Quantitative Isolation and Estimation of Cell Wall Material from Dehulled Pea (*Pisum sativum*) Flours and Concentrates[1]

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ABSTRACT

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Cell wall material (CWM) from soaked field pea cotyledons was quantitatively isolated (6.85 g/100 g of peas, db) by repeatedly sieving and washing the macerated peas. Purified CWM contained no starch and had a low protein content (5.30%). The composition of pea CWM and pea hulls was compared. Pea CWM contained mainly pectic substances and hemicellulose, whereas the major constituent of pea hulls was cellulose. CWM contained 44.3% neutral detergent fiber. Therefore, CWM contents of pea cotyledons or air-classified protein concentrates were estimated by multiplying their respective neutral detergent fiber values by 2.26. The CWM value for pea cotyledons obtained by the isolation procedure was in good agreement with the calculated value. When calculated CWM values were summed with the other constituents of either flours or concentrates, the sum of components was nearly 100%.

The study of cell wall material (CWM) has become an important area of research, especially after the recent findings on the relationship between dietary fiber and disease. A deficiency of dietary fiber has been implicated in several diseases, including diverticular disease of the large bowel and carcinoma of the colon (Burkitt 1973, Burkitt and Trowell 1975). In light of these findings, accurate quantitation and characterization of CWM is especially important.

Neutral detergent fiber (NDF) is widely used as an estimate of the cell wall fiber (Robertson 1978). NDF includes detergent-insoluble portions of the cell wall, which are classified mainly as hemicellulose, cellulose, and lignin. However, as a measure of dietary fiber, it has a serious limitation in that pectin and gums,

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which must be included in the dietary fiber by definition, are solubilized during the NDF determination (Bailey et al 1978).

Several methods for the isolation of CWM, some of which are relatively quantitative, have recently been developed. Selvendran and Du Pont (1980) obtained CWM by sequential extraction of wet ball-milled flours with 1% (w/v) aqueous sodium deoxycholate, phenol-acetic acid-water (2:1:1,w/v/v), and 90% (v/v) aqueous dimethyl sulfoxide. Several investigators (Ballance and Manners 1978, Fincher 1975, Mares and Stone 1973) have prepared CWM by wet-sieving ground barley or wheat flour in a 70% ethanol medium. Potato CWM has been prepared (Haydar et al 1980, Hoff and Castro 1969) by filtration of macerated and sonicated potato slices. Rice cell walls were prepared by successive digestion of water-extracted and defatted flour with α-amylase, glucoamylase, and protease followed by filtration through a glass bead-bedded filter (Shibuya and Iwasaki 1978).

The objective of this investigation was to develop a method for the isolation of CWM from field pea cotyledons using basically the wet-sieving principles of Hoff and Castro (1969). In addition, a method is proposed for the estimation of CWM based simply on NDF measurements. This technique was used to measure CWM in pea flours and allowed us to follow the fractionation of CWM during the air classification of pea flour.

MATERIALS AND METHODS

Dry Processing

Smooth field peas (*Pisum sativum* L. cv. Trapper) were obtained from farmers in Saskatchewan in 1978. Dehulling was accomplished with a resinoid disk, abrasive dehuller (Oomah et al 1981) and also a Currier plate mill. Air aspiration separated the cotyledons from the hull. Protein concentrates were prepared by pin milling the dehulled seeds in an Alpine pin mill model 250 CW and fractionating (vane setting = 25) the flour into a protein and a starch concentrate using an Alpine air classifier type 132 MP (Vose et al 1976).

Isolation of CWM

Dehulled peas (200 g) were soaked in distilled water (800 ml) for 16 hr at 1°C. The peas plus imbibition medium were then homogenized (10 min) in a Sunbeam eight-speed blender (highest setting) in a freezer (−12°C). The slurry was then washed through a bank of 250, 150, 75, and 44-μm screens, using a large quantity of distilled water. Each screen was rubbed gently by hand in the presence of water until no more material would pass. The rubbing action dislodged starch and protein particles that were held up in the cellular structure. The material on the sieves was combined and reground (10 min) in the blender, after 300 ml of distilled water (4°C) was added, and then sieved again. This was repeated once more, omitting the 250-μm screen. At no point in the procedure did the temperature rise above 28°C. The material on the sieves was combined and freeze-dried.

