Reduction of Phytic Acid Concentration in Protein Isolates by Acylation Techniques

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The interaction of phytic acid (PA) with proteins is dependent on the charges and conformation of the proteins and the ionic strength of the solution. Hence, changes in these parameters brought about by acylation could change the extractability, precipitation and interactions of PA with protein and minerals and consequently the PA concentration of protein isolates from phytate-containing foods. This paper summarizes studies in rapeseed and navy bean flours which demonstrate that a high degree of succinylation or acetylation can be used to separate the proteins from the PA and to prepare low phytate protein isolates of good functional properties. The separation of PA from the protein in rapeseed flour occurred during the extraction stage, while that in navy bean flour occurred at the isoelectric precipitation stage.

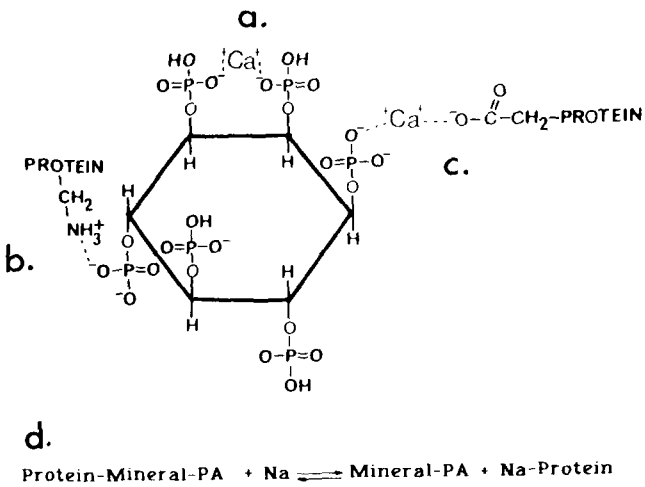

FIG. 1. Interactions of phytic acid with minerals and proteins.

Phytic acid (PA) has been considered an antinutrient because, in large concentrations, it can reduce the bioavailability of minerals (1-5). However, in small concentrations, PA may also have some beneficial effects; these include slowing the rate of starch digestibility and lowering the blood glucose response (6-8), controlling dental caries (9) and cancer (10) and improving the oxygen-providing ability of red blood cells (11). Hence, methods for reducing to a suitable level the PA in foods, especially protein isolates from oilseeds and legumes, have been the subject of numerous investigations (1-5). PA is closely associated with the proteins in these plant products (2) and is very often co-isolated with the proteins.

Acylation is a method commonly used to enhance the solubility and other functional properties of proteins (12). If acylation can also separate proteins from PA, then it can be used not only for improving the functionality but also for lowering the PA concentration of protein isolates.

PROTEIN-PHYTIC ACID INTERACTIONS AND PROTEIN ACYLATION

According to the Anderson (13) model, at neutral pH and at pHs commonly encountered in foods, PA is negatively charged. Therefore, it is very reactive with many positively charged groups such as cations and proteins. Cations can bind to one or two phosphate groups of PA molecule (Fig. 1a) (14) while the protein interacts with the PA dependent on pH (1,3,4).

At low pH, below the isoelectric pH of the proteins, the positively charged groups of proteins such as terminal amino, ϵ-amino group of lysine and imidazole group of histidine can directly form a binary complex with the negatively charged PA (Fig. 1b) (1,6).

At intermediate pH (pH 5-10), above the isoelectric pH, the proteins have a net negative charge and can form a ternary complex with multivalent cations and PA (Fig. 1c). The binding sites include the ionized carboxylic group and unprotonated imidazole group of histidine (1). Some binary complexes may still exist at intermediate pH because the lysyl and arginyl residues of the proteins are still positively charged at these pHs.

