

Probiotic Preparation Has the Capacity To Hydrolyze Proteins Responsible for Wheat Allergy

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ABSTRACT

This study was aimed at showing the capacity of probiotic VSL#3 to hydrolyze wheat flour allergens. Hydrolysis was investigated either by the use of baker's yeast bread treated with digestive enzymes and VSL#3, an experimental design that mimicked the activity of probiotics during gut colonization, or by the use of VSL#3 as a starter for dough fermentation, an experimental design that mimicked the predigestion of wheat flour proteins during food processing. Albumins, globulins, and gliadins extracted from wheat flour and chemically acidified and started dough and total proteins extracted from breads were analyzed by immunoblotting with pooled sera from patients with an allergy to wheat. Hydrolysis of wheat flour proteins was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional electrophoresis (2DE). Mass spectrometry matrix-assisted laser desorption and ionization–time of flight was used to identify some immunoglobulin E (IgE)–binding proteins. As shown by immunoblotting with sera from allergic patients, several IgE-binding proteins persisted after treatment of baker's yeast bread by pepsin and pancreatin. The signal of all these IgE-binding proteins disappeared after further treatment by VSL#3. As shown by SDS-PAGE and related immunoblotting and 2DE analyses, when VSL#3 was used as a starter for bread making, it caused a marked degradation of wheat proteins, including some IgE-binding proteins such as the putative transcription factor APFI and wheat α -amylase inhibitors. Indeed, the IgE-binding profile of the bread manufactured by VSL#3 was largely different from that of baker's yeast bread. The IgE-binding proteins that persisted in the bread made with VSL#3 were completely degraded by pepsin and pancreatin.

Currently, the clinical classification of food allergy diseases is based mainly on the presence or absence of food-specific immunoglobulin E (IgE) antibodies (25). IgE-mediated food reactions are characterized by the presence of a rapid onset of symptoms after ingestion of a single, well-defined food, whereas non-IgE-mediated disorders develop hours to days after food ingestion. IgE-mediated food allergy is by far the most common and studied type of disease. There is good general knowledge of the physiological mechanisms, and reliable diagnostic tools have been identified; however, proactive treatments are still lacking (13).

The IgE-mediated allergic state has been definitively linked to atopy, e.g., the predisposition to develop an allergic-type response to common environmental antigens (allergens). The number of patients suffering from IgE-mediated hypersensitivity reactions after ingestion of foods is increasing throughout the world and corresponds to ca. 6% of infants and children younger than 3 years of age and ca. 3% of the adult population (20, 27, 37, 51).

Wheat flour (*Triticum aestivum* and *Triticum durum*) is frequently consumed in the daily diet (e.g., leavened baked goods, pasta) and constitutes one of the most frequent causes of food allergy. Depending on the route of

exposure, wheat allergy may induce several clinical manifestations: classical food allergy affecting the skin, gut, or respiratory tract; exercise-induced anaphylaxis; occupational rhinitis or asthma; and contact dermatitis. IgE antibodies play a fundamental role in all these clinical manifestations (1, 32). Immunoblotting with the sera of patients suffering from allergic symptoms after wheat ingestion indicated that different protein components contain IgE-reactive epitopes; low-molecular-weight glutenin subunits; and, especially, albumins, globulins, and gliadins (2, 35, 44). Before coming into contact with their target organ, wheat flour allergens undergo extensive modifications either during food processing or, after being ingested, during proteolytic digestion in the gastrointestinal tract (19). Consequently, two major hypotheses should be investigated: (i) could selected probiotics, after gut colonization, degrade the cereal protein components that are responsible for wheat allergy? and (ii) could selected probiotics, during long-time fermentation, affect the native structure of wheat flour allergens and related IgE-reactive epitopes?

Probiotics are described as live microorganisms that, when administered in adequate numbers, confer a health benefit on the host (14). VSL#3 (VSL Pharmaceuticals, Gaithersburg, Md.) is a highly concentrated (ca. 11.5 log cells per g) mixture of probiotic lactic acid and bifidobacteria that has been shown to colonize the intestine and pro-

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mote several beneficial effects for the host, such as remission of ulcerative colitis in patients intolerant or allergic to 5-aminosalicylic acid (16, 29). Recent studies (28, 48) indicate that probiotic bacteriotherapy has a great potential for controlling the allergenic inflammation associated with food allergy. These reports were concerned with food allergies determined by cow's milk proteins, but, to our knowledge, no studies have considered the effect of probiotics on IgE-mediated allergy to wheat flour. Hydrolysis of wheat proteins has been studied during sourdough fermentation. Glutenin hydrolysis and gluten depolymerization have been shown to be pH-dependent because of the activation of cereal enzymes, which have their optimum activity at pH values ≤ 4.0 (46). A recent study showed that *Lactobacillus sanfranciscensis*, a key sourdough lactic acid bacterium, does not harbor a proteinase but that it has genes encoding for the peptide transport systems and intracellular peptidases (47). Nevertheless, compared with a chemically acidified dough, selected sourdough lactobacilli caused extensive hydrolysis, especially of the gliadin fractions, and the resulting bread (2 g of gluten) was tolerated by celiac patients during an in vivo acute challenge (11). Previously (9), it has been shown that proteolysis by a VSL#3 preparation has the capacity to eliminate any traces of toxic gliadin contaminants in gluten-free products for celiac patients.

This study was aimed at investigating the capacity of a VSL#3 probiotic preparation to degrade wheat protein allergens. Hydrolysis was investigated either by the use of baker's yeast bread treated with digestive enzymes and VSL#3, an experimental design that mimicked the activity of probiotics during gut colonization, or by the use of VSL#3 as a starter for dough fermentation, an experimental design that mimicked the predigestion of wheat flour proteins during food processing.