Quantitative Estimation of CWM

Pea flours or air-classified fractions were analyzed for NDF. Because purified CWM was found to contain only 44.3% NDF, the CWM of flours or fractions was calculated using the formula: CWM = NDF × 100/44.3.

Photomicroscopy

Sections of pea cotyledon were prepared by soaking tissue in distilled water (4°C) and then fixing these in 10% acrolein followed by 1% osmic acid. Following dehydration, they were embedded in Spurr low viscosity epoxy resin. Sections (1–2μm) were mounted on slides and stained with polychrome stain (Sato and Shamoto 1973).

CWM in the flour or concentrates was stained with ruthenium red (Jensen 1962). The powder (5 mg) was added to methanol (5 ml) in a test tube and mixed until completely dispersed. The tube was centrifuged and the methanol decanted. The residue was resuspended in 0.2 ml of methanol, and 1 ml of ruthenium red (1:1,000 in water) was added. The contents of the tube were mixed well, heated at 45°C for 20 min, centrifuged, decanted, and washed twice with distilled water (8 ml).

Starch was stained with an I_2/KI solution. Photomicrographs were obtained on a Carl Zeiss Universal microscope.

Analytical Methods

Protein. Nitrogen was determined by standard AOAC macro-Kjeldahl methods, and N × 6.25 was used to calculate protein.

Starch. Starch was assayed by using a modification of the dual enzyme semimicro method of Banks et al (1970) using α-amylase and amyloglucosidase (Tenase and Diazyme L-100, respectively; Miles Laboratories, Inc., Elkhart, IN). The chromogen used in conjunction with the glucose oxidase-peroxidase system was 2,2′-Azino-Di-(3-ethyl benzthiazoline sulfonic acid) (Sigma Chemical Company, St. Louis, MO).

Sugars. Oligosaccharides soluble in 80% methanol (sucrose, raffinose, stachyose, and verbascose) were quantitated by gas liquid chromatography, essentially using the procedure described by Vose et al (1976).

Lipid. Neutral lipids were extracted with hexane in a Soxhlet apparatus; polar lipids were similarly extracted from the hexane-extracted flours with chloroform/methanol (2:1,v/v).

Fiber. NDF was determined using AACC method 32-20. To obtain reproducible results on finely ground material (pin-milled), the glass wool filter bed used in filtering the neutral detergent digest must be prepared carefully. Thus, glass wool (3 g) was solidly packed into coarse glass frit filters and successively washed with hot concentrated HCL and large amounts of water.

Acid detergent fiber (ADF), lignin, and crude fiber were determined according to AOAC methods 7.055, 7.058, and 7.050, respectively. Hemicellulose and cellulose values were determined by difference using NDF and ADF values, ie, hemicellulose content equals NDF minus ADF and cellulose content equals ADF minus lignin.

Water Absorption Capacity. Determinations were made using a combination of the methods of Sollars (1972) and Sosulski (1962). A sample (5 g) was weighed into a 250-ml centrifuge tube, and distilled water (100 ml) was added. After shaking (1 hr), the sample was centrifuged (1,200 × *g*) for 25 min. Tubes were placed on an incline at 45°C and allowed to drain for 10 min. The water retained was determined by weighing and was expressed as a percentage of the original sample weight.

Pectic Substances. The 0.5% ammonium oxalate-oxalic acid (0.25% of each) extraction (24 hr at 90°C) and ethanol precipitation procedure of Dever et al (1968) was used to obtain the pectin fraction.

