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CHEMICAL DEGLYCOSYLATION OF CARCINOEMBRYONIC ANTIGEN FOR AMINO ACID SEQUENCE STUDIES

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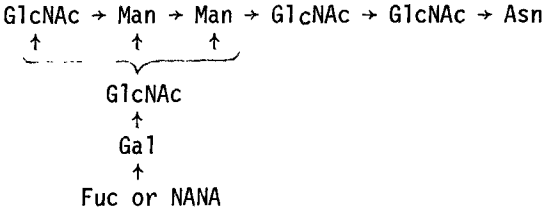
Summary

Two chemical methods are compared for the deglycosylation of the highly glycosylated glycoprotein, carcinoembryonic antigen (CEA). Solvolysis of CEA in anhydrous HF removed all of the carbohydrate except for N-glycosidically linked GlcNAc. CEA deglycosylated by HF solvolysis has a single polypeptide chain, which has been sequenced in high yield through the first 30 amino terminal residues. It retained 15% of its original antigenic activity in a radioimmunoassay and was able to inhibit up to 85% of the binding of intact CEA to anti-CEA. CEA deglycosylated by mild methanolysis had 3% carbohydrate remaining and was concomitantly split into at least 10 peptides, which appear to be amenable to further sequence studies.

Carcinoembryonic antigen (CEA) is a glycoprotein of molecular weight 180,000 comprising 60% by weight of carbohydrate. CEA was first described by Gold and Freedman (1) as a component of fetal gut and adult colonic mucosa (2, 3). Several CEA crossreacting antigens (4, 5, 6) with wide tissue distribution have been identified, among which nonspecific crossreacting antigen (NCA) (7), biliary glycoprotein I (BGPI) (8, 9), and a tumor antigen related to CEA (TEX) (10) have been partially characterized. The utility of the CEA radioimmunoassay in the diagnosis of cancer and monitoring of cancer therapy depends on an adequate knowledge of the structural relationships between CEA and its crossreacting antigens isolated from both benign and malignant tissue.

Considerable progress has been made in the determination of the carbohydrate structure of CEA (11, 12, 13), for which a general unit structure has been proposed (14):



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The extensive and heterogeneous degree of glycosylation in CEA, however, has hampered amino acid sequence studies. Although it has been possible to determine the amino terminal sequences of several tryptic peptides (15), it is clear that extensive sequencing depends on the prior deglycosylation of CEA. Recent work in this laboratory has centered on two chemical methods of deglycosylation. One is solvolysis in anhydrous hydrogen fluoride, a deglycosylation method described by Mort and Lamport (16). This is an extension of the technique which has been previously used to remove protecting groups from peptides synthesized on solid phase supports (17). The other is a mild treatment with methanolic hydrogen chloride, which under harsher conditions has been routinely used for the carbohydrate analysis of glycoproteins (18). This report compares the results of the two deglycosylation methods on CEA and presents initial amino terminal sequence results on deglycosylated CEA.

Materials and Methods

CEA was purified from the liver metastasis of a colonic adenocarcinoma from a single patient according to Coligan *et al.* (19) with a final purification step on a concanavalin A Sepharose column (20). A small amount (< 1 μg) of ^{125}I-labeled CEA was added as a tracer to each sample before deglycosylation.

The apparatus used for HF treatment was purchased from Peninsula Laboratory, Inc. (San Carlos, CA).

Methanolic hydrogen chloride (1.8 N) was prepared according to Pritchard and Todd (21) and heated at 80°C for 17 hr. Preheated methanolic hydrogen chloride is converted to an equilibrium mixture of methyl chloride, water, and hydrogen chloride (0.1 N) in methanol. Pritchard and Todd (21) have previously shown that the reagent is a mild deglycosylation reagent.