MATERIALS AND METHODS

Sera of patients with food allergy to wheat. Sera were obtained from 10 patients with a food allergy to wheat, suffering from various gastrointestinal symptoms (e.g., diarrhea, abdominal pain, bloating) after ingesting foods containing wheat. Food allergy to wheat was established by evidence of IgE-dependent sensitization with the CAP-FEIA system (Pharmacia, Uppsala, Sweden), by skin prick tests and by standardized double-blind placebo-controlled food challenges (3). Specific IgE antibodies to barley, rice, cereals, gluten, wheat, and α -amylase were tested (UniCAP-specific IgE, Pharmacia & Upjohn, Uppsala, Sweden). Human sera were pooled and used in immunoblotting experiments. The use of pooled sera allowed us to develop a unique immunological map, because it targeted a large number of immunogenic epitopes (42, 43). All IgE-binding bands recognized by single sera were present when pooled sera were examined in immunoblotting experiments. Pooled sera from healthy subjects were used as the negative control.

Probiotic preparation. A freeze-dried preparation of VSL#3 (VSL Pharmaceuticals) (ca. $11.5 \log$ cells per sachet) was used in this study. It contained *Streptococcus thermophilus* (isolated from fermented milk), *Lactobacillus plantarum* (from silage), *Lactobacillus acidophilus* (from the human intestine), *Lactobacillus casei* (from cheese), *Lactobacillus delbrueckii* spp. *bulgaricus*

(from fermented milk), and *Bifidobacterium breve*, *Bifidobacterium longum*, and *Bifidobacterium infantis* (all from the human intestine). After isolation, all the species were identified by rRNA 16S gene sequencing.

Bread making. Type O wheat flour (*T. aestivum*), variety Svevo, cultivated in the south of Italy, was used. The characteristics of the wheat flour were as follows: moisture, 12.8%; protein (nitrogen $\times 5.70$), 10.7% of dry matter; fat, 1.8% of dry matter; ash, 0.6% of dry matter; and total soluble carbohydrates, 1.5% of dry matter.

Two types of bread (baker's yeast and VSL#3 breads) were manufactured. Baker's yeast bread was made by a very short fermentation without probiotic bacteria and consisted of the following: a mixture of 125 g of wheat flour and 64 ml of tap water (containing 1.5% baker's yeast), which was allowed to ferment for 2 h at 37°C and baked at 250°C for 15 min. VSL#3 bread was made by a preliminary long fermentation with probiotic bacteria and consisted of the following: a mixture of 125 g of wheat flour and 297 ml of tap water (containing a cell concentration of the probiotic preparations of ca. $9.0 \log$ CFU g⁻¹). The semiliquid dough was incubated for 24 h at 37°C under stirring conditions (ca. 200 rpm). After fermentation, water was removed by freeze-drying, and ca. 125 g of this pretreated wheat flour was mixed with 64 ml of tap water (containing 1.5% baker's yeast), allowed to ferment for 2 h at 37°C, and baked at 250°C for 15 min.

A semiliquid dough, without a bacterial inoculum, was chemically acidified to pH 4.0 with a mixture of lactic and acetic acids (molar ratio, 4:1), freeze-dried, and used as the control for electrophoresis analyses.

Extraction of wheat flour and bread proteins. After dough fermentation by probiotic preparation or chemical acidification, wheat flour proteins were separately extracted following the method originally described by Osborne (33) and further modified by Weiss et al. (50). Extracted albumins, globulins, and gliadins were stored at -20°C and used for electrophoresis.

Total proteins were extracted from wheat flour, baker's yeast, and VSL#3 breads. Samples (100 mg) were suspended in 4 ml of 0.2 N HCl and homogenized with a Sterilmixer Lab (PBI International, Milan, Italy); then, 2 ml of 0.6 M Tris-HCl, pH 7.4, containing 30% (wt/vol) glycerol, 6% (wt/vol) sodium dodecyl sulfate (SDS), and 6% (vol/vol) 2-mercaptoethanol, was added. Samples were heated for 5 min at 100°C. After centrifugation ($12,000 \times g$, 20 min), the supernatant (total protein extract) was stored at -20°C and used for electrophoresis. Protein concentration of the extracts was determined by the Bradford method (6) with bovine serum albumin as the standard.

Bread digestion. Both baker's yeast and VSL#3 breads were subjected to sequential protein hydrolysis by digestive enzymes according to the method described by Pasini et al. (36). Briefly, 100 mg of bread was suspended in 4 ml of 0.2 N HCl (pH 2) containing 0.05 mg ml⁻¹ of pepsin (2,500 U/mg from a porcine source) (Sigma-Aldrich, St. Louis, Mo.) and homogenized in a Sterilmixer Lab (PBI International). After 30 min of incubation at 37°C, 1.15 ml of a solution of 1 M boric acid and 0.5 N NaOH, adjusted to pH 6.8 with 5 N HCl and containing 0.25 mg ml⁻¹ of pancreatin (from a porcine source) (Sigma-Aldrich), was added. The resulting pH was 7.6. Pancreatic digestion lasted 150 min. Only the baker's yeast bread was further incubated with ca. $9.0 \log$ CFU ml⁻¹ of VSL#3 preparation for 24 h at 37°C, under stirring conditions, to mimic hydrolysis by probiotics colonizing the gastrointestinal tract. Digested samples were recovered after 30 min of pepsin attack and were further treated with either pan-

