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International Journal of Food Microbiology 84 (2003) 1–12
INTERNATIONAL JOURNAL OF Food Microbiology
www.elsevier.com/locate/ijfoodmicro

Proteolytic activities in *togwa*, a Tanzanian fermented food

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Received 30 April 2001; received in revised form 18 July 2002; accepted 19 July 2002

Abstract

Proteolytic activities were investigated in sorghum-based *togwa* prepared by natural fermentation and using starter cultures previously isolated from the native product, i.e., *Lactobacillus brevis*, *Lactobacillus cellobiosus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Pediococcus pentosaceus*, and *Issatchenkia orientalis* in coculture with either *L. brevis* or *L. plantarum*.

Both proteinase and aminopeptidase activities were substantially higher in naturally fermented *togwa* than in those with starters (14–30%, 12–70%, respectively). A variable but substantial part of the proteinase activity followed the particulate fraction of *togwa*; aminopeptidase activity was mainly in that fraction. The breakdown of relatively high molecular mass protein (64 kDa) in *togwa* was detected by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE); the products were mainly in the 14–30 kDa range. Reversed-phase fast-protein liquid chromatography (RP-FPLC)-protein/peptide patterns changed during fermentation with some variation between *togwa* of different cultures. Supplementation of gruel with malt increased the concentration of total protein [from 9.5% to 11.0% (w/w) on dry weight basis)] and of most of the free amino acids. Fermentation had no effect on total protein content; however, the concentration of most of the amino acids was reduced, except for the proline content that increased. Natural fermentation also increased the concentration of glutamic acid and ornithine. Fermentation by *P. pentosaceus* increased aspartic acid, while *L. cellobiosus*, *L. fermentum*, and *L. brevis* in coculture with *I. orientalis* increased the concentration of citrulline.

Keywords: Sorghum; *Togwa*; Proteolysis; Starter cultures; Lactic acid bacteria; Yeasts

1. Introduction

The protein component of cereals constitutes the major portion of dietary proteins in developing countries (Lorri and Svanberg, 1993). In sorghum, kafirins and glutelins are major (storage) proteins, while albumins and globulins (enzyme proteins) are minor components (Anibaba et al., 1997; Youssef, 1998). Nutritionally, kafirin is of poor quality, as it contains no lysine and little tryptophan and threonine (Guiragossian et al., 1978), and it is the least digestible protein in sorghum (Hamaker et al., 1995). This may account for the poor nutritional quality of sorghum in comparison with most other cereals (Hamaker et al., 1995).

Cereal flour, lactic acid bacteria (LAB) (Spicher and Nierle, 1988), and yeasts (Ogrydziak, 1993) contain proteinases and peptidases. Cereal malts are used to initiate spontaneous fermentation in a number

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doi:10.1016/S0168-1605(02)00387-2

of African indigenous foods (Lorri and Svanberg, 1995; Steinkraus, 1996). The proteolytic activities in cereals increase considerably during germination (Gahlawat and Sehgal, 1994; Anibaba et al., 1997), and an improvement in the composition of some of the amino acids in sorghum has been observed (Mbugua et al., 1992). A significant decrease in dry matter with a concomitant increase in the content of soluble proteins and free amino acids, and in vitro protein digestibility have been reported for germinated millet grains (Pawar and Pawar, 1997).

Fermentation of African indigenous cereal-based gruels has also been reported to improve the nutritional quality, protein digestibility, and availability of amino acids (Mbugua et al., 1992; Lorri and Svanberg 1993; Steinkraus, 1996). Amino acids and peptides stimulate the growth and fermentative activity and tolerance of yeasts as well as proteolysis and lactate production by LAB, and these factors affect the sensory and nutritional quality of fermented foods (Collar et al., 1992; Martinez-Anaya, 1996). Many yeasts produce extracellular proteinases and some are used commercially for controlling chill-haze formation in beer and wine (Ogrydziak, 1993). The proteolytic/peptidolytic activity of LAB can contribute to debittering or liberation of bitter peptides (Habibi-Najafi and Lee, 1996) and bioactive peptides (Meisel and Bockemann, 1999). The reported improvement of the in vitro protein digestibility of *togwa*, a Tanzanian indigenous fermented food (Lorri and Svanberg, 1993), suggests that proteolysis takes place during the preparation of the product. A variety of substances that are protein in nature, referred to as bacteriocins, are produced by LAB and have inhibitory effect on other microorganisms (Stiles, 1994). *Togwa* has been reported to inhibit the growth and toxin production by enteropathogenic bacteria (Svanberg et al., 1992; Kingamkono et al., 1995, 1998).

Togwa is a cereal- or cassava-based fermented food widely produced in Tanzanian homes for use directly as a weaning food or diluted as refreshment (Mugula et al., 2001). Traditional fermentation is spontaneous and initiated by addition of either sorghum or millet malt and/or back-slopping gruel at ambient temperature. LAB and yeasts are the predominant microorganisms found in *togwa* (Mugula et al., 2001). The aim of the present study was to investigate the proteinase and aminopeptidase activities, the changes in proteins, their degradation products, and amino acids in sorghum-based *togwa*, prepared by natural and controlled fermentations with pure cultures of single or mixed strains of the LAB and yeasts isolated from native *togwa*.

