Interference of Phytic Acid with Extraction of Proteins from Grain Legumes and Wheat with Acetic Acid†

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Formation of insoluble phytic acid-protein complexes in different legumes and various cultivars of wheat under acidic pH were compared by Kjeldahl nitrogen and aluminum lactate polyacrylamide gel electrophoresis (AL-PAGE). Seed proteins extracted in 5 M acetic acid (HAc) were treated with various concentrations of phytic acid solution. The most precipitation occurred in faba beans and the least in wheat. Among legumes, field bean showed very little precipitation with phytic acid. Slow-moving (in AL-PAGE) HAc soluble proteins of the legumes were found to be more sensitive to form insoluble complexes with phytic acid compared with those of medium mobility. Recovery (solubilization) of the bound proteins from their insoluble complexes by NaOH treatment varied selectively, however, the stability of these proteins remained unaffected during the recovery process. Among various cultivars of wheat more of the low-mobility proteins were recovered for cvs. Fielder, HY 320, and Norstar, whereas in the case of Katepwa and Glenlea more of the fast-moving bands were recovered by NaOH treatment.

INTRODUCTION

Consumption of foods with high fiber content has increased dramatically over the past few years. Some of the natural high fiber materials contain other constituents which affect the nutritional quality of the food. The most common of these constituents is phytic acid (Lolas and Markakis, 1975; Lolas et al., 1976; Reddy et al., 1982). In processed foods phytic acid would have the capacity to bind minerals and/or proteins to form insoluble complexes (O'Dell et al., 1972; O'Dell and de Boland, 1976; Okubo et al., 1976; Omossiye and Cheryan, 1979; Honig et al., 1984, Lasztity, 1990) which would decrease the bioavailability of these ingredients (Erdman, 1981; Thompson, 1986; Carnovale et al., 1988; Juliano et al., 1991). The mechanism(s) of the interaction of food constituents with phytic acid have not been fully elucidated. In this context, we investigated the effect of extrinsic phytic acid on the acetic acid extractable proteins of some grain legumes and wheat. Changes in the electrophoretic band patterns due to in vitro addition of phytic acid are reported in this paper.

MATERIALS AND METHODS

Plant Material. Grain samples used in this study were field bean (*Phaseolus vulgaris*, cv. Loop, Canada), broad bean (*Vicia faba*, cv. BF 2/2, Sudan), field pea (*Pisum sativum*, cv. Century, Canada), chickpea (*Cicer arietinum*, cv. Bold Seed Segregate, acc. 5712, India), lentil (*Lens culinaris*, cv. Turkish red, Syria), and common wheat (*Triticum aestivum*, Sub spp. *vulgare*, cvs. Katepwa, Fielder, Glenlea, HY 320, and Norstar, Canada).

Legumes were dehulled manually and the cotyledons were milled on Udy Cyclone Mill (Udy Corporation, Boulder, CO) to pass a 40 mesh screen. Wheat flour (straight grade, average extraction rate = 72%) was prepared using Buhler pneumatic laboratory mill.

Preparation of Protein Extract. Protein solution for electrophoresis was prepared by agitating 0.1 g of seed (cotyledon) meal or flour in 0.5 mL of 5 M acetic acid. Low pH (2.00) extractant (5 M HAc) was the solvent of choice mainly because both the proteins and phytic acid were more soluble at low pH (Carnovale et al., 1988; De Meester, 1972) and the straight HAc extract would contain all Osborne solubility classes of proteins. The seed meal-HAc slurry was allowed to stand for at least 2 h at room temperature (21 ± 2 °C) with occasional shaking to facilitate extraction.

