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Deglycosylation by gaseous hydrogen fluoride of mucus glycoproteins immobilized on nylon membranes and in microtiter wells

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Strongly reacting antibodies specific for defined mucin gene products are often directed against the mucin protein backbone of the heavily glycosylated serine/threonine rich regions. A prerequisite for the use of such antibodies is often the complete removal of the oligosaccharides from the protein. This paper describes an efficient one-step deglycosylation method using gaseous hydrogen fluoride on nylon blotting membranes and microtiter wells.

***Keywords*: mucin, glycoprotein, deglycosylation, hydrogen fluoride, trifluoromethanesulfonic acid**

***Abbreviations*: HF, hydrogen fluoride; TFMSA, trifluoromethanesulfonic acid; NBT, nitro blue tetrazolium; BCIP, bromo-chloro-indolyl-phosphate; BSA, bovine serum albumin; DELFIA, dissociation enhanced lanthanide fluoroimmunoassay**

Introduction

Mucins or mucus glycoproteins are a heterogeneous group of proteins having more than 50% of the molecular mass as oligosaccharides *O*-linked to serine or threonine [1]. To the group belong the 'classical' gel forming mucins, exemplified in this paper by the MUC2 mucin. The gel forming mucins are secreted as large polymers covering all mucosal surfaces in the body where they are a vitally essential part of the barrier between the internal and external milieu. Another type of mucin, here represented by the MUC1 mucin, does not form polymers but is instead bound to the membrane of epithelial cells [1]. Typical for mucins are the so called mucin domains to which most of the oligosaccharides are localized. These domains are dominated by the amino acids serine, threonine and proline that often occur in tandem repeats encoded by VNTR sequences. Since the repeated sequences give rise to repetitive antibody epitopes, the best antibodies with an acceptable specificity for an entire mucin are directed against the protein part of these domains. A problem concerning tandem repeat antibodies is, however, that glycosylation of the mucin usually blocks the antibody epitopes. Mucin molecules, except at pre-Golgi biosynthesis stages, must therefore be deglycosylated before such antibodies can be used. In this paper gaseous hydrogen fluoride (HF) has been used on samples immobilized on blotting membranes and in microtiter wells. This seems to yield a more complete deglycosylation than the previously described method for membranes where trifluoro-methanesulfonic acid (TFMSA) was used [2, 3].

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Materials and methods

Materials

MUC1 mucin (H-CanAg) was prepared from the cell line COLO 205 [4]. Insoluble mucins including MUC2 were purified from the 6 M guanidinium chloride insoluble pellet of LS 174T cells by washing six times in 6 M guanidinium chloride, reduction, alkylation and three rounds of CsCl density gradient ultracentrifugation [5, 6]. A crude MUC2 preparation was obtained by only pelleting, one washing step and reduction. An equally crude, radiolabeled MUC2 preparation was obtained in the same way from LS 174T cells metabolically labeled with ^{35}S-Met as described before [7], using a 2 h pulse-labeling followed by 48 h chase with fresh medium. The MUC3 mucin (SBG1) was prepared from human bile [8]. The human sera 477 and 739 were obtained from breast cancer patients [9]. The monoclonal antibody HMFG-2 against MUC1 [10], and the polyclonal antiserum α-MUC2TR against the tandem repeat of MUC2 [7] have been defined before. An antiserum called

