydrin. A procedure for pre-column derivatization of amino acids with Fmoc-Cl and HPLC for the separation of the derivatives with fluorometric detection was used to verify the data on free amino acids. Although a few of the protein amino acids could not be discerned following the latter method, the profiles obtained by these two methods compared fairly well (data not shown). There was 100% recovery of the standards. The values show high reproducibility with an average coefficient of variability for all amino acids of 5.3.

The very high acidic amino acid content of kinema was of particular interest. Glutamic acid was the most abundant amino acid, followed by aspartic acid, together representing 30.8% of total residues. The total basic amino acids (i.e. lysine, histidine and arginine) constituted

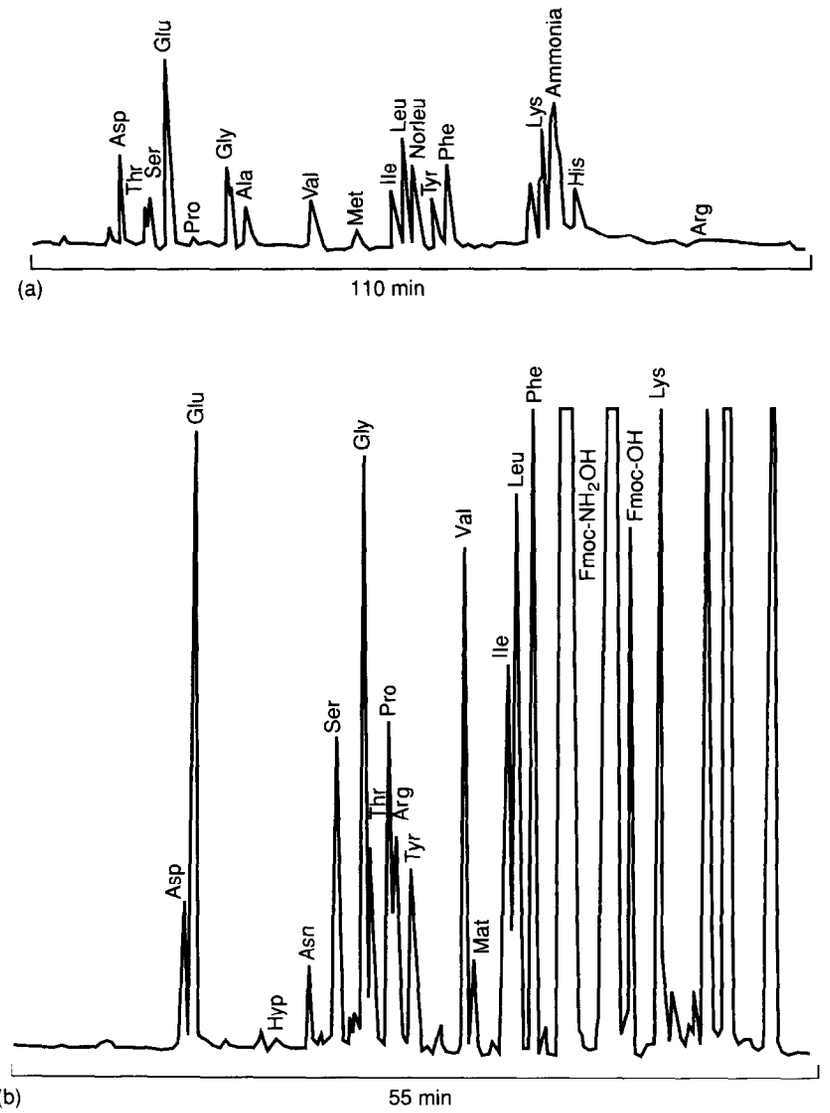

Fig. 1. Reversed-phase high-performance liquid chromatographic profiles of amino acids of kinema produced by fermentation of soybeans with *Bacillus subtilis* at 37°C for 48 h: (a) total amino acids derivatized with ninhydrin; (b) free amino acids derivatized with 9-fluorenylmethylchloroformate. Peaks are labelled with three-letter abbreviations for amino acids.

only 15.1% of the total. Aromatic amino acids and proline accounted for 13.0% and 5.6%, respectively, of the total. Tryptophan, cyst(e)ine and methionine were the major limiting amino acids in kinema.