2. Materials and methods

2.1. Characterization of LAB and yeasts

Duplicate samples of *togwa* (10 ml) were homogenized with 90 ml sterile peptone physiological saline solution (5 g peptone, 8.5 g NaCl, 1000 ml distilled water, pH 7.0 + 0.2). The homogenate was decimal diluted, and the relevant dilutions surface plated. M17 agar (Merck, Darmstadt, Germany) plates containing 0.1% (w/v) glucose were incubated aerobically and MRS agar (Merck) plates containing 0.1% (w/v) natamycin (Delvocid, Delft, The Netherlands) were incubated anaerobically (BBL Gas Pak, H_2, and CO_2; Becton-Dickinson, Cockeysville, MD, USA) for 48 h at 30 °C for the isolation of lactic acid bacteria (LAB). A total of 120 representative colonies was randomly picked from higher dilution plates of various fermentation stages and confirmed to be Gram-positive and catalase-negative. For subsequent purification and subculturing, M17 and MRS agar and broths were used. The pure bacterial cultures were inoculated into appropriate broth, incubated for 24 h at 30 °C, centrifuged (Kubota 2010, Kubota, Tokyo, Japan) at 3000 rpm for 15 min and the supernatant decanted. The cell pellets were resuspended either in sterile MRS or M17 broth containing 10% (v/v) glycerol. The suspension was aseptically transferred into sterile cryo-tubes containing acid-washed glass beads and stored at −80 °C until required for identification. Yeasts were isolated after incubation for 3–5 days at 25 °C on wort agar (WA, Merck) containing 0.01% (w/v) sterile oxytetracyline (Merck) or on Rose Bengal chloramphenicol agar (RBCA, Oxoid, Basingstoke, Hampshire, England) containing 0.01% (w/v) chloramphenicol (selective supplement, Oxoid). Purification and subculturing was done using potato dextrose agar (PDA, Oxoid) and yeast extract–malt extract (YM) broth. The purified yeast cultures were stored on PDA slants at 4 °C until required for identification.

The bacteria were characterized by microscopic examination and by conventional biochemical and physiological tests. The cultures were examined for colony and cell morphology; motility, cell arrangement, Gram reaction; catalase reaction; growth in broth at 10, 15, 40, and 45 °C; growth in presence of 2%, 4%, and 6.5% (w/v) NaCl; production of ammonia from arginine; production of dextran from sucrose; and production of carbon dioxide from glucose using Gibson's litmus milk. These tests were done according to procedures described by Harrigan and McCance (1990). The production of carbon dioxide was also determined in MRS and M17 broth, after incubation at 30 °C for 24 h, using an infrared gas analyzer (ADC 225 MK3, The Analytical Development, Hertfordshire, UK) connected to a Chromatopac (C-R3A, Shimadzu Analytical Instruments, Kyoto, Japan) according to Narvhus et al. (1992). Preliminary grouping for selection of 30 isolates for API tests was based on the abovementioned morphological, physiological, and biochemical characteristics. The fermentation pattern among carbohydrates was determined by using the API 50 CH gallery with the API 50 CHL medium (Bio Mérieux, Marcy-l'Etoile, France). Anaerobiosis in the inoculated tubes was obtained by overlaying with sterile paraffin oil. The inoculated galleries were incubated at 30 °C and the observations were made after 24 and 48 h. The identification of the isolates was facilitated by the use of a computer programme, APILAB PLUS, version 3.2.2. (Bio Mérieux) and reference to Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986) and Wood and Holzapfel (1995).

The yeast isolates were identified by using the Simplified Identification Method (SIM) described by Deak and Beuchat (1996), with additional standard taxonomical methods (Kurtzman and Fell, 1998), the use of ID32C diagnostic kits (Bio Mérieux), assisted by a computer software (API LAB PLUS version 3.2.2, Bio Mérieux). For the SIM, the tests included the fermentation patterns among D-glucose, fructose, raffinose, maltose, D-galactose (Merck), lactose, sucrose (BDH, Poole, England); and the assimilation patterns among xylose, melibiose, rhamnose, trehalose, mannitol, arabinose, citrate, soluble starch, cellobiose, D-ribose, melezitose, DL-lactate, L-sorbose, lactose, sucrose, galactose, and raffinose (Merck); erythritol, 2-ketogluconate, and α-methyl-D-glucoside (Sigma, St. Louis, MO, USA). Other tests included starch formation, cycloheximide (Sigma) resistance, urease activity, assimilation of potassium nitrate (Merck), L-lysine, and cadavarine (Sigma); growth at 37 and 40 °C; growth in 60% glucose–yeast extract agar, growth in the presence of 16% NaCl, growth in vitamin-free medium, growth in media containing 1% acetic acid, potassium sorbate or benzoate. The formation of mycelium and pseudohyphae was examined by microscopy of Dalmau plates; ascospore formation on Gorodkova agar, acetate agar, and YM agar; and the cell morphology on YM broth culture wet mounts.

2.2. Preparation of samples

Sorghum flour was mixed with water (1:9 w/v) and boiled for 20 min to gruel. Naturally fermented samples were prepared by supplementing the gruel, cooled to around 30 °C, either with sorghum malt alone or with sorghum malt followed by back-slopping with 1:9 (v/v) of native *togwa*, and 100-ml quantities in 250 ml screw-capped bottles were incubated at 30 °C. For controlled fermentation samples, the malt was added when the gruel was at 55–58 °C and were then left to cool for 30 min. They were then autoclaved at 121 °C for 15 min and cooled down to 30 °C prior to inoculation.