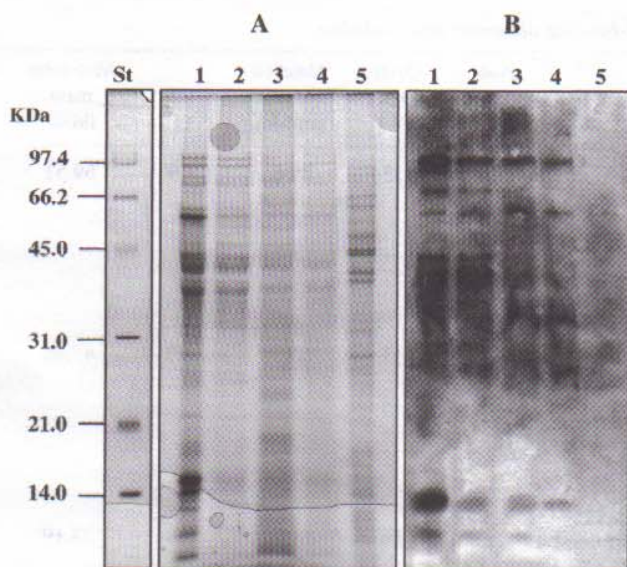


FIGURE 1. SDS-PAGE analysis (A) and related immunoblot (B) of total proteins extracted from wheat flour and baker's yeast bread during *in vitro* digestion. (St) Protein standards; lane 1, wheat flour; lane 2, undigested baker's yeast bread; lanes 3, 4, and 5, baker's yeast bread after sequential digestion by pepsin, pancreatin, and VSL#3, respectively.

creatic digestion for 150 min or incubation with VSL#3 for 24 h. These samples were then subjected to total protein extraction as described elsewhere.

Electrophoresis. Albumin, globulin, and gliadin fractions extracted from wheat flour and total proteins extracted from undigested and digested breads (10 to 20 μ l, containing ca. 15 μ g of protein) were diluted 1:1 with sample buffer, treated at 100°C for 5 min, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the Laemmli procedure (30).

Two-dimensional electrophoresis (2DE) was performed by the Immobiline-polyacrylamide system as described by Bjellqvist et al. (5). Aliquots of 30 μ g of wheat proteins were used for the electrophoretic run. Isoelectric focusing was carried out on Immobiline strips, providing a nonlinear pH gradient of 3.0 to 10.0 (for albumins and globulins) and a linear pH gradient of 6.0 to 11.0 (for gliadins) (IPG strips, Amersham Pharmacia Biotech, Uppsala, Sweden) by IPG-phor, at 20°C. The voltages were as follows: 0 to 300 V for 1 h, 300 to 500 V for 3 h, 500 to 2,000 V for 4 h, and a constant 8,000 V for 4 h. Following electrophoresis, IPG strips were equilibrated for 12 min against buffer A (6 M urea, 30% [vol/vol] glycerol, 2% [wt/vol] SDS, 0.05 M Tris-HCl [pH 6.8], and 2% [wt/vol] dithiothreitol) and for 5 min against buffer B (6 M urea, 30% [vol/vol] glycerol, 2% [wt/vol] SDS, 0.05 M Tris-HCl [pH 6.8], 2.5% [wt/vol] iodoacetamide, and 0.5% bromophenol blue). Electrophoresis for the second dimension was carried out in a Laemmli system on 13.5% polyacrylamide gels (13 cm by 20 cm by 1.5 mm) at a constant current of 40 mA per gel and at 15°C for approximately 5 h, until the dye front reached the bottom of the gel (30). Gels were calibrated with two molecular mass markers: comigration of the extracts with human serum proteins for a molecular mass range of 200 to 10 kDa. The electrophoretic coordinates used for the serum were as described by Bjellqvist et al. (5). Gels were silver stained as described by Hochstrasser et al. (22). The protein maps were scanned with an Image Scanner and analyzed by Image Master 2D v.3.01 computer software (Amersham Pharmacia Biotech). Three gels were ana-

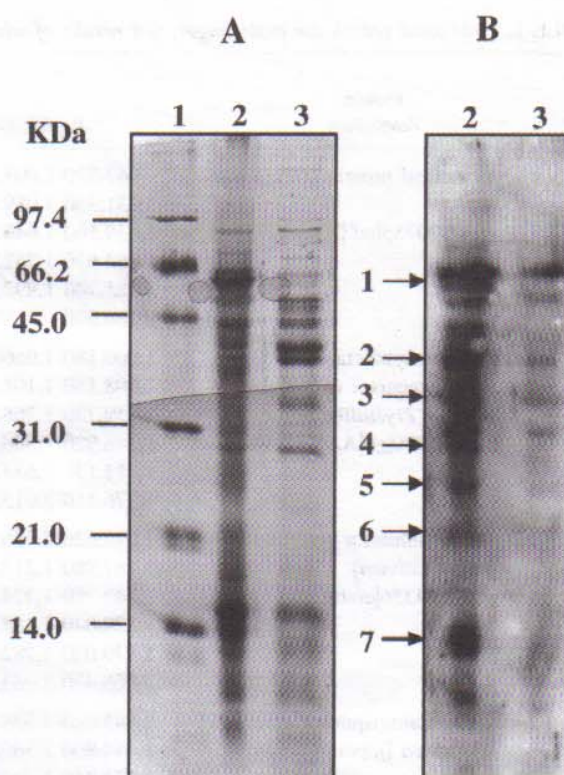


FIGURE 2. SDS-PAGE analysis (A) and related immunoblot (B) of wheat albumins and globulins. Lane 1, protein standards; lane 2, chemically acidified dough (control); lane 3, dough started with VSL#3 for 24 h at 37°C. Numbers in panel B refer to proteins subjected to mass spectrometry matrix-assisted laser desorption and ionization-time of flight analysis (see Table 1).

lyzed, and spot intensities were normalized as described by Bini et al. (4). Only hydrolysis factors with statistical significance where the *P* value was <0.05 were reported.

