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A New Method for Determination of Insoluble Cell Walls and Soluble Nonstarchy Polysaccharides from Plant Materials

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A two-step enzymatic procedure was established for accurate determination of insoluble plant cell walls (including polysaccharides, lignin, and wall proteins), the major component of "dietary fiber". Cytoplasmic proteins are efficiently eliminated by Pronase with presence of sodium lauryl sulfate and 2-mercaptoethanol, and starch is enzymatically removed by amyloglucosidase after solubilization in hot 90% DMSO and limited breakdown by Termamyl in boiling 30% DMSO. Cell walls of a good standard of purity are obtained with respect to their protein content (1–8%). The method allows the quantitative gravimetric determination of the plant cell walls with a good precision (cv = 5.6%), whatever the wall percent from very low contents (semolina, 1.4%, cv = 5.9%) to very high ones (wheat straw, 84.4%, cv = 0.9%). The technique is rapid and operates under mild conditions suitable for chemical preservation of wall polysaccharides. A separate determination of soluble nonstarchy polysaccharides by gas–liquid chromatography of alditol acetates from monosaccharides obtained after acid hydrolysis showed that they represent in most samples a very low proportion of total "dietary fiber" but in cereal ones (endospermic parts only) they constitute ~20–40%.

Since Trowell (1976) reported the beneficial action of so-called "dietary fiber" in man for prevention of intestinal diseases in developed countries, considerable literature has arisen reporting experiments on man and animal fed fiber-supplemented diets (Cummings et al., 1978; Bertrand et al., 1981; Nyman and Asp, 1982), food and feed composition tables (Englyst et al., 1982; Carré and Brillouet, 1986), and methodology of fiber determination (Schweizer and Würsch, 1979; Selvendran and DuPont, 1980; Englyst and Cummings, 1984; Asp et al., 1983; Prosky et al., 1984).

Although the biochemical significance of recently developed methods for the determination of dietary fiber has considerably improved as compared to the former crude fiber method, they are still proposed on nutritional grounds in accordance with Trowell's definition (1976) contrary to other plant nutrients (proteins, starches, ...) that are basically measured by chemical [e.g., DMSO–hydrochloric acid method for starches (Boehringer, 1976)] techniques prior to application of methods approximating their nutritional behavior [e.g., α-amylolysis for starches (Tollier et Guilbot, 1971)]. An excellent biochemical procedure has been developed by Selvendran (1975) for preparation of plant cell walls that was not initially designed for nutritional purposes; it provides a cell wall residue of optimum purity with respect to residual intracellular proteins and starch but could hardly be fitted to heavy-user requirements due to its length.

The aim of the present study was therefore to develop a rapid and accurate sequential enzymatic method for gravimetric determination of plant cell walls of general applicability to raw plant materials and processed food and feedstuffs. The related soluble nonstarchy polysaccharides will also be considered. Biochemical aspects of the technique (purity of residues, preservation of chemical structures, ...) will be emphasized.

EXPERIMENTAL SECTION

Samples. Description of the various products studied in the present work is presented in Tables I and II. These materials were ground in an IKA grinder with tap water refrigeration for not longer than 3 min; the ground material was sifted through a 0.5-mm screen, and coarser particles were reground until all the sample passed through a 0.5-mm sieve.

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Table I. Description of the Plant Material

no.	product	description
		Monocotyledons
	Gramineae	
1	wheat flour (*Triticum vulgaris* L.)	white wheat flour (T55, Westhove Co.)
2	wheat bran (*Triticum vulgaris* L.)	coarse wheat bran
3	wheat straw (*Triticum vulgaris* L.)	
4	semolina (*Triticum durum* L.)	low extraction S3 (cv. Agathe; INRA, Technologie Céréales, Montpellier)
5	oats (*Avena sativa*)	whole grain
6	sorghum (*Sorghum bicolor*)	whole grain (cv. NK121; INRA, Plantes Fourragères, Lusignan)
7	corn draff (*Zea mays*)	
7′	corn germ meal (*Zea mays*)	
8	bagasse (*Saccharum officinarum* L.)	
	Palms	
9	coprah meal (*Cocos nucifera*)	
		Dicotyledons
	Leguminosae	
10	soya bean meal 1 (*Glycine max* L.)	dehulled seed (solvent extracted)
11	soya bean meal 2 (*Glycine max* L.)	dehulled seed (solvent extracted)
12	soya bean hulls (*Glycine max* L.)	
13	faba bean (*Vicia faba* var. minor L.)	whole seed
14	common bean (*Phaseolus vulgaris* L.)	whole seed (cv. Ain Temouchent, Algeria)
15	pea fiber (*Pisum sativum* L.)	fibrous residue after protein and starch extraction
16	groundnut meal (*Arachis hypogea* L.)	
17	white lupin meal (*Lupinus albus* L.)	dehulled seed (cv. Lucky 811; INRA, Plantes Fourragères, Lusignan; solvent extracted)
18	lucerne (*Medicago spp.*)	
	Chenopodiaceae	
19	sugar beet pulp (*Beta vulgaris* L.)	
	Cruciferae	
20	rapeseed meal (*Brassica mannensis* L.)	whole seed (solvent extracted)
	Compositae	
21	sunflower meal (*Helianthus annuus* L.)	
22	lettuce (*Lactuca sativa* L.)	INRA, Bioconversion, Narbonne
	Euphorbiaceae	
23	cassava (*Manihot esculenta* Crantz)	skinned dried tubers
	Rubiaceae	
24	coffee bean hulls (*Coffea canephora* P. ex. Fr.)	SIRAD, Montpellier
	Vitaceae	
25	grape pomace (*Vitis vinifera* L.)	INRA, Bioconversion, Narbonne
	Rutaceae	
26	citrus pulp (*Citrus spp.*)	
	Oleaceae	
27	olive pulp (*Olea europea*)	pulp obtained by pression and hexane extraction
		Mixed Foods
28	Grany (soft cookies, Heudebert)	müesli (26%) (oats, wheat bran, coconut, corn flakes, ...), wheat flour, pulp from various berries (17%)
29	Chocapic (soft cookies, Nestlé)	wheat whole meal (46%), cocoa, chocolate
30	Grelines (soft cookies, Nestlé)	cereals (58%) (barley, oats, wheat), fruits (9%) (grapes, apple)
31	Cracottes (extruded toasts, Diepal)	wheat whole meal (70%), rye flour
32	Capt'n Crokies (extruded toasts, Quakers)	oats, chocolate
33	Glucottes (extruded toasts, SHN, Dijon)	corn flour, wheat bran, rice, amylomaize starches
		Feedstuffs and Digesta
34	milk goat concentrate	maize (whole grain) (65%), soya bean meal (11%), dextrose (9.5%), urea (3%), molasses (5%), CWM (6.5%)
35	swine feedstuff	CANA, Ancenis
36	dairy cow concentrate	CANA, Ancenis
37	coprah meal diet	maize starch (42.4%), coprah meal (33.2%), casein (15.1%), maize oil (4.5%)
38	beet pulp diet	sugar beet pulp (35%), corn starch (39%)
39	in vitro (Rusitec) digested beet pulp	sugar beet pulp fermented in a Rusitec (48 h; INRA, LTAA, Nantes)
40	in vitro (Rusitec) digested wheat straw	wheat straw fermented in a Rusitec (48 h; INRA, LTAA, Nantes)

The influence of particle size on the yield of insoluble cell wall polysaccharides was studied by ultramilling some of the above samples in liquid nitrogen with a Spex 6700 freezer mill (Spex Industries Inc., Metuchen) for 2.5, 10, and 20 min (Lomax et al., 1983). The particle size distribution was analyzed in 4% ammonium thiocyanate in 2-propanol with a Coulter counter.