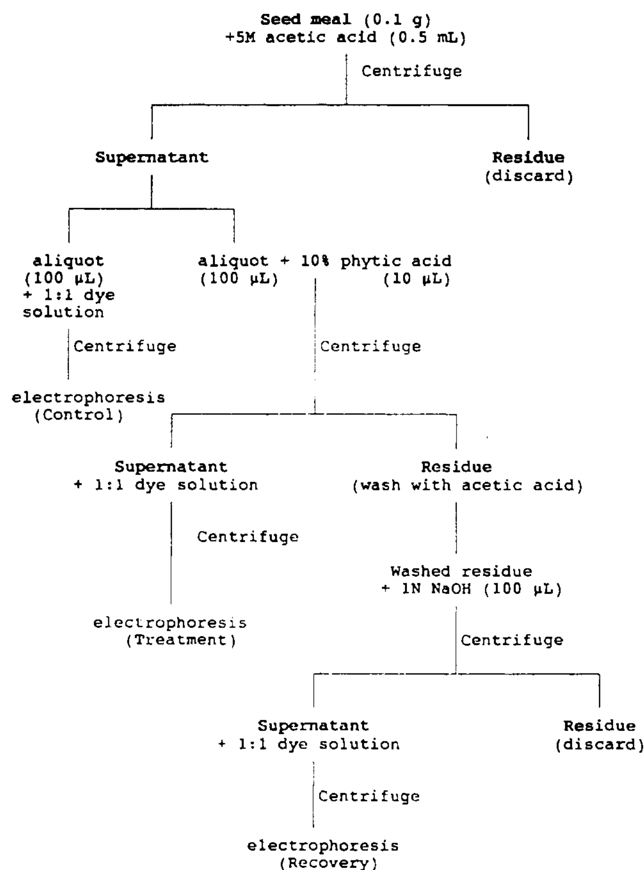

Figure 1. Steps involved in the preparation of protein solutions.

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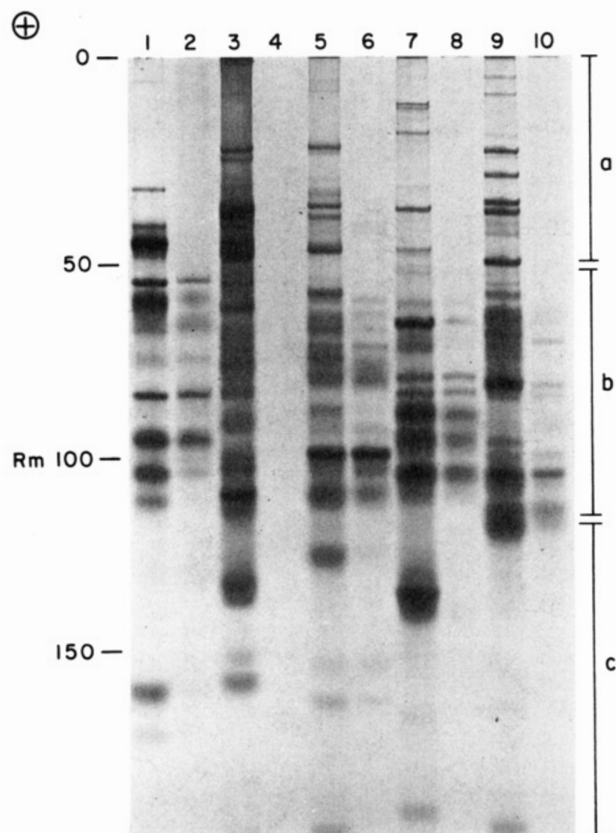

Figure 2. Precipitation of proteins by the addition of phytic acid to extracts of various legumes: Lanes 1 and 2 represent field bean; 3 and 4, faba bean; 5 and 6, pea; 7 and 8 chickpea; and 9 and 10, lentil. Odd numbers represent samples before and even numbers after the addition of phytic acid.

