Isolation and Characterization of Glucosamine-containing Storage Glycoproteins from the Cotyledons of *Phaseolus aureus*¹

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**ABSTRACT**

Cotyledons of *Phaseolus aureus* contain protein-bound glucosamine which is metabolized during germination. The glucosamine is present in storage glycoproteins, and these are concurrently metabolized along with the glucosamine. These glycoproteins are associated with protein bodies. Characterization of the glucosamine-containing storage proteins showed them to be identical with vicilin and legumin, the major storage proteins of the Leguminosae. *Phaseolus aureus* vicilin has a sedimentation constant of 8.0S and is made up of four nonidentical subunits. It contains 0.2% glucosamine and 1% mannose. Legumin has a sedimentation constant of 11.3S and is made up of three nonidentical subunits. It contains about 0.1% glucosamine.

Glucosamine-containing proteins occur in a variety of plants (17). Indeed, when plant tissues are incubated with radiolabeled glucosamine, it becomes incorporated into a variety of glycoproteins, both intracellular and extracellular (21, 22). Only a few of these glycoproteins have been characterized (12, 23). Recently, Pusztaï and Watt (18) characterized one such protein from the cotyledons of *Phaseolus vulgaris* and called it "glycoprotein II." Racusen and Foote (19) observed a similar protein in the cotyledons of this species and suggested that it was a storage protein. However, they were unable to show that either the protein or the glucosamine disappeared from the cotyledons during germination and subsequent growth. The present study was undertaken to determine whether or not protein-bound glucosamine is present in the related species *Phaseolus aureus* and to characterize the proteins in which it occurs.

**MATERIALS AND METHODS**

**Plant Material.** Seeds of *Phaseolus aureus* were sterilized for 5 min in commercial bleach diluted 1 to 10 (about 0.5% hypochlorite) and rinsed with distilled water. They were allowed to imbibe water for 24 hr at room temperature. The cotyledons were removed, or the seeds were planted in moist vermiculite and allowed to grow in the dark. In some experiments cotyledons were removed from dry seeds, sterilized, and planted on moist sterile sand.

**Extraction of Proteins.** The cotyledons were homogenized either in 0.4 M NaCl or in 20 mM sodium borate, pH 9.0, using 2 ml of medium per g of tissue. The homogenate was centrifuged at 30,000g for 20 min, and the supernatant was collected. The pellet was extracted three times with the same medium, and the extracts were combined with the initial supernatant.

**Quantitative Determination of Protein.** Proteins were usually assayed according to the procedure of Lowry et al. (13) using bovine serum albumin as a standard. The protein content of peak III glycoprotein was also determined with ninhydrin (27) after acid hydrolysis of the protein.

**Quantitative determination of hexosamine.** The samples to be assayed for protein-bound hexosamine were precipitated with 7.5% trichloroacetic acid, washed with 5% trichloroacetic acid, alcohol, and ether. The dried samples were resuspended in 1 N HCl. Samples to be assayed for total hexosamine were homogenized in 1 N HCl. Hydrolysis was carried out at 90 C for 24 hr. Under these conditions, there was no detectable loss of hexosamine. Hexosamines were separated from interfering chromogens by passage through a small Dowex 50 cation exchange column according to the method of Boas (4). This step is absolutely necessary to obtain reproducible results. Eluates from the columns were dried by evaporation under vacuum at 85 C, and the residue was dissolved in water. The hexosamine content was determined by the method of Elson-Morgan as modified by Johnson (11).

**Quantitative Determination of Carbohydrates.** Carbohydrates were measured with the anthrone reagent (28) using mannose as a standard.

**Identification of Amino Acids, Sugars, and Aminosugars.** An analysis of the amino acids was performed on an acid hydrolyzate (6 N HCl, 110 C, 24 hr) with an automated amino acid analyzer according to the method of Moore and Stein (16). Identification of the neutral sugars was done in the laboratory of Dr. Peter Albersheim, University of Colorado, Boulder, using separation of the acetylated sugar alcohols by gas chromatography (1).

The hexosamine present was tentatively identified as glucosamine by cochromatography on the automated amino acid analyzer and on paper using ethyl acetate-pyridine-water (8:2:1) as a solvent. In this solvent, glucosamine has an Rg of 0.76 and galactosamine has an Rg of 0.69 (9). (Rg is the mobility with respect to glucose.)