2.3. Preparation of starter cultures

Cultures of LAB (*Lactobacillus brevis*, *Lactobacillus cellobiosus*, *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Pediococcus pentosaceus*), and a yeast (*Issatchenkia orientalis*) isolated from native *togwa*, were used. LAB were cultivated by streaking on MRS agar (Merck) plates and incubated anaerobically (BBL, Gas Pak, H_2, and CO_2; Becton-Dickinson) at 30 °C for 24 h. A colony was picked from each pure culture plate, grown successively in MRS broth before centrifugation at $655 \times g$/15 min (Kubota 2010, Kubota). The pellet was washed in peptone physiological salt solution, centrifuged again and redistributed in peptone physiological salt solution. This procedure achieved an inoculum containing about 10^9 colony forming units (cfu)/ml, determined as viable counts on MRS agar. Pure cultures of *I. orientalis* were cultivated by streaking on Rose Bengal chloramphenicol agar (RBCA, Merck), incubated at 30 °C for 48 h

and the picked colony was inoculated into 10 ml of YM broth [3 g yeast extract (Oxoid), 3 g malt extract (Oxoid), 5 g peptone (Difco), 10 g glucose (Merck), 1000 ml distilled water, pH 6.9] and incubated at 30 °C for 24 h. These cultures were centrifuged and washed as described above. This procedure achieved an inoculum containing about 10^7 cfu/ml, as viable count on RBCA.

2.4. Fermentation

In controlled fermentation, 100 ml of sterile gruel was inoculated with 1 ml of LAB or 1 ml of yeast suspension. Mixed fermentation by LAB and yeast was initiated using 1 ml of each inoculum. Inoculated samples were thoroughly mixed (Vortex Gene-2, Model G-560E, Scientific Industries, Bohemia, NY, USA) and incubated at 30 °C. At 0, 8, 12, and 24 h of fermentation, samples were withdrawn for freeze-drying (Drywinner, model DW 6-85, Heto-Holten, Allerød, Denmark), below 0.6 mbar at −85 °C, and for protein extraction.

2.5. Protein extraction

Protein was extracted by a modification of the method of Hamaker et al. (1995). Samples (200 mg) of freeze-dried *togwa* were extracted for 16 h on an orbital shaker (280 rpm, Gallenkamp, UK) at 25 °C with 6 ml of a pH 10 buffer containing 0.0125 M sodium borate (Merck), 1% sodium dodecyl sulphate (SDS, Koch-Light Laboratories, Colnbook-Bucks, England), and 2% 2-mercaptoethanol (Merck). The mixture was centrifuged at 8160 × *g* for 10 min at 4 °C (Beckman J2-MC, Beckman Instruments, CA, USA) and the supernatant freeze-dried.

2.6. Activity of proteinases

The samples were assayed for proteinase activity using fluorescent casein as a substrate, by a modification of the method of Twining (1984) and Tobiassen et al. (1997). The incubation mixture consisted of 25 μl of reconstituted freeze-dried *togwa* (1:9 w/v) centrifuged at 11300 × *g* (Miramax Model 230, International Equipment, Needham Heights, MA, USA) for 5 min (particle-free supernatant) or not centrifuged, 60 μl of 500 mM sodium phosphate buffer pH 6.7, 3 μl of 10% sodium azide, and 60 μl of 0.5% fluorescent casein (Sigma). The mixture was incubated at 30 °C for 24 h. The reaction was stopped by addition of 400 μl of trichloroacetic acid (Sigma), and after 1 h at room temperature, the samples were centrifuged at 11300 × *g* (Miramax Model 230) for 5 min. A 400 μl portion of the supernatant was mixed with 2.6 ml of 500 mM Tris buffer (pH 8.5). The control consisted of uncentrifuged and unincubated mixture to which the stopping solution and buffer were added. Fluorescent casein, phosphate buffer, and sodium azide were also added to 25 μl of trypsin (type IX, five times crystallised, with activity of 15200 units/mg protein, Sigma) at concentration of 5 μg/ml, and the conditions of the assay were as for *togwa* samples. The proteolytic activity in samples was determined as trypsin equivalents (ng trypsin/min/ml) using a Perkin-Elmer LS-5 luminescence spectrophotometer (Perkin-Elmer, Norwalk, CT, USA) set for 1000 arbitrary fluorescence units at 525 nm (emission) and 490 nm (excitation).

2.7. Activity of aminopeptidases

Aminopeptidase activity was assayed under conditions as outlined for caseinolytic activity, but using 0.4 mM H–Ala–amino–4-methylcoumarin (Bachem, Bubendorf, Switzerland) as a substrate. The stock solution (10 mM) was dissolved in dimethyl sulphoxide. The enzyme activity in samples was determined by quantifying the concentration of the released 7-amino-4-methylcoumarin (nmol/min/ml). The released 7-amino-4-methylcoumarin and its standard (Sigma) were measured, according to Goossens et al. (1992), by using a Perkin-Elmer LS-5 luminescence spectrophotometer (Perkin-Elmer) set for 1000 arbitrary fluorescence units with 2.5 μM 7-amino-4-methylcoumarin and fluorescence at 440 nm (emission) and 370 nm (excitation).