Immunoblotting by sera of patients with food allergy to wheat. Albumins, globulins, and gliadins extracted from wheat flour and total proteins extracted from digested and undigested breads were analyzed by immunoblotting to detect the IgE-binding capacity of pooled human sera of patients with a food allergy to wheat (8). A semidry blotting technique was used. Protein bands separated by SDS-PAGE were transferred onto nitrocellulose sheets with a Trans-blot Cell (Bio-Rad Laboratories, Milan, Italy) by a transfer buffer containing 48 mM Tris, pH 9.2, 39 mM glycine, 20% methanol, and 0.1% SDS. The voltage was 50 V for 5 h. Blotting bands were visualized by soaking the membranes for a few minutes in Ponceau S (0.1% in 3% trichloroacetic acid) and then marking them with a pencil before destaining with water. Membranes were blocked with Tris-buffered saline (TBS) containing 0.05% (vol/vol) Tween 20 (TBS-T) and 5% (wt/vol) skim milk powder (M-TBS-T) for 2 h and were incubated overnight with pooled human sera, diluted 1:20 in TBS-T. After washing five times with M-TBS-T, blots were incubated for 1 h with a monoclonal antihuman IgE peroxidase-conjugate antibody (Sigma-Aldrich), diluted 1:5,000 in M-TBS-T. After four washes in M-TBS-T and one in TBS, bound IgE antibodies were visualized by chemiluminescence with the Supersignal Detection kit (Pierce Biotechnology Inc., Rockford, Ill.), according to the instructions provided by the manufacturer. The procedure was carried out at room temperature.

TABLE 1. ProFound search for mass fingerprint results of wheat IgE-binding albumins and globulins

No. ^a	Protein description	Peptide masses	Probability (%)	Coverage (%)	Matched measured peptides	pI	Molecular mass (kDa)
1	Hypothetical protein (<i>Oryza sativa</i>) T gi 50920755 ref XP_470738.1	947.370 1,003.300 1,016.390 1,031.330 1,089.440 1,326.520 1,639.560 1,646.620 1,668.620 1,684.670 1,752.750 1,769.780 1,985.780 1,993.800 2,013.820 2,086.920	97	15	7/16	8.9	69.51
2	Glutamine synthetase leaf isozyme precursor (<i>Mesembryanthemum crystallinum</i>) T gi 4884970 gb AAD31898.1	1,060.180 1,066.220 1,082.190 1,098.150 1,101.750 1,133.680 1,159.770 1,268.780 1,293.260 1,346.920 1,360.980 1,499.080 1,612.130 1,633.130 1,670.120 1,776.110 2,015.350	76	13	6/17	6.7	47.46
3	Xylanase inhibitor protein 1 (<i>Triticum aestivum</i>) T gi 20804336 emb CAD 19479.1	1,018.620 1,036.650 1,150.760 1,187.780 1,212.810 1,229.830 1,242.780 1,274.800 1,308.900 1,339.840 1,355.830 1,387.830 1,570.030 1,782.310 1,960.330 2,066.430 2,083.480	93	16	5/17	8.9	33.19
4	Putative transcription factor APFI (<i>O. sativa</i> [japonica cultivar group]) T gi 50939569 ref XP_479312.1	1,145.610 1,338.750 1,367.700 1,444.800 1,503.890 1,568.850 1,584.860 1,600.810 1,743.970 1,794.000 1,821.030 1,853.040	52	16	4/12	6.4	29.60
5	Unknown protein (<i>Arabidopsis thaliana</i>) T gi 23198268 gb AAN1 5661.1	991.700 1,021.660 1,117.770 1,145.780 1,189.860 1,230.840 1,307.950 1,476.110 1,479.070 1,495.020 1,602.240 1,613.150 1,792.220 1,960.490	54	32	6/14	5.7	22.33
6	Endogenous α -amylase-subtilisin inhibitor (WASI) T gi 123975 sp P16347 IAAS_WHEAT	1,060.260 1,066.280 1,082.240 1,098.240 1,182.780 1,318.920 1,334.940 1,340.890 1,366.960 1,450.020 1,509.120 1,562.100 1,613.080 1,627.230 1,701.180 1,802.300 1,971.380 1,978.540 2,083.480	100	55	8/19	6.8	19.62
7	α -Amylase inhibitor 0.19 (0.19 AI) (0.19 α -AI) T gi 123963 sp P01085 IAA1_WHEAT	1,023.650 1,060.690 1,105.790 1,111.670 1,221.780 1,249.940 1,292.850 1,499.120 1,514.120 1,613.180 1,831.310 1,902.420 1,986.600	62	33	3/13	6.7	13.32

^a Protein designations correspond to those indicated in the immunoblot of Figure 2.

Mass spectrometry matrix-assisted laser desorption and ionization-time of flight. Slices of gel were destained by several washings with 5 mM NH_4HCO_3 -acetonitrile (ACN) (50:50) and were subsequently dried with pure ACN. The gel slices were rehydrated for 45 min at 4°C in 20 μl of digestion buffer containing 10 ng μl^{-1} of trypsin and 5 mM NH_4HCO_3 (49). The excess of protease solution was removed, and the volume was adjusted with 5 mM NH_4HCO_3 to cover the gel slices. Digestion was allowed to proceed overnight at 37°C. The mass spectrometer used was a Tofspec SE (Micromass, Manchester, UK) equipped with a delayed extraction unit. The laser wavelength was 337 nm, and the accelerating voltage was 20 kV (18). Peptide spectra were obtained in reflectron mode in the range of 800 to 4,000 Da. The peptide solution was loaded onto the matrix-assisted laser desorp-

tion and ionization target plate by mixing 1.5 picoliters of each solution with the same volume of a matrix solution, which was prepared by dissolving 10 mg ml^{-1} alpha-cyano-4-hydroxycinnamic acid solution in 40% ACN-0.1% trifluoroacetic acid (vol/vol) and then allowed to dry. External calibration was done with the standard fragment ions generated from adrenocorticotrophic hormone 18-39 and angiotensin I. Each mass spectrum was generated by accumulating data from 100 to 120 laser shots (15). Database searches were done by searching the peptide masses against the nonredundant National Center for Biotechnology Information database with the search program ProFound (<http://www.prowl.rockefeller.edu/cgi-bin/ProFound>) from Rockefeller University (New York, N.Y.) and ProteoMetrics (Winnipeg, Manitoba, Canada).