Amino terminal sequences were performed on a Beckman Model 890C sequencer according to Shively *et al.* (15). Amino acid and amino sugar analyses were performed on a Beckman 121M analyzer according to Del Valle and Shively (manuscript submitted). Carbohydrate analyses were performed on the trimethylsilyl derivatives of sugar methyl glycosides as described by Pritchard and Todd (21). SDS polyacrylamide gel electrophoresis was performed according to Laemmli (22).

HF deglycosylation was performed according to Mort and Lamport (16). Thoroughly dried CEA (82 mg) and 3 ml of anisole scavenger[1] were added to the Kel-F reaction vessel and treated with *ca.* 10 ml of anhydrous liquid HF (previously dried over cobalt trifluoride). Solvolysis was allowed to

[1]Several trials were attempted without the addition of anisole scavenger as suggested by Mort and Lamport (16); however, the product was yellow to brown in appearance and insoluble in a wide range of denaturing agents. The addition of anisole is therefore strongly recommended. For a discussion of its role as a scavenger see Mort and Lamport (16).

proceed for 2 hr at room temperature before complete removal of HF (calcium oxide trap). The product had a white and rubbery appearance. It was solubilized in 44% formic acid with 2% by weight sodium dodecylsulfate (SDS) and dialyzed extensively versus 44% formic acid to remove excess anisole and SDS. The dialysate was centrifuged to remove insoluble material (8.7 mg).

Methanolic hydrogen chloride deglycosylation. CEA (44 mg) dissolved in 1 ml of water was aliquoted to ten 50 ml ampoules and slowly mixed by constant vortexing at room temperature with 40 ml of pretreated methanolic hydrogen chloride. The ampoules were sealed and heated at 80° C for 17 hr with intermittent vortexing. At first the CEA is present as a fine suspension but becomes almost entirely soluble by the end of the 17 hr period. The sample was dried under a stream of nitrogen, dissolved in water, and lyophilized (yield 45 mg).

Results and Discussion

HF Solvolysis

The results of chromatography of HF treated CEA on Sephadex G50 in 44% formic acid are shown in Figure 1A. Pool 1 yielded 48 mg of a dried, lyophilized powder whose amino acid composition is essentially unchanged from intact CEA (Table 1). The expected yield of deglycosylated CEA is 49 mg (43% protein plus 17% carbohydrate times 82 mg starting material). Based on the work of Mort and Lamport (16), HF solvolysis at room temperature removes all but N-glycosidically linked GlcNAc from glycoproteins. Since there are about 80 carbohydrate chains in CEA (23) each N-glycosidically linked to asparagine residues, one would expect HF treated CEA to contain about 16% GlcNAc by weight. Based on the amino acid analysis data shown in Table 1 CEA contains 32% GlcNAc before and 17% GlcNAc after HF treatment. The carbohydrate analysis shown in Table 1 shows that HF treatment effectively removes all of the sialic acid, fucose, and galactose, most of the mannose, and the expected portion of the GlcNAc.

Carbohydrate and amino acid analyses were also performed on Pool 2, which had an anomalously high dry weight (121 mg). It contained 9% amino acids and 4% GlcNAc by weight but gave no identifiable peaks on carbohydrate analysis. The latter result may be due to the alkylation of anisole by glycosyl fluorides (16).