2.8. Separation of proteins and their degradation products by SDS-PAGE

A 0.05 g freeze-dried protein extract sample was added to a 5 ml mixture containing 10% Tris (hydroxymethyl aminomethane hydrochloride) buffer (pH 8.8) and 1% sodium dodecyl sulphate (SDS). The mixture was shaken to dissolve the sample. The mixture (1 ml)

was mixed with 20 μl of 2% 2-mercaptoethanol, heated in steam for 3 min, cooled in ice before adding 10 μl of bromothymol blue. Proteins and peptides were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with PhastSystem (Pharmacia Biotechnology, Uppsala, Sweden) and silver-stained according to the protocols based on the procedures of Heukeshoven and Dernick (1985) and Blum et al. (1987) and supplied with PhastSystem Owner's Instruction Manual (1987). The system consists of a separation and control unit, a development unit and high-performance Phastgel separation media. The proteins and peptides in gels were fixed and sensitised at 30 °C using 5% glutaraldehyde, 30% ethanol, 1.5% iso-propanol and 0.03% sodium acetate. The gels were washed with reagent water, 10% ethanol, and 5% acetic acid, and stained with 0.4% silver nitrate in reagent water. The gels were developed in 2.5% sodium carbonate containing 2.0% formaldehyde; nonspecific background staining reducer (2.5% sodium thiosulphate and 3.7% Tris–HCl, pH 8.8 (Sigma), and then preserved with 10% acetic acid and 10% glycerol in reagent grade water.

The electrophoretograms were used to quantify the molecular mass of proteins with an included molecular mass marker protein (Pharmacia), using a computerised densitometer (Colour Image Scanner Model JX-330 with scanner software Sharp Twain/Win, version 2.2x, Sharp, Japan) and Labscan, version 2.01 (Pharmacia). The scanned images were analysed and bands quantified with Image Master 1D Elite, version 2.01 (Pharmacia) software.

2.9. Separation of proteins and their degradation products by RP-FPLC

Proteins and peptides soluble in 0.065% trifluoroacetic acid were separated by a modification of the method of Stepaniak and Fox (1996) by reversed-phase fast-protein liquid chromatography (RP-FPLC), with FPLC equipment (Pharmacia) and a UV detector operating at 214 nm. A 10-mg sample was dissolved in 1.6 ml of 0.065% trifluoroacetic acid (eluant A) and centrifuged at 17 600 × *g* for 10 min (Miramax, Model 230). Supernatant (0.5 ml) was loaded onto a Source 15RPC ST column (Pharmacia, diameter 4.6 mm; length 100 mm) and eluted at a flow rate of 1.0 ml/min by using a 0.050% TFA in (80%) acetonitrile (Rathburn, Walkerburn, Scotland) (eluant B) gradient from 0% to 15% between 3 and 30 min, from 15% to 30% between 30 and 50 min and from 30% to 100% between 50 and 60 min. Unfermented gruel served as control and was treated as described above.

2.10. Total protein content

Protein contents were determined by the Kjeldahl method (AOAC, 1990). A freeze-dried sample (0.1 g) was digested at 420 °C in 3.00 ml concentrated sulphuric acid, 1.5 g potassium sulphate, and 7.5 mg selenium as a catalyst, until the solution cleared. The solution was distilled, sodium hydroxide and a mixture of methyl red and bromocresol green indicators in boric acid added, and the concentration of total protein was determined by titration with 0.05 M hydrochloric acid (% protein=% nitrogen × 6.25) using a Tecator Kjeltec Auto Sampler System Analyzer (Model 1035, Sweden). Skim milk was used to calibrate the instrument.

2.11. Amino acid content

The distribution of free amino acids in *togwa* was determined by automated reversed-phase high-performance liquid chromatography (RP-HPLC) and the extraction and deproteination were carried out according to Ardö and Polychroniadou (1999) with modifications. Two grams of freeze-dried *togwa* was extracted and deproteinated in 10.0 ml of 0.1 M hydrochloric acid solution containing 0.5 μmol/ml L-norvaline (Merck) and piperidine-4-carboxylic acid (Merck) as internal standards in a 25-ml centrifuge tube and homogenized on Ultra-Turrax (PRO Scientific, Monroe, USA) for 5 min at 20 000 rpm. The covered tube (Para-Film) was kept for 30 min in an ultrasonic bath (Bransonic B-220, Carouge, Geneva, Switzerland) and centrifuged at 2200 × *g* for 10 min at 4 °C. Two millilitres of supernatant were added to 2.00 ml of 400 g/l trichloroacetic acid. The suspension was mixed on a Vortex mixer (Scientific Industries) then kept in an ice bath for 10 min and centrifuged at 14 800 × *g* for 10 min at 4 °C. The pH of the clear supernatant was adjusted to 7.5 with NaOH before the derivatization of the amino acids. The solution was filtered through a 0.45-μm membrane (Advantec MFS, Pleasanton, CA, USA) before injection. Amino

acid standards (L-amino acid kit, LAA-21) were obtained from Sigma.