**Fractionation of Soluble Proteins on DEAE-cellulose and Determination of S Values on Sucrose Gradients.** Two grams of cotyledons were homogenized in 20 mM sodium borate buffer (pH 9.0), and the homogenate was centrifuged at 30,000g for 20 min. The soluble proteins were passed through

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This page contains detailed scientific information about the isolation and characterization of glucosamine-containing storage glycoproteins from the cotyledons of *Phaseolus aureus*. The research was conducted by Mary C. Ericson and Maarten J. Chrispeels and was published in Plant Physiology in 1973. The abstract outlines the study's objectives, methods, and findings, focusing on the characterization of specific glycoproteins and their associated proteins. The experimental procedures include the extraction of proteins, quantification of protein, and carbohydrate content, as well as amino acid and sugar analysis. The scientific approach and methodology are described in detail, providing a comprehensive understanding of the study's methodology and outcomes.
a Sephadex G-50 column equilibrated with 25 mM sodium phosphate buffer, pH 7.0, and then loaded on a DEAE-cellu-
lose (Whatman DE-52) column. At this point the proteins were not in solution; the column was eluted with a 0 to 0.4 M or
0.025 to 0.4 M gradient of NaCl in 25 mM sodium phosphate,
pH 7.0, which solubilized the proteins and allowed for com-
plete recovery. About 100 fractions of 5.0 ml each were col-
clected. The fractions were assayed for total protein content, and
the carbohydrate content was assayed directly, as well as in the
trichloroacetic acid precipitated material. Fractions from peaks
III and IV (Fig. 4) were concentrated in dialysis bags covered
with Sephadex G-200. Proteins were concentrated to about 2
mg/ml for gel electrophoresis, amino acid analysis, and su-
crose gradient determination of S values. Samples for sucrose
gradients were dialysed against 25 mM sodium phosphate buffer
containing 0.3 M NaCl and layered on linear 5 to 20% sucrose
gradient. The sucrose was dissolved in the dialysis buffer.
The tubes were centrifuged for 18 hr at 40,000 rpm in the SW
41 rotor of a Spincro ultracentrifuge. A small amount of sheep
hemoglobin was added as a marker, and its location was de-
termined by measuring the absorbance at 420 mm. Sedimenta-
tion coefficients were calculated using the method of Martin
and Ames (14).

Analytical Gel Electrophoresis. Electrophoresis of peak III
protein was performed using 7.5% polyacrylamide gels with
tris buffer at pH 8.9 according to the procedure of Davis (7).
The gels were run for 2 hr at 3 mA/gel and then stained with
1% amido schwarz in 7% acetic acid. The proteins present in
peaks III and IV were dissociated with sodium dodeyl sulfate,
and the molecular weights of the subunits were determined by
electrophoresis in 10% acrylamide gels containing sodium
dodecylsulfate. The procedure of Weber and Osborn (31) was
used, but modified in two ways. First, the gel buffer was used at
half the recommended concentration, and second, in order to
ensure complete and permanent reduction of disulfide bonds,
40 mM dithiothreitol was substituted for 1% mercapto-
ethanol during incubation; following the 2-hr incubation under
these reducing conditions, the protein solutions were boiled
for 2 min to ensure complete dissociation, and the gels were
prerun with buffer containing 10 mM sodium thioglycolate.
Samples containing 10 to 80 ,ug of protein were made dense
with glycerol and loaded on the gels. The gels were run at 8
mA/gel for 8 to 12 hr and then stained with 0.25% Coomassie
blue in methanol-water-acetic acid (5:5:1). Bovine serum
albumin, chymotrypsin, and ribonuclease A were used as stan-
dards.

Isolation of Protein Bodies. Five grams of imbibed coty-
ledons were homogenized with a mechanical razor blade chopper
in 10 ml of medium (0.6 M sucrose 0.01 M phosphate, 
pH 6.0), and the homogenate was filtered through Miracloth.
Half the homogenate was layered on a linear 50 to 90% (w/v)
sucrose gradient and centrifuged for 45 min at 27,000 rpm
in the SW 27 rotor of a Spincro ultracentrifuge. After cen trif-
ugation the gradient was fractionated by pumping it from
the bottom; 18 fractions of 2 ml each were collected. The pro-
tein content of each fraction was determined, and the frac-
tions containing protein bodies were combined. The sucrose
concentration was lowered to about 0.6 M, and the protein
bodies were sedimented by centrifugation at 45,000 rpm
for 20 min. This pellet was fixed for electron microscopy (see
below) or dissolved in 0.3 M NaCl in 25 mM phosphate, pH 7.0,
for further characterization of the proteins.