α-MUC2N3 was raised in a rabbit against a synthetic peptide, CPKDRPIYEEDLKK, based on amino acids 1167–1180 on the N-terminus of the human MUC2 apoprotein. A New Zealand White rabbit was immunized once with 500 μg peptide conjugated via Cys to 400 μg KLH in Freund's complete adjuvant, and twice with 250 μg peptide conjugated to 200 μg KLH in Freund's incomplete adjuvant. The intervals between the immunizations were two weeks. Using a similar protocol, the antiserum α-MUC3TR was raised against a synthetic peptide based on the MUC3 tandem repeat region (TTTEIPSHSTPSFTSSIC). A New Zealand White rabbit was immunized once with 250 μg peptide conjugated to KLH together with Freund's complete adjuvant, and twice with 200 μg peptide conjugated to KLH together with Freund's incomplete adjuvant. Alkaline phosphatase conjugated anti-rabbit and anti-mouse antibodies were from DAKO (Glostrup, Denmark). Hydrogen fluoride (HF) was from Fluka (Buchs, Switzerland), trifluoromethanesulfonic acid (TFMSA) from Aldrich (Steinheim, Germany), the nylon (PVDF) membranes (Immobilon-P) were from Millipore (Bedford, MA) and the microtiter well strips (MaxiSorp C12) from Nunc (Roskilde, Denmark). Nitro blue tetrazolium (NBT) and bromo-chloro-indolylphosphate (p-toluidine salt) (BCIP) were from Sigma (St. Louis, MO).

Blotting onto membranes

Slot blots were performed using nylon membranes and a slot blot apparatus (Schleicher and Schuell, Dassel, Germany). Samples for Western blot were separated on 6% SDS-PAGE (reducing conditions) using a 3% stacking gel. After electrophoresis, the proteins were electrophoretically transferred (Sartoblot II-S) to the blotting membrane using 48 mM Tris, 39 mM glycine, 0.0375% SDS and 10% methanol (pH 8.3) as transfer buffer. The membranes were washed several times in water after blotting and dried in a vacuum dessicator before HF or TFMSA treatment.

HF treatment

Hydrogen fluoride treatment was performed in a HF-apparatus (Peptide Institute Inc., 4-2-1 Ina Minoh-Shi, Osaka 562, Japan) consisting of two teflon containers connected via a vent equipped tubing system to a HF gas cylinder and to a water jet and a rotary pump. One container was used as a HF store, in which gaseous HF from the cylinder was condensed by cooling the container with a dry ice-ethanol bath. HF for about five treatments was transferred each time. The nylon membranes or micro titer well strips were placed into the secondary container, air was evacuated and the container cooled on a dry ice-ethanol bath. The connection to the store (brought to room temperature) was thereafter opened and kept open until about 5 ml fluid HF had condensed in the bottom of the cooled container (fluid level not reaching the samples). The cold bath was removed allowing the formation of gaseous HF and exposing the samples over night at room temperature. The HF was removed by a water jet suction for about 2 h followed by a rotary pump (connected via a NaOH trap) for about 1 h. Since HF gas is extremely corrosive and toxic, the whole equipment was kept in a fume hood and when handled frequently checked for leakages using NH_3 gas (from 25% NH_3 in water); NH_4F is visible as white smoke.

TFMSA treatment

Blotting membrane strips were exposed to anhydrous TFMSA, 50–100 μl cm^{-2}, for 6 h. The treatment was performed in glass tubes with teflon sealing on wet ice and under agitation. TFMSA in contact with water yields very toxic fumes, formed when the deglycosylation reaction is terminated by putting the membranes into 50 mM Tris-HCl, pH 8.0.

Assay of membranes

HF treated membranes and controls were wet quickly in methanol and placed into 50 mM Tris-HCl pH 8.0, whereas TFMSA treated membranes showed hydrophilic properties and could be placed immediately into 50 mM Tris-HCl pH 8.0. All membranes were washed twice in PBS (136.8 mM NaCl, 2.7 mM KCl, 1.76 mM KH_2PO_4, 10.14 mM Na_2HPO_4); blocked over night at 37 °C in PBS, 2% bovine serum albumin (BSA), 0.05% NaN_3; washed once in TBST (150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.05% Tween-20); incubated 1–2 h at room temperature in assay buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1.5% BSA, 20 μM diethylenetriaminepentaacetic acid, 0.01% Tween-40, 0.05% NaN_3) with 5% HMFG-2 antibody hybridoma supernatant, 1% α-MUC2TR, 2% α-MUC2N3 or 1% α-MUC3TR serum; washed three times in TBST; incubated 1–2 h at room temperature in alkaline phosphatase conjugated anti-mouse or anti-rabbit antibody diluted 1:500 in assay buffer; washed six times in TBST; washed twice in TBS (150 mM NaCl, 20 mM Tris-HCl pH 7.5); and finally developed in 0.3 mg ml^{-1} NBT, 0.15 mg ml^{-1} BCIP, 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$. Color density of slot blots was measured using a video camera from KAPPA Messtechnik (Gleichen, Germany) and software from Bildanalys (Stockholm, Sweden). Radioactively labeled samples were assayed by exposure to film (Kodak BioMax MR) over night at −80 °C, and the intensity was measured by video densitometry.