Table I. Protein Content of Acetic Acid Extract before and after Phytic Acid Addition[a]

plant material	cultivar	HAc extract	super-natant[b]	residue[b]	% removal[c]
field bean	Loop	99.46[d]	40.76[d]	36.14[d]	36.33
broad bean	BP 2/2	97.80	10.11	79.70	81.47
pea	Century	85.90	30.04	51.90	60.41
chickpea	Bold Seed	76.16	31.40	38.04	49.94
lentil	Turkish red	78.10	29.66	45.40	58.13
wheat	Katepwa	112.16	101.57	8.06	7.16

[a] Average of three replicates. [b] After addition of phytic acid. [c] Different data obtained using 1 g of seed meal. [d] Milligram/gram of seed meal.

Phytic Acid Treatment and Electrophoresis. The clear supernatant obtained after centrifugation at 8850*g* for 10 min at room temperature was carefully removed in 100-μL aliquots in two separate centrifuge tubes. One aliquot was mixed 1:1 with the tracking dye solution (0.5% methyl green in 8.5 mM aluminum lactate, pH 3.1 containing 40% sucrose) and centrifuged further for 5 min at the same speed. Ten microliters of the clarified supernatant was used in each slot for electrophoresis.

The second aliquot (100 μL) of the extract was mixed with 10 μL of freshly prepared 1, 2.5, 5, or 10% phytic acid (inositol hexaphosphoric acid, dodecasodium salt from rice, FW 923.8, Sigma Chemical Co., St Louis, MO) solution (in 5 M HAc). The resulting mixture was agitated using vortex mixer. After 30 min the resulting mixture was centrifuged at 8850*g* for 10 min at room temperature. Clear supernatant was decanted into a new centrifuge tube, and the residue was saved for the determination of protein recovery. The supernatant was mixed 1:1 with the tracking dye solution and centrifuged further, and depending upon the protein content, 10.5–11.5 μL of the final supernatant was used for electrophoresis. Differences in protein solubility were estimated from the differences in electrophoretic patterns.

The residue obtained after the addition of phytic acid was suspended in 100 μL of 1 N NaOH. The mixture was well agitated using a vortex mixer and centrifuged at 8850*g* for 10 min at room

Table II. Protein Bands Affected by Phytic Acid Treatment[a,b]

Rm	field bean	broad bean	pea	chickpea	lentil
5	•	•	•	•	o
6	o	•	o	•	•
10	•	•	•	•	o
12	•	•	•	o	•
13	•	•	•	o	•
16	•	•	•	o	•
19	•	•	•	o	•
20	•	•	•	•	o
22	•	•	•	o	•
24	•	•	•	•	o
25	•	•	•	•	o
30	•	•	•	•	o
33	o	•	•	•	•
34	•	•	o	•	•
36	•	•	•	•	o
38	•	•	o	o	o
39	•	o	•	•	•
40	•	•	o	o	o
41	o	•	•	•	•
43	•	o	•	o	o
45	•	•	•	•	o
47	o	o	o	•	•
48	•	•	•	o	•
51	•	o	•	•	o
53	•	•	•	+	•
54	•	•	•	+	•
56	++	•	•	•	o
57	•	o	•	•	•
58	•	o	•	•	•
59	•	•	++	•	•
62	++	•	•	•	•
63	•	o	•	+	•
64	•	•	•	•	+
65	•	o	•	•	•
66	•	•	•	•	+
68	•	•	•	+	•
69	++	•	++	•	+
70	•	o	•	•	++
73	•	•	++	+	++
74	•	o	•	•	•
76	++	•	++	•	•
80	•	•	++	++	•
83	•	•	•	•	++
85	++	+	•	++	•
86	•	•	•	•	++
88	•	•	+	•	•
92	•	+	•	•	•
93	•	•	•	•	++
96	++	•	•	++	o
100	•	•	+++	•	•
101	•	•	•	•	++
103	•	•	+	++	++
104	++	•	•	•	•
109	•	o	+	+	•
111	+	•	•	•	•
112	•	o	•	•	•
114	•	•	•	•	++
119	•	•	•	•	+
126	•	•	+	•	•
132	•	o	•	•	•
135	•	•	•	+	•
150	•	o	•	•	•
158	•	o	+	•	•
160	+	•	•	•	•
163	•	•	+	•	•
169	+	•	•	•	•
196	•	o	+	•	o

[a] Phytic acid (1 mg to HAc extract of 100 mg grain sample). [b] Symbols are as follows: •, absent; o, present in control, absent after treatment; +, present as a faint band after treatment; ++, present with some loss of color intensity; +++, intensity unaffected. Rm = relative mobility.