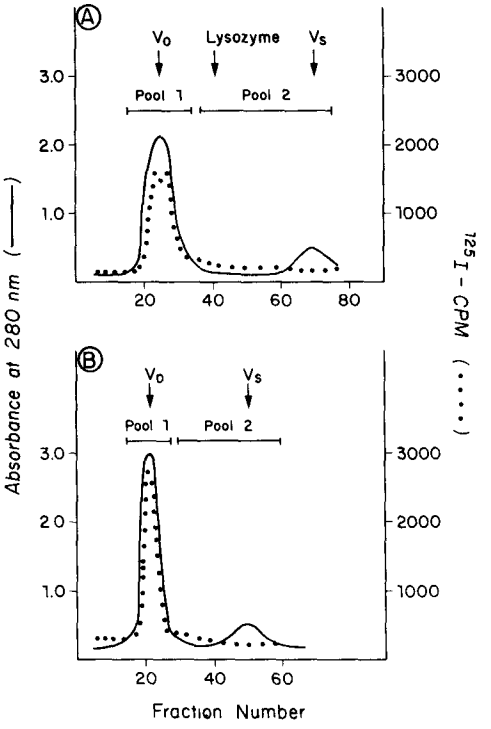

Fig. 1A: Sephadex G50 (150 mesh) column chromatography of HF deglycosylated CEA in 44% formic acid. The sample (6 ml, one half of the total) was loaded onto a 1.5 x 50 cm column and 0.9 ml fractions were collected. The column was calibrated with CEA (V_0, excluded volume) lysozyme (MW 12,000) and $^{57}CoCl_2$ (Vs, included volume).

Fig. 1B: Sephadex G25 (150 mesh) column chromatography of methanolyzed CEA in 0.1 M acetic acid. The sample (1.25 ml) was loaded onto a 1.5 x 50 cm column and 1.5 ml fractions were collected. The column was calibrated with CEA (V_0, excluded volume), $^{57}CoCl_2$ (Vs, included volume), and β-lactalbumin (MW 18,000), which coeluted with CEA.

Table 2 presents the results of the first 30 Edman degradation cycles on HF deglycosylated CEA. The sequence agrees with previously published results (26) through position 24 but is different from the results reported by Chu *et al.* (27) for positions 25-30. The high absolute yield (71%) and repetitive yield (98%) obtained suggests that deglycosylated CEA is more amenable to extended sequence determination than intact CEA. In addition these results demonstrate that HF solvolysis does not cleave peptide bonds. It has previously reported that HF may cleave methionyl peptide bonds

Table 1

Amino Acid and Carbohydrate Composition of CEA Before and After Deglycosylation[a]

	Before	After HF Treatment	After Methanolysis
	Mole Percent		
Tyrosine	3.5	4.7	4.5
Phenylalanine	1.5	2.3	2.5
Lysine	2.8	2.5	2.5
Histidine	1.7	1.6	1.7
Arginine	3.7	4.2	3.8
Aspartic Acid	16.0	14.5	15.1
Threonine	9.4	9.3	9.8
Serine	12.0	11.4	11.0
Glutamic Acid	10.9	10.8	10.9
Proline	11.0	10.7	9.8
Glycine	6.7	5.9	5.6
Alanine	6.4	6.3	6.8
Valine	4.5	5.3	5.4
Methionine	0.0	0.0	0.0
Isoleucine	3.8	3.0	2.8
Leucine	6.4	7.4	7.1
	Weight Percent		
Amino Acids[b]	34.1	32.0	57.0
Glucosamine[b]	16.3	6.6	3.9
Glucosamine[c]	16.3	2.6	1.1
Mannose	9.0	1.0	0.4
Galactose	9.4	0.0	0.0
Fucose	8.5	0.0	0.0
NANA	1.8	0.0	0.0

[a]Average of duplicate analyses.

[b]Results obtained from amino acid analyzer. Percent recoveries of amino acids and glucosamine for CEA before treatment were recalculated to the same value of glucosamine obtained by carbohydrate analysis.

[c]Determined by gas chromatography of the trimethylsilyl derivative of the methyl glycoside.

(16, 17), but since CEA contains no methionine, this was not a problem. We are currently engaged in obtaining specific peptide fragments from HF deglycosylated CEA and expect that less difficulties will be encountered during the purification and sequencing stages than with our previous studies with intact CEA.

HF deglycosylated CEA retains 15% by weight of its original antigenic activity as measured by radioimmunoassay and is able to inhibit up to 85% of the binding of intact CEA to anti-CEA.

