CHARACTERIZATION OF THE GLYCOPEPTIDE BOND IN LEGUMIN FROM *PISUM SATIVUM* L

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1. Introduction

Legumin and vicilin, the reserve storage globulins in pea cotyledons, are glycoproteins containing neutral and amino sugars. The amino sugar has been characterized as glucosamine and the neutral sugars are principally mannose [1]. In native glycoproteins, glucosamine usually occurs as an acetylated derivative (*N*-acetyl glucosamine) and is frequently involved in glycopeptide linkages [2]. Acetylated amino sugars are involved in glycopeptide linkages with the hydroxy amino acids serine or threonine or alternatively, with peptidyl asparagine [3]. The glycopeptide linkage in plant glucosamine-containing glycoproteins is considered in [4–7].

To extend our studies concerning the biosynthesis of glucosamine-containing glycoproteins [1,8–10], it is necessary to determine the nature of the glycopeptide linkage. Exogenously-supplied glucosamine is incorporated into legumin and vicillin in developing pea cotyledons [1]. We now report that some of this exogenously-supplied glucosamine may be isolated from legumin as a GlcNAc–Asn glycopeptide linkage.

2. Materials and methods

2.1. *Sources of materials*

D-[U-^{14}C]glucosamine (254 μCi/mmol, 50 μCi/238 μl) was purchased from Amersham Searle Corp., Arlington Heights, IL and 1-*N*-B-L-aspartyl–2-deoxy-B-D-glucopyranosylamine (GlcNAc–Asn) was purchased from Bachem Chem., Marina Del Ray, CA. Protease type VI was purchased from Sigma, St Louis, MI and proteinase K was purchased from Beckman Inst., Co., Palo Alto, CA.

2.2. *Preparation of [^{14}C]glucosamine-labeled legumin*

70 cotyledons were collected from developing peas (18–21 days post anthesis) and injected with 1.5 μl [^{14}C]glucosamine/cotyledon. Following a 4 h incubation, legumin was isolated from the cotyledons as in [10]. Recovered legumin (71.9 mg protein) was suspended in 20 ml 0.2 M NaCl 100 mM sodium phosphate buffer pH 7.0 and incubated with 9.0 mg protease type VI at 37°C for 48 h. The sample was overlaid with a small amount of toluene to prevent bacterial growth. An additional 9 mg protease was added after 48 h and the incubation continued for an additional 48 h. After each protease addition, the medium was readjusted to pH 7.0. Protease digestion was terminated by incubation in boiling water for 5 min and the undigested residue was centrifuged out. The supernatant from this initial digestion was evaporated to dryness in vacuo, suspended in 3.0 ml 0.1 M pyridine acetate, pH 5.0 and applied to a Sephadex G-15 column (1.5 X 105 cm). Fractions, 4.0 ml, were collected and monitored for radioactivity, carbohydrate and amino nitrogen. The fractions containing ^{14}C radioactivity were pooled, evaporated to dryness in vacuo, and suspended in 50 mM Tris–HCl buffer, pH 7.5 and redigested as above. Redigestion, followed each time by fractionation of products on G-15, was repeated 5 times. Digestion 2 was completed in 10 ml buffer, digestions 3 and 4 in 5.0 ml buffer and digestion 5 in 3.0 ml buffer at 47°C for 72 h with 6.0 mg proteinase K, of which 3 mg was added after 24 h incubation.

2.3. *Alkali digestion*

^{14}C-Labeled glycopeptides, isolated after 2 proteolytic digestions, were dissolved in either 1 ml 0.1 N NaOH or H_2O and incubated at 37°C for 72 h. Additional samples were dissolved in 1 ml 1.0 N NaOH or H_2O and incubated for 6 h at 100°C. After incubation, the samples were adjusted to pH 7.0 with 2.0 N HCl and the products analysed by paper chromatography in solvent system A.

2.4. *Acid hydrolysis*

[^{14}C]Glycopeptides, isolated after 5 proteolytic digestions, were dissolved in 2.0 ml 2.0 N HCl and placed in boiling water for 20 min. Following incubation, the mixture was then diluted to 8.0 ml with H_2O and passed through a column (1 × 25 cm) of Dowex X-8 (200–400 mesh acetate resin). The glycopeptides were washed from the column with H_2O. The effluent was evaporated in vacuo and the resulting residue was dissolved in H_2O and analyzed by paper chromatography in solvent systems A and B.