Enzymes and Chemicals. Pronase from *Streptomyces griseus* (31 nKat/mg; 40 °C; bovine serum albumin as substrate; pH 7.5) was from Boehringer (Mannheim, West Germany). Heat-stable α-amylase (E.C. 3.2.1.1; Termamyl 60L) from *Bacillus licheniformis* (22.8 μKat/mL; 40 °C; 1% waxy maize β-limit dextrin as substrate; pH 7.0) was obtained from Novo A/S (Copenhagen, Denmark). Amyloglucosidase (E.C. 3.2.1.3) from *Aspergillus niger* (117 nKat/mg; 40 °C; 25 mM maltose as substrate; pH 4.5) was purchased from Merck (Darmstadt, West Germany). A 1-nKat portion of enzyme liberated 1 nmol of reaction product per s (Folin-positive amino acids and peptides equivalent to 1 nmol of tyrosine for Pronase and reducing sugars equivalent to 1 nmol of glucose for amyloglucosidase or maltose for Termamyl 60L). Enzymes were checked for contaminating activities on the following substrates: crude sugar beet arabinan (Koch-Light 52867), polygalacturonic

Table II. Protein, Starch, and Ash Contents of Starting Materials

no.	water	protein,[a] N × 5.7	starch[a]	ash[a]
1	14.2	8.8	76.0	0.6
2	11.2	13.3	20.0	6.9
3	9.3	3.1	0.1	6.1
4	12.7	12.3	63.0	0.6
5	9.3	7.6	65.4	3.2
6	13.2	11.7	62.6	1.8
7	9.5	18.0	0.2	5.9
8	7.5	6.6	nd	4.1
9	9.7	nd[b]	0.5	7.7
10	11.3	43.5	0.3	6.4
11	11.9	50.5	1.1	6.5
12	10.9	11.4	0.1	5.0
13	16.2	28.0	33.2	3.6
14	12.4	25.0	38.7	4.4
15	8.2	2.7	0.3	2.9
16	11.7	52.3	2.0	4.9
17	13.5	43.4	0.2	4.9
18	9.6	17.7	0.2	8.0
19	11.2	9.5	nd	6.8
20	10.6	35.6	0.6	6.7
21	10.0	33.7	0.2	7.1
22	12.4	22.3	0.1	13.7
23	11.3	1.3	80.7	1.0
24	10.7	9.2	0.1	7.5
25	5.5	11.1	nd	4.7
26	10.1	7.0	1.0	7.9
27	5.2	5.1	0.1	2.7
28	4.1	7.4	23.5	2.6
29	2.7	7.1	34.5	2.3
30	4.4	6.6	46.8	1.9
31	4.4	10.0	56.2	3.3
32	2.8	7.9	37.1	4.0
33	7.7	2.4	63.2	1.8
34	12.7	18.5	31.4	7.3
35	13.1	13.7	35.7	8.1
36	13.8	19.6	26.0	11.0
37	10.5	13.8	nd	7.8
38	10.7	17.2	31.6	8.0
39	11.3	26.0	nd	22.8
40	13.4	4.1	nd	11.2

[a] Percent dry basis. [b] nd = not determined.

acid (ICN 102771), larchwood arabinogalactan (Sigma A-2012), carboxymethylcellulose sodium salt (Sigma C-4888, medium viscosity), oat spelt xylan (Sigma, X-0376), and hemicellulose B from wheat bran (Brillouet and Mercier, 1981). Amylomaize starch (Eurylon) was from Roquette Frères (Lestrem, France) while wrinkled pea starch was from our department (Colonna and Mercier, 1984).

Sodium lauryl sulfate (=sodium dodecyl sulfate, SDS), 2-mercaptoethanol, and dimethyl sulfoxide (DMSO) were of the highest grade available (Merck).

Analytical Methods. Moisture contents were determined by drying at 130 °C for 2 h; all yield and composition calculations were made on a moisture-free basis. Proteins (N × 5.7) were determined by the Kjeldahl procedure. Starch was measured as glucose in supernatant from amyloglucosidase treatment by the glucose oxidase–peroxidase–ABTS system (Boehringer, 1976). Ashes were measured by incinerating overnight at 550 °C.

Soluble nonstarchy polysaccharides were hydrolyzed with 2 M trifluoroacetic acid (TFA) for 1.25 h at 120 °C. (Albersheim et al., 1967). Insoluble cell wall polysaccharides were hydrolyzed by pretreatment with 72% sulfuric acid for 0.5 h at 25 °C (particle size ~80 μm) followed by dilution to 1 M and heating at 100 °C for 2 h (Saeman et al., 1954; Hoebler et al., 1986). Liberated neutral sugars were derivatized into their alditol acetates (Sawardeker et al., 1965) and analyzed by GC according to the procedure of Blakeney et al. (1983) as modified by Hoebler et al. (1988). Inositol was used as the internal standard.

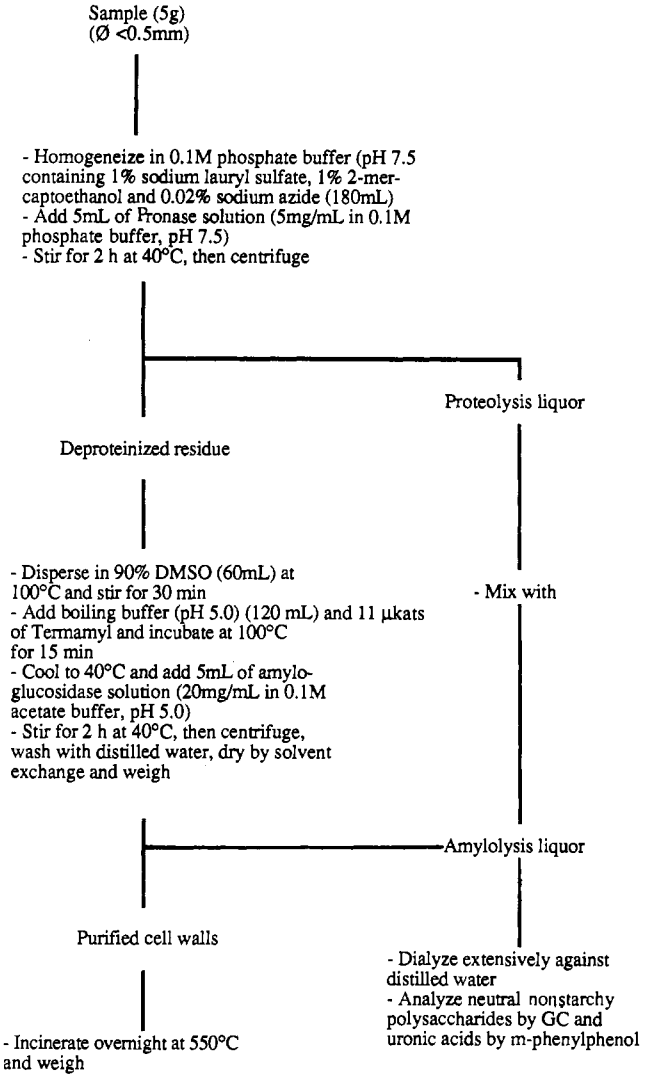

Figure 1. Purification procedure of insoluble cell walls and soluble nonstarchy polysaccharides.

Soluble nonstarchy polysaccharides were also analyzed automatically for their neutral sugar and uronic acid contents by the orcinol (Tollier and Robin, 1979) and the *m*-phenylphenol (MHDP) (Blumenkrantz and Asboe-Hansen, 1973; Thibault, 1979) methods, respectively. Orcinol responses were corrected for uronic acid interferences. Galacturonic acid was used as a standard for dicotyledonous plants (products 10–27) and for products 38–39 while glucuronic acid was preferred for all other materials. Arabinose was chosen for orcinol standardization. Uronic acids occurring in the insoluble cell wall polysaccharides were automatically analyzed by the MHDP technique after dispersion in 72% sulfuric acid at 25 °C (Ahmed and Labavitch, 1977) followed by hydrolysis in 2 N H_2SO_4 at 100 °C (2 h), using same calibration as for soluble polysaccharides.

Determination of Insoluble Cell Walls and Soluble Nonstarchy Polysaccharides. The sequential enzymatic procedure is shown in Figure 1.

Sample Preparation. Native or freeze-dried samples (moisture content <20%) were milled for 3 min and sieved on a 0.5-mm screen (see the Experimental Section). Fatty materials can be processed without preliminary defatting providing they pass a 0.5-mm sieve.