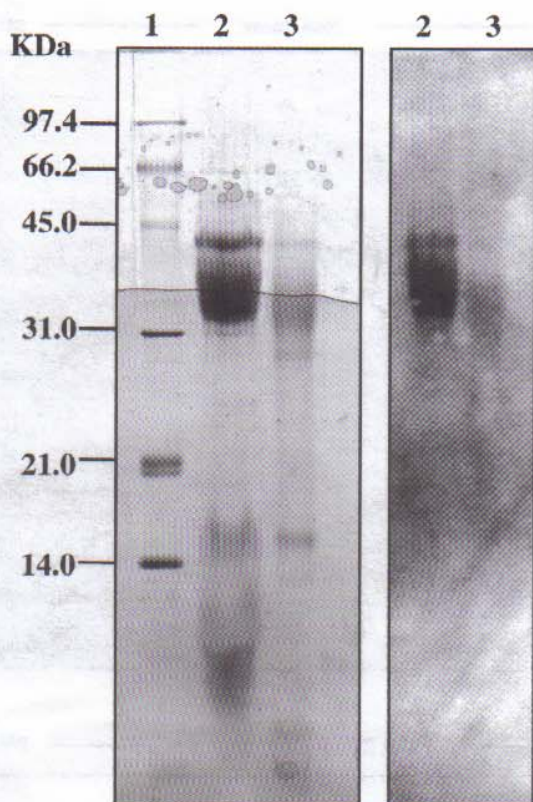


FIGURE 3. SDS-PAGE analysis (A) and related immunoblot (B) of wheat gliadins. Lane 1, protein standards; lane 2, chemically acidified dough (control); lane 3, dough started with VSL#3 for 24 h at 37°C.

RESULTS

Digestion of baker's yeast bread. Baker's yeast bread was manufactured and subjected to sequential hydrolysis by pepsin, pancreatin, and VSL#3 to mimic the digestive process, including the potential activity of probiotic microorganisms. After digestion, total wheat flour proteins were extracted and used for analyses.

The SDS-PAGE and related immunoblot analysis of the total proteins extracted from nonprocessed wheat flour were characterized by several bands of very different molecular sizes (Fig. 1A). Moreover, the IgE-binding protein profile of the baker's yeast bread was only in part similar to that of wheat flour (Fig. 1B). Protein bands with molecular sizes of (i) ca. 14 kDa and (ii) 45 to 97 kDa showed a weaker reaction with respect to wheat flour extract. Compared with the undigested baker's yeast bread, the combined activity of pepsin and pancreatin caused a decrease of the signal intensity of several IgE-binding proteins, mainly located in the range from 31 to 45 kDa. Allergenic α -(sulfur-rich) and ω -(sulfur-poor) prolamins have been identified within the above molecular mass ranges (41). Nevertheless, several IgE-binding proteins persisted after hydrolysis by pepsin and pancreatin, especially at molecular mass values of ca. 14, 60, and 97 kDa. The signals of all the above IgE-binding proteins disappeared after further treatment for 24 h at 37°C by ca. 9.0 CFU g^{-1} of VSL#3 preparation.

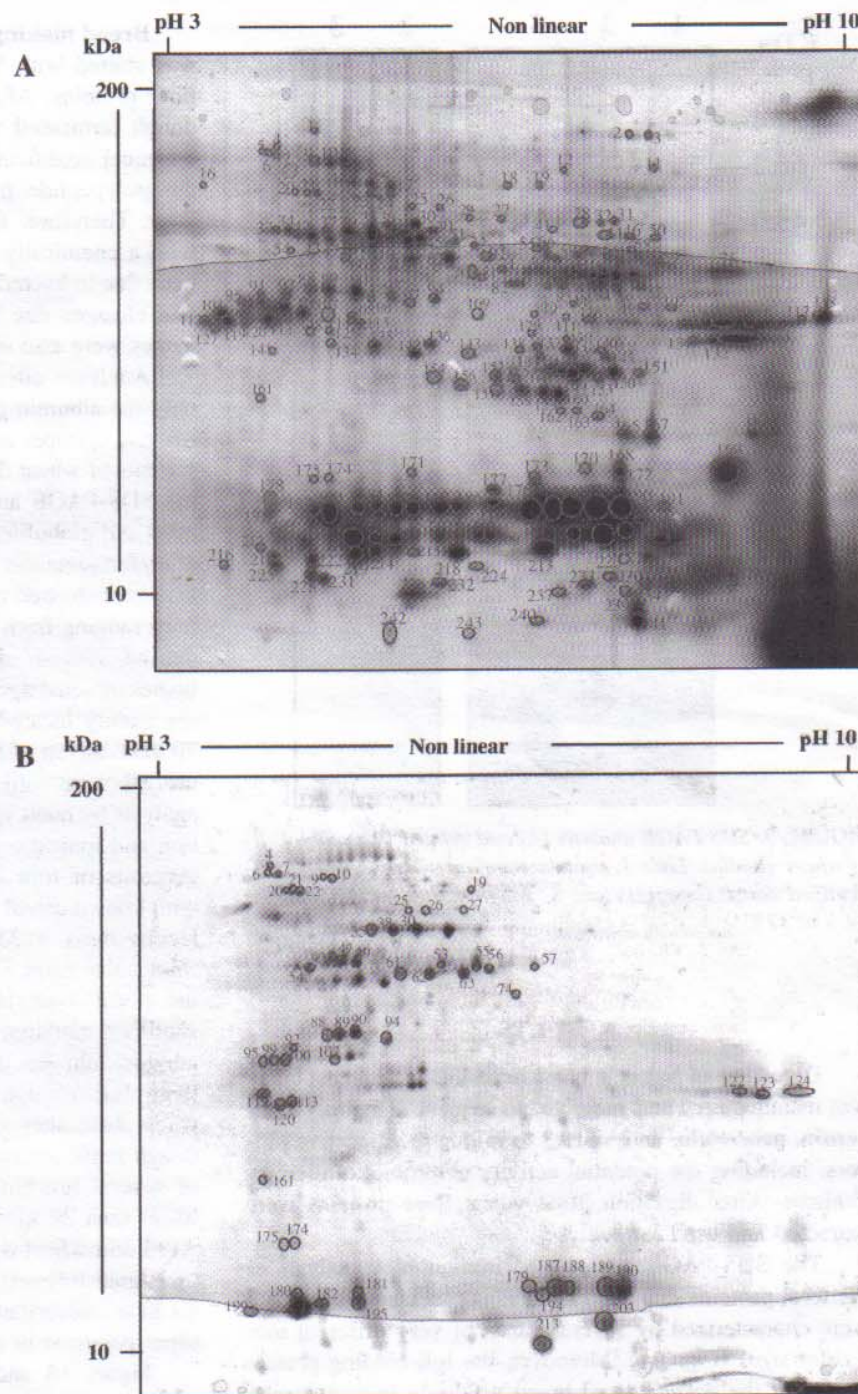
Bread making by VSL#3 preparation. Bread making was started with VSL#3 to mimic predigestion of wheat flour proteins. After 24 h of fermentation, the pH of the dough fermented with VSL#3 was ca. 3.8. Biological or chemical acidification may cause a direct modification of the polypeptide pattern compared with nonacidic wheat flour. Therefore, fermented dough was always compared with a chemically acidified (pH 4.0) dough to find variations due to bacterial proteolysis only. This method ensured that changes due to proteolysis by flour endogenous enzymes were also excluded.