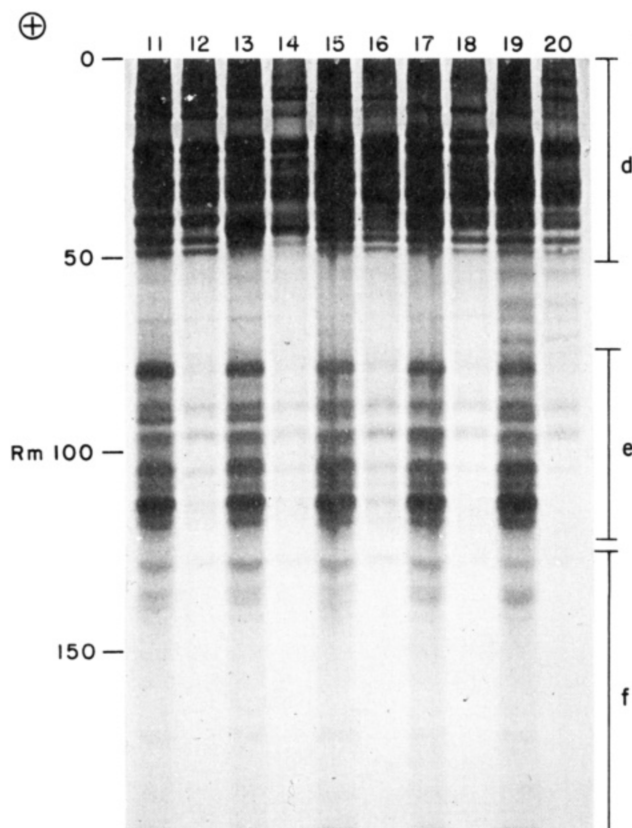

Figure 3. Precipitation of proteins by the addition of phytic acid to extracts of various cultivars of wheat: Lanes 11 and 12 represent katepwa; 13 and 14, glenlea; 15 and 16, norstar; 17 and 18, HY 320; and 19 and 20, fielder. Odd numbers represent samples before and even numbers after the addition of phytic acid.