Quantitative analysis of the ethanol-soluble fraction indicated that the free amino acid content was only 0.2% of the total dry mass of unfermented soybeans. This value increased significantly ($P < 0.05$) after fer-

Table 1. Amino acid profiles of processed soybeans (g kg^{-1} dry sample) in kinema making

Amino acid	Total (protein-bound and free) amino acids in fermented beans (Bs, 37°C, 48 h)	Free amino acids				
		Unfermented beans (soaked and cooked) (mean ± SEM)	Fermented beans (mean ± SEM)			
			Bs, 37°C, 48 h	(Bs + Ef, 37°C, 48 h)	(Bs + Ef + Cp + Gc, 37°C, 48 h)	(Bs, 45°C, 18 h)
Glycine	17.76	0.02c ± 0.00	5.08a ± 0.11	5.08a ± 0.03	3.97b ± 0.17	0.34c ± 0.00
Alanine	16.25	0.03c ± 0.00	4.73a ± 0.42	4.48a ± 0.06	2.71b ± 0.20	0.52c ± 0.05
Valine	19.91	0.08e ± 0.00	7.05b ± 0.12	7.17a ± 0.37	4.68c ± 0.30	1.18d ± 0.08
Leucine	30.97	0.10d ± 0.00	9.50a ± 0.13	9.76a ± 0.20	7.22b ± 0.34	2.49c ± 0.08
Isoleucine	18.11	0.06c ± 0.00	6.22a ± 0.33	5.88a ± 0.12	3.87b ± 0.25	0.88c ± 0.06
Serine	15.78	0.08c ± 0.00	3.24a ± 0.22	3.34a ± 0.06	1.51b ± 0.13	0.27c ± 0.00
Threonine	13.10	0.13d ± 0.00	2.79b ± 0.19	3.57a ± 0.06	1.80c ± 0.17	0.37d ± 0.03
Aspartic acid	44.93	0.10c ± 0.00	6.25a ± 0.22	6.07a ± 0.12	3.67b ± 0.31	0.20c ± 0.00
Glutamic acid	80.59	0.11e ± 0.00	21.06a ± 0.49	18.84b ± 0.19	14.55c ± 0.58	2.02d ± 0.19
Asparagine	ND	0.12c ± 0.00	2.87a ± 0.05	3.36a ± 0.19	1.49b ± 0.09	0.27c ± 0.02
Glutamine	ND	0.04c ± 0.00	0.54b ± 0.00	0.43b ± 0.00	1.34a ± 0.07	0.47b ± 0.04
Lysine	29.42	< DL	8.23a ± 0.28	8.28a ± 0.15	6.44b ± 0.26	1.85c ± 0.17
Histidine	12.58	0.09d ± 0.00	4.52a ± 0.07	4.34a ± 0.07	3.50b ± 0.12	1.66c ± 0.12
Arginine	19.59	0.27a ± 0.00	0.22a ± 0.02	0.12b ± 0.00	0.09b ± 0.00	0.09b ± 0.00