RESULTS AND DISCUSSION

Isolation of Pea CWM

Pea cotyledon cells were nearly spherical in shape and had an average diameter of approximately 90 μm (Fig. 1). Starch (54%) and protein (24%) were the major constituents of the cells. Figure 1 shows that pea starch granules varied in size, with mean dimensions of approximately 22 × 15 μm. Protein bodies were much smaller and were rigidly packed between starch granules. Cell walls (2–7μm thick) were distinct and often separated, suggesting that the fixing procedure might have dissolved some of the intercellular cementing material, which is comprised mainly of pectic substances (Kertesz 1951).

The isolation of the CWM by a simple sieving procedure was based on the observation that cell walls did not break down readily when soaked peas were macerated in a blender. Figure 2a illustrates a piece of CWM after the soaked peas were macerated for 10 min. It is many times the size of other released intracellular components (starch and protein bodies) and contains only a few starch granules. Particle-size analysis of CWM following exhaustive water-washing to remove all free intracellular material is shown in Table I. After the cells were macerated for 10 min, the majority of cell wall particles were greater than 150 μm. Macerating the cells twice more for 10 min reduced most CWM particles to sizes between 75 and 150 μm. Very little CWM was less than 75 μm and therefore the

amount of CWM passing through the 44-μm screen was considered negligible. Thus the isolation procedure was quantitative and reproducible. Yield of CWM was 6.85% of the dry weight of the dehulled seed, and the standard deviation was 0.05. Selvendran and Du Pont (1980) reported dry weight yields of 3.8 and 8.6% CWM in dehusked oats and rye flour, respectively, and 52.5% in wheat bran. Peeled potato tubers contained 5.2% CWM on a dry weight basis (Haydar et al 1980).

Figure 2b illustrates isolated CWM. Iodine staining showed that virtually no starch was present. Ruthenium red staining (Fig. 2c) illustrated that intercellular pectin-cementing material was still present in the isolated CWM. Cell walls stained faint pink, whereas intercellular areas stained bright red.

Previous investigators have obtained CWM by ethanol sieving of ground barley or wheat flour (Fincher 1975, Mares and Stone 1973). They observed that the method was not quantitative because some of the CWM passed through the 75-μm bolting cloth. We have also observed that dry grinding of seeds before CWM isolation resulted in CWM of a very fine and unmanageable particle size. Figure 2d illustrates CWM in pin-milled flour. Quantitative isolation of CWM from this flour would be nearly impossible because many CWM particles were about the same size as the starch granules. In a soaked pea, however, CWM proved to be very resilient and resistant to the cutting action of the blades in the blender, and this permitted quantitative isolation.

The isolation of pectin-containing substances is often preceded by boiling the tissue in 70% ethanol (McCready 1970). Pectinases and other enzymes are inactivated by such a treatment. To minimize enzymatic activity during our isolation procedure, the temperature was kept as low as practically possible. To determine whether enzymatic activity would reduce the yield of CWM, the dehulled peas were boiled for 2 hr in 70% ethanol before isolation of CWM by the usual procedure. The pretreatment appeared to toughen the cell walls, and some cells were not broken down by the shearing action of the blades. The final product therefore contained some starch (5.7%) and had a higher protein content (10.1%) than did the CWM prepared by the usual procedure (Table II). After its starch and protein content was taken into account, the yield of CWM was 6.5%. This was comparable to the yield obtained without pretreatment, and therefore this step was not considered necessary.

Table II gives the composition of CWM in comparison to pea hull, which is the other "fiber" source present in field peas. Pea hulls are used as a dietary fiber supplement in white bread, and a commercial product is being sold in Canada (Satin et al 1978). Pectic substances and hemicellulose were the major components of CWM, whereas cellulose was predominant in pea hulls. Hoff and Castro (1969) and Dever et al (1968), using the same analytical procedure for pectin, obtained 55 and 15% pectic substances in potato CWM and mature corn root CWM, respectively. Protein content of CWM was comparable to that of CWM isolated from oat flour (6.1%, Selvendran and Du Pont 1980), rice endosperm (3%, Shibuya and Iwasaki 1978), and barley endosperm (4.9%, Ballance and Manners 1978). Starch was not detected in CWM or pea hulls; ash and lipid were very low in both. Hemicellulose and lignin contents of pea hulls were comparable to those reported by Schaller (1978), but our cellulose content was substantially higher. Pea CWM contained 11.4% lignin; oat CWM contained 9.4% (Selvendran and Du Pont 1980). CWM absorbed approximately 18 times its weight of water and in this respect is similar to pectinic acid, which has been found to absorb 15 times its weight of water (Kertesz 1951).

