A CRYSTALLINE BEAN SEED PROTEIN IN COMBINATION WITH PHYTIC ACID

BY JAQUES BOURDILLON

(From the Division of Laboratories and Research, New York State Department of Health, Albany, New York)

(Received for publication, October 5, 1950)

A protein combined with phytic acid has been extracted from the dried seeds of Phaseolus vulgaris, variety Great Northern, by the following method.

Extraction—100 gm. of bean flour were stirred with 100 ml. of N NaCl, followed by 800 ml. of water. The suspension was centrifuged and the sediment discarded. The pH of the turbid supernatant solution was lowered to 4.6 with about 7.0 ml. of N HCl, which produced a precipitate. The suspension was heated to 50° and the precipitate was removed by centrifugation. The pH of the supernatant fluid was lowered to 4.2 with about 4.0 ml. of N HCl, followed by 1 volume of water. The precipitate formed was collected by centrifugation and dissolved in 40 ml. of N NaCl, and the solution clarified by filtration in a Büchner funnel, through coarse paper covered with Filter-Cel, with the help of moderate suction (to avoid frothing and clogging). To the filtrate were added 400 ml. of water and the precipitate thus produced was collected by centrifugation.

Crystallization at pH 4.0—The precipitate was dissolved in 200 ml. of a buffer solution made from 3 volumes of N NaCl, 1 volume of N sodium acetate, 3 volumes of N acetic acid, and 1 of water. The solution was warmed to 50°, about 120 ml. of warm methanol were slowly added to it, and the container was placed in a bucket filled with water at 50° and allowed to cool slowly at room temperature. After 12 to 24 hours, the material crystallized in the form of elongated dodecahedra with slightly rounded edges (Fig. 1, left). These crystals were strongly anisotropic. Recrystallization was carried out several times by the same method. The material was finally dialyzed against distilled water and dried from the frozen state. The yield was 4 gm.

Crystallization at pH 5.3—1 gm. of the above powder was dissolved in 200 ml. of 0.5 N NaCl, the pH was adjusted to 5.3, and 100 ml. of methanol were added. The amorphous precipitate thus produced changed in a few hours at room temperature into rather coarsely shaped bisphenoids (Fig. 1, right), which were not birefringent. Microscopic quadrangular bipyramids of sharp outline formed when the amorphous precipitate was allowed to stand under a cover-slip. These two crystalline forms and the absence
of birefringence show that under these conditions the protein crystallized in the cubic system.

**General Properties**—The material prepared at pH 4.0 or 5.3 had the general properties of a protein. In the dry state it was a white powder which dissolved easily in water at pH above 7 or below 3.5 and yielded clear, faintly yellowish solutions. Between pH 7 and 3.5 it required a salt molarity of 0.1 to 0.4 for solution. It was precipitated by alcohol or acetone and could be dried with these solvents without denaturation. It was irreversibly precipitated by trichloroacetic acid and by boiling.

The complex nature of this material was first shown in the following observation. When the protein prepared by crystallization at pH 4.0 was heated to boiling in buffers of various pH and of 0.01 ionic strength, it remained insoluble in the pH range of approximately 4 to 6.5, and showed maximum flocculation at 5.0. If the ionic strength was 0.1, the substance first dissolved fairly easily, but soon precipitated as the temperature was raised; there were three flocculation maxima at pH 4.0, 5.0, and 5.6, respectively; the first two appeared as the temperature neared the boiling point, the third one only upon cooling. Finally, in buffers of 1.0 ionic strength, turbidity and flocculation occurred only upon boiling, and only below pH 4.2.

**Chemical Analysis**—Preparation I, obtained by crystallization at pH 4.0, gave the following information: carbon (Van Slyke and Folch) 49.8 per cent, nitrogen (Kjeldahl) 15.7 per cent, carboxyl nitrogen (Van Slyke)
0.17 per cent, phosphorus (phosphomolybdate) 0.487 per cent, sulfur (Carius) 0.30 per cent, sugar (carbazole) 4.8 per cent, and ash 0.44 per cent. Preparation IIA, obtained by the same procedure, contained 0.478 per cent phosphorus and 4.3 per cent total sugar. Preparation IIB (which was Preparation IIA crystallized twice at pH 5.3) gave 0.122 per cent phosphorus and 4.5 per cent total sugar.

Complete removal of the phosphorus was not achieved by repeated crystallization at pH 5.3, but only by prolonged dialysis in a slightly alkaline medium. Dialysis was also the simplest way of isolating the phosphorus-containing fraction; procedures causing the precipitation of the protein were found less suitable because most of the phosphorus was precipitated.