Derivatization of the amino acids was carried out by mixing 150 μl of borate buffer (0.4 M, pH 10.4), 10 μl *o*-phtaldialdehyde/3-mercaptopropionic acid reagent (OPA/MPA, Agilent Technologies, Switzerland) reagent and 5 μl sample solution for 1 min. Ten microlitres of 9-fluorenylmethylchloroformate (FMOC) in acetonitrile was added and the solution was mixed for 1 min, and 1 μl of the solution was injected directly into the HPLC system (Perkin-Elmer pump series 410; auto injector ISS 200; column oven series 200; fluorescence detector LC 240; Turbochrom LC terminal version 4.1 software and Interface 900 series; Hypersil ODS 250 × 4 mm id column and 4 × 4 mm id precolumn), as OPA derivates are not stable.

Chromatographic conditions were as follows: mobile phase solvent A, 30 mM NaOAc, pH 7.20 + 0.25% tetrahydrofuran + 0.1 M titriplex III ($C_{10}H_{14}N_2Na_2O_8{\cdot}2H_2O$, Merck); solvent B, 100 mM NaOAc, pH 7.20 + 80% acetonitrile + 0.1 M titriplex III; flow rate, 1.0 ml/min; column temperature 42 °C. The derivatized amino acids were separated by a 24-min stepwise linear gradient from 3.3% to 40% B over 20 min and 40% to 100% B over 4 min. Column cleanup with 100% B and reequilibration required 10 min. The detector parameters were set to detect the OPA derivates at the start of the programme at excitation 340 nm and emission 455 nm then changed at 22 min to detect FMOC derivates at excitation 266 nm and emission 313 nm.

2.12. Statistical analysis

The data obtained were subjected to analysis of variance (SAS/stat, 1996), and mean differences determined by Duncan's multiple range test ($P < 0.05$).

3. Results

3.1. Phenotyping of starter cultures isolated from togwa

Among the 120 LAB isolates, rods accounted for 90%, cocci 10%, dextran producers 36%, CO_2 producers 70%, while 62% of the isolates were able to grow at 45 °C, and 34% tolerated 6.5% NaCl. The cocci were homofermentative, grew at 10–45 °C and hydrolysed arginine. The bacteria isolates identified by the use of a computer programme, APILAB PLUS, version 3.2.2. (Bio Mérieux) and reference to Bergey's Manual of Systematic Bacteriology (Sneath et al., 1986) and Wood and Holzapfel (1995) were tentatively identified as: *L. brevis*, *L. cellobiosus*, *L. fermentum*, *L. plantarum*, *P. pentosaceus*, and *W. confusa*. The species were isolated from all stages of fermentation, and *L. plantarum* dominated the final stages of fermentation.

The yeast isolates identified by using the SIM procedure described by Deak and Beuchat (1996), as well as reference to the standard taxonomic key

Table 1
Proteinase and aminopeptidase activities[a] in *togwa* prepared by natural fermentation and using starter cultures

Starter culture	Proteinase activity (ng trypsin/min/ml)		Aminopeptidase activity (nmol AMC[b]/min/ml)	
	Total	Supernatant	Total	Supernatant
Naturally fermented (NF)[c]	1019a	502a	92.3a	4.4a
Lactobacillus brevis (LB)	237b	34d	51.7bc	1.6c
L. cellobiosus (LC)	306b	106c	25.4c	1.7c
L. fermentum (LF)	242b	44d	28.6c	1.2c
L. plantarum (LP)	141c	104c	10.2d	1.4c
Pediococcus pentosaceus (PP)	270b	87c	61.5b	1.3c
LB + *Issatchenkia orientalis* (LBIO)	271b	139b	69.4b	2.5b
LP + *I. orientalis* (LPIO)	217b	136b	9.6d	1.3c

Values are means of triplicate determinations (on a dry weight basis).
Figures in columns with the same letter are not significantly different ($P < 0.05$).
[a] Per milliliter of enzyme preparation; total = uncentrifuged *togwa*; supernatant = centrifuged *togwa*.
[b] AMC = released 7-amino-4-methylcoumarin.
[c] Started by addition of malt and back-slopping.

outlined by Kurtzman and Fell (1998) and the use of ID32C diagnostic kits (Bio Mérieux), assisted by a computer software (API LAB PLUS version 3.2.2, Bio Mérieux), were tentatively identified as: *I. orientalis* (50%), *S. cerevisiae* (23%), *C. tropicalis* (10%), and *C. pelliculosa* (17%). The species were isolated from all stages of fermentation.

3.2. Enzymatic activities

Table 1 shows the proteinase and aminopeptidase activities in *togwa*. Particle-free supernatants had less activity in comparison with uncentrifuged samples. A variable but substantial part of the proteinase activity followed the particulate fraction of *togwa* and amnio-