Assay of mucins immobilized in microtiter wells

Serial dilutions of MUC2 mucin in water were lyophilized in microtiter wells and HF treated or kept as controls. The mucin material was then coated onto the plastics by adding 200 μl of 100 mM Tris-HCl pH 7.5, PBS, 0.05% NaN_3 and incubating over night at 37 °C. The samples were assayed by dissociation enhanced lanthanide fluoroimmunoassay

(DELFIA) [11]. The strips were washed twice in washing buffer (5 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.005% Tween-20, 0.02% NaN_3) and once in 150 mM NaCl; blocked over night at 37 °C in 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 2% BSA, 90 μM $CaCl_2$, 4 μM EDTA, 0.02% NaN_3; incubated on a shaker at room temperature with 1% α-MUC2TR serum in assay buffer for 1–2 h; washed three times in washing buffer; incubated 1 h on a shaker at room temperature with europium (Eu^{3+})-labeled anti-rabbit antibody in assay buffer and washed six times in washing buffer. Enhancement solution (200 μl) was added followed by incubation on a shaker for 5 min to release Eu^{3+} [11]. Fluorescence was measured in an Arcus fluorometer (Pharmacia Wallac, Sweden).

Results

HF treatment of purified mucins slot blotted onto membranes

To compare deglycosylation of mucins with HF and TFMSA, purified MUC1, MUC2 and MUC3 mucins were serially diluted and slot blotted onto nylon membranes. The membranes were treated with gaseous HF at room temperature over night or with TFMSA on ice for 6 h, stained with rabbit antisera or a monoclonal antibody, all against tandem repeat domains, followed by an alkaline phosphatase conjugated secondary antibody, and developed with the NBT/BCIP substrate. Results of color density measurements are shown in Figure 1. The α-MUC2TR serum against the MUC2 tandem repeat showed strong and specific reactivity with HF treated MUC2 mucin, whereas practically no reactivity was observed with nontreated. The reactivity with TFMSA treated MUC2 mucin was non-specific, as preimmune serum reacted as strongly as the immune serum. This non-specific reactivity was probably due to proximal *N*-acetylgalactosamine (GalNAc) remaining on the mucin, as TFMSA treatment is known to leave the GalNAc attached to the protein core [2, 3]. Untreated MUC3 mucin showed strong non-specific reactivity with the α-MUC3TR serum (stronger with preimmune than immune serum for unknown reasons), probably due to reactivity with carbohydrate epitopes. The HF treated MUC3 mucin stained with the same serum showed an expected dilution curve, although at a relatively low intensity. The MUC3 mucin from bile is known to have very long saccharide side chains with a mean number of 50 sugar residues per chain [8] as compared to about 5 on the MUC2 mucin used in this paper (unpublished results). This may explain why

