Facile Cleavage of Complex Oligosaccharides from Glycopeptides by Almond Emulsin Peptide: N-Glycosidase*

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Almond emulsin peptide: N-glycosidase has been partially purified by using a new 3H-labeled 5-dimethylaminonaphthalene-1-sulfonoyl-octaglycopeptide substrate derived from ovalbumin. The enzyme hydrolyzes the β-asparaglycosylamylase linkage of both high mannose and biantennary complex glycopeptides, as shown by the isolation of the corresponding carbohydrate-free peptides containing aspartic acid and intact oligosaccharides with the core di-N-acetylgalactosamine moiety at the reducing end. Complex glycopeptides appear to be the preferred substrates. The location of the oligosaccharide on the peptide backbone and its chain length are major determinants for enzymatic activity. Glycosylated asparagine residues are hydrolyzed less favorably if present at the carboxyl- or NH2-terminal position of a peptide chain. Glycopeptides containing long, bulky oligosaccharide chains are cleaved by peptide:N-glycosidase at least 15-fold faster than their corresponding endo-β-N-acetylglucosaminidase H-modified, peptide-GlcNAc counterparts.

Endo-β-N-acetylglucosaminidase H was the first well defined endoglycosidase capable of releasing intact high mannose oligosaccharides from glycoproteins and glycopeptides (1, 2). It has been a valuable tool for investigating oligosaccharide structure (3–6), glycoprotein biosynthesis (7–10), and structure-function relationships (11,12) of N-glycosylated proteins. However, glycopeptides and glycoproteins which contain complex oligosaccharide chains are resistant to hydrolysis by Endo-H1 (2), and there has been a need for another class of carbohydrate-cleaving enzymes with a broader substrate specificity.

The possibility that such an enzyme exists was suggested by the studies of Takahashi and Nishibe (13, 14), who demonstrated in almond emulsin an N-glycosidase which could release intact oligosaccharides from defined bromelain and ovalbumin glycopeptides by hydrolysis at the β-asparaglycosylamylase junction. Asialoglycopeptides of the complex type and asialotransferrin were reported to be susceptible to the N-glycosidase, but neither specificity studies nor isolation and characterization of reaction products were performed.

To more fully characterize the almond emulsin peptide:N-glycosidase, we have partially purified the enzyme and examined its specificity with glycopeptides prepared from ovalbumin and IgM. Our data demonstrates that both high mannose and complex glycopeptides are hydrolyzed at the β-asparaglycosylamylase linkage by peptide:N-glycosidase and that complex glycopeptides appear to be the preferred substrates for this enzyme. Studies performed with complex glycopeptides and with Endo-H-modified ovalbumin glycopeptides demonstrate that both the oligosaccharide chain length and the position of the carbohydrate in the peptide are important structural determinants.

MATERIALS AND METHODS

Enzyme Preparation and Assay—Peptide:N-glycosidase was partially purified (300-fold) from 10% of crude almond emulsin using DE52, CM52, and Sephareryl S-200 column chromatography.2 Assays were conducted at pH 5.1 with [3H]dansyl-ovalbumin octaglycopeptide (330 nmol/mL; specific activity, 48 × 106 dpm/nmol) by the general procedure developed for the determination of Endo-H (1, 15); the procedure was modified by eluting the reaction product with 1.5 mL of 0.4 M sodium dodecyl sulfate at 70 °C for 30 min. The enzyme was obtained in a 3% yield with a specific activity of 438 nmol of [3H]dansyl-octapeptide formed/min/mg of protein.

The peptide:N-glycosidase preparation was devoid of α-mannosidase but contained traces of β-galactosidase and β-N-acetylgalactosaminidase activity. These contaminating glycosidases did not significantly affect the experimental results; only very minor amounts of galactose and N-acetylgalactosamine were liberated during incubation of peptide:N-glycosidase with IgM glycopeptides, and the compositional analysis of the liberated oligosaccharide was essentially the same as the parent glycopeptide (Table I). Protease activity was not detected during prolonged incubation (24 h) of purified peptide:N-glycosidase with methyl-14C-glycinated hemoglobin (16) or α-casein (17). Furthermore, the recovery of carbohydrate-free peptides from large scale enzyme digests was satisfactory, and no peptide fragments were detected.

Takahashi and Nishibe (14) have reported that their preparation consists of three related enzymatic forms, which were purified 30-, 136-, and 99-fold. The forms differed in the length of peptide chain preferred for optimal cleavage. We have not accumulated any data that would directly prove or disprove their findings. Our methods appear to produce a more homogeneous preparation, but further substrates are being developed to test their hypothesis.

Preparation of Glycopeptides—IgM (Ga) was isolated from the plasma of a patient with Waldenstrom's macroglobulinemia, as reported earlier (18). The Fab, fragment containing the Asn-171 complex oligosaccharide was obtained by hot tryptic digestion of IgM (5 g), according to Florent et al. (19) and purified on Bio-Gel A-0.5m (Bio-Rad). Reduced S-carboxymethylated Fab, was digested for 6 h at 37 °C with 1.5% trypsin (w/w) at pH 8.6 in 1 m urea, 0.1 M NaCl. Soluble glycopeptides were purified on a column (38 × 238 cm) of Sephadex G-30 equilibrated in 0.1 M NH4HCO3, 1% 1-butanol, followed by a column (1.5 × 110 cm) of Dowex 50-X2 (16–48 ρ). Equilibration buffer (pH 2.9) was followed by a gradient to 0.4

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1 The abbreviations used are: Endo-H, endo-β-N-acetylgalactosaminidase H; PNGase, peptide:N-glycosidase; CHO, intact oligosaccharide moiety; ConA, concanavalin A; CHO(+), carbohydrate-free; CHO(+)Asn, Asn with intact oligosaccharide; NR, nonretarded; R, retarded; dansyl, 5-dimethylaminonaphthalene-1-sulfonoyl.
TABLE 1
Amino acid and carbohydrate composition of glycopeptides