Isolation of Phytic Acid—2.5 gm. of the protein crystallized at pH 4.0 were dissolved in 10 ml. of water, followed by enough NaOH to bring the pH to about 8. The solution was dialyzed for several days against 250 ml. of water. The dialysate was treated with BaCl₂ solution added dropwise until no more precipitate formed. The precipitate was sedimented, washed with water, suspended in 5 ml. of water, and dissolved with a few drops of n HCl. The barium was precipitated with an excess of H₂SO₄, and the supernatant solution made alkaline to pH 10.5 with NaOH and treated with 3 volumes of methanol. The precipitate formed was washed in 80 per cent methanol, followed by pure methanol and dried at 120°. The white powder was readily soluble in water and gave negative tests for nitrogen and carbohydrates (carbazole and Molisch tests). The carbon and phosphorus content was as follows:

\[
\begin{align*}
\text{C}_6\text{H}_4\text{O}_{12}\text{P}_4\text{Na}_{42}. & \quad \text{Calculated. C 7.80, P 20.14} \\
& \quad \text{Found. " 7.98, " 20.14} \\
\text{C}_6\text{H}_4\text{O}_{12}\text{P}_4\text{Na}_{42}.3\text{H}_2\text{O}. & \quad \text{Calculated. C 7.36, P 19.02}
\end{align*}
\]

The results are in agreement with the assumption that the phosphorus was obtained as anhydrous sodium phytate. The isolation of inositol was not attempted for lack of sufficient material.

Recombination of Phytic Acid and Protein—Crystallization of the protein at pH 5.3 took place as easily when the original phytic acid was still present as when it had been completely removed by dialysis. Crystallization in the form of dodecahedra at pH 4.0, on the other hand, was never achieved in the absence of phytic acid. If a sample of the complex obtained at pH 4.0 was caused to crystallize at pH 5.3, and if the crystals and the mother liquor were then acidified to pH 4.0 and treated with more methanol, all of the phytic acid was carried down with the protein. The precipitate, dissolved in buffer at pH 4.0, could then be made to crystallize again in the form of dodecahedra. The crystals, however, were usually accompanied by a variable amount of amorphous matter, some of which
proved to be irreversibly precipitated. That time played a part in the success of the operation was shown by the following example: 50 mg. of Preparation II were suspended in 5 ml. of water, dissolved by making the solution alkaline to pH 7.7 with NaOH, and allowed to stand at room temperature. 1 ml. samples were removed at intervals and acidified with HCl to pH 5.4, which precipitated all of the protein and all of the phosphorus. The sediments were then dissolved in acetate buffer and treated by the method described for crystallization at pH 4.0. The results are shown in Table I.

Recrystallization at pH 4.0 was similarly performed by mixing the phosphorus-free protein with its own dialysate, with the sodium phytate extracted from it, or with commercial sodium phytate. In general, the results were better with phytic acid added in considerable excess, but the appearance of amorphous matter could not be avoided. The formation of non-protein nitrogen or of inorganic phosphorus was never observed.

Table I

<table>
<thead>
<tr>
<th>Time left at pH 7.7 (min.)</th>
<th>Crystallization at pH 4.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st attempt</td>
</tr>
<tr>
<td></td>
<td>All amorphous</td>
</tr>
<tr>
<td>8</td>
<td>&quot;</td>
</tr>
<tr>
<td>30</td>
<td>&quot;</td>
</tr>
<tr>
<td>110</td>
<td>&quot;</td>
</tr>
<tr>
<td>295</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

Dissociation Curve—Fig. 2 shows (Curve I) the acid- and base-binding power of the original complex, (Curve II) of protein twice crystallized at pH 5.3 and deprived of most of its phosphorus, and (Curve III) of protein after complete removal of phosphorus. The protein solutions, of approximately 3 per cent concentration in 0.5 N KCl, were treated with increasing amounts of 0.075 N HCl or KOH in 0.5 N KCl. The data are corrected for the titration of the solvent. Measurements were made with the glass electrode. The apparent isoelectric point of the complex is about pH 4.8, that of the protein alone about 7.1. The phosphorus-free protein possesses but little base-binding power.

The difference between Curves I and III along the ordinates is about twice that to be expected from the base-binding power of phytic acid alone (1, 2). This discrepancy cannot be considered significant, however, since the difference would be considerably affected by a slight systematic error in the position of either curve.

Molecular Weight—The molecular weight of the original sample was
studied by the osmotic pressure method (3). Protein concentrations ranged from 0.7 to 2.4 per cent in 0.2 to 0.5 M buffers. The pressures read ranged from 10 to 45 mm. of toluene. The results, corrected by -1.7 per cent to give the molecular weight of the protein without phytic acid, are shown in Table II. The mean molecular weight at neutrality was

![Fig. 2. Acid- and base-binding power of phytic acid protein measured in 0.5 N KCl. Phosphorus content, Curve I, 0.48 per cent; Curve II, 0.13 per cent; Curve III, none.](http://www.jbc.org/)

**Table II**

*Molecular Weight of Phytic Acid Protein Obtained from Osmotic Pressure Measurements at Various pH Values*

<table>
<thead>
<tr>
<th>pH</th>
<th>mol. wt.</th>
<th>pH</th>
<th>mol. wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>150,000</td>
<td>5.7</td>
<td>282,000</td>
</tr>
<tr>
<td>3.3</td>
<td>211,000</td>
<td>6.9</td>
<td>174,400 ± 2900*</td>
</tr>
<tr>
<td>3.9-5.1</td>
<td>600,000 (Approximately)</td>
<td>8.8</td>
<td>167,000</td>
</tr>
</tbody>
</table>

* Standard deviation of mean of five measurements.