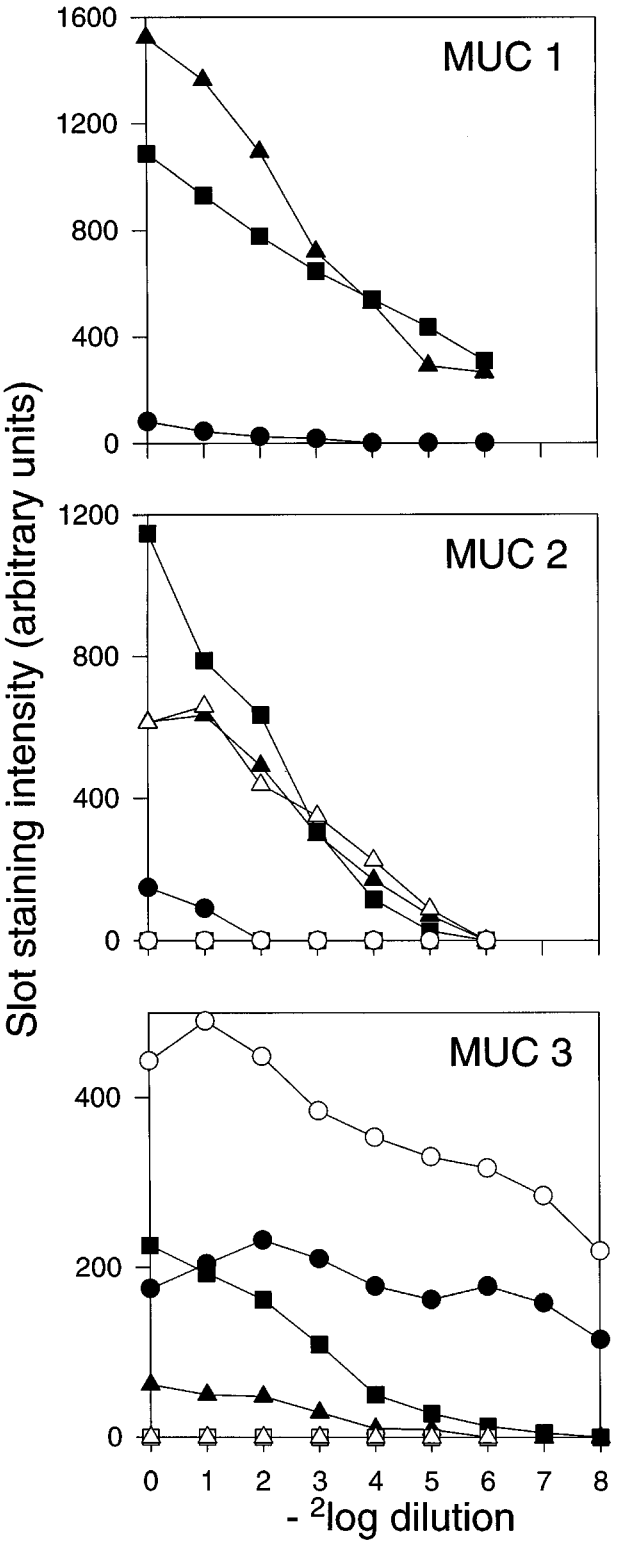

Figure 1. Immune stained slot blots of the purified MUC1, MUC2 and MUC3 mucins in serial 1:2 dilutions. The membranes were treated with gaseous HF or liquid TFMSA, or left as untreated controls, prior to assay. The primary antibodies used were the monoclonal antibody HMFG-2 for the MUC1 mucin, the α-MUC2TR antiserum for the MUC2 mucin and the α-MUC3TR antiserum for the MUC3 mucin. (■) monoclonal antibody/immune serum, HF treated mucin; (▲) monoclonal antibody/immune serum, TFMSA treated mucin; (●) monoclonal antibody/immune serum, untreated mucin; (□) preimmune serum, HF treated mucin; (Δ) preimmune serum, TFMSA treated mucin; (○) preimmune serum, untreated mucin.

HF treatment gave a relatively weak antibody reaction, although still markedly stronger than TFMSA treatment. The monoclonal antibody HMFG-2 showed a stronger reaction with the MUC1 mucin from a colon carcinoma cell line after the TFMSA than after the HF treatment. This mucin has oligosaccharides with a mean length of about 15 residues [4]. The HMFG-2 is directed against the non-glycosylated PDTR epitope, which is sterically blocked when long oligosaccharide chains are present on the adjacent amino acids. That the HMFG-2 reaction was stronger after TFMSA treatment indicates a more efficient deglycosylation by HF than TFMSA, as this monoclonal antibody is known to react better if proximal GalNAcs are left on the MUC1 mucin (Drs. Joyce Taylor-Papadimitriou and Joy Burchell, personal communication).