Phenylalanine	29.55	0.15d ± 0.00	11.82a ± 0.09	11.09a ± 0.17	8.91b ± 0.35	4.58c ± 0.37
Tyrosine	17.11	0.14c ± 0.00	4.86a ± 0.06	4.31a ± 0.00	2.80b ± 0.22	2.91b ± 0.25
Tryptophan	6.32	< DL	< DL	< DL	< DL	< DL
Cyst(e)ine	6.01	0.13 ± 0.00	< DL	< DL	< DL	< DL
Methionine	6.24	0.04e ± 0.00	3.15a ± 0.06	2.82b ± 0.00	2.13c ± 0.09	0.75d ± 0.07
Proline	22.83	0.09d ± 0.00	3.54a ± 0.03	3.33b ± 0.07	2.78c ± 0.09	0.25d ± 0.00
Hydroxyproline	ND	< DL	0.44a ± 0.00	0.52a ± 0.05	0.27b ± 0.00	0.11c ± 0.00
Ammonia	6.49	0.10c ± 0.00	1.14a ± 0.00	1.22a ± 0.00	1.19a ± 0.05	0.52b ± 0.00
EAA_7[a]	147.30	0.56d ± 0.01	48.76a ± 1.12	48.57a ± 0.98	35.05b ± 1.76	12.10c ± 1.22
EAA_{10}[a]	185.79	0.92d ± 0.02	53.50a ± 1.29	53.03a ± 1.02	38.64b ± 1.88	13.85c ± 1.27
Basic[b]	61.59	0.36d ± 0.00	12.97a ± 0.34	12.74a ± 0.22	10.03b ± 0.38	3.60c ± 0.30
Acidic[b]	125.52	0.37d ± 0.00	30.72a ± 0.71	28.70a ± 0.48	21.05b ± 1.21	2.96c ± 0.29
Total charged[b]	187.11	0.73d ± 0.01	43.69a ± 1.03	41.44a ± 1.90	31.08b ± 1.57	6.56c ± 0.58
Hydrophilic[b]	215.99	0.94d ± 0.00	49.72a ± 1.23	48.35a ± 0.72	34.43b ± 1.97	7.20c ± 0.65
Hydrophobic[b]	128.21	0.57d ± 0.02	42.60a ± 0.66	41.03a ± 0.81	29.61b ± 1.64	12.79c ± 1.26
Apolar[b]	111.10	0.43d ± 0.01	37.74a ± 0.69	36.72a ± 0.79	26.81b ± 1.33	9.88c ± 1.01
R_1[b]	1.68	1.65a ± 0.03	1.17b ± 0.00	1.18b ± 0.00	1.16b ± 0.00	0.56c + 0.00
R_2[b]	1.94	2.19a ± 0.07	1.32b ± 0.00	1.32b ± 0.00	1.28b ± 0.00	0.73c ± 0.00
R_3[b]	1.46	1.28a ± 0.03	1.03b ± 0.00	1.01b ± 0.00	1.05b ± 0.00	0.51c ± 0.00
R_4[b]	1.68	1.70a ± 0.06	1.16b ± 0.00	1.13b ± 0.00	1.16b ± 0.00	0.66c ± 0.00
Total AA-N (g kg^{-1} dry sample)[c]	58.97	0.36d ± 0.00	14.31a ± 0.30	14.08a ± 0.23	10.40b ± 0.50	3.08c ± 0.25
Total protein (g kg^{-1} dry sample)[d]	355.83					