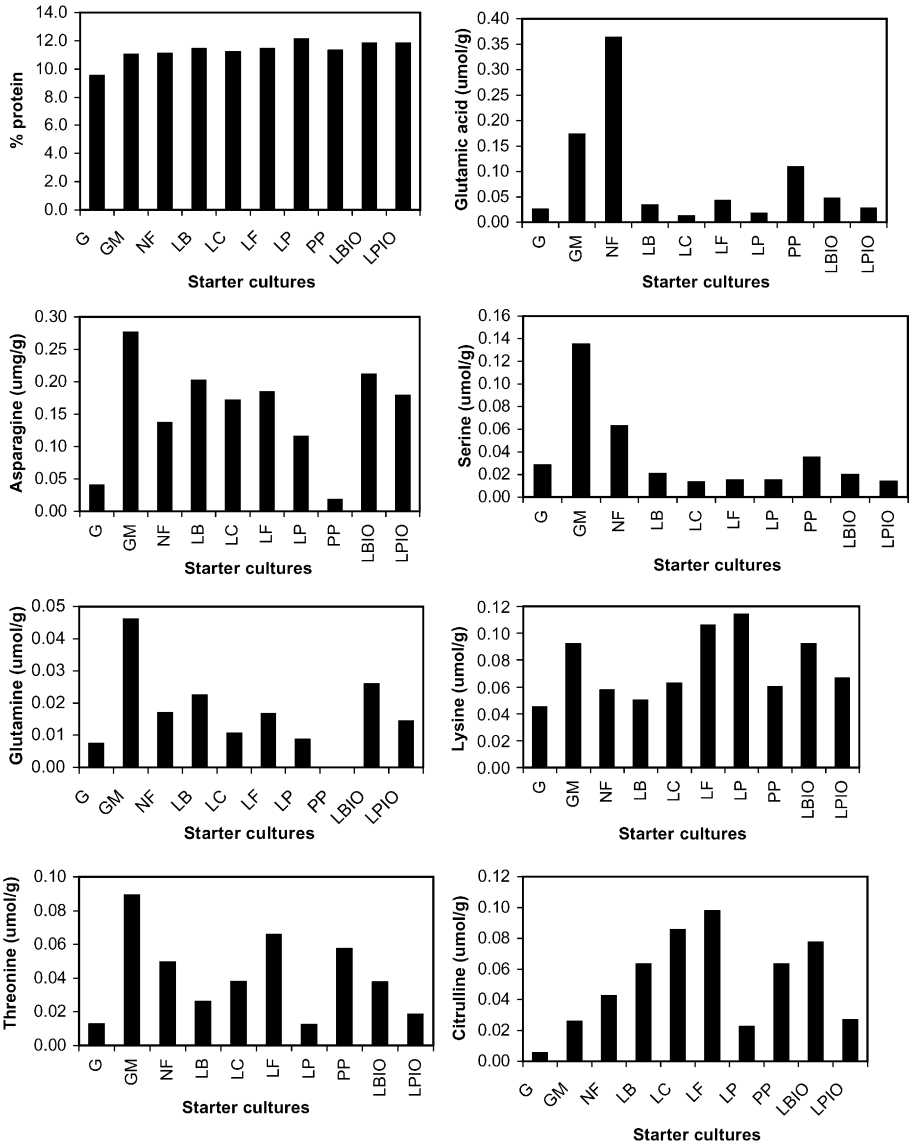

Fig. 1. Total protein and amino acid content of gruel (G), gruel with added malt (GM) and togwa fermented naturally (NF), and by *L. brevis* (LB), *L. cellobiosus* (LC), *L. fermentum* (LF), *L. plantarum* (LP), and *P. pentosaceus* (PP), and *I. orientalis* in co-culture with either *L. brevis* (LBIO) or *L. plantarum* (LPIO). Values are means of triplicate determinations.