Proteolysis. Sample (5 g) was weighed with 0.1-mg ac-

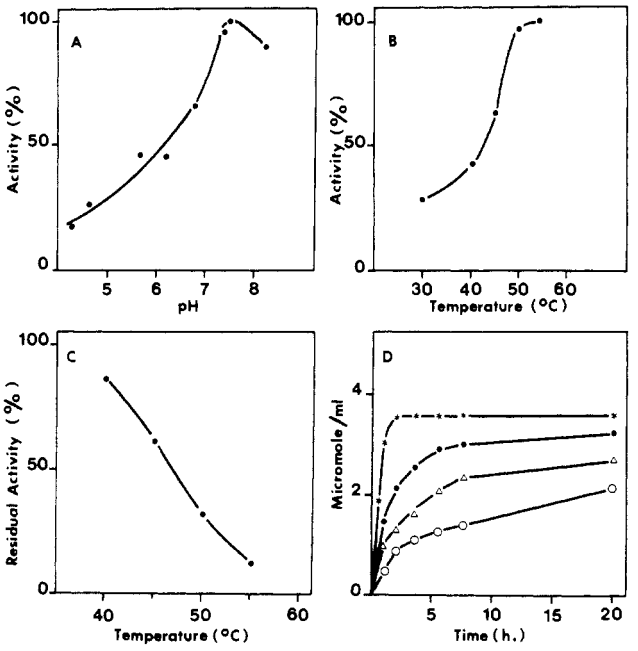

Figure 2. Working parameters of Pronase. Effect of pH (A) and temperature (B) on Pronase activity. (C) Heat stability of Pronase. (D) Influence of the Pronase to bovine serum albumin ratio on the proteolysis rate: (○) 1/1000; (△) 1/500; (●) 1/200, (*) 1/50.

curacy in a stoppered 250-mL Erlenmeyer flask fitted with a magnetic bar. Phosphate buffer (pH 7.5) (180 mL, 0.1 M) containing 1% sodium lauryl sulfate, 1% 2-mercaptoethanol (w/v), and 0.02% sodium azide was added and the resultant mixture homogenized by gentle magnetic stirring (no clumps). Pronase solution (5 mL, 5 mg/mL in 0.1 M phosphate buffer, pH 7.5) was added and the mixture placed in an oscillating water bath (40 °C) for 2 h (100 oscillations/min). The mixture was transferred to a centrifuge bottle, the Erlenmeyer flask was rinsed with 15 mL of distilled water, and the washings were added to the centrifuge bottle and centrifuged for 25 min at 15000*g* (20 °C). The proteolysis supernatant was kept overnight at ambient temperature (under hood).

Amylolysis. The pellet from proteolysis was resuspended in hot (100 °C) 90% dimethyl sulfoxide (60 mL) and transferred into a stoppered 250-mL Erlenmeyer flask fitted with a magnetic bar. The mixture was homogenized by gentle magnetic stirring (no clumps), placed in an oscillating boiling water bath (100 °C) for 30 min (100 oscillations/min), and stirred from time to time with a magnetic stirring device. Boiling 0.1 M acetate buffer (pH 5.0, 150 mL) was added and the resultant solution mixed thoroughly; 0.5 mL of Termamyl 60L was added, and the resultant solution was mixed and allowed to remain in the oscillating boiling water bath for an additional 15 min. The Erlenmeyer flask was withdrawn and left on the bench to cool to 40 °C. Amyloglucosidase solution (5 mL, 20 mg/mL in 0.1 M acetate buffer, pH 5.0) was added and the flask placed in an oscillating water bath (40 °C) for 2 h. Contents were transferred to a centrifuge bottle, rinsed, and centrifuged as described above. The amylolysis supernatant was kept overnight at ambient temperature, pooled with the proteolysis supernatant, and dialyzed extensively against distilled water (20 °C).

Gravimetric Determination of Insoluble Cell Walls. The final pellet was washed extensively with distilled water with intermittent centrifugation, resuspended in 50 mL of absolute ethanol, and filtered through a weighed fritted glass crucible (porosity 4, o.d. pores 10–16 μm). It was dried by acetone and then diethyl oxide (50 mL each),

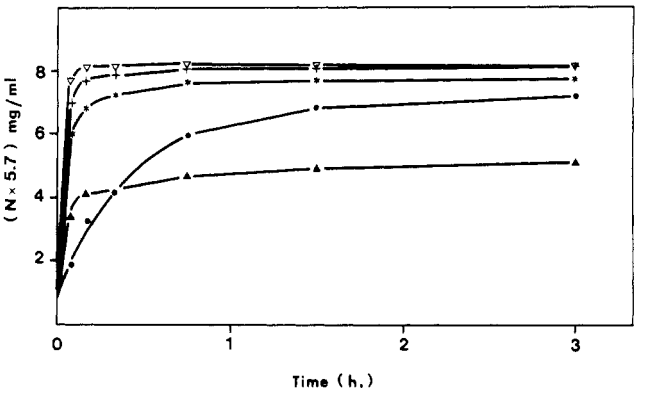

Figure 3. Kinetics of solubilization–proteolysis of proteins from soya bean meal (product 11) by various combinations of reagents: (●) Pronase; (▲) sodium lauryl sulfate; (*) sodium lauryl sulfate + 2-mercaptoethanol; (+) Pronase + sodium lauryl sulfate; (▽) Pronase + sodium lauryl sulfate + 2-mercaptoethanol.

placed in a vacuum oven (0.50 psi) overnight at 70 °C, cooled in a desiccator, and weighed with 0.1-mg accuracy. Finally, the pellet was incinerated overnight at 550 °C and weighed with 0.1-mg accuracy.

$$\text{insol cell walls (\%)} = \frac{\text{wt of residue} - \text{wt of ashes}}{\text{wt of sample}}$$

Separate Determination of Soluble Nonstarchy Polysaccharides. Mixed dialyzed supernatants from proteolysis and amylolysis were subjected to colorimetric analysis of uronic acids by the *m*-phenylphenol technique and to GC analysis of neutral constituent monosaccharides.

$$\text{sol nonstarchy polysaccharides (\%)} = \frac{\text{wt of (neutral + acidic) polysaccharides}}{\text{wt of sample}}$$

Light Microscope Examinations. Purified cell walls were observed under light microscope using Fast Green coloring agent for examination of residual proteins and I_2–KI for starch (Locquin and Langeron, 1978).

RESULTS AND DISCUSSION

Proteolysis. We chose Pronase for removal of cytoplasmic proteins since Morrison (1973) has shown that Pronase, a mixture of endo- and exopeptidases able to degrade proteins up to the amino acid level, was the most efficient agent for removal of intracellular proteins in grass samples as compared to pepsin and sodium lauryl sulfate. Pronase has been also occasionally used by others for successfull preparation of cell walls with low nitrogen content (Selvendran, 1975; Brillouet and Carré, 1983).

Determination of Optimum Working Parameters for Pronase. The pH and temperature optima, the thermal stability, and the influence of Pronase to bovine serum albumin ratio on the rate of proteolysis were studied, and the data are shown in Figure 2. The activity of Pronase was determined (pH 7.5, 40 °C) by measuring by the Lowry method the release from bovine serum albumin (BSA) of amino acids and peptides soluble in 10% trichloroacetic acid. The enzyme exhibited maximum activity at pH 7.5 and at 55 °C in a 10-min assay. So, Pronase works better around neutral, in contrast to pepsin (Schweizer and Würsch, 1979; Asp et al., 1983) or Alcalase (Asp et al., 1983), which is more favorable to the chemical stability of wall polysaccharides since pectic substances may undergo alkaline β-elimination (Albersheim et al., 1960) from one side and arabinofuranosyl linkages occurring in some polysaccharides (arabinoxylans, arabinans,

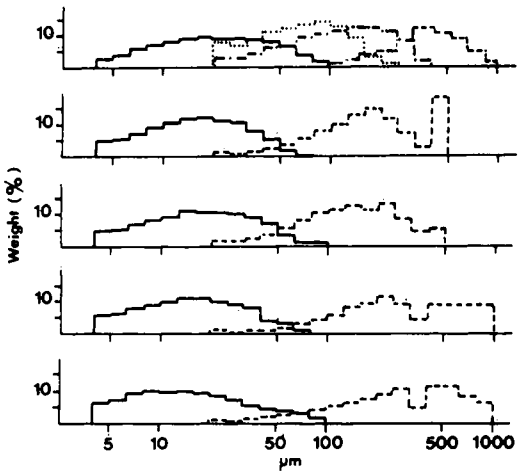

Figure 4. Particle size distribution of starting (- - -) and 20-min-milled (liquid N_2) (—) materials. Top to bottom: wheat bran (2); rapeseed meal (20); lucerne (18); corn germ meal (7′); white lupin meal (17). For wheat bran, 1-min- (-·-), 2.5-min- (···), and 10-min- (—) milled products are also shown.

