Glycoprotein Metabolism in the Cotyledons of Pisum sativum during Development and Germination

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ABSTRACT

The glycoprotein nature of legumin and vicilin, the reserve globulins in the cotyledons of Pisum sativum was studied. Legumin from mature seed was found to contain 1% neutral sugars (mannonse and glucose) and 0.1% amino sugar (glucosamine), whereas vicilin contained 0.3% neutral sugar (mannonse) and 0.2% amino sugar (glucosamine). On the basis of the incorporation of 14C-labeled glucosamine, it appeared that not all of the component subunits of the reserve glycoproteins are glycosylated to the same extent. In addition, it has been established that glycosylation occurs after peptide synthesis. During seed development there was a change in neutral sugars and amino sugar ratio in vicilin. During germination, the neutral sugars and the amino sugar content of the glycoproteins declined. These findings are discussed in relation to the synthesis and degradation of the glycosyl component of the glycoproteins.

Glucosamine has been reported to be a constituent of the glycoproteins of many seeds (10). Additionally, on the basis of the incorporation of radioactive glucosamine, this amino sugar is apparently a constituent of the glycoproteins occurring in root tissue (15). The glucosamine containing glycoproteins from the seeds of Phaseolus vulgaris (11-14) and Phaseolus aureus (3) have been characterized and they correspond in part to the cotyledonary reserve proteins. As part of our studies on the metabolism of the reserve proteins of Pisum sativum, we have investigated the glycoprotein nature of legumin and vicilin. Similar to Phaseolus, the reserve proteins of Pisum sativum have been found to contain glucosamine and mannose. We have followed the changes in the glucosamine-containing glycoproteins during seed development and germination. These changes are described below.

MATERIALS AND METHODS

Plant Material. Pea seeds (Pisum sativum L. cv. Burpeeana) were purchased from W. Atlee Burpee Company, Clinton, Iowa. Pea plants were grown in sterile vermiculite (Terralite) irrigated with Hoagland’s solution in a growth chamber at a day temperature of 24 C and a night temperature of 13 C, a 12-hr photoperiod, and a light intensity of 2,500 ft-c. The flowering date was recorded and cotyledons were obtained from the developing seeds at varying periods of time after flowering. For germination studies the seedlings were grown in the dark at 25 C in sterile vermiculite irrigated with deionized H2O, and cotyledons were obtained from these seedlings after various periods of seed germination.

Protein Extraction. Protein was extracted from the cotyledons of developing and germinating peas and fractionated as described elsewhere (2). The legumin and vicilin fractions obtained after dialysis were suspended in deionized H2O and lyophilized and stored at -10 C.

Isolation of Neutral and Amino Sugars from Protein. Sixty mg of protein (legumin or vicilin) were hydrolyzed with 1 N HCl for 1 hr in sealed tubes in an autoclave at 124 C. After hydrolysis, the samples were filtered and the filtrates were evaporated in a rotary evaporator in vacuo. The hydrolysate residues were taken up in 2 ml of 0.3 N HCl and applied to a Dowex 50 (200, H+ form) resin columns (8 x 70 mm) prepared as described by Gardell (5). The sugars were eluted with a total volume of 20 ml of 0.3 N HCl in 2 ml fractions.

The neutral sugars were eluted in the initial three fractions, while the amino sugars were contained in the remaining seven fractions. The neutral sugar content in the initial three fractions was determined by the anthrone sulfuric acid method of Yemm and Willis (20). The amino sugar content in the remaining seven fractions was determined by the modified Rondle and Morgan method (16). For this purpose, the 2-ml fractions of the eluate were neutralized to the phenolphthalein end point. One ml of acetyl acetone reagent (2% acetyl acetone in 0.5 N Na2CO3) was added, and the mixture was heated in a boiling water bath for 20 min and cooled to room temperature. One ml of Ehrlich’s reagent (0.8 g of recrystalized p-dimethylaminobenzaldehyde in 30 ml of ethanol and 30 ml of concentrated HCl) was added; the mixture was heated for 10 min at 60 to 65 C. cooled to room temperature, and the absorbance was measured at 530 nm. Glucosamine hydrochloride was used as a standard.

Identification of Carbohydrates. One hundred µl of the concentrated samples of neutral and amino sugar fractions were spotted on Whatman No. 1 chromatogram paper strips of 6.25 cm width, and the sugars were separated by single dimensional descending chromatography using the solvent system 1-propanol-ethyl acetate-water (7:4:2) as recommended by Koshiyama (6). After 18 hr, the paper strips were dried and rechromatographed once again in the same direction with the same solvent. After another 18 hr, the paper strips were dried at 90 C for 5 min and sprayed with alkaline acetyl acetone reagent (0.5 ml of solution B [5 ml of 50% aqueous KOH in 20 ml of ethanol] in 10 ml solution A [1% acetyl acetone in 1-butanol]) and dried at 105 C for 5 min. The papers were again sprayed with Ehrlich’s reagent (1 g of p-dimethylaminobenzaldehyde in 30 ml of ethanol and 30 ml concentrated HCl and diluted with 180 ml of 1-butanol before use) as described by Partridge (9). The RF values were calculated and compared with the standard sugars for identification of the sugars present in the protein hydrolysates.