Table 2

Amino Terminal Sequence of HF Deglycosylated CEA[a]

Position	GC[b]	TLC[c]	HPLC[d]	Conclusion
1		lys	lys	lys
2	ile/leu	ile/leu	leu	leu
3	thr	thr	thr	thr
4	ile/leu	ile/leu	ile	ile
5		glu	glu	glu
6			ser	ser
7	thr	thr	thr	thr
8	pro	pro	pro	pro
9	phe	phe	phe	phe
10		asn	asn	asn
11	val	val	val	val
12	ala	ala	ala	ala
13		glu	glu	glu
14		gly	gly	gly
15		lys	lys	lys
16		glu	glu	glu
17	val	val	val	val
18	ile/leu	ile/leu	leu	leu
19	ile/leu	ile/leu	leu	leu
20	ile/leu	ile/leu	leu	leu
21	val	val	val	val
22			his	his[e]
23		asn	asn	asn
24	ile/leu		leu	leu
25	gly	gly	gly	gly
26	phe	phe	phe	phe
27	ile/leu	ile/leu	leu	leu
28	pro		pro	pro
29		ser	ser	ser
30			thr or gln	thr or gln

[a]Results obtained on a Beckman 890C sequencer. HF deglycosylated CEA (1.6 mg or 16.7 n mole based on an MW of 96,000) gave 71% absolute yield of PTH-leucine on cycle 2 with a repetitive yield of 98% based on the yields at cycles 2, 18, and 19.

[b]Gas chromatographic analysis according to Pisano and Bronzert (24).

[c]Thin-layer chromatographic analysis according to Summers *et al* (25).

[d]High pressure liquid chromatographic analysis according to Shively *et al* (15).

[e]Also confirmed by a weakly positive spot test according to Easley (26).

Methanolysis

Figure 1B presents the results of the purification on Sephadex G25 of CEA deglycosylated with methanolic hydrogen chloride. Pool 1 yielded 15 mg of dried, lyophilized material whose amino acid composition remained essentially unchanged from intact CEA (Table 1). Since the amount of sugar

remaining is 3%, the calculated yield of deglycosylated CEA is 20 mg (43% protein plus 3% carbohydrate times 44 mg of starting material). Compared to the HF deglycosylation procedure, methanolysis results in a more nearly complete removal of carbohydrate, including GlcNAc (see Table 1).

SDS polyacrylamide gel electrophoresis (not shown) of methanolyzed CEA revealed 3 major peptide bands ranging in molecular weight from 5,000 to 30,000. Amino acid sequence determination indicated there were at least 10 peptides. In contrast to HF solvolysis, these results show that a number of peptide bond cleavages occur during methanolysis. In contrast to sequence results on peptides obtained from intact CEA (15), we were able to obtain extended sequences on the deglycosylated peptides. We are currently attempting to separate and purify the peptides obtained by methanolysis.

A rather interesting difference between HF deglycosylated CEA and methanolyzed CEA is solubility. Methanolyzed CEA peptides are initially very soluble in water and become slightly less soluble after repeated lyophilization. HF deglycosylated CEA is soluble in $\geq$ 44% formic acid, but insoluble in 8 M urea, 8 M guaninidium hydrochloride, 0.1-2% SDS, Triton X-100, or a combination of solubilizing reagents over a wide range of pH. Attempts to determine the molecular size of HF deglycosylated CEA were not entirely successful. SDS polyacrylamide gel electrophoresis or gel filtration on Bio-Gel P-300 in 44% formic acid show that extensive oligomerization has occurred: molecular weight values of (95,000)n where n is 1, 2, or 3 are obtained. The size determinations are hampered by the low solubility of the oligomers and the relatively high percentage (17%) of remaining carbohydrate. Although we are unable to explain the mechanism of oligomerization, the high yield of a single amino-terminal sequence demonstrates that amino-termini are not involved, nor are new free amino-termini formed.

It is our hope that the findings presented here will enable a more rapid determination of the amino acid sequence of CEA and other similar

glycoproteins, which due to their high degree of carbohydrate substitution have been difficult to sequence.

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