Electron Microscopy. Protein bodies were fixed for 15 min
in 2% glutaraldehyde buffered with 100 mM phosphate (pH 7.4),
rinsed, and postfixed in 1% osmium tetroxide (same buffer).
The pellet was dehydrated through an ethanol-water and an ethanol propylene oxide series and embedded in
Epon. Sections were stained with uranyl acetate and lead
citrate. The electron microscopy was done with a Phillips 200
electron microscope.

RESULTS AND DISCUSSION

Metabolism of Hexosamine. When mungbean cotyledons
were homogenized either in a high salt medium (0.4 M NaCl)
or in an alkaline buffer (20 mM sodium borate, pH 9.0), the
extracted proteins contained approximately 0.7% hexosamine.
Most (83%) of the protein-bound hexosamine present in the
solution was solubilized in this way. The hexosamine-containing
proteins were not soluble at neutral pH or when salt was
omitted from the homogenization medium, suggesting that
the hexosamine was bound to the globulins. The protein-bound
hexosamine disappeared from the cotyledons of germinating
beans, decreasing from 32 ,g/plant to 2 ,g/plant during 9
days of growth in the dark (Fig. 1). Total protein content and
fresh weight of the cotyledons decreased concomitantly. That
the protein-bound hexosamine was indeed metabolized and not
merely solubilized is shown in Figure 2. The data show that
the total hexosamine in the tissue disappeared at the same rate
as did the protein-bound hexosamine.

The mobilization of the protein-bound hexosamine was de-
pendent on some factor(s) from the embryo. Removal of the
plant axes before imbibition results in an inhibition of storage
protein degradation (5, 29). Axis removal also inhibited the
metabolism of protein-bound hexosamine (Fig. 3). The dis-
appearance of protein from the cotyledons was inhibited,
whereas the fresh weight of the cotyledons increased when
the axes had been removed prior to imbibition. All these re-
results suggest that hexosamine is an integral part of one or
more storage proteins which are metabolized and utilized
during germination and subsequent growth.

To characterize these proteins, we fractionated the alkaline-
soluble proteins from imbibed cotyledons by means of a
DEAE-cellulose column. The column was loaded with 48 mg
of protein and eluted with a 0 to 0.4 M NaCl in 25 mM phosphate,
pH 7.0. The protein was distributed in 4 peaks (I, II, III, and
IV, Fig. 4). Proteins from peaks I and II were relatively un-
charged, if the starting NaCl concentration in the eluting
buffer was 25 mM they were not retained on the column and
eluted as one peak. These proteins had rather small sedi-

Fig. 1. Temporal changes in fresh weight, protein content and
protein-bound glucosamine in cotyledons during 10 days of growth.
The proteins soluble in 0.4 M NaCl contained 83% of the protein-
bound glucosamine. The fresh weight of the cotyledons is about
twice that of the dry weight.
Fig. 2. Temporal changes in total and protein-bound glucosamine content of mung bean cotyledons during germination. Cotyledons were harvested at the times indicated and homogenized in 1 N HCl (for total glucosamine) or in 0.4 M NaCl (for protein-bound glucosamine). The proteins were precipitated with trichloracetic acid, washed, and their glucosamine content was determined.

Fig. 3. Effect of embryo removal on the temporal changes in fresh weight, protein content and protein-bound glucosamine in mung bean cotyledons. Embryos were removed from dry seeds and the cotyledons were sterilized, imbibed and incubated on moist sand at room temperature. Controls were germinated in vermiculite at room temperature.

Fig. 4. Fractionation on DEAE-cellulose of the proteins present in an extract of mung bean cotyledons. Beans were allowed to imbibe for 24 hr. Proteins were extracted from the cotyledons and separated on a DEAE-cellulose column (see "Materials and Methods"). The concentration of NaCl was determined by titration with standard 0.010 N AgNO₃ using K₂Cr₂O₇ as an indicator (20). Fractions of 5 ml were assayed for protein and carbohydrate. Carbohydrates were assayed in the trichloracetic acid-precipitable material.