Analyses after dough fermentation took into account only the albumin-globulin and gliadin fractions, since IgE-reactive epitopes are mainly contained in these protein components of wheat flour (2, 35, 45). Figure 2A and 2B shows the SDS-PAGE and related immunoblot analysis of albumins and globulins extracted from wheat dough before and after fermentation by the VSL#3 preparation. Pooled human sera showed an IgE-binding pattern that included proteins ranging from 10 to 80 kDa. Other authors (2) showed that ca. 70% of all wheat-allergic patients have IgE antibodies directed against the group of albumins-globulins that are mainly located in the migration zone between 30 and 70 kDa. Seven of the IgE-binding proteins detected in Figure 2B were subjected to tryptic digestion and sequence analysis by mass spectrometry matrix-assisted laser desorption and ionization-time of flight (Table 1). The mass fingerprints of four proteins could be matched significantly with known cereal proteins: xylanase inhibitor protein (molecular mass = 33.19), putative transcription factor APFI (molecular mass = 29.60), and two proteins belonging to the wheat α -amylase inhibitor protein family (α -amylase-subtilisin inhibitor, WASI, molecular mass = 19.62, and α -amylase inhibitor 0.19, molecular mass = 13.32). The other three IgE-reactive proteins represented not-yet-described wheat flour allergens. Hydrolysis by VSL#3 during wheat dough fermentation caused the disappearance of the signals of several IgE-binding proteins at molecular mass values lower than 30 kDa, including putative transcription factor APFI and wheat α -amylase inhibitors, and the decrease of the signal intensity in the migration zone between 30 and 80 kDa. Nevertheless, IgE-reactive albumin-globulin epitopes persisted in the fermented dough.

Figure 3A and 3B shows the SDS-PAGE and related immunoblot analysis of gliadins extracted from wheat dough before and after fermentation by VSL#3 preparation. Although a marked decrease of the signal intensity in the migration zone between 30 and 45 kDa was found, IgE-reactive gliadin epitopes slightly persisted in the dough fermented by VSL#3.

Overall, 2DE analysis was used to further characterize the hydrolysis of albumin-globulin and gliadin polypeptides by VSL#3 during dough fermentation. A total of 243 albumin-globulin polypeptides were identified in the chemically acidified dough used as the control and were distributed in the pI and molecular mass ranges of 4.02 to 9.9 kDa and 6.0 to 83 kDa, respectively (Fig. 4A). This separation was in substantial agreement with other electrophoresis analyses of albumins and globulins of wheat flour (12,

FIGURE 4. 2DE analysis of albumin and globulin polypeptides of chemically acidified dough (control) (A) and of a dough started with VSL#3 for 24 h at 37°C (B). Albumin and globulin polypeptides are indicated by numbered red ovals.



39, 41, 50). Of the 243 polypeptides identified (Fig. 4A), 183 showed hydrolysis factors higher than 80%, 34 were degraded by 30 to 60%, and only 16 had hydrolysis factors lower than 30% (Fig. 4B). A total of 84 gliadin polypeptides were identified by 2DE analysis and were widespread throughout the pI range of 6 to 11 (Fig. 5A). Seventy-nine of the 84 gliadin polypeptide spots were degraded after dough fermentation with VSL#3 compared with the control (Fig. 5B). Most of the polypeptides degraded (65 of the 79) had hydrolysis factors higher than 80%, and only eight showed hydrolysis factors lower than 40%.