<table>
<thead>
<tr>
<th>Amino Acid or Carbohydrate</th>
<th>Ovalbumin ConA-R octaglycopeptide</th>
<th>IgM undecaglycopeptide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No treatment</td>
<td>After Endo-H</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Asp</td>
<td>1.00</td>
<td>1.00</td>
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<tr>
<td>Asn</td>
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<td></td>
</tr>
<tr>
<td>Thr</td>
<td>0.96</td>
<td>0.92</td>
</tr>
<tr>
<td>Ser</td>
<td>0.88</td>
<td>0.92</td>
</tr>
<tr>
<td>Hse</td>
<td>0.34</td>
<td>0.42</td>
</tr>
<tr>
<td>Val</td>
<td>1.16</td>
<td>1.09</td>
</tr>
<tr>
<td>Ile</td>
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<td></td>
</tr>
<tr>
<td>Leu</td>
<td>1.88</td>
<td>1.97</td>
</tr>
<tr>
<td>Tyr</td>
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<td>0.96</td>
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<tr>
<td>Lys</td>
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<tr>
<td>Gln</td>
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<tr>
<td>GlcN</td>
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<tr>
<td>GlcN10GlcN'</td>
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<tr>
<td>Man</td>
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<td>Fuc</td>
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<tr>
<td>NeuAc</td>
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</table>

- Hydrolysis in 6 M HCl for 20 or 24 h; values are uncorrected for destruction.
- Hydrolysis by aminopeptidase M.
- Carbohydrate oligosaccharide released by peptide:N-glycosidase.
- Molar ratios are based on the following: ovalbumin glycopeptide, Asp: 1.00; IgM glycopeptide, Asp:Fuc = 3.00:1.00.
- Ratio after borohydride reduction.
- Phenol-sulfuric acid method (29).

RESULTS AND DISCUSSION

Ovalbumin ConA-R octaglycopeptide was used to confirm and extend previous studies with peptide:N-glycosidase (13, 14). The products of hydrolysis by peptide:N-glycosidase were separated on a column of Sephadex G-25 (not shown) and analyzed (Table I). Enzymatic cleavage at the glycopeptide linkage was confirmed by the identification of a CHO(-)-aspartic acid-containing octapeptide and of an intact oligosaccharide with GlcNAc on the reducing end. The di-N-acetylchitobiosyl core was conserved, since subsequent isolation of the (Man)₆₆(GlcNAc) fraction on Bio-Gel P-4, yielded 1 mol of free GlcNAc after Endo-H treatment.

Oligosaccharide release from glycopeptides was quantitated by the phenol-sulfuric acid method (25). The glycopeptides were purified by the aforementioned conditions for trypsin-released fragments. Hydrolysis by aminopeptidase M.

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strongly influenced by the position of the CHO(+)-Asn moiety. Fractions 0, relative fluorescence of original ovalbumin octapeptide-GlcNAc; fractions. 0, relative fluorescence of 30-pL aliquots; absorbance at 544 nm. The bar marks fractions combined for further study. Inset, release of free N-acetylglucosamine with time as compared to the theoretical maximum (Morgan-Elson assay).

The products of the reaction between peptide:N-glycosidase and the complex undecaglycopeptide of IgM at Asn-171 (for sequence see Table II) were separated on Sephadex G-25 (Fig. 3) and characterized (Table I). Two peaks were observed: a phenolsulfuric acid-positive, fluorescamine-negative peak, representing the intact oligosaccharide, and a retarded fluorescamine-positive peak of CHO(-)-undecapeptide. The composition of the peaks indicates that deglycosylation of the undecaglycopeptide was complete. Aminopeptidase cleavage of the CHO(-)-undecapeptide demonstrated the expected molar ratios of amino acids in a 75% yield. The released oligosaccharide moiety was intact, and GlcNAc was present on the reducing end in the anticipated ratio of 1:3, based on its known structure (18, 33).

Peptide:N-glycosidase is an enzyme with great potential for structural analysis of glycopeptides. Its specificity apparently will accommodate carbohydrate structures as diverse as intact high mannose and biantennary complex oligosaccharide chains, as well as a single GlcNAc resulting from prior Endo-H cleavage. The presence of fucose on the N-acetylglicosamine residue proximal to asparagine in IgM glycopeptides (18, 33) was not a hindrance, in view of the rapid cleavage illustrated in Fig. 2. Release of intact oligosaccharide chains from glycopeptides would allow simple Edman degradation of the peptide portion, as well as sequence studies on the carbohydrate moiety. These preliminary studies indicate that any

**Table II**

<table>
<thead>
<tr>
<th>IgM position*</th>
<th>Amino acid sequence*</th>
<th>Cleavage*</th>
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<tbody>
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<td>Asn-171</td>
<td>Tyr-Lys-Asn-Ser-Asp-Ile-Ser-Thr-Arg</td>
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<tr>
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<td>CHO</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tyr-Lys-Asn-Ser-Asp</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>His-Thr-Asn</td>
<td>9.3</td>
</tr>
</tbody>
</table>

* Data of Putnam et al. (32) and F. W. Putnam, personal communication, 1981.

* Reaction conditions: 43.5 nmol of glycopeptide and 21.75 units of enzyme/N-glycosidase in 75 μl of 0.01 m sodium acetate (pH 5.1), 0.1 m NaCl at 37 °C for 1 h. Reaction was terminated with 0.825 ml of 0.2 m sodium citrate, pH 2.2. Unhydrolyzed glycopeptides were quantitated on the amino acid analyzer.
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REFERENCES