Quantitative Estimation of CWM

The recent literature has tended to equate NDF values with the cell wall content of a plant material (Robertson 1978, Southgate 1976). The NDF value is reported to approximate the sum of the hemicellulose, cellulose, and lignin contents. These components are largely digested by microbial fermentation in animals. A fundamental objection, however, is that the method underestimates total dietary fiber because pectic substances and gums, which are decomposed and fermented by bacteria in the colon (Werch and Ivy

TABLE I
Particle Size Distribution of Hydrated Cell Wall Material After Extensive Washing of Macerated Pea Cotyledons

Sieve Size[a] (μm)	Macerating Time (min)		
	10	20	30
>250	14.8	0	0
>150	47.2	23.6	10.7
> 75	36.3	72.8	83.9
> 44	1.8	3.6	5.4

[a] Tyler mesh sizes 60, 100, 200, and 325, respectively.

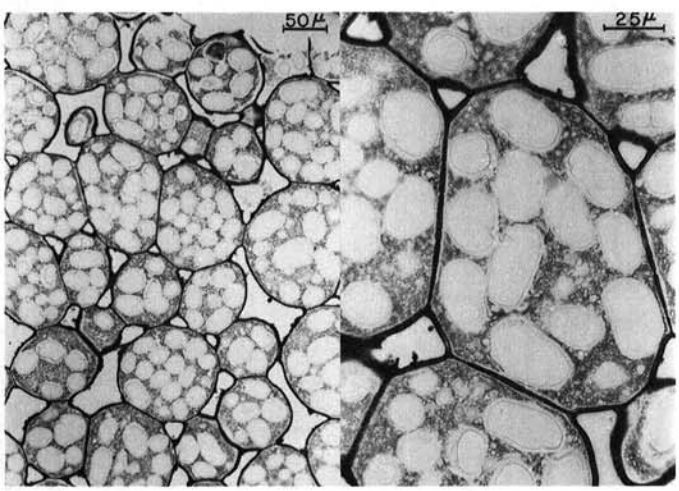

Fig. 1. Pea cotyledon sections at two magnifications, illustrating starch granules, protein bodies, and cell walls.

TABLE II
Composition[a] and Physical Properties of Pea Cell Wall Material (CWM) and Pea Hulls

Component	CWM	Hulls[b]
Pectic substances	26.3	16.8
Hemicellulose	22.3	7.5
Lignin	11.4	1.4
Cellulose	10.6	68.8
Protein	5.30	3.80
Ash	1.32	1.96
Neutral lipid	0.24	0.31
Polar lipid	0.10	0.60
Starch	0	0
Crude fiber	16.0	58.4
Water absorption	1,816	296
Color	White	Light tan

[a] Percent dry matter.
[b] Obtained by hand-dissection.

TABLE III
Fiber Analysis[a] of Pea Flours and Protein Concentrates[b]

Sample	Crude Fiber	Acid Detergent Fiber	Neutral Detergent Fiber	Cell Wall Material[c]
High protein peas (28.5%)				
Flour	1.7	2.4	3.14	7.1
Concentrate	2.3	3.3	5.59	12.6
Low protein peas (14.5%)				
Flour	1.4	2.4	4.26	9.6
Concentrate	3.0	4.5	7.77	17.6

[a] Percent dry basis.
[b] Prepared by pin milling and air classification.
[c] Neutral detergent fiber × 2.26.

1941), are lost during the extraction procedure.