2.5. *Chromatographic procedures*

Samples were applied to Whatman 3 MM paper and chromatographed in solvent mixtures containing: (A) 1-butanol/pyridine/H_2O (40/30/40): (B) 1-butanol/acetic acid/H_2O (12/3/5).

Radioactive areas of chromatograms were determined by cutting the chromatograms into 1 × 4.5 cm strips and counting each of these strips in 4 ml scintillation cocktail (8.0 gm PPO, 1 liter Triton X-100, 2 liters toluene) in a Beckman LS-100 scintillation counter. GlcN and GlcNAc–Asn were detected by spraying chromatograms with a solution of 0.2% (w/v) ninhydrin in acetone, followed by heating at 100°C for 5 min. GlcNAc was detected using Ehrlichs reagent as in [1].

2.6. *Analytical methods*

Carbohydrate was determined by the anthrone method [11] using D-mannose as a standard. Amino nitrogen was determined by the ninhydrin method [12] using L-leucine as a standard.

3. Result and discussion

The products of protease digestion of labeled legumin were fractionated by exclusion chromatography on Sephadex G-15. The distribution of radioactivity, carbohydrate and amino nitrogen are shown in fig.1. Over 95% of the radioactivity applied to the column was recovered in the pooled glycopeptide fractions. Since anthrone does not react with glucosamine or *N*-acetylglucosamine, the association of [^{14}C]glucosamine with anthrone-positive material indicates that the label was incorporated into carbohydrate-containing components. This agrees with the report that exogenously-applied [^{14}C]-glucosamine is incorporated into reserve glycoprotein [1]. The radioactivity associated with glycopeptides was distinctly separated from α-amino nitrogen fractions, indicating that the [^{14}C]glucosamine did not undergo conversion to amino acids during the incubation period.

Paper chromatography of untreated ^{14}C-labeled glycopeptides indicated that radioactivity was associated with components showing little mobility in solvent system A. Milk alkali treatment had no major influence on the chromatographic mobility of the radioactivity. In contrast, strong alkali treatment of labeled glycopeptides released radioactivity which migrated to the center of the chromatogram. The heterodispersion of radioactivity in the chromatogram implied that oligosaccharide components of varying degrees of complexity have been released from the glycopeptides [13].

Resistance of the [^{14}C]glycopeptides to mild alkali hydrolysis tends to prelude the involvement of serine or threonine in the glycopeptide linkage, while the susceptibility of the glycopeptide to strong alkali is consistent with the occurrence of a GlcNAc–Asn linkage [2]. However, the demonstration of a specific type of linkage cannot be based solely on B-elimination studies, but relies on the isolation of the putative linkage compound from the glycopeptide in question [14].

When the components produced by mild acid hydrolysis of the ^{14}C-labeled legumin glycopeptides were neutralized and subjected to paper chromatography in solvent System A (fig.2), the majority of radioactivity was localized in a peak which co-chromatographed with authentic GlcNAc–Asn. A second radioactive component co-chromatographed with glucosamine. A similar chromatographic profile was observed with acid hydrolysates of labeled legumin glycopeptides in solvent system B.