At very high pHs (pH > 10), the interaction between protein and PA is diminished. The lysyl and arginyl residues lose their positive charges and, consequently, their ability to form the binary complex. The ternary complex is also destabilized as the ionic strength increases at very high pH. Increased sodium ion concentrations may shift the equation (Fig. 1d) to the right, forming insoluble Ca phytate and soluble sodium protein (1,4,6). A single protein molecule can bind several molecules of PA and cations (15). A change in protein conformation may, however, change the accessibility of binding sites to PA and consequently the number of PA molecules eventually bound to the proteins.

From the foregoing, it appears that the degree of protein-PA interactions is affected by the protein charges and conformation and ionic strength of the solution at a given pH.

Acylation with acetic or succinic anhydride at alkaline pH introduces a neutral acetyl or anionic succinate group in the nucleophilic groups of amino acid residues of proteins such as the ϵ-amino group of lysine, the sulfhydryl group of cysteine, the phenol group of tyrosine and the imidazole group of histidine (16-18). Because of the increase in the net negative charges and the introduction of the bulky acetyl and succinate groups to the proteins, the protein unfolds and its propensity to dissociate to subunits increases (16,17). These, in turn, help improve the functional

properties of the proteins including solubility, emulsifying and fat absorption capacities.

Because PA appears to bind to the same groups in the protein as the acylating agents and since the PA-protein interactions are dependent on protein charges and conformation, we hypothesized that the extensive changes in protein charges and conformation brought about by acylation could affect the interactions, extractability and precipitation of PA, minerals and proteins. In addition, the change in ionic strength associated with addition of alkali during acylation could also have an effect. Hence, through proper manipulation of acylation conditions, it may be possible to separate the PA from the proteins and to prepare low phytate protein isolate of good functional properties.

To support this hypothesis, we conducted studies on dehulled, solvent extracted rapeseed flour (RF) and navy bean flour (NBF) containing 4.9 and 1.5% PA and 46 and 21% protein, respectively; the results are summarized in this paper. We determined the extractability, precipitation and interactions of the proteins, PA and minerals in the presence of acylating agents and, based on these, were able to prepare low phytate protein isolates of good functional properties (19-21).

RAPESEED FLOUR

Aqueous dispersions of RF (20%, w/v) were acylated with various levels of succinic anhydride (0.0183-0.186 g/g protein) or acetic anhydride (0.0183-0.186 ml/g protein) for one hr at pH 8.5 at room temperature as previously described (19). The supernatant obtained after centrifugation is extract I. Redispersion of the residue in deionized water (20%, w/v) followed by adjustment to pH 8.5, stirring for one hr and centrifugation provided extract II.

The effect of succinic anhydride on the extractability of nitrogen, PA, minerals (free and bound) and the degree of chemical modification is shown in Figure 2. In the first extract, while nitrogen extractability