An example of this underestimation is that the NDF value of pea cotyledons is 3.10% (SD = 0.14), whereas the actual yield of the isolated CWM (dietary fiber) gave a value of 6.85% (SD = 0.05). This discrepancy must be due to solubilization of the pectic substances and other constituents of the cell wall by the detergent system employed for NDF analysis. The NDF value of purified CWM was only 44.3% (SD = 0.85), which suggested that an estimate of CWM in pea cotyledons or fractions obtained therefrom could be derived by multiplying the NDF of the material by the factor 100/44.3. For pea cotyledons, the calculated value of CWM was found to be 7.0%, which is in very good agreement with the actual CWM content found by the isolation procedure. The calculated value (NDF × 2.26) assumes that CWM is the only component in the pea cotyledon that contributes to the NDF value. This assumption was substantiated by the fact that pea protein

TABLE IV
Total Component Analysis[a] of Pea Flours and Concentrates

Sample	Protein	Starch	Cell Wall Material[b]	Lipid[c]	Ash	Sugars[d]	Sum of Components
High protein pea (28.5%)							
Flour	28.5	49.7	6.6	3.0	2.8	7.47	98.1
Concentrate	60.2	4.9	11.7	6.0	5.5	12.51	100.8
Low protein pea (14.5%)							
Flour	14.5	59.8	9.0	4.1	3.3	7.85	98.6
Concentrate	40.9	7.3	16.4	9.7	7.2	15.14	96.6

[a] Percent dry weight.
[b] Neutral detergent fiber × 2.26, minus ash, protein, and lipid content of cell wall material.
[c] Neutral plus polar lipid.
[d] Sum of sucrose, raffinose, stachyose, and verbascose.