Protein-bound Hexosamine is in Storage Proteins. Extracts from cotyledons obtained from beans which had been allowed to germinate for 1, 4, and 7 days were fractionated on DEAE-cellulose columns to determine whether the hexosamine-con-
taining proteins were metabolized during germination. The results (Fig. 6) show that the glucosamine-containing proteins (peak III and IV) rapidly disappeared from the cotyledons. These data suggest that the glucosamine is bound to specific storage proteins which are metabolized during germination. The storage proteins present in the cotyledons of the Leguminosae are localized in special cellular organelles—the protein bodies or aleurone grains (30). To determine whether the hexosamine-containing glycoproteins are also associated with the protein bodies, these organelles were isolated on isopycnic sucrose gradients. The protein bodies had a characteristic density of 1.26 to 1.28 g/cm³ and banded in the lower third of a linear 50% to 90% sucrose gradient. Protein bodies from sunflower cotyledons have a similar density (25). The band was clearly visible by the naked eye, and its position in the gradient was determined by protein determination (Fig. 7A). No other bands of cellular organelles were observed in these gradients. An electron micrograph of the isolated protein bodies is shown in Figure 8. The protein bodies appeared to be free of contaminating cellular organelles, but they were not intact. The protein of isolated protein bodies contained 0.65% hexosamine. When the protein bodies were solubilized and fractionated on DEAE-cellulose columns, they contained almost exclusively peak III and IV proteins (data not shown). When the protein body proteins were sedimented on rate zonal sucrose gradients, two peaks with approximate S values of 8 and 11 were obtained (Fig. 7B).

Fig. 5. Distribution of soluble proteins on a sucrose gradient. Soluble proteins from 1-day germinated cotyledons were layered on a linear 5% to 20% sucrose gradient and centrifuged for 18.5 hr at 40,000 rpm. Fractions (0.4 ml) were collected and assayed for protein.

Fig. 6. Disappearance of the glucosamine containing glycoproteins during germination. Cotyledons were harvested after 1, 4, and 7 days of germination, homogenized in borate buffer, pH 9.0, and the soluble proteins were fractionated on a DEAE cellulose column eluted with a NaCl gradient. Protein was determined by the method of Lowry et al. (13). Chloride was measured titrimetrically and the profiles have been normalized with respect to the NaCl concentration of the fractions.

Fig. 7. Isolation of protein bodies on isopycnic sucrose gradients and characterization of their proteins by rate zonal centrifugation. A: Fractionation of a homogenate of cotyledons (from beans germinated for 1 day) on a linear 90% to 90% sucrose gradient. The peak closest to the bottom contains the protein bodies, while the other cellular organelles and the soluble proteins remained at the top. B: Rate zonal centrifugation of the protein body proteins on a linear 5 to 20% sucrose gradient. The small peak closest to the bottom represents the 11.3S legumin, the big peak in the middle of the gradient is the 8.0S vicilin.
Identification of the Hexosamine-containing Proteins. The elution profile from the DEAE-cellulose column suggested to us that peak III and IV may correspond to vicilin and legumin, the two main storage proteins found in the various species of Leguminosae, because similar elution profiles have been obtained for the storage proteins of peas (8) and vetch (26). To confirm this idea, we determined the sedimentation constants of these two major protein fractions. When the total salt soluble proteins of cotyledons were separated on a rate zonal sucrose gradient, we obtained 3 peaks (A, B, and C, Fig 5). Samples of protein from peaks III and IV of the DEAE-cellulose column were concentrated and sedimented on gradients with sheep hemoglobin as a marker (Fig. 9). Calculations based on an S value of 4.15 for sheep hemoglobin indicated S values of 8.0 for peak III (identical to B) and 11.3 for peak IV (identical to A). Peaks III and IV, therefore, probably correspond respectively to vicilin and legumin as identified by Danielsson (6).
Fig. 9. Sucrose gradient sedimentation of (A) purified peak III protein and (B) purified peak IV protein. Glycoproteins from peaks III and IV were collected and dialyzed against 25 mM sodium phosphate buffer, pH 7.0, containing 0.3 M NaCl. Samples (0.2 ml) were loaded on the gradients (5 to 20% sucrose in the same buffer and salt) and centrifuged for 18 hr at 40,000 rpm in the SW 41 rotor of a Spinco ultracentrifuge. Protein was determined by the method of Lowry et al. (13). Sheep hemoglobin was used as a marker and located by measuring absorbance at 420 nm.