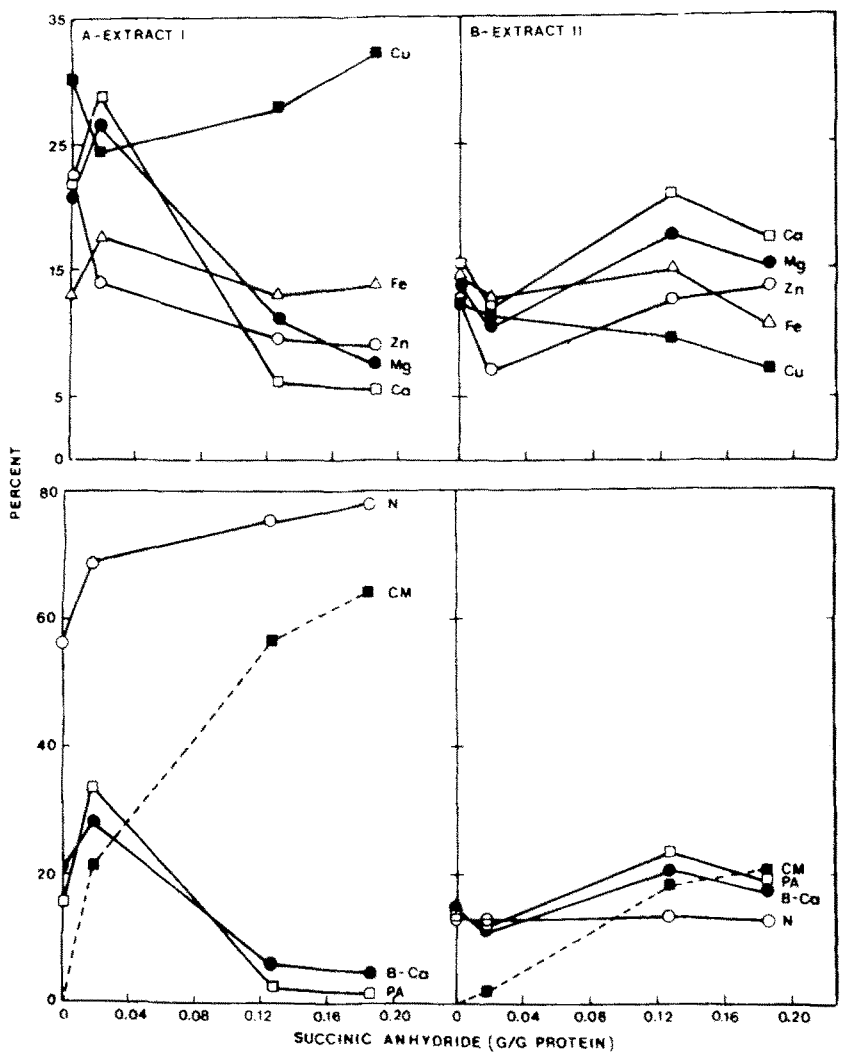

FIG. 2. Percent chemical modification (CM) and extractability of nitrogen (N), phytic acid (PA), bound Ca (B-Ca) and total minerals upon treatment of rapeseed flour with various levels of succinic anhydride (19).

increased with degree of modification, PA extractability increased at a low degree and decreased at a high degree of modification. The extractability of the major minerals such as Fe, Ca and Mg as well as the bound Ca followed the pattern of PA, suggesting that these minerals are extracted closely associated with PA. Bound Ca was calculated as total Ca (analyzed by atomic absorption spectroscopy) minus free or ionic Ca (estimated by a Ca ion electrode in a Beckman Ion Analyzer).

At a low level of acylation (21%), the slight unfolding of the proteins may have resulted in exposure of additional binding sites to the PA and, consequently, an increase in the extractability of the soluble ternary complex of nitrogen, PA and minerals. On the other hand, at a high level of modification (63%), extensive changes in protein conformation caused by increased negative charges may have caused steric hindrance to binding of the PA with the proteins and hence reduction in PA extractability. In addition, at a high level of succinylation, the ternary complex may have been destabilized in part due to the excessive amount of alkali (NaOH) added to the sample to maintain the pH at 8.5 during acylation. About four times more alkali was used in the highly succinylated samples in comparison with the control and the samples succinylated at low levels. As hypothesized, the high ionic concentrations probably dissociated the ternary complex to form soluble proteinate and insoluble Ca phytate.

The nitrogen content in extract II of unmodified RF and RF modified with different levels of succinic anhydride did not differ significantly, while the PA content tended to increase with the level of succinic anhydride (Fig. 2). As in extract I, the extractabilities of bound Ca and total Ca, Mg and Fe followed that of PA. This suggests that in extract II, the PA was solubilized not only as a ternary complex with proteins and minerals but also as a mineral-PA complex. The decrease in ionic strength related to the use of only deionized water in the second extract may have been responsible for the PA solubilization.