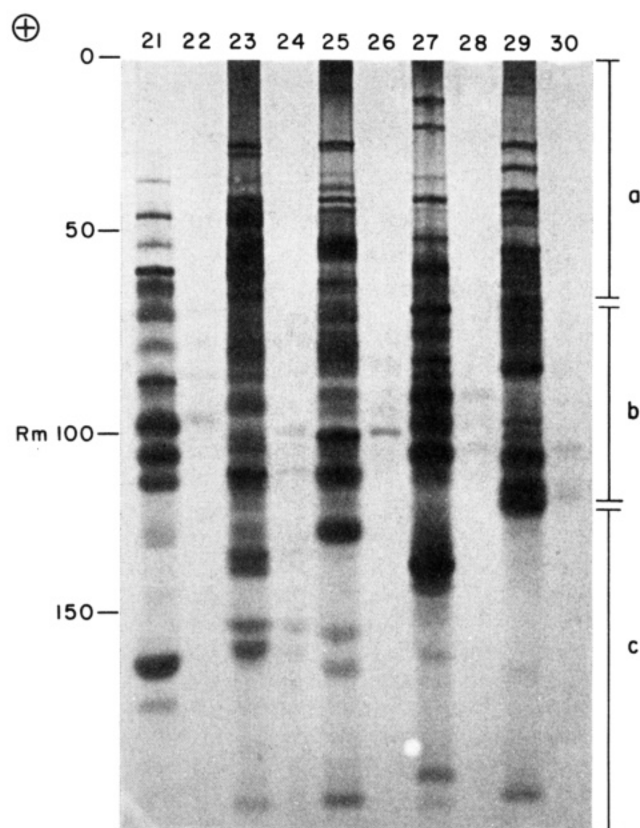

Figure 4. Solubilization of the precipitated legume proteins in 0.1 N NaOH: Lanes 21 and 22 represent field bean; 23 and 24, faba bean; 25 and 26, pea; 27 and 28, chickpea; and 29 and 30, lentil. Odd numbers represent untreated extracts, and even numbers represent solubilized proteins of the precipitate.

temperature. Clear supernatant was mixed 1:1 with the tracking dye solution and centrifuged again at the same speed for 5 min. Appropriate volume adjusted according to the protein content of the clarified supernatant–dye mixture (20–25 μL) was used for AL–PAGE. A volume of the untreated control samples was kept constant (10 μL) in all experiments. A schematic diagram of the procedure is presented in Figure 1.

AL-PAGE was carried out in a locally constructed electrophoresis unit (Hussain et al., 1988). The gel solution contained acrylamide (10 g), bisacrylamide (0.5 g), ascorbic acid (0.1 g), ferrous sulfate (0.0015 g), glycerol, (15 mL), and aluminum lactate (8.5 mM) + lactic acid to pH 3.1 (85 mL). The gel mixture was polymerized with 100 μL of 3% hydrogen peroxide solution. Electrophoresis was carried out under the following conditions: running buffer, aluminum lactate, pH 3.1; running temperature, 20 °C; running current, 12 mA; running time, ca. 6 h (until the dye front reached the lower end of the gel). The gel was stained overnight with Coomassie Brilliant Blue (CBB) R-250. If necessary the background stain was removed using CBB G-250 solution prepared according to Blakesley and Boenzi (1977). Destained gel was rinsed with detergent soap and water to remove precipitated CBB particles and was photographed using an orange filter.

The reproducibility of the entire procedure was tested using several extracts of the same sample and all our results were reproducible.

Relative Mobility. Relative mobility (Rm) of the protein bands was calculated according to Hussain et al. (1988) using the following equation:

$$\text{Rm}(x) = \frac{\text{migration distance of band } (x)}{\text{migration distance of the reference band}} \times 100$$

An intense band appearing in the center of the pea electrophoretic pattern (Figure 2, lanes 5 and 6) was assigned an arbitrary mobility of 100 and relative mobility of the other bands was calculated using the above equation. The relative amount of protein in corresponding band (based on mobility) was estimated subjectively from the intensity of the stained band and reported as +, ++, and +++ (+++ is most intense).

Protein Determination. A larger sample of seed meal (1 g) was extracted in 5 mL of acetic acid for nitrogen determination. Nitrogen content of protein extracts before and after the addition of phytic acid was determined according to AACC method 46-12 (AACC, 1984) using the Kjeltec system I equipment (Tecator AB, Hoganas, Sweden). Protein content was calculated by multiplying the nitrogen value by 5.44 for legumes and 5.7 for wheat (Sosulski and Imafidon, 1990). Results reported were the average of three replicates.

RESULTS AND DISCUSSION

Addition of phytic acid to the clarified 5 M acetic acid (HAc) extracts of selected legumes precipitated a large portion of the proteins (Table I). Extract of broad bean (*V. faba*) showed a strong phytic acid–protein interaction. Addition of phytic acid (0.2 g) to the protein extract prepared from 100 g of the dehulled broad bean, removed 81.5% of the soluble protein as an insoluble precipitate. Further additions of phytic acid did not increase the amount of precipitate. The lowest amount of precipitation occurred in the extract of wheat despite the fact that the protein content of these extracts was higher than those of the legumes (Table I). The weaker binding between phytic acid and proteins of wheat could be related to the limited availability of cationic groups (Lasztity, 1990). Protein removal based on Kjeldahl nitrogen was different for other legumes: 60% in pea, 58% in lentil, 50% in chickpea, and 36% in field bean. A small loss of protein, during Kjeldahl determination of the supernatant and the residue fraction, most probably was the result of an incomplete transfer to samples into the digestion tubes.