Digestion of VSL#3 bread. After long-time fermentation by VSL#3, water was removed by freeze-drying, and the predigested wheat flour was processed under the conditions used for the manufacture of baker's yeast bread. VSL#3 bread had a profile of total proteins (Fig. 6A and 6B) clearly different from that of baker's yeast bread (Fig. 1A and 1B). VSL#3 bread was characterized by IgE-binding proteins mainly located in the range of 21 to 31 kDa, while baker's yeast bread showed a higher number of IgE-binding proteins that were widespread throughout the range of 14 to 97.4 kDa. It is well known that baker's yeast does

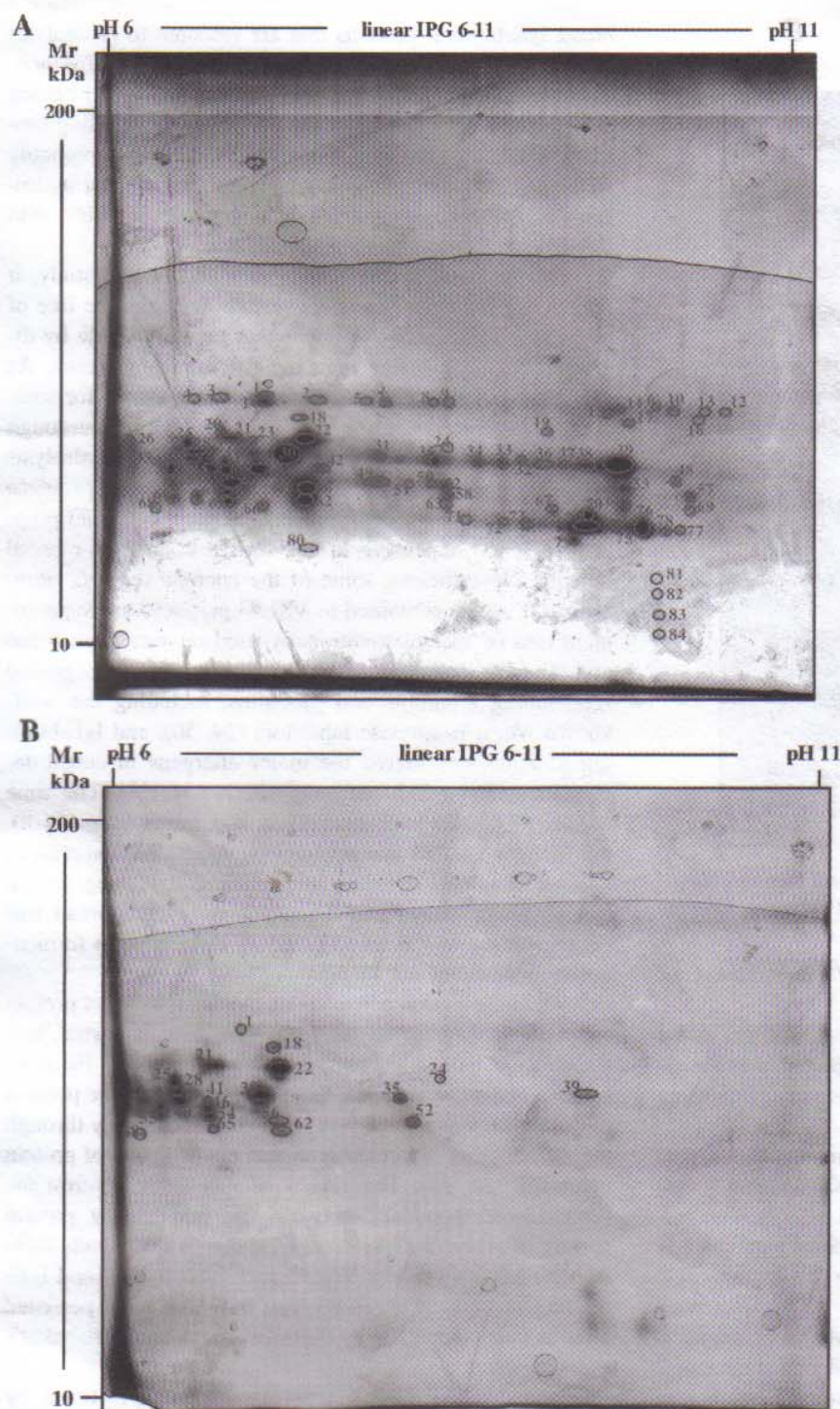


FIGURE 5. 2DE analysis of the gliadin polypeptides of a chemically acidified dough (control) (A) and of a dough started with VSL#3 for 24 h at 37°C (B). Gliadin polypeptides are indicated by numbered red ovals.

not cause appreciable proteolysis during dough fermentation (17), while VSL#3 preparation was shown to be capable of intense proteolytic activity (9). Notwithstanding the effect of baking, this finding suggested that proteolysis by VSL#3 during dough fermentation influences the native structure of wheat flour allergens and related IgE-reactive epitopes. The signals of the IgE-binding proteins contained in VSL#3 bread disappeared after digestion with pepsin and pancreatin (Fig. 6B). The same effect by digestive enzymes

was not found for baker's yeast bread, which showed persistent IgE-binding proteins (Fig. 1B).

DISCUSSION

Currently, the only treatment for food allergy is the elimination of the offending food from the diet. Several therapeutic modalities, such as peptide immunotherapy, DNA immunization with immunostimulatory sequences, and anti-IgE therapy, are under investigation to provide an