Since peak III contained most of the salt-soluble protein-bound hexosamine, we attempted to characterize it more exhaustively. The single symmetrical peak obtained on the sucrose gradient (Fig. 9A) suggested a high degree of purity after chromatography on DEAE-cellulose. A single diffuse band was obtained when the protein was subjected to electrophoresis on an acrylamide gel (Fig. 10). Glycoproteins often give diffuse bands on acrylamide gels; this is thought to be due to the microheterogeneity of the carbohydrate portion of the glycoprotein (24).

Electrophoresis of peak III protein on sodium dodecyl sulfate acrylamide gels yielded four clearly definable bands (Fig. 10), suggesting that the protein may have four nonidentical subunits. The molecular weights of these subunits are summarized in Table I. On close examination, a few hairline bands could be seen between the origin and the first band. That these were due to reforming of bonds between subunits is suggested by the fact that, when the vigorous reducing conditions described under "Materials and Methods" were used, these bands were bigger and more numerous, and they also occurred on the gels with the standards. A fifth reproducible band was seen between bands 2 and 3, but this band was so faint that it could be seen only on gels loaded with large amounts of protein. Therefore, this band is probably either a contaminant or the result of overloading. To determine whether or not any of the subunits may have been due to contamination from Peak IV, the protein in this peak was also concentrated and subjected to sodium dodecyl sulfate-acrylamide gel electrophore-

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Table I. Molecular Weights of Subunits Obtained on Sodium Dodecyl Sulfate Gels

<table>
<thead>
<tr>
<th>Band</th>
<th>Mobility Relative to Bromphenol Blue</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vicilin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.244</td>
<td>63,500</td>
</tr>
<tr>
<td>2</td>
<td>0.316</td>
<td>50,000</td>
</tr>
<tr>
<td>3</td>
<td>0.473</td>
<td>29,500</td>
</tr>
<tr>
<td>4</td>
<td>0.532</td>
<td>24,000</td>
</tr>
<tr>
<td>Standards</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>0.225</td>
<td>68,000</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>0.500</td>
<td>25,700</td>
</tr>
<tr>
<td>Ribonuclease</td>
<td>0.703</td>
<td>13,700</td>
</tr>
<tr>
<td>Legumin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.278</td>
<td>56,000</td>
</tr>
<tr>
<td>2</td>
<td>0.350</td>
<td>44,000</td>
</tr>
<tr>
<td>3</td>
<td>0.640</td>
<td>16,500</td>
</tr>
</tbody>
</table>

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The amino acid and carbohydrate composition of peak III protein are shown in Table II. The percentage of neutral sugar and the percentage of hexosamine in all of the experiments reported above are based on the amount of protein as determined by the method of Lowry et al. (13) using bovine serum albumin as a standard. However, all of the values in Table II are expressed on a dry weight basis. The values based on dry weight can be compared with those previously presented (based on the Lowry assay), if the following correction factor is used: values based on dry weight = 3 × (values based on Lowry protein). Values based on dry weight of the protein are the most accurate.
rate, since they give results identical to those based on measurements of the ε-amino nitrogen content of hydrolyzed glycoprotein (using ninhydrin and an average molecular weight of 129 for the amino acids).

The hexosamine cochromatographed with glucosamine, but not with galactosamine, on the automatic amino acid analyzer and by paper chromatography, and was tentatively identified as glucosamine.

**CONCLUSION**

These data strongly suggest that the glucosamine-containing proteins present in the cotyledons of *Vicia faba* and metabolized during germination are vicilin and legumin. The amino acid composition obtained for peak III is in close agreement with that obtained for *Vicia faba* vicilin (15). The number and size of vicilin subunits in different species have been reported by many investigators (3, 8, 10, and 32). However, no two reports agree, indicating that vicilin may be a heterogeneous protein and that there may be considerable variation from species to species. The data obtained for peak IV (legumin) are in close agreement with those recently reported by Bailey and Boulter (2) and by Millerd et al. (15). The sedimentation constants of 8.0S for vicilin and 11.3S for legumin are also in agreement with those obtained by other workers.

It seems more than likely that peak III is identical with "glycoprotein II," as characterized by Puszta1 and Watt (18) and with the porcine of Racusen and Foote (19). As a result, we can state with some assurance that the major storage proteins of *Phaseolus aureus* are actually glycoproteins which contain small amounts of glucosamine and neutral sugars.

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**LITERATURE CITED**