When RF was acylated by different levels of acetic anhydride, the extractability pattern as well as the actual concentration of nitrogen and PA extracted at the highest level of acetylation were similar to that of the succinylated samples (19). However, two differences can be noted. At the highest level of acylating agent used, i.e., 0.186 g succinic anhydride or ml acetic anhydride/g protein, rapeseed protein was modified by 63% with succinic anhydride but 87% with acetic anhydride, suggesting the greater reactivity of the latter. Nevertheless, at the same level of modification, i.e., 63%, acetylation was not effective, whereas succinylation was with respect to increasing the nitrogen and decreasing the PA extractability. Since succinic anhydride introduces a long, negatively charged succinyl side chain while acetic anhydride introduces a neutral, shorter side chain, greater electrostatic repulsion and conformational change in the protein as well as steric hindrance to protein-PA binding may have occurred with succinylation than acetylation at the same level.

Figure 3 summarizes the total extractability of the

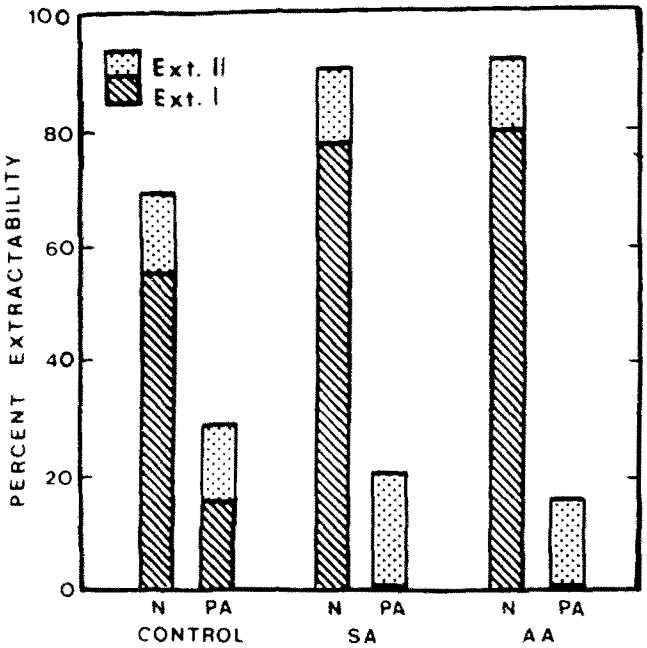

FIG. 3. Percent total extractability of nitrogen (N) and phytic acid (PA) in the unmodified control and rapeseed flour succinylated (SA) or acetylated (AA) at the highest level (0.186 g succinic anhydride or ml acetic anhydride).

nitrogen and PA in the unmodified control and the RF succinylated or acetylated at the highest level; the extracts of RF acylated at low levels were no longer of interest since they contain high concentrations of PA. Note the higher yield of nitrogen and lower yield of PA in the highly acylated samples in comparison with the control. Since 93% of the total extracted PA came from the second extract, processing of the first extract separately from the second extract assured preparation of protein isolate with low PA concentration.

The extracts were adjusted to various pHs and the precipitation yields of nitrogen, PA and bound Ca were determined. The unmodified and highly succinylated samples had similar precipitation patterns, i.e., as the pH decreased, there was an increase followed by a decrease in nitrogen and PA precipitation yields, whereas the bound Ca decreased continuously (Fig. 4) (20). The pattern for the acetylated extracts was also similar to that of the succinylated extracts. These results suggest that much of the extracted PA coprecipitated with the proteins, especially at the isoelectric pH, with little or no mediation by Ca and other minerals. Obviously, the reduction in negative charges of the proteins as the pH was lowered resulted in the destabilization of the ternary complex and formation of binary complex. A slight shift in the isoelectric pH of the highly acylated samples, especially the first extract, was expected due to the increased negative charges after acylation.