Bs, *Bacillus subtilis*; Ef, *Enterococcus faecium*; Cp, *Candida parapsilosis*; Gc, *Geotrichum candidum*; ND, not determined; DL, detection limit (0.01 g kg^{-1} dry sample); AA-N, amino acid nitrogen; EAA, essential amino acids.

Values with standard error of measurements (SEM) were obtained from three replicates. Means within a row sharing common letter were not significantly different ($P < 0.05$).

[a] Calculated according to the method of Lee *et al.* (1978). EAA_7: valine, leucine, isoleucine, threonine, lysine, phenylalanine and methionine. EAA_{10}: EAA_7 plus histidine, arginine and tryptophan.

[b] Calculated according to the method of Barrantes (1973, 1975): ratio 1 (R_1), hydrophilic/hydrophobic; ratio 2 (R_2), hydrophilic/apolar; ratio 3 (R_3), total charged/hydrophobic; ratio 4 (R_4), total charged/apolar.

[c] Calculated according to the method of Heidelbaugh *et al.* (1975).

[d] Calculated according to the method of Horstmann (1979).

mentation. The free amino acids of kinema (Fig. 1(b)) accounted for approximately 26% of the total amino acid content. Processing of soybeans with *Bacillus subtilis* produced a very high proteolytic activity with subsequent ammonia production and a corresponding increase in pH (Sarkar *et al.*, 1993). The results indicate that fermentation led to an overall increase in free amino acids and ammonia by 60- and 40-fold, respectively. There was a relative enhancement of free acidic, hydrophobic and apolar amino acids and depletion of free basic, hydrophilic and sulphur-containing amino acids. The sum of free charged amino acids remained unchanged. The net decrease in some individual amino acid levels after fermentation suggests that they were metabolized to a greater extent than they were replaced by proteolytic activity. The decrease was particularly pronounced for arginine and cyst(e)ine. In addition, levels of some amino acids (alanine, isoleucine, serine, aspartic acid, asparagine, arginine, tyrosine, methionine and hydroxyproline) were significantly ($P < 0.05$) depleted when yeasts contributed to the *Bacillus* fermentation of soybeans (traditional fermentation). These results suggest a preferential uptake of arginine and cyst(e)ine by *Bacillus* and a strong demand for alanine, isoleucine, serine, aspartic acid, asparagine, arginine, tyrosine, methionine and hydroxyproline by yeasts. These findings are consistent with the nutritional requirements for amino acids described for a bacterium and a yeast during wheat sour dough fermentation (Collar & Martinez, 1993). Amino acid assimilation by yeasts was indicated by the smaller increase of some individual components (glycine, leucine, glutamine, lysine, histidine, phenylalanine and proline) during fermentation of samples with yeasts compared to those without. However, *Enterococcus faecium*, which had no detectable effects on the proteolytic activity, ammonia production or final pH value of the *Bacillus* fermentation of soybeans (Sarkar *et al.*, 1993), had no influence on either individual (except valine, threonine, glutamine, arginine, methionine and proline) or overall amino acid content of the *Bacillus*-fermented beans. The overall free amino acid content of kinema produced at 45°C for 18 h (the improved method) was approximately 5-fold less compared to that of kinema produced at 37°C for 48 h (the optimized conventional method).

Free 4-hydroxyproline was not detected in unfermented beans, but was found after fermentation. Indeed, this unique amino acid is rarely present in its free state in plant tissues. Soybean seed coat contains 77% of the total 4-hydroxyproline in the seed (Cassab *et al.*, 1985). This is present as a conjugated structural protein (Lamport, 1977).

There appeared to be a preferential accumulation of certain amino acids in the beans according to processing method. Barrantes (1973, 1975) suggested grouping amino acids into total charged, hydrophilic, hydrophobic and apolar classes, then comparing the ratio of the frequencies of occurrence of these groups. We used this method in the present study, and the results are summarized in Table 1. The data indicate a significant ($P < 0.05$) increase in hydrophobicity and decrease in total charges of the free amino acids after fermentation.

The EAA profile of kinema (Table 2) shows considerable amounts of all EAAs (FAO/WHO/UNU, 1985; FAO/WHO, 1990). Lee *et al.* (1978) attempted to classify the EAAs for predicting the nutritional quality of meat and meat products. They placed the EAAs in groups of either seven or ten amino acids. Kinema had EAA_7 and EAA_{10} values of 41.4% and 52.2%, respectively, of total protein. The EAA profile of kinema was compared with two high-quality protein foods (eggs and cow's milk) and also with that of the reference pattern established by FAO/WHO/UNU (1985) and FAO/WHO (1990). These results indicate that kinema proteins are a good source of almost all EAAs and that the EAA score is as high as that of egg and milk proteins.

Table 2. Comparison of the essential amino acid (EAA) composition of kinema with two high-quality animal foods and the suggested EAA pattern of requirements for humans

EAA	EAA (mg AA g^{-1} total protein)			
	Requirements for preschool child (2–5 years)[a]	Kinema	Animal products[a]	
			Eggs	Cow's milk
Histidine	19	35	22	27
Isoleucine	28	51	54	47
Leucine	66	87	86	95
Lysine	58	83	70	78
Methionine + cyst(e)ine	25	34	57	33
Phenylalanine + tyrosine	63	131	93	102
Threonine	34	37	47	44
Tryptophan	11	18	17	14
Valine	35	56	66	64
Total	339	532	512	504

[a]Data from FAO/WHO/UNU (1985) and FAO/WHO (1990).