Reduction of non-specific background by HF treatment

TFMSA treated purified MUC2 mucin and nontreated purified MUC3 mucin showed strong non-specific reactivity (Figure 1). However, after HF treatment no non-specific staining was observed. The non-specificity was probably due to carbohydrate epitopes on the mucins. Our experience is that problems with non-specific background are increased when using antisera on nonpurified mucin materials. It has also been observed that HF treatment can markedly reduce these problems. In order to illustrate this, material from LS 174T cells insoluble in 6 M guanidinium chloride was pelleted, washed once, dissolved by the reduction of disulfide bonds and applied to nylon membranes. One membrane was subjected to HF treatment followed by staining with the α-MUC2TR antiserum, whereas the other was nontreated and stained with α-MUC2N3, an antiserum reacting also with fully *O*-glycosylated MUC2 mucin (Figure 2). On the HF treated samples, the α-MUC2TR antiserum showed a strong and specific reactivity with the nonpurified mucin. On the nontreated membrane, however, preimmune and immune sera gave essentially the same staining, showing that the relatively weak specific reactivity of the α-MUC2N3 antiserum was hidden in the high non-specific background. To demonstrate that the background in this case could be reduced by purification of the sample, the α-MUC2N3 antiserum was tested against a nonHF treated MUC2 preparation equally pure as the one used in Figure 1. The non-specific background was drastically reduced compared to the crude samples (Figure 2). HF