For both the unmodified and the acylated samples, the nitrogen precipitation yields at the isoelectric pH were low (54-55% in extract I and 63-76% in extract II). This was reflected in the low overall yields when protein isolate eventually was prepared from the raw

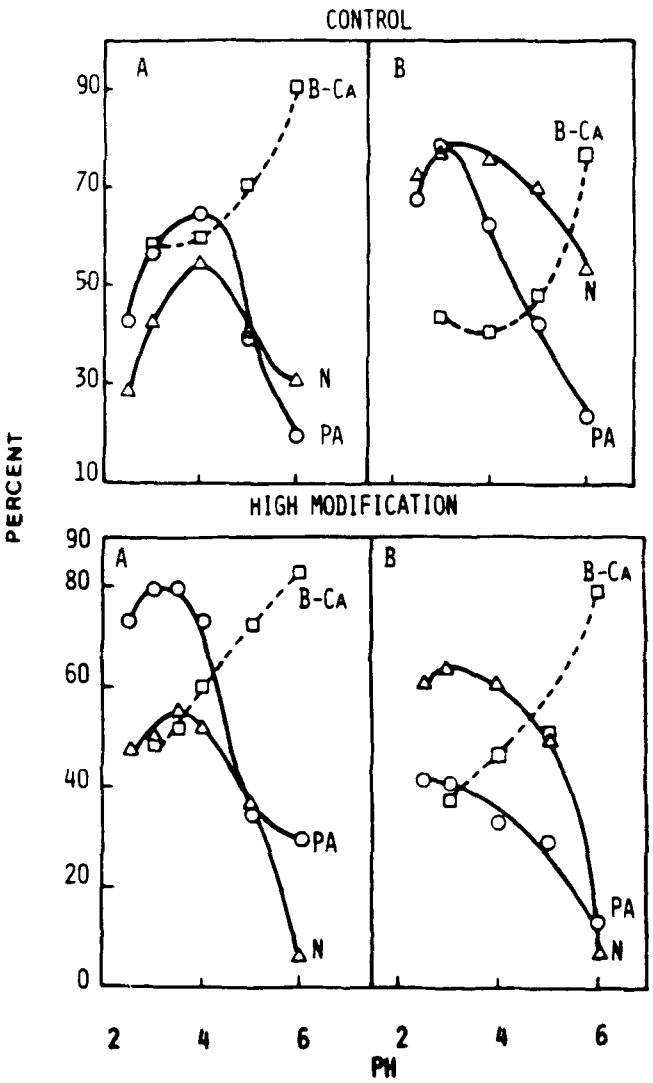

FIG. 4. Percent bound Ca (B-Ca) and precipitation yield of nitrogen (N) and phytic acid (PA) of unmodified and highly succinylated rapeseed flour extracts (20). A=extract I, B=extract II.

material by isoelectric precipitation (Fig. 5) (20). To improve the nitrogen yields, the protein extract was dialyzed against distilled water to remove the residual acylating agent and non-proteinaceous constituents of the extract, and the retentate was then freeze dried. However, twice as much PA was recovered by using the dialysis technique as by isoelectric precipitation. Addition of 0.1 M EDTA to extract II, which is higher in PA concentration, can effectively lower the total PA recovered by dialysis techniques but only to levels similar to that obtained by isoelectric precipitation (Fig. 5). Since PA appears to be extracted partly in the form of the ternary complex, complexation of EDTA with the minerals prevented the association of the still negatively charged proteins and PA and allowed diffusion of PA out of the dialysis bag. Ultrafiltration of the protein extracts gave recoveries similar to those of the dialysis technique.