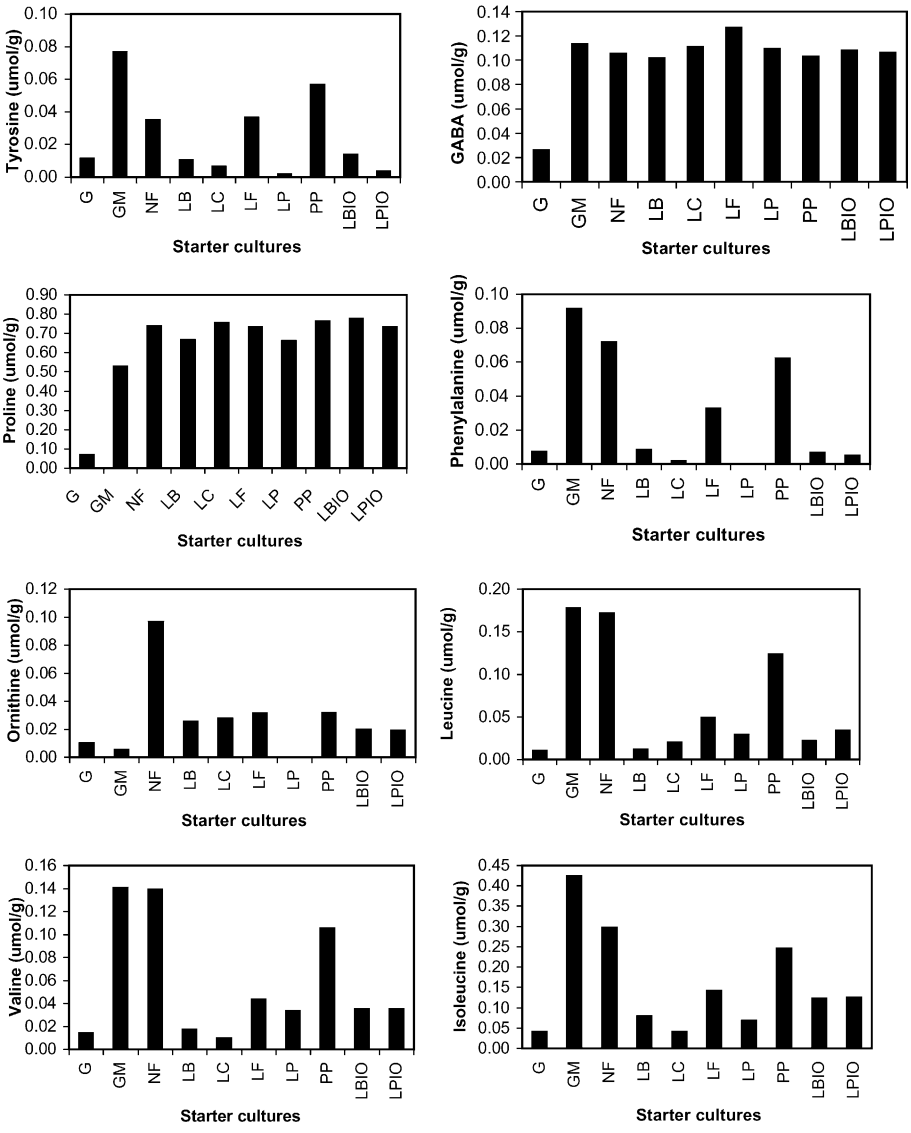

Fig. 1 (*continued*).

peptidase activity was mainly in that fraction. Naturally fermented samples had more enzymatic activity than those fermented by starter cultures. Samples fermented by *L. plantarum* and *L. plantarum* in coculture with *I. orientalis* had the least aminopeptidase activity. There was no significant difference in proteinase activity in *togwa* fermented by starter cultures except *L. plantarum*, which indicated the least activity. Cocultures of LAB and yeasts generally indicated enhanced enzymatic activity of the particle-free supernatant.

3.3. Changes in proteins and their degradation products

The RP-FPLC chromatograms of proteins and peptides extracted from sorghum *togwa* indicated that fermentation caused small detectable changes in their

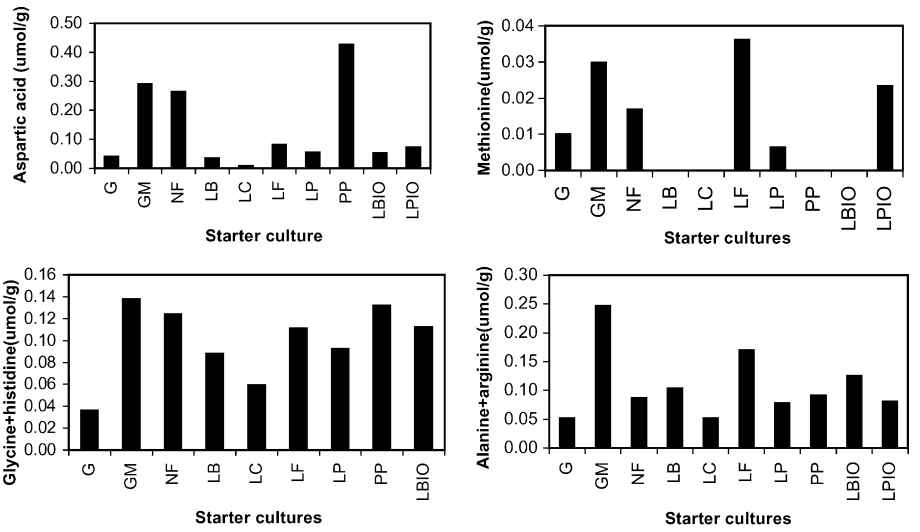

Fig. 1 (*continued*).

profiles (results not shown). Natural and fermentation with starter cultures brought about an increase in the second peak between 8 and 24 h. Fermentation did not cause any detectable change in the profile of the peaks eluted at the end of the gradient.

The SDS-PAGE banding pattern indicated that *togwa* preparation resulted into the breakdown of high molecular mass protein (about 64 kDa) as indicated by the clearing of the gel near the point of application (results not shown). The molecular mass of the bands in the fermented samples ranged from 12.3 to 54.5 kDa. Most of the bands had molecular masses ranging from 14 to 30 kDa. There was no difference in electrophoretogram banding patterns observed between *togwa* produced by natural or controlled fermentations.

3.4. Protein and amino acid content

There was a slight increase in total protein content from 9.5% to 11.0% (on dry weight basis) after supplementing gruel with malt (Fig. 1). Fermentation did not cause any significant change in the total protein content in *togwa*. The protein content ranged from 11.0% in naturally fermented samples to 12.1% in samples fermented by *L. plantarum*.

The composition of free amino acids in natural and controlled fermented samples is shown in Fig. 1. Generally, the supplementation of gruel with malt significantly ($P<0.05$) increased the concentrations of the amino acids. Although glycine, histidine, alanine, and arginine could not be separated under the chromatographic conditions used, their combined results indicated a similar trend. Natural and controlled fermentation increased the concentration of proline while it reduced that of most of the amino acids. Natural fermentation significantly increased the concentration of glutamic acid and ornithine. Fermentation by *P. pentosaceus* increased aspartic acid, while *L. cellobiosus*, *L. fermentum*, and *L. brevis* in coculture with *I. orientalis* increased the concentration of citrulline in *togwa*; however, in this last case, concentrations were generally low. Fermentation by *L. fermentum* slightly increased the content of essential amino acids methionine and lysine, and *L. plantarum* slightly increased the content of lysine.