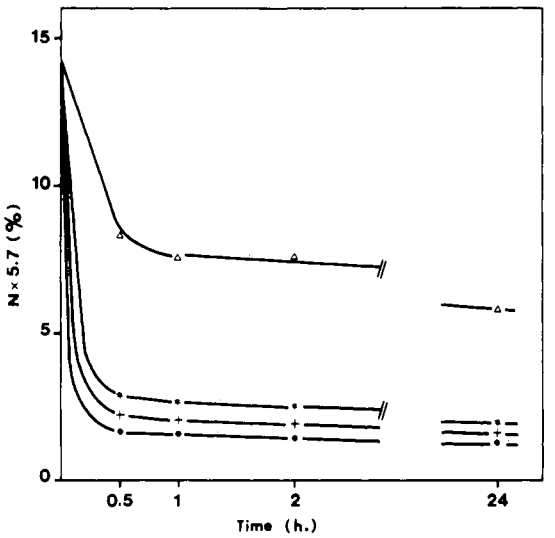

Figure 5. Influence of the particle size on the residual protein content of wheat bran (2). Duration of milling in liquid N_2: (*) 2.5 min; (+) 10 min; (●) 20 min. (△) Starting bran (<0.5 mm).

...) may be split under acidic conditions from other side (Asp et al., 1983). The residual activity after 2-h incubation at pH 7.5 at various temperatures was tremendously reduced above 40 °C, an 85% recovery being measured at 40 °C. On the other hand, the rate of proteolysis was strongly influenced by the ratio (pronase/BSA), the highest ratio 1/50 giving a maximum extent of degradation within 2 h; a 1/100 ratio was finally selected for limitation of the assay cost, which corresponds for the plant materials richest in proteins, e.g. defatted cake from groundnut (product 16, ~50% N × 5.7), to 25 mg of Pronase in a 5-g assay. This amount will be kept throughout the study whatever the protein content of the sample.

The following conditions were preliminarily chosen for the proteolysis step and tested on soya bean meal (product 11): pH 7.5, presence of 0.02% sodium azide as bactericide, temperature 40 °C, solid to liquid ratio 1/37.

Influence of Pronase in Admixture with Various Chemicals on Nitrogen Solubilization. To help solubilization of storage proteins (protein bodies) and improve the rate and extent of proteolysis, the efficiency of Pronase treatment was tested in adjunction with some detergents and a reducing agent. A soya bean meal (product 11,

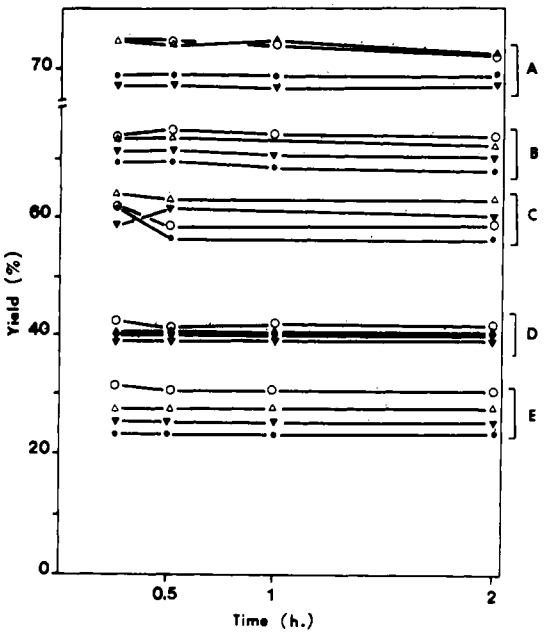

Figure 6. Influence of the particle size in a 2-h assay on the yield in cell walls (corrected for residual proteins, N × 5.7). Duration of milling in liquid N_2: (○) unmilled; (△) 2.5 min; (▼) 10 min; (●) 20 min. Key: (A) wheat bran (2), (B) corn germ meal (7′); (C) lucerne (18); (D) rapeseed meal (20); (E) white lupin meal (17).

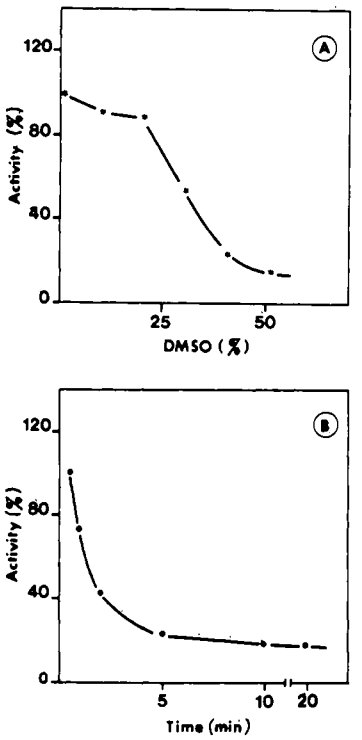

Figure 7. Working parameters of Termamyl in dimethyl sulfoxide: (A) effect of increasing concentration of DMSO in the reaction medium on activity; (B) residual activity as a function of time in hot (100 °C) 30% DMSO.

~50% N × 5.7, particle size <0.5 mm) was submitted to action of Pronase alone and in admixture with sodium cholate, Tween 80, and sodium lauryl sulfate or (sodium lauryl sulfate + 2-mercaptoethanol), each chemical being at 1% final level. The activity of Pronase with the presence of each chemical was ~80% of the reference. After 2-h incubation in an oscillating water bath, the medium was centrifuged and the pellet extensively washed with distilled water and dried by solvent exchange through

Table III. Influence of the Milling in Liquid N_2 on the Yield of Cell Wall Noncellulosic Polysaccharides

	starting material (<0.5 mm)[a,b]								20 min milled[a,b]							
product	Rha	Fuc	Ara	Xyl	Man	Gal	Glu	total	Rha	Fuc	Ara	Xyl	Man	Gal	Glu	total
rapeseed meal	0.3	0.2	5.1	2.1	0.2	1.8	1.6	11.5	0.3	0.2	4.5	1.8	0.2	1.6	1.5	10.2
corn germ meal	0.2	tr.	11.5	11.9	0.2	2.4	29.2	55.5	0.1	tr.	12.1	14.6	0.2	2.8	22.3	52.3
lucerne	0.4	0.1	1.8	4.5	0.4	1.2	1.5	10.1	0.3	0.1	1.6	3.8	0.4	1.0	1.4	8.6
white lupin meal	0.3	0.2	4.0	1.8	tr.	16.0	0.3	22.7	0.3	0.2	2.7	1.2	tr.	9.2	0.3	14.0

[a] After hydrolysis by 2 M trifluoroacetic acid (120 °C, 1.25 h). [b] Percent of dry matter of starting sample.