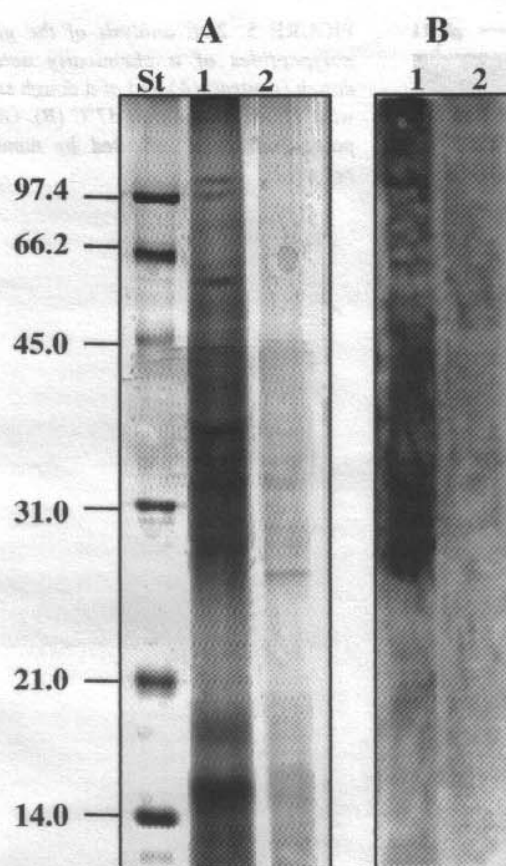


FIGURE 6. SDS-PAGE analysis (A) and related immunoblot (B) of total proteins extracted from VSL#3 bread during in vitro digestion. (St) Protein standards; lane 1, undigested VSL#3 bread; lane 2, VSL#3 bread digested by pepsin and pancreatin.

effective treatment and prevention of food allergies in the near future (7). This study showed the capacity of probiotic microorganisms such as VSL#3 to degrade IgE-binding proteins of wheat flour.

In the healthy gut, the immune system is able to create a balance between the protective mucosal immunity and systemic tolerance. In persons with food allergy, this balance is impaired, and oral tolerance of dietary antigens is not achieved or maintained (23, 40). The nonimmunologic and immunologic defense barriers of the gut are increased by probiotic bifidobacteria and lactobacilli (e.g., suppression of lymphocyte proliferation, increase of interleukin-2 and interleukin-12 secretion, and decrease of T-cell helper [type] 2 cytokine secretion) (10, 26, 31, 38). Besides possessing the above effects, probiotics may cause a degradation of food allergens at the intestinal lumen (48). Intact, rather than intestinally processed, cow's milk proteins have been shown to stimulate peripheral blood mononuclear cells to release proinflammatory cytokines in patients with cow's milk allergy (21). Indeed, cow's milk proteins degraded by lactobacilli may generate nonallergenic peptides from the native protein (45). VSL#3 preparation is a very complex mixture of bifidobacteria and lactobacilli, and its capacity to colonize the gut has also been shown (16, 29). During in vitro digestion for 24 h at 37°C, ca. $9.0 \log \text{CFU g}^{-1}$ of VSL#3 probiotic bacteria completely hydrolyzed the wheat

bread IgE-binding proteins that are resistant to proteolysis by digestive enzymes. Similar results were found by incubating them for 12 h, but a shorter time (e.g., 4 h) caused a degradation of ca. 50% of the persistent IgE-binding proteins. Therefore, the role of probiotic bacteria is probably weakened at the intestinal level, where the time for hydrolysis of IgE-binding proteins is scarcely compatible with the digestive process.

On the basis of the results obtained in this study, it seems that for wheat bread to be safe, it should be free of or contain IgE-reactive epitopes that are degradable by digestive enzymes before reaching the intestinal lumen. As previously shown (9), VSL#3 is a suitable starter for sourdough fermentation. Compared with traditional sourdough starter cultures, it also showed a more extensive hydrolysis of wheat albumins, globulins, and gliadins in the presence of 1 log cycle fewer cell numbers. Certainly, VSL#3 preparation is too expensive to be used as a starter for bread making. Nevertheless, some of the bacteria (e.g., *L. plantarum*, *L. casei*) contained in VSL#3 preparation are parental strains of species traditionally used in sourdough bread making (17). During dough fermentation, VSL#3 degraded IgE-binding albumins and globulins, including the well-known wheat α -amylase inhibitors (24, 50), and IgE-binding gliadins, considered the major allergens in cereal-dependent exercise-induced anaphylaxis (34, 35). The time required to effect such hydrolysis was rather long (24 h), but the freeze-dried and predigested dough still maintained suitable rheology characteristics for bread making during baker's yeast fermentation. The volume of this bread was ca. two thirds of the bread made by baker's yeast fermentation alone (data not shown).

The information provided by the IgE-mediated protein spectrum of raw or fermented wheat flour is partial, and the effect of baking has to be considered carefully. Far from being a general way to decrease allergenic risk, the process of baking is just as likely to increase allergenicity through the introduction of neoantigens and modification of protein solubility (36, 43). The results of this study confirm the limited correspondence between the IgE-binding protein spectra of wheat flour or fermented dough and breads started with baker's yeast or VSL#3 and show that several IgE-binding proteins (e.g., α -amylase inhibitor 0.19) persisted after in vitro hydrolysis by digestive enzymes of the baker's yeast bread.

Cereal-baked goods are currently manufactured by very accelerated processes in which the long-time fermentation by sourdough starter cultures, a cocktail of acidifying and proteolytic lactic acid bacteria with yeasts, is almost totally replaced by the use of chemical leavening agents, baker's yeast leavening agents, or both. The proteolytic activity of sourdough lactobacilli and VSL#3 preparation was clearly proven to have an effect on human gluten tolerance due to the presence of a pool of specialized peptidases (9, 11). Together with the results of this study, it may be suggested that traditional protocols of bread making, which include long-time fermentation (e.g., 24 h at 37°C) with selected starters, are suitable to cause an extensive degradation of the wheat flour proteins involved in allergy and

intolerance. VSL#3 activity markedly influenced the wheat IgE-binding proteins, which, consequently, resulted in in vitro degradation by digestive enzymes.

Food production is becoming increasingly complex, with several different food products being made in the same facility, and carries the risk of cross-contamination of allergenic wheat compounds. Under these circumstances, a biotechnology like VSL#3 treatment, especially as a starter for dough fermentation, should at least eliminate the danger of the presence of traces of wheat allergens in wheat-free baked goods.

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