4. Discussion

The proteinase and aminopeptidase activities, SDS-PAGE electrophoretogram banding patterns, and RP-FPLC chromatograms of proteins and peptides extracted from *togwa* indicated the presence of proteolytic activities in *togwa*. Most proteinase and aminopeptidase activities were observed in naturally

fermented samples. This suggests that endogenous cereal and malt enzymes, and possibly also those produced by LAB and yeasts, are involved in natural fermentation to produce *togwa*. A proteolytic system that allows the degradation of proteins is crucial for growth, and the conversion of larger peptides to small peptides and free amino acids and the subsequent utilization of these amino acids is a central metabolic activity in fermentation microorganisms such as LAB (Christensen et al., 1999). The observed lower proteinase activity in particle-free supernatants compared to the total activity may be accounted for by the removal of lactic acid bacteria with cell-bound proteinases. Among the LAB, lactobacilli have been reported with particularly high cell-bound proteinase activities (Christensen et al., 1999). Intracellular location of peptidases in LAB has been suggested (Law and Haandrikman, 1997; Christensen et al., 1999). A part of the peptidase activities in supernatants may arise from autolysed cells or cell fragments with attached enzymes (Gobbetti et al., 1996). The extracellular system of lactic bacteria is limited mainly to cell-bound serine-type proteinases (Law and Haandrikman, 1997; Christensen et al., 1999). Very few yeasts are strongly proteolytic and some have caseinolytic activity, for example *Yarrowia lipolytica* and a number of strains of *Candida* and *Saccharomyces* spp. have been reported to produce extracellular proteinases with various applications in the food industry, including gelatin liquefaction (chill proofing of beer and wine-haze reduction) (Ogrydziak, 1993).

Supplementation of gruel with malt significantly improved its protein and free amino acid content. Obatolu et al. (2000) reported that malting improved the nutritional quality of cereal-based complementary foods. The low amount of protein and essential amino acids in sorghum *togwa* indicate the need for complementing it with proteinaceous foods in order to improve its nutritional quality for use as a weaning food. In the present work, natural fermentation increased the content of glutamic acid, proline, and ornithine. Fermentation by *L. fermentum* slightly increased the content of essential amino acids methionine and lysine, and *L. plantarum* slightly increased the content of lysine, a limiting amino acid in sorghum. Fermentation of cereals by LAB has been reported to increase free amino acids and their derivatives by proteolysis and/or by metabolic synthesis (Collar and Martinez, 1993). The microbial mass can also supply low molecular mass nitrogenous metabolites by cellular lysis (Collar et al., 1992; Collar and Martinez, 1993; Gobbetti et al., 1994). The free amino acids enhance the growth of yeasts (Gobbetti et al., 1994) and contribute directly or as precursors of flavour development during cereal-based fermentation (Damiani et al., 1996). In this study, fermentation did not cause any significant change in the amount of protein in *togwa*. The concentration of most of the free amino acids, including the essential amino acids, was reduced by both natural and controlled fermentation. This shows the utilisation of the amino acids for growth of the fermentation microorganisms and production of metabolites. Considering the nutritional requirement for amino acids by LAB, the present results may indicate that amino acids and availability is a growth-limiting/-controlling factor for LAB in *togwa* fermentation. It appears also that a satisfactory *togwa* process is dependent on the proteolytic contribution by malt and fermentation organisms.

According to Kunji et al. (1996), several metabolic properties of LAB serve special functions, which directly or indirectly have impact on processes such as flavour development in foods. The degradation of proteins such as caseins plays a crucial role in the development of texture and flavour. Certain peptides contribute to the formation of flavour and undesirable bitter-tasting peptides that can lead to off-flavour. The proteolytic system of LAB can also contribute to the liberation of bioactive peptides (Meisel and Bockemann, 1999). The bioactive peptides produced during food processing could also play a significant role in the enzymatic processes involved in food preparations (Gobbetti et al., 1997). Specifically, peptides may also have inhibitory activities towards microbial proteolytic enzymes in food spoilage (Gobbetti et al., 1997). Other accepted and potential benefits in probiotic-containing foods include aiding in lactose digestion, anticolon cancer effect, minimizing effects of small bowel bacterial overgrowth, immune system modulation, reduction of allergenicity, assimilation of cholesterol, and resistance to enteric pathogens (Sanders and in't Veld, 1999). *Togwa* has been reported to inhibit the growth and toxin production by enteropathogenic bacteria (Svanberg et al., 1992; Kingamkono et al., 1995, 1998), and was found effective in the dietary management of diarrhoea in children (Willumsen et

al., 1997). Some food-derived peptides are claimed to be health-enhancing components, which can be used for functional food and pharmaceutical preparations (Meisel and Bockemann, 1999).

The results of this study show that there are proteolytic activities in *togwa*. The supplementation of gruel with malt enhanced its free amino acid content, while natural and controlled fermentations reduced the content of most of the free amino acids in *togwa*. Further research work is proposed to identify the products of protein degradation in *togwa*, and to assess their potential probiotic and other applications.

Acknowledgements

This study was supported by grants from the Norwegian Council of Universities' Committee for Development Research and Education (NUFU, Project 26/96) through the Agricultural University of Norway and Sokoine University of Agriculture, and by the Lånekassen of Norway. We are grateful to Kari Olsen for assistance with the HPLC analyses of the amino acids.

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