Revision of the Structure for an Endo-β-N-acetylglucosaminidase H Substrate Using a Novel Modification of the Smith Degradation*

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\[(\text{Man})_n(\text{GlcNAc})_2\text{Asn}\] was shown in a previous study (Trimble, R. B., Tarentino, A. L., Plummer, T. H., Jr., and Maley, F. (1978) J. Biol. Chem. 253, 4508-4511) to be hydrolyzed by α-mannosidase to \(6(\text{Man})_n(\text{Man})_1 \rightarrow 6(\text{Man})_n(\text{Man})_1 \rightarrow 6(\text{Man})_n(\text{Man})_1 \rightarrow 4\text{GlcNAcββ} \rightarrow 4\text{GlcNAc-Asn}\). The latter is the most effective substrate for endo-\(\beta\)-N-acetylglucosaminidase H tested to date. By employing a new and highly sensitive modification of the Smith degradation, it is shown that this compound is in reality \(6(\text{Man})_n(\text{Man})_1 \rightarrow 3(\text{Man})_1 \rightarrow 4\text{GlcNAcββ} \rightarrow 4\text{GlcNAc-Asn}\). The method entails the conversion of a glycosyl asparagine derivative to its corresponding dimethylaminonaphthalene sulfonyl analogue, which, after periodate oxidation is treated directly with Dowex 50\(^+\) to eliminate the modified carbohydrate residues. The dansylated products, which are eluted from the resin with ammonium hydroxide, can be identified rapidly by thin layer chromatography.

Our previous study (1) on the substrate specificity of endo-\(\beta\)-N-acetylglucosaminidase H (2) extended the range of activity of this enzyme to asparaginyl glycopeptides containing only a single mannosyl residue. Although its rate of hydrolysis was \(10^5\) to \(10^6\)-fold slower than that of \((\text{Man})_n(\text{GlcNAc})_2\text{Asn}\), \(\text{Man(\text{GlcNAc})_2Asn}\) was eventually hydrolyzed to completion. One of the compounds prepared for this study by a limited α-mannosidase digestion of \((\text{Man})_n(\text{GlcNAc})_2\text{Asn}\) was \((\text{Man})_n(\text{GlcNAc})_2\text{Asn}\), with a structure determined from compositional and methylation analyses to be that of A in Fig. 1. The proposed structure was based in part on that described for a similar compound isolated from an ovalbumin digest (3) but with the peripheral mannosyl linked \(1 \rightarrow 3\).

However, it is evident from the structures of A and B in Fig. 1 that a methylation analysis would not distinguish between these compounds, and while our originally proposed structure of this compound was A, it could also be B. A means to resolve this problem presented itself in the form of the Smith degradation (4), which should yield \(\text{Man(\text{GlcNAc})_2Asn}\) from A but \(\text{GlcNAc-Asn}\) from B. In the course of this study, which proved eventually that B and not A is the correct structure, a novel and interesting modification of the Smith degradation was uncovered, one that should be valuable for the structural analysis of glycosyl asparagine derivatives and possibly glycopeptides.

**EXPERIMENTAL PROCEDURES**

The various asparagine-containing oligosaccharides used in these experiments were prepared as described earlier (1) and dansylated with [\(\text{Me}^-\text{3H}\)]dimethylaminonaphthalene-1-sulfonil chloride (New England Nuclear) which had been diluted with unlabeled dansyl chloride. The specific radioactivity of the dansyl-Asn oligosaccharides was from 13 to 60 \(\times 10^6\) cpm/\(\mu\)mol.

The modified Smith degradation was conducted as follows: 10 to 50 nmol of a \(\text{H}\)-labeled dansylated glycosyl asparagine derivative (larger than \(\text{GlcNAcAsn}\)) were incubated overnight at \(4^\circ\)C with 0.2 ml of a solution containing 50 mm sodium acetate, pH 4.5, and 30 mm sodium periodate. An additional 0.1 ml of the latter was then added, and the incubation was continued for another 24 h at \(4^\circ\)C. The solution was brought to 2 ml with water and passed through a column of Dowex 50\(^+\)-X12 (100-200 mesh) (5). The column was washed with water until the eluate was neutral and then eluted after 18 to 24 h at room temperature with 5-ml aliquots of 5\% NH\(_2\)OH. Most of the radioactivity (40 to 60\% of that added to the column) was eluted in the first fraction, which was concentrated in vacuo to dryness in a 50-ml pear-shaped flask. The residue was taken up in 0.1 ml of water and 10 \(\mu\)l were applied to a thin layer cellulose (0.1-mm) glass plate (20 cm \(\times\) 20 cm), which was developed in 1-butanol/ethanol/H\(_2\)O (2:1:1). Although the products in most instances were clearly identified on exposure of the chromatogram to UV light, even greater sensitivity could be achieved by fluorography after the region with the tritiated compounds had been irrigated with a solution containing 2-methylthiophenol, tolune, and diphenyl-o-xazole (5).

\text{GlcNAcAsn-dansyl} and \(\text{GlcNAcAsn-dansyl}\) were treated with periodate as above, and the eluted products were chromatographed on plastic-backed polyethyleneimine paper (Polygram cel 300 PEI, Brinkmann) as described earlier (6).