These results provide valuable information on optimal kinema production conditions in order to produce high-quality kinema with elevated amino acid content.

ACKNOWLEDGEMENTS

This work was financed by the International Foundation for Science, Stockholm, Sweden (E/1479-2) and the Crawford Fund for International Agricultural Research, Melbourne, Australia. The authors are grateful to Dr J. H. Bradbury, Co-ordinator of Asia Pacific Food Analysis Network in the Australian National University, Canberra, Australia, for coordination of this work.

REFERENCES

Allred, M. C. & MacDonald, J. L. (1988). Determination of sulfur amino acids and tryptophan in foods and food and feed ingredients: collaborative study. *J. Assoc. Off. Anal. Chem.*, **71**, 603–606.

Barrantes, F. J. (1973). A comparative study of several membrane proteins from the nervous system. *Biochem. Biophys. Res. Commun.*, **54**, 395–402.

Barrantes, F. J. (1975). The nicotinic cholinergic receptor. Different compositions evidenced by statistical analysis. *Biochem. Biophys. Res. Commun.*, **62**, 407–414.

Cassab, G. I., Nieto-Sotelo, J., Cooper, J. B., van Holst, G. J. & Varner, J. E. (1985). A developmentally regulated hydroxyproline-rich glycoprotein from the cell walls of soybean seed coats. *Plant Physiol.*, **77**, 532–535.

Collar, C. & Martinez, C. S. (1993). Amino acid profiles of fermenting wheat sour doughs. *J. Food Sci.*, **58**, 1324–1328.

FAO/WHO (Expert Consultation) (1990). Protein Quality Evaluation. FAO/WHO Nutrition Meetings, Report Series 51. Food and Agriculture Organization/World Health Organization, Rome.

FAO/WHO/UNU (Expert Consultation) (1985). Energy and Protein Requirements. FAO/WHO Nutrition Meetings, Report Series 724. Food and Agriculture Organization/World Health Organization, Geneva.

Finlayson, A. J. (1964). Amino acid recoveries in the analysis of some feed samples. *Can J. Plant Sci.*, **45**, 184–188.

Haynes, P. A., Sheumack, D., Kibby, J. & Redmond, J. W. (1991). Amino acid analysis using derivatisation with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. *J. Chromatogr.*, **540**, 177–185.

Heidelbaugh, N. D., Huber, C. S., Bernarezyk, J. F., Smith, M. D., Rambaut, P. C. & Wheeler, H. O. (1975). Comparison of three methods for calculating protein content of foods. *J. Agric. Food Chem.*, **23**, 611–613.

Horstmann, J. H. A. (1979). A precise method for the quantitation of proteins taking into account their amino acid composition. *Anal. Biochem.*, **96**, 130–138.

Lamport, D. T. A. (1977). Structure, biosynthesis and significance of cell wall glycoproteins. In *Recent Advances in Phytochemistry*, Vol. II, eds F. A. Loewus and V. C. Runeckless. Plenum Press, New York, pp. 79–115.

Lee, Y. B., Elliott, J. G., Rickanarud, D. A. & Hagberg, E. C. (1978). Predicting protein efficiency ratio by the chemical determination of connective tissue content in meat. *J. Food Sci.*, **43**, 1359–1362.

Minero-Amador, A., Stewart, K. A. & Guillou, A.-M. (1992). Comparison of the amino acid composition and protein contents of two northern adapted *Asparagus* cultivars. *J. Agric. Food Chem.*, **40**, 2395–2403.

Moore, S. (1963). On the determination of cystine and cysteic acid. *J. Biol. Chem.*, **238**, 235–237.

Sarkar, P. K. & Tamang, J. P. (1995). Changes in the microbial profile and proximate composition during natural and controlled fermentation 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.

Snedecor, G. W. & Cochran, W. G. (1989). *Statistical Methods*, 8th edn. Iowa State University Press, Ames.

Tamang, J. P., Sarkar, P. K. & Hesseltine, C. W. (1988). Traditional fermented foods and beverages of Darjeeling and Sikkim—a review. *J. Sci. Food Agric.*, **44**, 375–385.