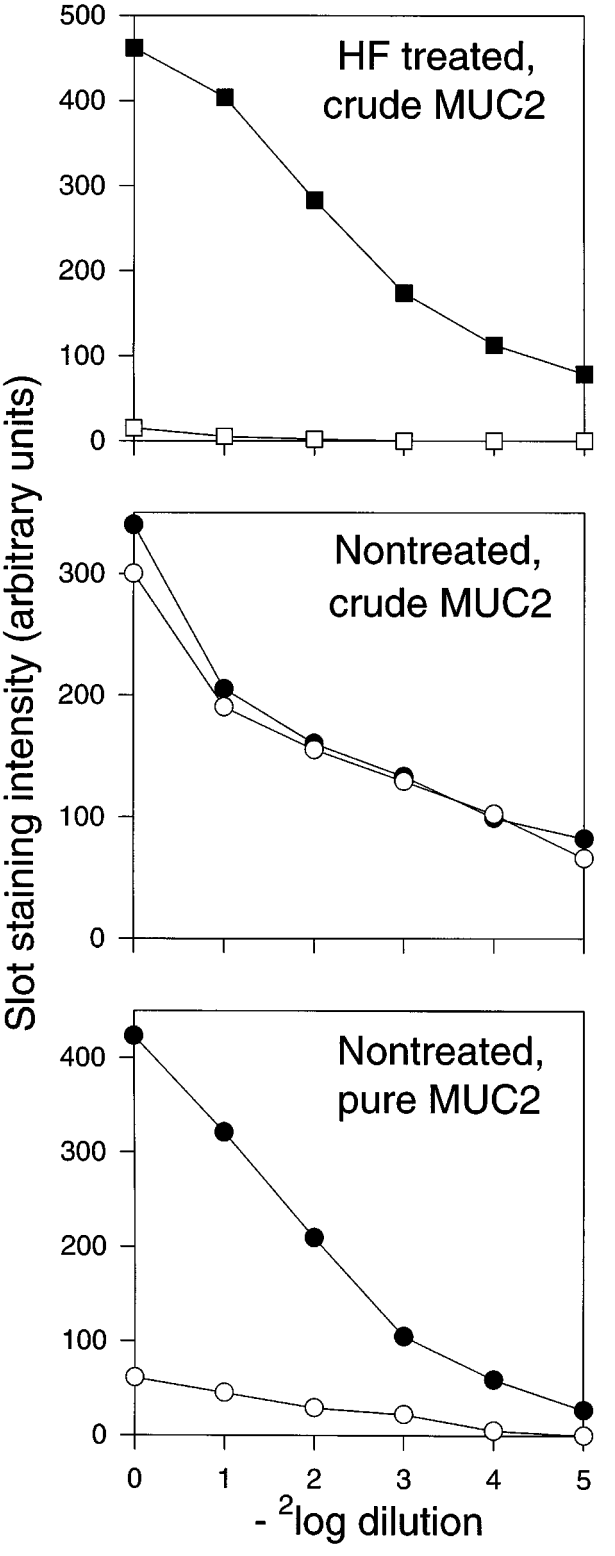

Figure 2. HF treated and untreated slot blots of crude and purified insoluble mucins from LS 174T cells. Serial 1:2 dilutions starting with material from 0.5 cm^2 of cells. The crude preparation included all material from the LS 174T cells that was insoluble in 6 M guanidinium chloride, whereas the purified sample was obtained after three rounds of CsCl density gradient ultracentrifugation of mucins solubilized by reduction. The *α*-MUC2TR antiserum was used on the HF treated membrane and the *α*-MUC2N3 antiserum on the nontreated membranes. (■) *α*-MUC2TR antiserum, HF treated mucin; (□) preimmune serum, HF treated mucin; (●) *α*-MUC2N3 antiserum, untreated mucin; (○) preimmune serum, untreated mucin.

treatment can thus reduce the nonspecific background when analysing crude samples, thus minimizing the needs for purification.

Effect of HF treatment on protein backbones

To analyze to what extent the HF treatment is destructive to the mucin apoproteins, insoluble mucins were extracted from ^{35}S-labeled LS 174T cells and slot blotted onto membranes. One membrane was HF treated whereas the other was kept as a nontreated control. The membranes were washed and autoradiographed, and the intensity was measured by video densitometry. As shown in Figure 3, HF treatment had little effect on the radioactivity of the slots, indicating that the proteins remained relatively intact on the blotting membrane. However, longer times for HF treatment than over night can usually not be recommended, as a significant protein epitope destruction has been observed after three days (not shown). Liquid HF can dissolve samples from the membrane and vigorous boiling, causing splashes, should be avoided during the HF evacuation step.

HF treatment of Western blotted mucins

As western blot is a commonly used technique, the possibility of using HF treatment for the deglycosylation of Western blotted mucins was investigated (Figure 4). Purified MUC1 mucin and two different breast cancer patient sera were separated by SDS-polyacrylamide gel electrophoresis and transferred to nylon membranes. The purified MUC1 mucin and the patient serum 739 showed reactivity only after HF treatment, revealing diffuse 'bands' on the gel typical for highly glycosylated compounds. The MUC1 mucin from the patient serum 477 on the other hand formed a smaller and more distinct band, indicating less glycosylation possibly explaining the weak antibody reaction observed also without HF treatment.