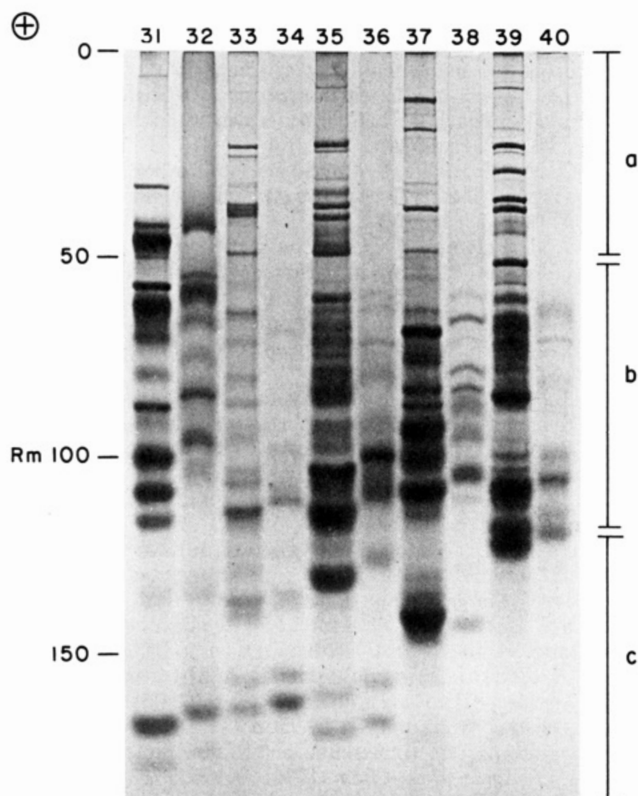

Figure 5. Solubilization of the precipitated legume proteins in 1 N NaOH: Lanes 31 and 32 represent field bean; 33 and 34, faba bean; 35 and 36, pea; 37 and 38, chickpea; and 39 and 40, lentil. Odd numbers represent untreated extract, and even numbers represent solubilized proteins of the precipitate.

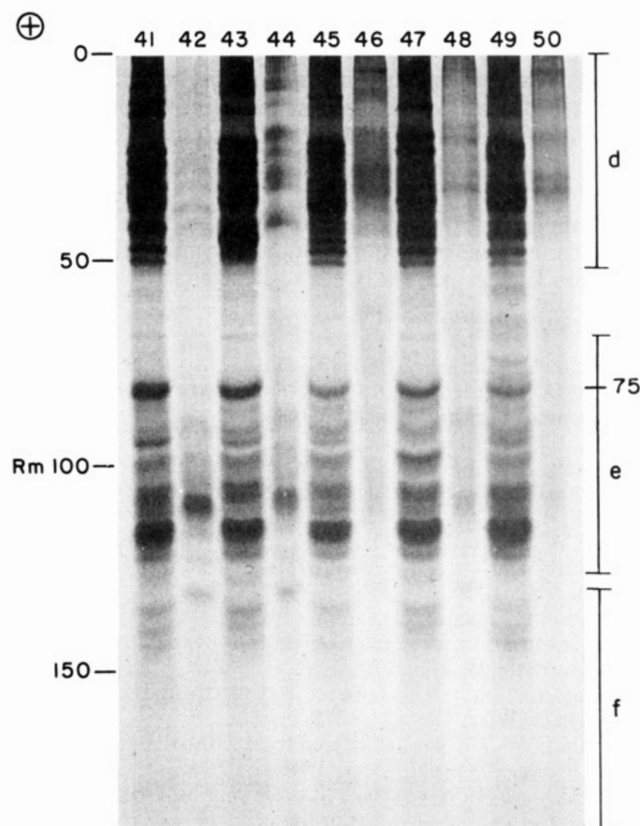

Figure 6. Solubilization of the precipitated wheat proteins in 1 N NaOH: Lanes 41 and 42 represent cultivar Katepwa; 43 and 44, Glenlea; 45 and 46, Norstar; 47 and 48, HY 320; and 49 and 50, Fielder. Odd numbers represent untreated extract, and even numbers represent solubilized proteins of the precipitate.