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Characterization of $^{14}$C-Glucosamine Labeled Protein. Five µl (1 µCi) of $^{14}$C-glucosamine (58 mCi/m mole) were injected into each cotyledon of 20 peas still in the pod attached to the plant and incubated for 6 hr in the light. The cotyledons were collected and homogenized, and the protein was isolated and fractionated as described (2).

$^{14}$C-Glucosamine labeled legumin and vicilin were dissociated with SDS and dithiothreitol according to the procedure of Palminteri et al. (8) and subjected to SDS gel electrophoresis as described by Weber and Osborn (18). After optical scanning, the gels were frozen with dry ice and transversely sliced into 1-mm thick slices by means of a Mickle gel slicer. The slices were placed in scintillation vials, and 1 ml of solution containing 10% piperidine and 1 mm EDTA in distilled H$_2$O was added. The vials and contents were placed in an oven at 60°C. After 18 hr or after complete evaporation of the added solution, 0.5 ml of H$_2$O was added to each vial, and the gel slices were allowed to hydrate. After 1 hr, 12 ml of scintillation fluid (8 g of PPO in 1 liter of Triton-X-100 and 2 liters of scintillation toluene) was added. Subsequently, the radioactivity in the gel slices was determined on a Beckman LS-100 liquid scintillation counter.

Confirmation that radioactivity in the protein was due to glucosamine was obtained by hydrolyzing the labeled legumin and vicilin and subjecting the hydrolysates to paper chromatography. The chromatography strips were cut into 2.5-cm long pieces, and each piece was further cut into smaller segments and placed in scintillation vials. Each piece was eluted with 1 ml of H$_2$O, and the radioactivity in each vial was measured by adding 12 ml of scintillation fluid. The location of radioactivity on the chromatogram was compared with the R$_f$ values of authentic glucosamine.

Influence of Cycloheximide on Incorporation of Amino Sugars and Neutral Sugars into Protein. Pea pods (24 days after flowering) were chilled immediately after harvest. Cotyledons were recovered from the developing seeds and sliced to 1 to 2 mm thickness and kept at 4°C. The cotyledon slices were rinsed thoroughly with 20 mm sodium citrate buffer, pH 5.5. Sixteen cotyledon slices were transferred to 5 ml of incubation medium containing $^{14}$C-glucosamine (2 µCi) or $^{14}$C-mannose (2 µCi) and $^3$H-amino acids (2.5 µCi) with and without cycloheximide (50 µg/ml) in 20 mm citrate buffer, pH 5.5.

The cotyledon slices were incubated in 25-ml flasks in a shaker bath at 30°C for 4 hr. After 4 hr incubation, the slices were rinsed thoroughly with cold deionized H$_2$O and homogenized in m NaCl, 20 mm phosphate buffer, pH 7. The protein was extracted and fractionated (2). Aliquots were taken from the different protein fractions, and the radioactivity was determined.

RESULTS AND DISCUSSION

Analysis of the material resulting from the hydrolysis of legumin and vicilin, prepared from mature dry peas, in HCl for 1 hr at 124°C, demonstrated the presence of amino and neutral sugars. Legumin contained 1% neutral sugars and 0.1% amino sugar, while vicilin contained 0.3% neutral sugars and 0.2% amino sugar. Paper chromatography of the hydrolysates indicated that legumin contained mannose, glucose, and glucosamine, and the vicilin hydrolysates contained mannose and glucosamine. The levels of glycosylation are considerably lower than those usually encountered in glycoproteins of animal origin (17) but are similar to those reported in seed proteins (3, 10).

Characterization of $^{14}$C-Glucosamine-Protein Association. Legumin and vicilin dissociate into several components following treatment with SDS and dithiothreitol suggesting that they are polymeric proteins containing discrete subunits (1, 2, 19). The low level of glycosylation of the reserve proteins might be due to the fact that not all of the components of the reserve proteins are glycosylated. A partial check of this possibility was provided by an analysis of $^{14}$C-glucosamine labeled vicilin and legumin prepared from seeds of different developmental stages. These labeled proteins were dissociated with SDS and dithiothreitol and subjected to SDS polyacrylamide gel electrophoresis. A comparison of the distribution of protein and radioactivity in the gels demonstrates that in legumin, at all stages of development, label was associated with component V (Fig. 1). There was much less radioactivity associated with the other components. In contrast, although most of the components of the dissociated vicilin became labeled, the majority of the radioactivity was associated with the slower migrating components I and II (Fig. 2). It should be noted that not all of the components of vicilin and legumin are synthesized simultaneously. The component composition varies with developmental stages of the cotyledon. However, at all stages of development the same specific subunits of legumin and vicilin, respectively, became labeled. This information provides preliminary support for the concept that the components of the reserve proteins show differing degrees of glycosylation (Figs. 1 and 2).