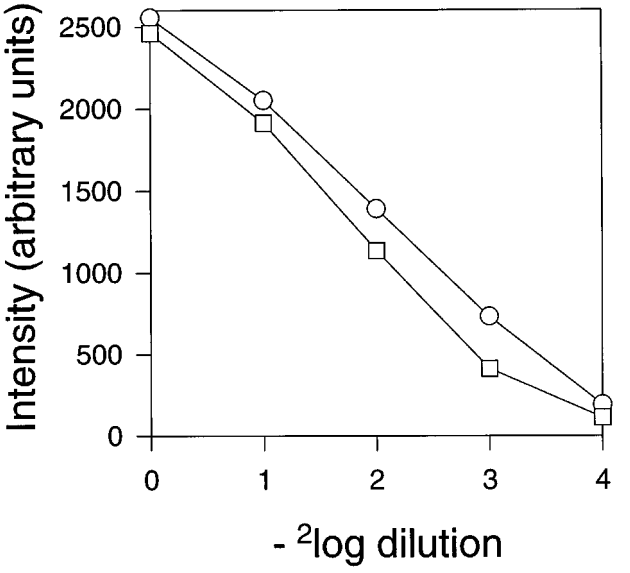

Figure 3. Comparision between the yield of HF treated (□) and untreated (○) slot blotted metabolically radiolabeled insoluble mucins from LS 174T cells. Serial 1:2 dilutions starting with material from 2 cm^2 of cells were slot blotted onto nylon membranes, which were HF treated or kept untreated before washing and exposure to an X-ray film. The values were obtained by video densitometry.

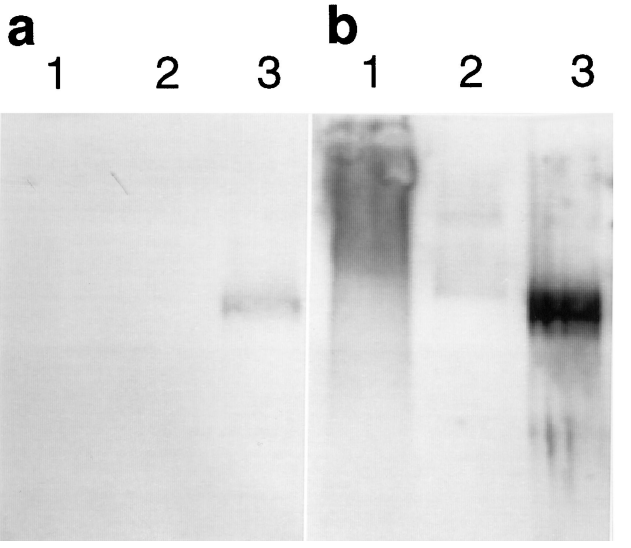

Figure 4. Untreated (a) and HF treated (b) Western blots after polyacrylamide gel electrophoresis of the MUC1 mucins purified from COLO 205 cells (lane 1) and the breast cancer patient sera 739 (lane 2) and 477 (lane 3). The monoclonal antibody HMFG-2 was used as primary antibody.

HF treatment of mucins adsorbed in microtiter wells

The possibility of performing HF deglycosylation on mucins adsorbed in microtiter wells was also tested using pure MUC2 mucin. After deglycosylation the reactivity was tested in a fluoroimmunoassay using the α-MUC2TR antiserum followed by europium-labeled anti-rabbit immunoglobulins (Figure 5). Strong specific antibody reaction was observed after HF treatment, whereas no reactivity occurred in nontreated controls.

Discussion

Liquid hydrogen fluoride in 10% methanol has previously been described as an effective technique for the deglycosylation of mucins in solution [12]. This method is applicable on a preparative scale when larger amounts of pure mucin material are to be analyzed, but not suitable for the assay of large numbers of fractions. In this paper we present a method where HF is used in the gaseous form for the deglycosylation of samples immobilized on blotting membranes and in microtiter wells. This is an analytical method, convenient for the analysis of fractions. To our knowledge, this is also the first deglycosylation method practically