Similarly protein loss observed as disappearance of bands was noticed when extracts with and without the addition of phytic acid were analyzed in 10% polyacrylamide gel at pH 3.1. On the basis of the mobility in 10% acrylamide gel, electrophoretic patterns could easily be divided into three main groups viz. (a) slow (Rm 0–50), (b) medium (Rm 50–110), and (c) fast (Rm 110–160) which in case of legumes may correspond to legumin, lectin, and albumins/nonstorage proteins, respectively (Bliss and Brown, 1983).

In legumes (Figure 2) proteins of slow (a) and fast (c) mobilities were rendered insoluble much more rapidly than those of medium (b) mobility (Table II). However in case of wheat (Figure 3) proteins of medium (e) and fast mobility (f) were more susceptible to phytic acid interaction. Partial removal seen as loss of stain intensity of proteins bands in regions b and d (Figures 2 and 3) would suggest a weaker phytic acid–protein association. In a separate experiment, gradual addition of 0.1 N HCl (in 10-μL aliquot) and deionized water to the clarified HAc extract did not cause any turbidity or change in the electrophoretic patterns (results not shown), implying that formation of insoluble complex was not due to lowering of pH or dilution.

A mild treatment with 0.1 N sodium hydroxide (100 μL) applied to the residue obtained after phytic acid addition solubilized the proteins of medium mobility (Figure 4). Increased concentration of NaOH (1 N/100 μL) resulted in an increased recovery of proteins of both medium and high mobility (Figure 5). In contrast, the slow moving proteins, presumably the high molecular weight proteins (Hussain and Bushuk, 1991) were difficult to solubilize from their insoluble precipitates. Addition of 5 N NaOH, extracted some of the slow moving proteins but this harsh treatment denatured other proteins and caused extensive background and tailing in the electrophoregrams (results not shown).

Viewed collectively, the results (Figures 4–6) indicated clearly that the proteins which were bound to phytic acid remained quite stable; mobility of bands, however, was slightly slower compared to their corresponding untreated samples. The decrease of mobility could result from chemical and physical changes due to the addition of NaOH. The electrophoresis results also indicate that the acetic acid soluble proteins of *V. faba* showed a greater sensitivity to phytic acid–protein interaction compared with other legumes and wheat. Among legumes, proteins of field bean showed most resistance to phytic acid interaction as 11–12 of the 15 (control) protein bands appeared in the electrophoregram (lane 2, Figures 2 and 5).

In wheat, proteins of fast mobility which correspond to the gliadin fraction according to the Osborne solubility fractionation (Osborne, 1907) tended to interact more with phytic acid than did the slow moving acetic acid soluble proteins (glutenins). Among the cultivars tested more of the slow-moving protein bands (d) were recovered in Norstar, HY 320, and Fielder, whereas more of the fast-moving proteins were recovered in Katepwa and Glenlea (Figure 6) which are known to have good dough properties. However one band (75 mm) was difficult to recover for all tested cultivars. Unlike wheat, the slow-moving proteins in legumes (Figure 5, region a) could not be recovered with 1 N NaOH treatment. Perhaps the number of cationic groups available for binding exceeds those present in the wheat.

In conclusion, all the legumes that were tested, irrespective of their species, showed a similar trend of

interaction with phytic acid (Table II); low-mobility proteins were more sensitive to phytic acid binding compared with those of the medium mobility. Phytic acid–protein interaction with acetic acid soluble wheat proteins was not as intense as in the case of legumes.

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