Table IV. Yield and Composition of Insoluble Cell Walls

no.	cell wall[a,b]	protein[c]	proteolysis efficiency	ash[c]	cell wall polysaccharides[c]
1	2.5 ± 0.1	4.8	98.7	nd	58.9
2	48.4 ± 0.5	4.8	82.0	3.9	55.6
3	84.4 ± 0.8	1.3	64.5	4.2	63.2
4	1.4 ± 0.1	3.6	99.6	1.6	94.5
5	29.4 ± 2.5	2.0	92.1	4.5	79.1
6	9.5 ± 0.1	18.1	85.5	2.5	42.5
7	34.5 ± 0.3	2.6	95.0	1.9	72.2
8	79.1 ± 0.8	0.9	99.0	3.6	64.2
9	51.8 ± 0.4	nd		3.2	69.7
10	21.3 ± 1.1	6.0	81.1	6.2	73.2
11	18.9	3.4	98.9	nd	nd
12	71.8 ± 0.1	4.2	72.8	3.6	78.0
13	21.9 ± 0.3	10.8	91.4	3.5	67.5
14	16.0 ± 0.5	7.7	94.8	2.9	69.7
15	56.9 ± 0.8	0.6	85.2	7.0	80.5
16	23.0 ± 0.7	8.2	96.2	5.2	58.1
17	26.4 ± 0.1	4.7	97.0	3.1	77.8
18	45.9 ± 7.0	5.5	83.6	13.3	55.5
19	46.7 ± 2.3	3.2	83.2	9.3	66.6
20	35.1 ± 1.6	7.2		8.4	49.8
21	36.0 ± 0.5	5.6	93.5	8.4	56.0
22	30.2 ± 1.2	1.6	97.8	9.1	67.9
23	4.9 ± 0.8	2.0	95.4	1.3	63.3
24	70.3 ± 1.5	6.7	46.8	4.5	49.6
25	69.2 ± 1.0	8.1	47.8	3.3	25.8
26	33.6 ± 1.1	1.3	92.9	14.0	69.9
27	71.0 ± 1.3	5.3	23.6	2.7	16.0
28	3.5 ± 0.6	nd		6.6	60.0
29	6.2 ± 0.3	nd		6.0	61.2
30	1.7 ± 0.2	nd		14.3	56.6
31	9.6 ± 0.4	6.3	93.0	13.1	59.1
32	4.2 ± 0.3	nd		12.8	37.9
33	13.6 ± 0.4	4.0	75.0	3.2	81.2
34	10.2 ± 0.2	3.6	97.3	19.5	58.8
35	21.1 ± 0.9	5.1	91.2	14.0	58.7
36	30.7 ± 2.4	6.0	89.3	13.3	57.2
37	24.3 ± 0.1	4.8	91.3	9.1	68.8
38	19.0 ± 0.1	5.3	93.0	14.6	64.2
39	48.8 ± 2.6	1.2	97.7	25.7	37.3
40	76.7 ± 0.6	3.8	27.0	4.2	64.4

[a] Percent of starting material, dry basis. [b] Corrected for ashes. [c] Percent of cell wall, dry basis.

Table V. Monosaccharide Constituent of Cell Wall Polysaccharides[a]

no.	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	U[b]	total
1			16.4	25.6	2.6	0	12.9	1.4	58.9
2	0.3		15.4	25.6	0.4	1.8	20.0	2.7	55.6
3	0.2		2.3	20.2	0.3	0.7	37.1	2.4	63.2
4		0.3	20.6	34.1	8.2	0.9	15.0	15.4	94.5
5	0.3		4.2	27.0	0.3	1.0	25.7	2.2	79.1
6	0.6		9.6	8.3	1.1	1.4	19.7	1.9	42.5
7			15.8	25.0	1.1	4.2	20.8	5.3	72.2
8	0.3	0.3	1.1	18.9	0.2	0.3	40.5	2.6	64.2
9	0.2		1.6	1.7	47.7	3.3	12.5	2.7	69.7
10	1.3	1.2	8.5	7.9	2.6	11.7	31.5	11.4	73.2
11	1.8	1.5	12.1	5.6	0.9	19.0	21.0	nd	
12	0.9	0.4	5.0	10.2	5.9	2.5	42.8	10.3	78.0
13	0.9		6.0	5.9	0.2	1.5	46.2	6.8	67.5
14	0.8	0.6	15.0	6.8	0.5	2.0	34.1	9.9	69.7
15	1.6	0.5	39.2	4.0	0.3	3.9	16.9	14.1	80.5
16	1.1	0.6	11.4	7.6	1.0	2.0	23.1	11.3	58.1
17	1.2	0.3	9.9	4.1	0.3	45.8	8.7	7.5	77.8
18	0.9	0.2	2.9	7.6	1.6	1.9	30.4	10.0	55.5
19	1.2		15.1	2.6	1.7	4.5	34.5	7.0	66.6
20	1.0	0.6	10.4	4.7	0.7	4.1	16.1	12.2	49.8
21	1.0	0.3	5.7	10.2	2.9	2.2	24.4	9.3	56.0
22	1.4	0.2	3.1	3.9	0.6	3.4	30.1	25.2	67.9
23	1.8	0.3	2.6	6.1	1.6	10.0	35.2	6.4	63.3
24	0.6		3.1	9.9	1.6	2.4	26.2	5.8	49.6
25	0.4		0.9	5.2	1.4	1.2	13.0	3.7	25.8
26	0.8	0.5	7.5	4.7	2.6	5.8	25.7	22.3	69.9
27	0.9		2.7	1.1	0.6	0.8	7.3	2.6	16.0
28	0		10.4	17.5	5.9	2.1	20.9	3.2	60.0
29	0.8		13.6	20.7	1.5	1.6	20.5	3.1	61.2
30			11.8	18.7	2.9	1.2	17.8	4.2	56.6
31	0.4		13.4	22.5	1.1	1.1	18.2	2.4	59.1
32	0.5		7.2	11.1	1.5	1.1	13.5	3.0	37.9
33	0		6.4	10.4	3.2	1.9	56.9	2.4	81.2
34	0.6	0.3	11.9	14.9	1.5	5.2	19.0	5.4	58.8
35	0.4		10.4	17.7	1.0	2.4	28.7	4.1	58.7
36	0.3		11.3	12.5	1.5	4.3	20.0	7.3	57.2
37	6.2		2.0	2.0	46.1	3.3	12.5	2.7	68.8
38	1.4		16.2	2.7	1.6	4.2	32.2	5.9	64.2
39	0.6		4.0	6.3	1.1	1.5	21.5	2.3	37.3
40	0.4		2.0	18.8	0.2	0.6	40.0	2.4	64.4

[a] Anhydro sugars as percent of cell walls, dry basis. [b] Uronic acids.

ethanol, acetone, and ether; the residual nitrogen content was determined by the Kjeldahl procedure on the deproteinized residue. Protein (N × 5.7) content: Pronase-treated meal, 20.8%; Pronase + sodium cholate treated meal, 15.4%; Pronase + Tween 80 treated meal, 15.4;, Pronase + sodium lauryl sulfate treated meal, 6.6%; Pronase + sodium lauryl sulfate + 2-mercaptoethanol treated meal, 3.4%. From these data, it can be concluded that the proteolytic action of Pronase is greatly enhanced with the presence of sodium lauryl sulfate, more so if 2-mercaptoethanol is added. The synergistic effect of the the three reagents (Pronase, sodium lauryl sulfate, 2-mercaptoethanol) was better demonstrated by analyzing (Kjeldahl) the nitrogen solubilized by various combinations of them (Figure 3). Pronase itself released nitrogen from soya bean meal at a moderate speed as did sodium lauryl sulfate. It must be noted that the solubilizing effect of sodium lauryl sulfate was strongly improved by addition of 2-mercaptoethanol. Nevertheless, the best result was obtained by a combination of the three components, therefore adopted for the continuation of the study.