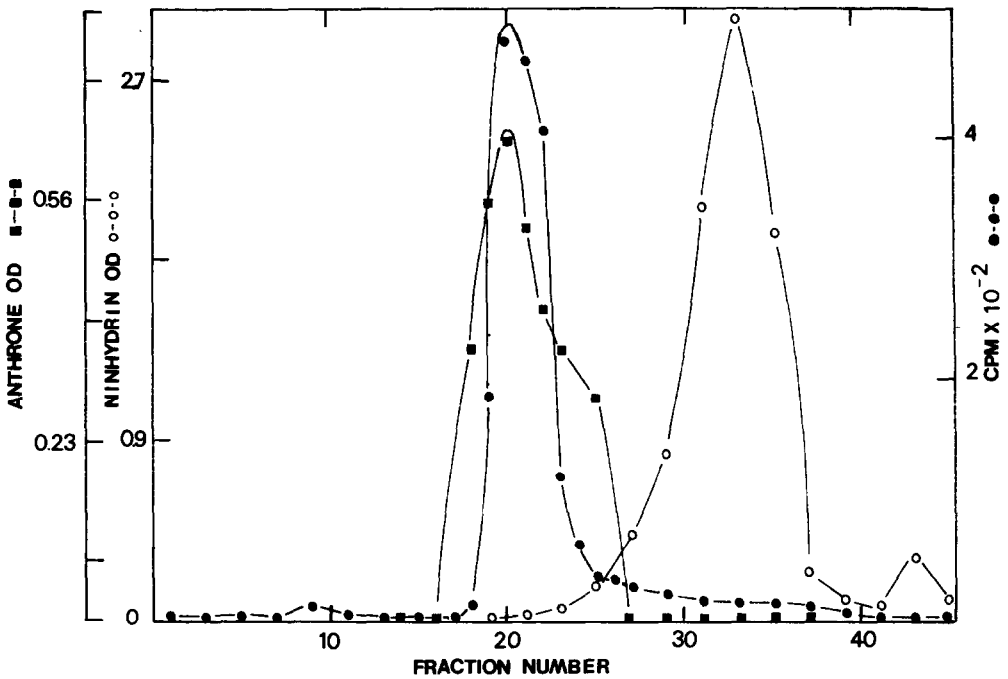

Fig.1. Analysis of legumin [^{14}C]glycopeptides on Sephadex G-15. Legumin glycopeptides were digested 5 times and applied to a Sephadex G-15 column (1.5 × 105 cm), equilibrated and eluted with 0.1 M pyridine acetate, pH 5.0. Fractions, 4.0 ml, were collected and analyzed for radioactivity (• – • – •), carbohydrate (– ■ – ■ –) and amino nitrogen (o – o – o).

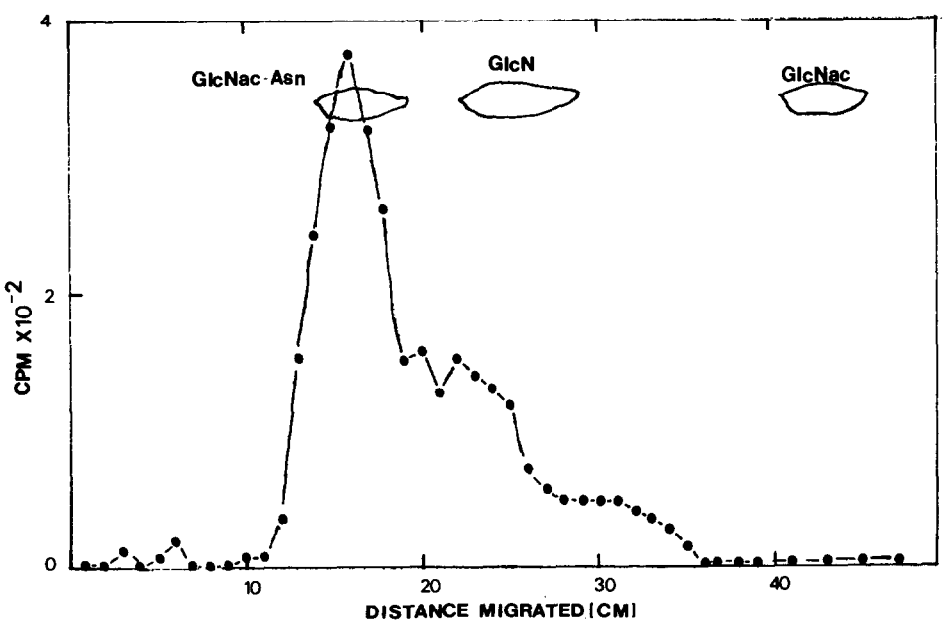

Fig.2. Paper chromatography of the products of mild acid hydrolysis of legumin [^{14}C]glycopeptides. Legumin glycopeptides were subjected to acid hydrolysis, as in section 2 and the products chromatographed on Whatman 3 MM paper in solvent system A for 24 h. Radioactivity (•–•–•) was determined and the distribution compared to the standard compounds, 1-*N*-B-L-aspartyl–2-deoxy-B-D-glycopyranosylamine (GlcNAc–Asn), glucosamine (GlcN), and *N*-acetyl glucosamine (GlcNAc).

4. Conclusion

[^{14}C]Glucosamine is incorporated into glycoprotein in the developing pea cotyledon, and a portion of this glucosamine is incorporated into a GlcNAc–Asn glycopeptide linkage. The existence of this linkage compound was demonstrated by the results of B-elimination studies and isolation of the ^{14}C-labeled GlcNAc–Asn linkage compound from mild acid hydrolysates of legumin glycopeptides.

Acknowledgements

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