That the radioactivity associated with legumin and vicilin was due to glucosamine and not products arising from its metabolism was confirmed by hydrolyzing the proteins in 1 N HCl. Such experiments indicated that over 70% of the radioactivity in the labeled protein was associated with glucosamine.

Time of Attachment of Carbohydrate Units to Peptide. It has been established that the reserve proteins in the pea cotyledon are glycoproteins in which the component subunits are not uniformly enriched in carbohydrate. The question is raised, are the carbohydrate units attached during or after peptide synthesis? In order to answer this question, pea cotyledon slices were incubated in a medium containing $[^3]$H]amino acids and either $[^14]$C-glucosamine or $[^14]$C-mannose; with or without cycloheximide which inhibits protein synthesis on 80S ribosomes. Amino acid incorporation into legumin was inhibited 90 to 100% by cycloheximide, whereas glucosamine and mannose incorporation were inhibited 11 to 35% and 36 to 40%, respectively (Table I). Carbohydrate incorporation into the glycoprotein legumin was less sensitive to inhibition by cycloheximide than was amino acid incorporation. Similarly amino acid incorporation into vicilin was inhibited by 90 to 94%, whereas glucosamine and mannose incorporation were inhibited by 66 to 74% and 40 to 58%, respectively.

If carbohydrate attachment occurred simultaneously with peptide bond formation, then inhibition of peptide bond formation by cycloheximide should have prevented the incorporation of the amino sugar and mannose into the glycoprotein. However, this is not the case, and we concluded that glycosylation occurs after peptide bond formation (Table I). This is consistent with the observations of Lew and Shannon (7) who showed that in horseradish peroxidase the carbohydrates are attached after peptide synthesis. The incorporation of glucosamine and mannose into vicilin was inhibited by cycloheximide to a greater extent than was the incorporation of the components into legumin. Because the incorporation of carbohydrates into the glycoprotein depends on the availability of acceptor sites, it is clear that the amount of sugar incorporated will depend, at least in part, on the rate of protein synthesis. We observed that amino acid incorporation into legumin was 10 times higher than that into vicilin, suggesting that legumin synthesis exceeds that of vicilin (Table I). This is the characteristic situation in the pea cotyledon where legumin is the principal globulin. The low rate of synthesis of vicilin will reduce the acceptor sites available for glycosylation; thus inhibition of protein synthesis will effectively lower the glycosylation.

Carbohydrate Content of Proteins during Seed Development. The neutral and amino sugars content of legumin preparations extracted from pea cotyledons at various stages of seed develop-
Fig. 1. Distribution of radioactivity and protein components following SDS polyacrylamide gel electrophoresis of 14C-glucosamine labeled legumin. The labeled legumin was prepared from developing pea cotyledons 18, 21, and 24 days after flowering and was dissociated with SDS and dithiothreitol.

Table I. Influence of Cycloheximide on Incorporation of 3H-Amino Acids and 14C-Glucosamine or 14C-Mannose into Protein

<table>
<thead>
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<th>experiment</th>
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<th>3H</th>
<th>14C</th>
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<td>1743</td>
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<tr>
<td>cycloheximide (50 µg/ml)</td>
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<td>0 (100)</td>
<td>2996 (11)</td>
<td>84 (95)</td>
</tr>
<tr>
<td>control</td>
<td>1358</td>
<td>2065</td>
<td>1036</td>
<td>923</td>
</tr>
<tr>
<td>cycloheximide (50 µg/ml)</td>
<td>868 (36)</td>
<td>161 (92)</td>
<td>616 (40)</td>
<td>83 (91)</td>
</tr>
</tbody>
</table>

Table II. Changes in Per Cent Sugar Content of Protein during Pea Seed Development

Cotyledons were collected from peas of different development age (15-33 days after flowering). Legumin and vicilin were extracted, and their neutral and amino sugar content were determined. I and II refer to values from duplicated experiments.
ment remained relatively constant (Table II). In vicilin, although the amino sugar content of the protein remained almost constant, there was a decline in the neutral sugar content of this protein during seed development.