Influence of Particle Size on Residual Nitrogen Content and Yield in Insoluble Cell Walls. Wheat bran (product 2, 13.3% N × 5.7, particle size <0.5mm) was chosen for a preliminary study on the influence of particle size on residual nitrogen content after Pronase treatment. The starting bran was ground in liquid nitrogen with a Spex mill (Lomax et al., 1983) for 2.5, 10, and 20 min, and the particle size distributions of resulting products is shown in Figure 4. Brans were submitted to Pronase in admixture with sodium lauryl sulfate and 2-mercaptoethanol under the above given conditions, and the washed residues were analyzed for nitrogen (Figure 5). The coarsest bran exhibited a two-slope kinetics: a very fast solubilization of nitrogen within 30 min followed by a slow removal up to 24 h. Reduction of the particle size highly improved the efficiency of the proteolytic treatment, the nitrogen content of the deproteinized residue being tremendously

Table VI. Characterization of Soluble Nonstarchy Polysaccharides

no.	precip (80% ethanol)[a,e]	neutral polysaccharides[a] orcinol S1	orcinol S2	GC S1	GC S2	uronic acids[a] S1	uronic acids[a] S2	neutral (GC) + acidic polysaccharides in (S1 + S2) c	d
1	0.8	0.20		0.75	0.10			0.8	24.2
2	0.6	0.30	0.50	0.70	0.45	0.05	0.10	1.3	2.6
3		0.50	1.00	0.45	0.45	0.20	0.40	1.5	1.7
4	0.1	1.10	0.80	0.45	0.15	0.10	0.24	0.9	39.1
5	0.1	0.80	3.90	0.80	0.30			1.1	3.6
6	0.1	0.60	1.10	0.40	0.10	0.30	0.85	1.6	14.4
7	0.2	0.90	0.70	0.10	0.20	0.10	0.20	0.6	1.7
8		0.80	0.70	0.30	0.15	0.30	0.15	0.9	1.1
9	0.1	0.40	0.10	0.80	0.25	0.10	0.06	1.2	2.3
10	6.8	0.20		0.55	0.35			0.9	4.0
12	4.2	1.10	0.40	1.20	0.10	0.15	0.05	1.5	2.0
13	2.0	0.90	1.10	0.35	0.15	0.15	0.25	0.9	3.9
14	4.4	0.60	1.70	0.35	0.15	0.05	0.20	0.7	4.2
15	7.1	0.70	5.10	0.75	1.30	0.05	0.50	2.6	4.4
16	5.2	0.50	0.10	0.35	0.20	0.10	0.05	0.7	2.9
17	9.2	1.60	7.60	0.70	3.35	0.10	0.70	4.8	15.4
18	5.8	2.00	2.70	0.65	0.30	0.50	0.10	1.5	3.2
19	22.0	2.20	4.50	4.65	1.05	2.55	2.15	10.4	18.2
20	4.6	1.50	2.70	0.85	0.30	0.10	0.20	1.4	3.8
21	7.8	0.30	0.40	0.35	0.35	0.05	0.05	0.8	2.2
22	0.1	0.90	1.10	0.85	0.10	0.10		1.0	3.2
23	0.1	0.10	0.90	0.15	0.15		0.20	0.5	9.2
24	12.0	1.30	0.80	0.65	0.60	0.05	0.30	1.6	2.2
25	5.2	0.70	0.90	0.65	0.30	0.05	0.20	1.2	1.7
26	10.8	1.60	1.30	1.10	0.45	0.60	0.85	3.0	8.2
27		1.50	4.90	1.00	0.70	0.30	0.65	2.6	3.5
28		0.60	0.60	1.25	0.10	0.20	0.10	1.6	31.4
29		1.80	1.30	1.65	0.30	0.25	0.15	2.3	27.0
30	2.5	1.20	1.10	1.30	0.25	0.10	0.15	1.8	51.4
31		1.50	2.30	1.40	0.60	0.05	0.15	2.2	18.6
32		1.50	1.10	1.50	0.30	0.15	0.15	2.1	33.3
33	3.8	0.80	0.40	1.30	0.30	0.05	0.10	2.3	14.5
34		0.40	0.50	0.35	0.10	0.10	0.10	0.6	5.5
35		0.70	0.90	0.70	0.25	0.20	0.10	1.2	5.4
36	6.4	0.40	0.40	0.65		0.05	0.15	0.8	2.5
37	6.2	0.70	0.60	0.90	1.53	0.24	0.06	2.7	10.0
38	5.6	3.30	1.00	0.70	0.14	0.60	0.15	1.6	7.8
39	5.2	0.30	0.60	0.50	0.40	0.05	0.10	1.0	2.0
40	0.1	1.00	1.50	0.15	0.15	0.60	0.90	1.8	2.3

[a] Percent of starting materials, dry basis. [b] Key: S1 = supernatant from proteolysis; S2 = supernatant from amylolysis. [c] Total nonstarchy polysaccharides (as determined by GC for neutral and *m*-phenylphenol for acidic polysaccharides) undergoing solubilization during both proteolysis and amylolysis treatments. [d] Percent of (c) in total dietary fiber [=solute nonstarchy polysaccharides + cell wall materials]. [e] Ethanol precipitation on mixed S1 and S2.

lowered with increased duration of milling. A very low (1.4%) level of remaining protein was obtained after 2 h for the finest (<100-μm) bran. Owing to these data, some products (corn germ meal 7′, white lupin meal 17, lucerne 18, rapeseed meal 20, particle size <0.5 mm) were ground in liquid nitrogen for 2.5, 10, and 20 min and submitted to Pronase. Particle size distribution of starting and 20-min-milled products is shown in Figure 4. It must be noted that discrepancies existed between measurement of particle size of starting products by screening on a sieve (<0.5 mm) and by the Coulter counter technique. Residues were weighed and analyzed for nitrogen and noncellulosic cell wall polysaccharides. Data are presented in Figure 6 and Table III. Yield in insoluble cell wall material after correction for residual protein (N × 5.7) was independent of length of Pronase treatment within 2 h. Conversely, a progressive reduction of particle size led to a corresponding decrease of insoluble cell wall polysaccharides for all tested materials but rapeseed meal. This was confirmed by the general lowering of noncellulosic wall polysaccharides after 20-min milling as compared to starting products, especially at the galactose level in the white lupin meal (product 17). We have previously extensively studied the constituting polysaccharides of cotyledonary cell walls of white lupin (Carré et al., 1985), which are essentially linear (1→4)-β-D-galactans linked to rhamnogalacturonan chains. We have already observed a similar loosening of lupin galactan, which was paralleled by a drop of wall protein (Carré and Leclercq, 1985); it is possible that this partial solubilization was due to an increased sensitivity of wall protein to Pronase. Indeed covalent linkages have been reported between pectic arabinogalactans and hydroxyproline-rich wall protein (Albersheim, 1976). Monocotyledonous samples (wheat bran 2; corn germ meal 7′) exhibited the same behavior. Mongeau and Brassard (1986) also noted a similar phenomenon on wheat bran of decreasing size. In conclusion, we think that an extensive disruption of cell structures, even if it improves the purity of residues on nitrogen basis, would be detrimental to the recovery of entire cell walls by partial solubilization of some wall polysaccharides, especially the pectic arabino-(1→4)-β-D-galactans. An optimum milling scheme would promote breakage of cell walls without producing too many fines but is not at present available. The standard particle size (<0.5 mm) must therefore be considered as a fair compromise between an optimum purity with respect to nitrogen and the keeping of cell wall integrity.

Amylolysis. Both amylases (Termamyl 60L, amyloglucosidase) used in this study were tested for contaminating activities on various polysaccharides (see the Ex-

Table VII. Monosaccharide Constituents of Soluble Nonstarchy Polysaccharides in Proteolysis (S1) and Amylolysis (S2) Liquors