The protein and PA contents of the protein isolate

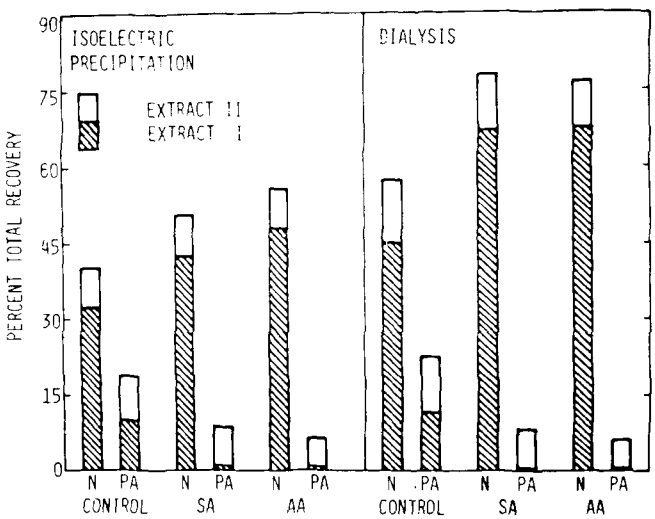

FIG. 5. Percent total recovery of nitrogen (N) and phytic acid (PA) in the protein isolates from the unmodified control and the succinylated (SA) or acetylated (AA) extracts at the highest level (0.186 g succinic anhydride or ml acetic anhydride/g protein).

from the unmodified RF extract I were 85.2 and 2.2% and from extract II, 68.9 and 6.3%, respectively. In contrast, the protein and PA contents of the protein isolate from the acetylated and succinylated RF extract I were 75.3-76.5% and 0.2-0.3% and from extract II, 57.5-60.7% and 2.8%, respectively.

The nitrogen solubility of the isoelectrically precipitated and dialyzed protein isolates differed greatly in the unmodified control but not much in the acylated samples (Fig. 6). In case of the unmodified control, the isoelectrically precipitated proteins had lower solubility than the dialyzed samples, but all solubilities were still lower than those of the acylated samples. On the other hand, the isoelectrically precipitated and dialyzed acylated samples both exhibited high solubilities. Similarly, the emulsifying and fat absorption capacities of the acylated proteins from the first extract were, respectively, 36-38% and 11-25%, higher than the unmodified control, but the whipping properties were over 50% lower (21).

Therefore, acylation is not only a good method for improving the nitrogen recovery and removing the PA from the proteins in rapeseed, but also for improving most of the functional properties of the protein isolates.

NAVY BEAN FLOUR

In contrast with rapeseed, the extraction of acylated navy bean protein did not result in the separation of nitrogen from the PA (Fig. 7). As the degree of acylation increased, both the PA and nitrogen extractability increased. However, when the extract was adjusted to the isoelectric pH, the PA in the unmodified control coprecipitated with the proteins but not with the acylated proteins (Table 1). These results suggest that acylation can help separate the PA from the

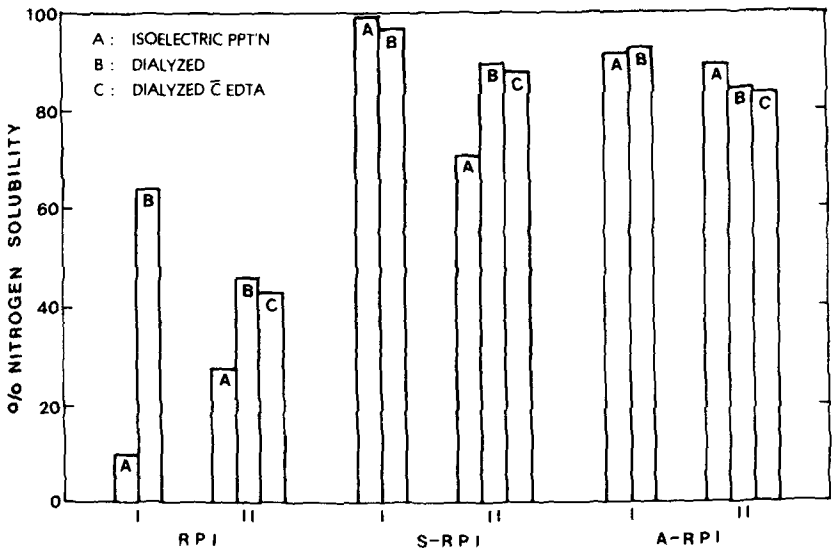

FIG. 6. Nitrogen solubilities of unmodified (RPI), succinylated (S-RPI) and acetylated (A-RPI) rapeseed protein isolates prepared by isoelectric precipitation or by dialysis of extracts I and II.