**RESULTS AND DISCUSSION**

As indicated previously (1), a limited α-mannosidase digestion of \((\text{Man})_n(\text{GlcNAc})_2\text{Asn}\) yielded \((\text{Man})_n(\text{GlcNAc})_2\text{Asn}\) and \((\text{Man})_n(\text{GlcNAc})_2\text{Asn}\). The structure of the tetramannosyl product was believed to be that of A in Fig. 1, based on a similar derivative isolated from a pronase digest of ovalbumin (3). However, the same methylated alditol acetates used to derive the structure in A would also be obtained from structure B. A possible means of distinguishing between A and B was suggested by the Smith degradation (4), wherein A should be converted to \(\text{Man(\text{GlcNAc})_2Asn}\) and B to \(\text{(GlcNAc)_2Asn}\).

The usual methodology employed in the Smith degradation is that of periodate oxidation, followed by sodium borohydride reduction and acid hydrolysis to remove the modified sugars. However, in the course of studies with \(\text{GlcNAcAsn-dansyl}\), it was found that on attempting to isolate the periodate oxidation product of this compound on Dowex 50\(^+\) by elution with NH\(_2\)OH, both asparagine and aspartic acid were present in the dansylated elution products (Fig. 2). This result contrasted with that obtained by purifying oxidized \(\text{GlcNAcAsn-dansyl}\) on Dowex 1 formate, which yielded the didehydro only, and suggested that Dowex 50\(^+\) was catalyzing the breakdown of the didehydro to the products observed in Fig. 2. The fact that dansyl-asparagine was present was surprising, since normal...
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resulting from a modified Smith degradation when applied layer polyethyleneimine chromatography of the dansylated products NAcAsn origin. The compounds were visualized by UV light.

oligosaccharides with Dowex 50-H\textsuperscript{+} suggested that this pro-

ative. Thus treatment of the dansylated oxidized asparagine in identifying the reaction products.

than those employed in Fig. 2, such as dansylated (Man\textsubscript{3})(GlcNAc)\textsubscript{2}Asn, (Man\textsubscript{4})(GlcNAc)\textsubscript{2}Asn, (Man\textsubscript{5})(Glc-

NAc)\textsubscript{2}Asn, (Man\textsubscript{6})(GlcNAc)\textsubscript{2}Asn, and (Man\textsubscript{7})(GlcNAc)\textsubscript{2}Asn. After these compounds were subjected to the periodate oxidation step of the Smith degradation, they were applied to

dow only aspartic acid would have been obtained by applying the Smith degradation to GlcNAcAsn or its dansylated derivative. Thus treatment of the dansylated oxidized asparagine oligosaccharides with Dowex 50-H\textsuperscript{+} suggested that this procedure could replace the reduction and acid hydrolysis steps required in the traditional Smith degradation, the net result being not only higher yields but greatly enhanced sensitivity in identifying the reaction products.

To confirm that the products resulting from the Dowex 50-H\textsuperscript{+} procedure were not an anomaly associated with the breakdown of GlcNAcAsn, the reaction was applied to (GlcNAc)\textsubscript{2}Asn, and as shown in Fig. 2, only GlcNAcAsn was obtained. This finding suggested that Dowex 50-H\textsuperscript{+} could successfully catalyze the acid hydrolysis of more complex carbohydrates than GlcNAcAsn. The reaction was therefore applied to even larger and more complex oligosaccharides than those employed in Fig. 2, such as dansylated (Man\textsubscript{3})(GlcNAc)\textsubscript{2}Asn, (Man\textsubscript{4})(GlcNAc)\textsubscript{2}Asn, (Man\textsubscript{5})(Glc-

NAc)\textsubscript{2}Asn, (Man\textsubscript{6})(GlcNAc)\textsubscript{2}Asn, and (Man\textsubscript{7})(GlcNAc)\textsubscript{2}Asn. After these compounds were subjected to the periodate oxidation step of the Smith degradation, they were applied to

Dowex 50-H\textsuperscript{+} columns. For the larger oligosaccharides it was necessary to use Dowex 50-H\textsuperscript{+}-X2 (100–200 mesh) to ensure maximal retention of the dansylated compounds to the resin.

On elution and thin layer chromatography of the oxidation products from (Man\textsubscript{3})(GlcNAc)\textsubscript{2}Asn-dansyl and (Man\textsubscript{4})(Glc-

NAc)\textsubscript{2}Asn-dansyl, it was found that these compounds had been converted in high yields (at least 50\%) to (Glc-

NAc)\textsubscript{2}Asn-dansyl (Fig. 3). The conversion of linear (Man\textsubscript{3})-

(GlcNAc)\textsubscript{2}Asn (lane 1) to mostly (GlcNAc)\textsubscript{2}Asn was anticipated from its structure (1); but the fact that (Man\textsubscript{4})-

(GlcNAc)\textsubscript{2}Asn (lane 2) yielded (GlcNAc)\textsubscript{2}Asn indicates that this compound, a product of the limited α-mannosidase di-

gestion of (Man\textsubscript{5})(GlcNAc)\textsubscript{2}Asn, is compound B and not A as reported previously (1). Of interest is the fact that (Man\textsubscript{3})(GlcNAc)\textsubscript{2}Asn (lane 3) and (Man\textsubscript{4})(GlcNAc)\textsubscript{2}Asn (lane 4) were converted primarily to (Man\textsubscript{5})(GlcNAc)\textsubscript{2}Asn, which in effect confirms the structures of the parent compounds (7).

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