no.		Rha + Fuc	Rib	Ara	Xyl	Man	Gal	Glc	U
1	S1	<0.01		0.22	0.15	0.02	0.19	0.17	
	S2			0.03	0.04		0.03	<0.01	
2	S1	0.12		0.25	0.08	0.03	0.10	0.11	0.04
	S2	<0.01		0.14	0.16		0.09	0.04	0.11
3	S1	0.02		0.06	0.08	0.07	0.18	0.10	0.13
	S2			0.02	0.04	0.31	0.03	0.04	0.40
4	S1	0.01	0.01	0.16	0.12		0.12	0.05	0.08
	S2	<0.01		0.03	0.03		0.06	0.03	0.24
5	S1			0.24	0.06	0.03	0.15	0.33	
	S2			0.04	0.04	0.09	0.03	0.06	
6	S1	0.03	0.01	0.11	0.11		0.02	0.14	0.29
	S2	<0.01		0.02	0.01		0.05	0.03	0.84
7	S1	0.07		0.20	0.12	0.04	0.10	0.35	0.11
	S2			0.04	0.04		<0.01	0.02	0.19
8	S1	0.02		0.04	0.31	0.02	0.08	0.12	0.28
	S2			0.02	0.19	0.02	0.02		0.16
9	S1	0.06		0.22	0.18	0.08	0.17	0.10	
	S2	0.01		0.06	0.05	0.06	0.06	0.02	
10	S1	0.07	0.06	0.14	0.03	0.05	0.17	0.04	0.04
	S2	0.03	0.01	0.08	0.03		0.18	<0.01	0.09
11	S1	nd	nd	nd	nd	nd	nd	nd	nd
	S2	nd	nd	nd	nd	nd	nd	nd	nd
12	S1	0.10		0.16	0.02	0.49	0.40	0.03	0.53
	S2	0.01		0.05	0.01		0.03	<0.01	0.15
13	S1	0.03	0.05	0.10	0.08		0.04	0.04	0.14
	S2	<0.01		0.02	0.02		0.07	0.02	0.22
14	S1	0.01	0.02	0.15	0.02	0.05	0.08	0.03	0.05
	S2			0.03	0.02		0.05	0.06	0.20
15	S1	0.02		0.33	0.05	0.10	0.21	0.02	0.04
	S2	0.05		0.87	0.11	0.02	0.18	0.05	0.48
16	S1	0.01	0.03	0.12	0.02	0.02	0.10	0.04	0.39
	S2	<0.01	0.01	0.08	0.04	<0.01	0.03	0.03	0.16
17	S1	0.02	0.07	0.16	0.04	0.03	0.39	0.03	0.08
	S2	0.08		0.46	0.10		2.69	0.02	0.65
18	S1	0.06	0.02	0.25	0.02	0.02	0.23	0.08	0.48
	S2	0.03		0.08	0.02		0.11	0.05	0.09
19	S1	0.15		3.68	0.02	0.03	0.68	0.09	0.56
	S2	0.05		0.77	0.02		0.22	<0.01	2.17
20	S1	0.04	0.07	0.45	0.06	0.02	0.15	0.07	0.12
	S2	<0.01		0.13	0.02		0.09	0.04	0.22
21	S1	0.03	0.05	0.12	0.02		0.10	0.03	0.03
	S2	0.04	0.01	0.11	0.05		0.14	0.02	0.06
22	S1	0.07		0.27	0.02	0.02	0.38	0.08	0.49
	S2	0.01		0.02	<0.01		0.04	0.01	
23	S1			0.03			0.06	0.04	0.02
	S2	<0.01		0.02	0.01		0.07	0.05	0.19
24	S1	0.08		0.19	0.03	0.07	0.21	0.07	0.07
	S2	0.03		0.11	0.06	0.17	0.13	0.08	0.87
25	S1	0.06		0.26	0.05	0.08	0.09	0.10	0.04
	S2	0.02		0.08	0.04		0.15	0.01	0.21
26	S1	0.06		0.51	0.09	0.05	0.26	0.11	0.58
	S2	0.04		0.14	0.03	0.04	0.17	0.03	0.84
27	S1	0.18		0.13	0.01	0.04	0.08	0.56	0.32
	S2								
28	S1	0.21		0.45	0.13	0.05	0.09	0.31	0.20
	S2	<0.01		0.02	0.02		0.04	0.01	0.10
29	S1	0.17		0.26	0.18	0.02	0.16	0.83	0.26
	S2	<0.01		0.06	0.10		0.06	0.07	0.18
30	S1	0.07	0.015	0.22	0.15		0.13	0.73	0.11
	S2			0.08	0.10		0.01	0.07	0.13
31	S1	0.07		0.49	0.40	0.03	0.15	0.28	0.07
	S2	<0.01		0.15	0.34		0.05	0.07	0.13
32	S1	0.12		0.30	0.05	0.02	0.16	0.86	0.17
	S2	0.04		0.13	0.10		0.01	0.14	0.17
33	S1	0.03	0.02	0.05	0.03	0.60	0.44	0.13	0.04
	S2	<0.01		0.05	0.06	0.37	0.39	0.03	0.11
34	S1	0.03		0.02	0.11	0.04	0.08	0.08	0.01
	S2	<0.01		0.03	0.01		0.05	0.02	0.10
35	S1	0.05		0.19	0.07	0.02	0.17	0.18	0.22
	S2			0.05	0.07		0.07	0.06	0.10
36	S1	0.02	0.03	0.23	0.07	0.03	0.19	0.09	0.05
	S2								
37	S1	0.09		0.32	0.29	0.04	0.06	0.07	0.14
	S2		0.08	1.10	0.01		0.29	0.05	0.06
38	S1	0.07	0.03	0.47	0.02		0.09	0.04	0.48

Table VII (Continued)

no.		Rha + Fuc	Rib	Ara	Xyl	Man	Gal	Glc	U
	S2			0.06	0.01		0.06	0.02	0.13
39	S1	0.07	0.03	0.18		0.03	0.11	0.07	0.03
	S2	0.04		0.14	0.02		0.15	0.05	0.10
40	S1			0.03	0.04	0.02	0.03	0.05	0.60
	S2	<0.01		0.03	0.04		0.06	0.03	0.90

perimental Section): Termamyl 60L was free of contaminants (maximum foreign activity $<2 \times 10^{-3}$% of major activity) while Merck amyloglucosidase exhibited a maximum of 0.5% only of other enzymatic activities.

Solubilization of Amylose and Prevention of Retrogradation. Starches rich in amylose (~60%) from amylomaize (Eurylon) and wrinkled pea (Colonna and Mercier, 1984) were chosen for the establishment of the solubilization–saccharification procedure. They were fully solubilized at 6% concentration by hot (100 °C) 90% dimethyl sulfoxide within 30 min as judged by absence of pellet on high-speed centrifugation and clarity of solutions. However, amylose severely retrograded when solutions were diluted to 30% DMSO with cold (20 °C) 20 mM acetate buffer (pH 5.0) prior to amyloglucosidase treatment. Dilution was therefore performed with boiling acetate buffer and Termamyl, a highly active and unusually heat-stable α-amylase from *B. licheniformis* (Asp et al., 1983), was added to prevent retrogradation of the amylose component of starch. The working parameters of Termamyl in DMSO were determined, and the data are shown in Figure 7. Termamyl 60L exhibited in 30% DMSO (40 °C, pH 5.0) half of its maximum activity in buffer alone but was perfectly stable up to 50% DMSO within 1 h at 40 °C (pH 5.0). Moreover, when Termamyl was incubated with 0.5% waxy-maize β-limit dextrin in 30% DMSO (pH 5.0) at 100 °C, it lost rapidly its activity but still retained ~20% of it after 15 min. In end, the optimum ratio (Termamyl 60L to starch) to prevent retrogradation was determined as follows: a 6% starch solution in hot 90% DMSO was diluted to 30% DMSO with boiling 20 mM acetate buffer (pH 5.0); various amounts of Termamyl 60L were added; and incubation at 100 °C proceeded for 15 min. Then, iodine reaction was tested on solutions and was negative when 50 μL of undiluted Termamyl 60 L (1 μKat; see the Experimental Section) was added to 100 mL of 2% starch solution in hot (100 °C) 30% DMSO (pH 5.0). Under these conditions, no retrogradation occurred after cooling the medium to 40 °C in 10 min.

Saccharification. The optimum quantity of amyloglucosidase to be added for complete saccharification of solubilized starch within 2 h was chosen by submitting Termamyl-treated amylomaize starch (above conditions) to various amounts of enzyme and by measuring the production of glucose by the GOD–POD–ABTS system. Maximum available glucose from the solubilized starch was obtained when a ratio of amyloglucosidase to starch of 2% (w/w; 2.3 μKat/g of starch) was used.

Precision of the Method. Semolina from durum wheat (product 4) was chosen for determination of the standard deviation of the cell wall measurement due to its very low fiber content. The average cell wall content was 1.485% (13 replicates) with a standard deviation of 0.087 and a coefficient of variation (cv) of 5.85%. Similar precision was obtained starting from 1 g (instead of 5 g) of semolina. These figures were confirmed by the cell wall analysis of 40 plant-derived materials (Table IV).

Application of the Method to 40 Plant-Derived Products. The method was applied to various materials of plant origin (Tables I and II), and the data are shown in Tables IV–VII. The insoluble cell wall content ranged from 1.4% (semolina product 4) to 84.4% (wheat straw 3), while the proportion of soluble nonstarchy polysaccharides fell between 0.5% (cassava 23) and 10.4% (sugar beet pulp 19).