In glycoproteins containing glucosamine, it is usually found that the amino sugar functions in forming the glycopeptide bond (between asparagine and N-acetylglucosamine) and it can also occur internally in the oligosaccharide component of the glycopeptide (17). Ericson and Chrispeels (4) have provisional information that the glycopeptide bond in vicilin from Phaseolus aureus contains glucosamine and asparagine. Thus, although the oligosaccharides have yet to be fully characterized, it appears that the glycopeptide linkage involves N-acetylglucosamine. If this is the case, then the higher ratio of neutral sugar to amino sugar content (Table II) indicates that the oligosaccharide component of legumin is of larger size than that of vicilin.

In contrast, vicilin, as demonstrated by the higher amino sugar content, appears to have more glycopeptide linkages than legumin. These glucosamine residues apparently initially have long oligosaccharides associated with them; during seed development, there appears to be a progressive decrease in the size of the oligosaccharides. The observation that the amino sugar content of vicilin is sustained while the neutral sugar content declines suggests that most of the glucosamine is associated with, or close to, the glycopeptide bond and is not in the oligosaccharide chain which becomes shortened during seed development.

Changes in Glycosylation during Seed Germination. Although there was some variability, the overall trend was for an increase in the neutral and amino sugars content in the legumin and vicilin preparations extracted from seeds on successive days of germination (Table III). With regard to legumin, we have previously shown (2) that not all of the components produced by SDS and dithiothreitol dissociation are degraded simultaneously. Component V, which is the one shown in this study to incorporate 14C-glucosamine preferentially, appears to persist

### Table III. Changes in Per Cent Sugar Content of Protein during Pea Seed Germination

<table>
<thead>
<tr>
<th>Time</th>
<th>Legumin</th>
<th>Vicilin</th>
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<tr>
<td></td>
<td>Neutral sugars</td>
<td>Amino sugars</td>
</tr>
<tr>
<td>days</td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>mg/100 mg protein</td>
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</tr>
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</tr>
<tr>
<td>12</td>
<td>2.027</td>
<td>2.633</td>
</tr>
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</table>

Fig. 2. Distribution of radioactivity and protein components following SDS polyacrylamide gel electrophoresis of 14C-glucosamine labeled vicilin. The vicilin was prepared from pea cotyledons 18, 21, and 24 days after flowering and was dissociated with SDS and dithiothreitol.
during germination. Thus in the case of legumin, it appears that the glycopeptide containing subunit may be more resistant to hydrolysis than the other components. This explanation does not hold for vicilin where we (2) have shown that components 1 and II (i.e., those which were most extensively labeled with glucosamine [Fig. 2]), are depleted more rapidly during germination than components IV and V. By following the change in neutral and amino sugar composition of the reserve proteins during germination, we hoped to obtain information concerning the mechanisms of degradation of the glycosyl moiety of the glycoprotein. Two methods of degradation are possible: (a) the carbohydrate residues could be released progressively from the free end of the oligosaccharide chain, and (b) the oligosaccharide side chain could be released from the peptide by a cleavage of the glycosyl peptide bond (presumably N-acetylglucosamine-asparagine (17)). If the former mechanism operated, there would be a progressive decline in the neutral sugar content of the glycoprotein. Since such a decrease was not observed, we speculated that the degradation of the glycosyl moiety involves an initial release of the oligosaccharide from the peptide. The observation that there is a progressive increase in the neutral and amino sugar content of the proteins suggests that the cleavage of the glycosyl peptide bonds is preceded by a degradation of peptide linkages. An alternative explanation for the increased neutral and amino sugar content is that the surviving proteins are glycosylated at later stages of germination. In view of the demonstrated (3) overall decline in glucosamine content, this is an unlikely possibility.

CONCLUSIONS

The reserve proteins, legumin, and vicilin of peas (Pisum sativum) appear similar to those from Phaseolus (11–14) in being glycoproteins containing mannose and glucosamine; additionally legumin appears to contain glucose.

The extent of glycosylation of the proteins from the pea cotyledon, although comparable to that observed in other legume seeds (3, 10), is much lower than that normally encountered in glycoproteins of animal origin (17). It is suggested that this low glycosylation may be attributable to the fact that all of the components of the polymeric proteins may not be glycosylated.

The constant ratio of amino sugars to neutral sugars of legumin during seed development suggests that in this globulin the glycosyl units are added as oligosaccharide components which do not undergo modification. In contrast, in vicilin there appears to be a progressive shortening of the oligosaccharide chain during seed development.

It appears that during seed germination the glycosyl units are released from the protein as complete oligosaccharides. However, the release of glycosyl residues is not so rapid as the hydrolysis of the peptide components. Consequently, during germination there is a progressive enrichment of the remaining globulins in neutral and amino sugars.

LITERATURE CITED