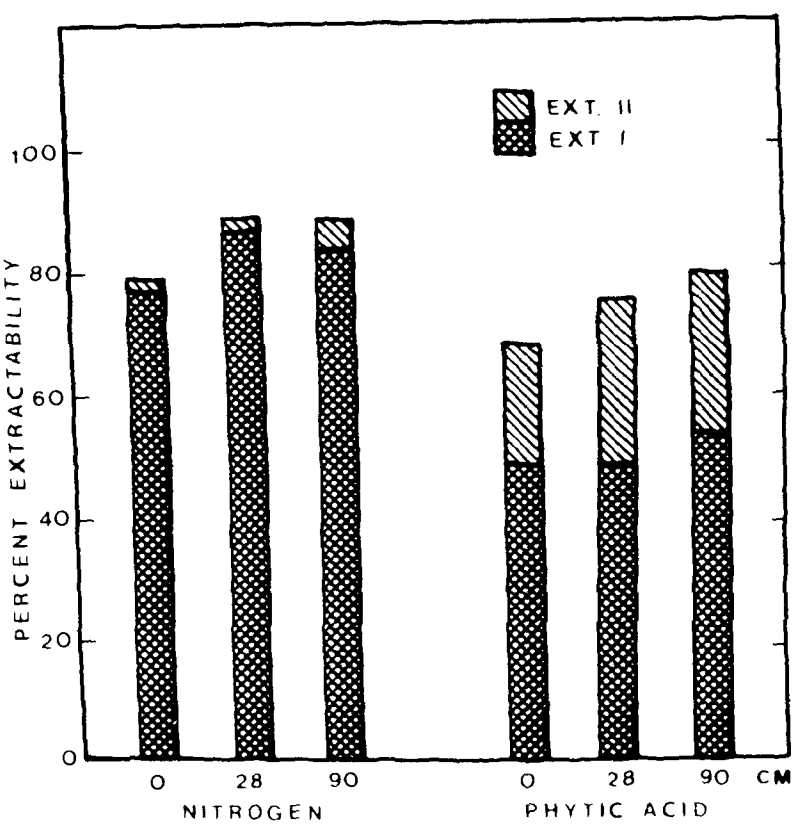

FIG. 7. Percent chemical modification (CM) and extractability of nitrogen and phytic acid in navy bean flour acylated with various levels of succinic anhydride.

TABLE 1.

Yield of Nitrogen and Phytic Acid from Extract I of Navy Bean Flour

g SA/g protein	% CM[a]	% PA ppted.		% N ppted.	
		pH 4	pH 3.5	pH 4	pH 3.5
0	0	68.4	90.3	89.6	90.8
1.5	90	5.6	9.3	89.0	94.5

[a]Chemical modification.

navy bean protein at the protein precipitation stage. The differences in the extractability and precipitation of proteins from RF and NBF may be due to differences in their protein charges, composition and conformation. Rapeseed protein is more heterogenous, consisting of many proteins of widely different isoelectric pH (22, 23), while navy bean protein is more homogenous, with most proteins having an isoelectric pH of about 4 (24).

We have demonstrated that protein acylation at a high level can be used to separate the proteins from the PA and to prepare in high yields low phytate protein isolate of good functional properties. However, the separation of the proteins from the PA may occur either at the extraction or the precipitation stage, depending on the nature of the proteins, their amino acid composition, charges and conformation. Therefore, for preparation of low phytate product, optimum acylation conditions for each protein source should be tested.

Acylated proteins have not reached the commercial stage, primarily due to limited data on their safety and toxicity. Mice (25) fed acetyl casein had lighter weights and smaller litters than those fed casein. However, there were no histological changes in the organs of mice fed acetyl casein for three generations. The acylated proteins are partly digested and the succinyl amino acids appear to be absorbed (26,27). Nevertheless, their metabolic fate is not clear. Therefore, acylated proteins, including those described here, probably should be tested further for biological effects before they can be used for human consumption.

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