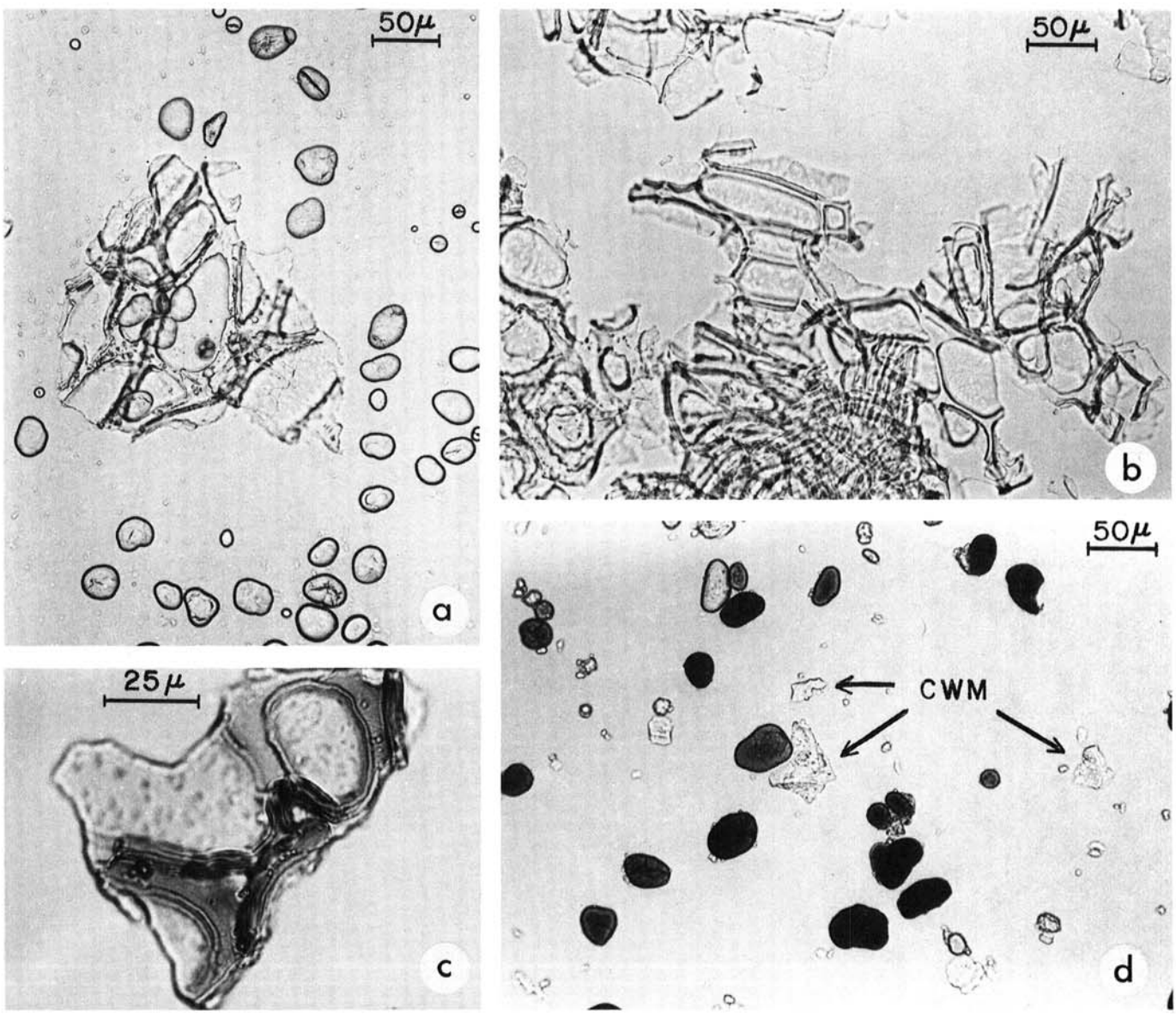

Fig. 2. a, Pea slurry after macerating soaked pea cotyledons for 10 min in a blender; **b,** purified cell wall material (CWM); **c,** CWM, showing differential staining with ruthenium red; **d,** pin-milled pea flour illustrating CWM (Starch stained with I_2/KI).

isolate (90.0% protein) and purified pea starch (97.2% starch) gave NDF values of 0.06 and 0.71%, respectively. The residual NDF of the pea starch may be due to the presence of contaminating CWM, which is very difficult to remove during the purification process (Vose 1977).

Calculated CWM values for pea flours with high and low protein contents and for their air-classified protein concentrates are shown in Table III. The protein concentrates from peas with high and low protein contents contained 12.6 and 17.6% CWM, respectively. Starch fractions contained lower quantities of CWM (4.2 and 6.4% for peas with high and low protein contents, respectively), indicating that the CWM had been preferentially segregated into the protein concentrate on air classification. Although the analysis of crude fiber, ADF, and NDF by the classical procedures showed the same trends, the fiber values were much lower and sound conclusions much less apparent. CWM values of the flours are 5–10% too high because of incomplete dehulling; however, the protein concentrates would be only slightly contaminated with hull material because hulls are nearly completely fractionated into the starch fraction during air classification (Vose et al 1976).

The protein, starch, CWM, lipid, ash, and simple sugar contents of the flours and concentrates are given in Table IV. CWM values were corrected for a small amount of ash, protein, and lipid in order not to count these constituents twice. When the calculated CWM values were summed with the values of the other constituents, the sum of components ranged from 96.6 to 100.8%. Other measures of fiber would not have made such a complete summation possible.

CONCLUSION

Purified pea CWM was quantitatively prepared by macerating soaked pea cotyledons in a blender followed by repeated sieving and washing of the CWM. Pea CWM contained small quantities of protein, lipid, and ash and larger quantities of hemicellulose, cellulose, lignin, and pectic substances. On the basis of the NDF value of the CWM (44.3%), the concentration of CWM in pea cotyledons or fractions obtained therefrom was estimated by multiplying their NDF value by the factor 2.26. The calculated value of CWM in pea cotyledons agreed very well with the value obtained by the isolation procedure. We are presently applying these CWM isolation and estimation techniques to a wide range of legume seeds to determine the overall applicability of the methods.

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