Insoluble cell walls were analyzed for their protein and ash levels and for their cell wall polysaccharides. The efficiency of the solubilization–proteolysis procedure was generally good (>80%) in case of materials rich in storage proteins (legume seed cotyledons, products, 11, 13, 14, ...) (Table IV). It was however lower for two groups of products, hulls and stems, having thick secondarized cell walls of low nitrogen content (3, 12, 24) and plant materials containing polyphenolics, especially tanins (25, 27), which are known to impair the solubility of proteins. The residual nitrogen content of purified cell walls was low (1–8%) for most of samples and corresponded to values usually reported for wall-inserted proteins (<10%) (Albersheim, 1976), the secondarized cell walls (3, 8) having trace amounts as compared to younger ones (1, 17). Our nitrogen figures are generally lower than previously reported values [e.g. 25% (N × 6.25) in cell wall residue from *Phaseolus vulgaris* (Asp et al., 1983) versus 7.7% in a similar bean flour (product 14)]. Chemical data are supported by light microscopic examination of residues stained by the Fast Green reagent. Walls were faintly colored in the junction area (middle lamella) while almost no Fast Green positive stuff was detected aside. Some tanin-containing samples (sorghum 6, faba bean 13) yielded highly contaminated residues as previously observed (Carré and Brillouet, 1986). Addition of sodium lauryl sulfate and 2-mercaptoethanol to Pronase does not allow a complete removal of intracellular proteins when tanins are present; it must be emphasized that in such cases erroneous measurements of the sulfuric acid lignin must be expected due to formation of artifactual lignin by tanin–protein complexes (Carré and Brillouet, 1986).

The ash content was also low (<15%) except in goat diet (34) and in residue from sugar beet pulp fermented in a Rusitec fermentor (39; 25.7%) (Czerkawski and Breckenridge, 1977). The composition of cell wall polysaccharides in their neutral + acidic sugar constituents is shown in Table V. Since Saeman hydrolysis was performed on walls, no discrimination was done between cellulosic and noncellulosic β-glucans; in addition, the rhamnose content would have been expected higher after trifluoroacetic acid hydrolysis (Selvendran et al., 1979). Raw materials belonging to the Gramineae (cereals) family (products 1–8) as well as processed products containing some cereals (28–36) exhibited a high pentosan content, especially at the xylose level, and a low proportion of galactose and uronic acid. Most samples from dicotyledonous plants (10, 11, 17, ...) had galactose and arabinose as major monosaccharide constituents. Their rhamnose and uronic acid content was also higher than in the cereal samples. Mannose was low in all materials but coprah meal (9) in which it represented approximately half of the walls. It is important to note that for both wheat flours (1, 4) that had a high starch content cell walls contained low quantities of glucose, reflecting the efficiency of our destarching treatment.

In an attempt to estimate the solubilizing effect of proteolytic and amylolytic treatments on cell wall polysaccharides, we have analyzed separately soluble non-

starchy polysaccharides (neutral + acidic sugars) arising in both proteolysis and amylolysis liquors (Table VI). Several techniques were applied (see the Experimental Section) for estimating their validity. There was no general agreement between orcinol and GC determinations of neutral sugars in the both media (S1, S2) except in some cases—e.g. for S1 products 3, 12, 15, ...—the situation being even worse for S2. Strong discrepancies were observed—e.g. for S1 product 38, for S2 18. When neutral (GC) + acidic polysaccharides are compared to the weight of the 80% ethanol precipitate, general disagreement was noted, some materials (products 3 and 28) giving no precipitate but containing detectable amounts of polysaccharides and others (24) having a far lower content as measured by GC then through ethanol precipitation. So, gravimetric (after ethanol precipitation) and colorimetric techniques are not suitable to determination of soluble nonstarchy polysaccharides, GC analysis of monosaccharides after acid hydrolysis being more advisable. The proportion of polysaccharides passing into solution was generally higher during the proteolysis step than for the amylolysis one except in some materials (15, 17). Soluble nonstarchy polysaccharides represented only a small portion (<4%) of the total determined material (insoluble cell wall + soluble polysaccharides) except for materials from endospermic parts of cereal grains (flours, products 1 and 4), processed foods containing large proportions of cereal flours (products 28–33), and sugar beet pulp (19). It is noteworthy that these soluble polysaccharides from Gramineae samples, which are essentially arabinoxylans (Table VII), accounted for ~25% of the total dietary fiber in both raw materials and processed foods (1, 4, 28, 29, 32). It is well established that arabinoxylans from cereal endosperms are partly soluble in buffers (Wilkie, 1979), and their present level indicates that addition of sodium lauryl sulfate and 2-mercaptoethanol in the proteolysis medium did not induce an extensive solubilization of these polymers as compared to water or buffer alone. Similar statement can be made for the beet pulp from which pectic arabinans (Hirst and Jones, 1948) were solubilized.

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Registry No. EC 3.2.1.3, 9032-08-0; Glu, 50-99-7; Rha, 3615-41-6; Fuc, 2438-80-4; Ara, 147-81-9; Xyl, 58-86-6; Man, 3458-28-4; Gal, 59-23-4; Pronase, 9036-06-0; lignin, 9005-53-2.

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Worldwide Contamination of Cereals by the *Fusarium* Mycotoxins Nivalenol, Deoxynivalenol, and Zearalenone. 1. Survey of 19 Countries

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By a rapid and sensitive method for simultaneous detection of nivalenol (NIV), deoxynivalenol (DON; vomitoxin), and zearalenone (ZEN), toxic metabolites of *Fusarium* species, their natural occurrence in cereals, foods, and feeds sampled from 19 countries was surveyed. Wheat, barley, oat, rye, corn, rice, and their products, in totaling 500 samples, were positive for NIV, DON, and ZEN in 244, 223, and 219 samples, though their contents varied depending on the commodities and sources. This is the first paper demonstrating the worldwide contamination of NIV, DON, and ZEN in agricultural products.

Trichothecene mycotoxins such as nivalenol (NIV) and deoxynivalenol (DON) and an estrogenic mycotoxin, zearalenone (ZEN), are produced by *Fusarium graminearum* Schwabe (teleomorph *Gibberella zeae* (Schw.) Petch.), one of the major causative fungi of head blight of wheat, barley, and other cereals. Contamination of cereals and feeds with these mycotoxins sporadically causes food- and feed-borne intoxication in man and farm animals, as reviewed by us (1983, 1985, 1986, 1987), Mirocha and Christensen (1974), Schoental (1985), and Joffe (1986).

Actually, several health scientists reported the natural occurrence of DON in Canada (Scott, 1983), the United States (Eppley et al., 1984; Shotwell et al., 1985), and South Africa (Marasas et al., 1979). Coexistence of NIV and DON was reported in Japan (Yoshizawa, 1983). Contamination of ZEN in animal feeds is a worldwide problem (Shotwell, 1977).

However, it is hard to compare the levels of these contaminants in agricultural commodities exactly, since the methods employed were different from one another and not adequate to detect the mycotoxins, particularly NIV, in cereals and foods, as pointed out by Tanaka et al. (1985a).

With the aim of clarifying the exposure levels of man and farm animals to these *Fusarium* mycotoxins, first, we developed an improved methodology for simultaneous detection of NIV, DON, and ZEN (Tanaka et al., 1985a,b). Second, we collected 500 samples of cereal and other agricultural products from 19 countries and districts. The detailed data on the products from Korea (Lee et al., 1985, 1986), China and the USSR (Ueno et al., 1986a,b), Poland (Ueno et al., 1986c), the U.K. (Tanaka et al., 1986), Canada (Tanaka et al., 1987b), and Japan (Tanaka et al., 1984) have already been reported in separate papers. Further surveys of products from West Germany, Italy, Nepal, Argentina, and others are presented in this paper, along with a summarized data of previous reports, in order to compare the level and frequency of occurrence of toxins.

MATERIALS AND METHODS

Samples. Wheat, barley, oat, rye, corn, and others, totaling 500 samples with each 40–50 g, were obtained from 19 countries under the cooperation of food scientists listed in the Acknowledgment. Most of the cereal samples were produced in 1983–1985 crop years and sampled randomly at respective agricultural stations. Four wheat samples from Xi-an, China, were collected at random from the

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