174,000. It rose to several times this value in the pH range of 3.9 to 5.1, and decreased again in more acid medium. Between pH 3.9 and 5.1, the results were erratic and suggested that the substance was not in a stable state of aggregation. Four attempts to measure the osmotic pressure of the phosphorus-free protein between pH 4 and 5 failed; the pressures observed were negligible and indicated considerable aggregation. Thus
Crytalline Protein with Phytic Acid

Phytic acid cannot be held responsible for the polymerization of the protein at pH 4 to 5.

High speed centrifugation and electron microscopy offered confirmatory evidence for the aggregation of the protein in moderately acid medium. Centrifugation of a 1 per cent solution in phosphate buffer, pH 7.35, for 70 minutes at 39,000 r.p.m. in an angle centrifuge yielded no sediment, whereas an appreciable gelatinous sediment, devoid of birefringence, formed under the same conditions in M acetate buffer, pH 4.2. Electron microscopy failed to reveal visible particles in a slightly alkaline water solution of the protein, whereas irregular masses were seen in a preparation buffered at pH 4.

From the sulfur content of 0.30 per cent, the minimum molecular weight would be 10,700, or one-sixteenth of 174,000. The phosphorus content of 0.48 per cent in the original material gives a molecular ratio of phytic acid to protein of 4.5:1.

Viscosity—The relative viscosity of the original complex was measured in 0.8 N KCl at 35° in an Ostwald type viscosimeter. At pH 5.0 the values were 1.87, 1.24, and 1.11 in concentrations of 10, 4, and 2 per cent, respectively. The reciprocal of these values (fluidity), plotted against concentrations, yields the straight line observed with most proteins (4). At pH 7.95 in 4 per cent concentration, the relative viscosity was 1.25; at pH 4.25, 1.24. The constancy of this value suggests that the polymerization which takes place at pH 4 to 5 is not chain-like, since this would be expected to cause considerable increase in viscosity (5).

Discussion

Most of the phosphorus of seeds is in the form of phytic acid (1, 6–9), and the ability of this acid to combine with animal as well as with plant proteins has been studied by several investigators (1, 7, 8). No crystalline complexes, however, seem to have thus far been reported. This property is not characteristic of phytic acid alone; Courtois and Barré (10) have shown that a purified almond protein, conamandin, forms similar complexes with orthophosphoric and pyrophosphoric acids, and with a number of phosphoric esters.

The structure of phytic acid is not definitely established, and the state of the phosphoric acid in it is uncertain. Courtois and Masson (11) favor the view that phytic acid contains 3 molecules of hydroxyphosphoric acid, \( H_3P_2O_9 \) (= \( 2H_3PO_4 + H_2O \)), rather than 6 of orthophosphoric acid, which would give it eighteen acid valences, and they suggest that the orthophosphoric structure may form during desiccation of the free acid or of acid salts through the loss of 3 \( H_2O \). (However, the C and P percentages of the phytate isolated here by precipitation from an alkaline
solution are in agreement with the orthophosphoric structure.) This uncertainty, and the possible structural lability of the acid, are mentioned because of the difficulties encountered here in attempting to recombine the dissociated protein-phytic acid complex.

The fact that this complex formed crystals at a different pH and of a different type from those of the protein alone indicates that it has a definite composition and is more specific, e.g., than the combination of crystalline ovalbumin with metaphosphoric acid (12). The apparent partial degradation with time of the phytic acid separated from the protein, and the need for an excess of the acid when attempts are made to recombine it with the protein to yield a crystalline product, suggested that only part of the acid remained in a state suitable for crystalline recombination. It has also been mentioned above that attempts at recombination produced variable amounts of insoluble protein. Since the protein, deprived of its phosphorus, appeared by the usual standards to be but little susceptible to denaturation, this insoluble fraction may have represented an irreversible combination in which the phytic acid was in a different state from that which yielded the crystalline complex. These observations suggest that the complex may have preexisted in the seed and was not an artifact of preparation.

**SUMMARY**

A new protein in combination with organic phosphorus has been extracted from the seeds of *Phaseolus vulgaris*. Experimental evidence is in agreement with the assumption that the phosphorus is present as phytic acid (inositolhexaphosphoric acid). The phytic acid-protein complex, the phosphorus content of which was 0.48 per cent, was crystallized at pH 4.0 in the form of anisotropic dodecahedra, and the protein alone at pH 5.3 in the form of isotropic bisphenoids and quadrangular bipyramids. The yield was 4 gm. of protein per 100 gm. of flour.

The molecular weight of the protein (from osmotic pressure measurements) was 174,000 in neutral medium, and approximately the same value at pH 2.4. At pH 4 to 5 the mean molecular weight was much higher; the existence of aggregation in this range was confirmed by high speed centrifugation.

The difficulties attending crystalline recombination *in vitro* of the protein with its original phytic acid or with commercial phytic acid suggested that the complex exists as such in the seed and is not an artifact of preparation.

**BIBLIOGRAPHY**

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