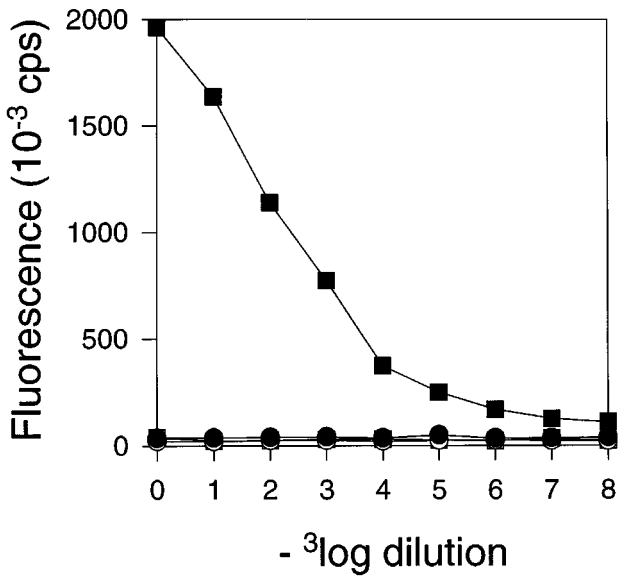

Figure 5. Fluoroimmunoassays of serial 1:3 dilutions of MUC2 mucin HF treated in microtiter wells. (■) *α*-MUC2TR antiserum, HF treated mucin; (●) *α*-MUC2TR antiserum, nontreated mucin; (□) preimmune serum, HF treated mucin; (○) preimmune serum, nontreated mucin.

usable directly on microtiter wells. On membranes TFMSA is an alternative, recommended to be used in combination with neuraminidase treatment and periodate oxidation – alkaline elimination [2, 3]. Our results indicate that TFMSA treatment can not be used as the only deglycosylation step for many antisera, due to high non-specific background. The monoclonal antibody HMFG-2, however, reacts well with TFMSA treated MUC1 due to the special properties of its epitope already discussed. Gaseous HF treatment, on the other hand, seems efficient enough to be recommended as a general one-step deglycosylation method. Such a method is often necessary for detection and quantification of different mucin gene products in for instance mucus, cell lysates and ultracentrifuge and chromatography fractions, as many of the mucin antibodies available are *O*-glycosylation sensitive.

In highly purified preparations, glycosylation insensitive antibodies directed against nontandem repeat regions can, if they exist, be used to circumvent deglycosylation. Such antibodies often give a weaker signal due to the low number of epitopes present on each molecule. Such an antiserum, α-MUC2N3, has been used on the purified MUC2 mucin in this paper, and showed specific reactivity well distinguishable from the non-specific background (Figure 2). However, this antiserum could still not be used on more crude preparations, due to a high non-specific background (Figure 2). It is our general experience that non-specific background often makes assays on nondeglycosylated material impossible on crude preparations such as for example mucus, cell lysates, cell culture media and fractions from first rounds of preparative ultracentrifugations. As illustrated in Figure 2, HF deglycosylation decreases such non-specific background drastically, probably by removing carbohydrate epitopes reacting with the antisera. The effective HF deglycosylation also enables the use of tandem repeat antibodies, which, due to their high number of epitopes, give stronger signals. Taken together, these two effects create a high 'signal to noise ratio', allowing the analysis of specific mucin gene products also in crude preparations. Such analyses are of interest in studies on mucus related diseases such as cystic fibrosis, chronic bronchitis, ulcus and ulcerative colitis. Despite its relative complexity, HF treatment is often a timesaving alternative to purification when dealing with great numbers of samples or fractions.

Since the conditions under treatment with gaseous HF are harsh, damage to the protein core could be expected. However, essentially no protein loss from the blotting membrane measured by metabolic radiolabeling was detected (Figure 3). It therefore seems probable that the proteins remained at least as fragments large enough not to be washed away from the membrane. This is in accordance with Western blots of HF treated mucins (not shown), showing diffuse bands indicating varying but limited numbers of random nicks in the protein backbone. These results suggest that antibody epitope destruction is a minor problem when gaseous HF treatment is used prior to immune assays. On the other hand, a substantial yield of totally intact proteins for chemical analyses can not be expected using the HF technique.

In summary, gaseous hydrogen fluoride was shown to be a very efficient deglycosylation agent for immobilized samples, usable both in microtiter wells and on nylon membranes, and suitable also on large membrane sheets such as Western blots. HF deglycosylation often gave stronger antibody staining and a markedly reduced non-specific background compared to TFMSA deglycosylation, which is an alternative on membranes. The effective removal of non-specific background by HF treatment makes it especially valuable when analyzing crude mucins.

Acknowledgments

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