

The recombinant N-terminal region of human salivary mucin MG2 (MUC7) contains a binding domain for oral *Streptococci* and exhibits candidacidal activity

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MG2 (the *MUC7* gene product) is a low-molecular-mass mucin found in human submandibular/sublingual secretions. This mucin is believed to agglutinate a variety of microbes and thus is considered an important component of the non-immune host defence system in the oral cavity. We have shown that MUC7 can bind to cariogenic strains of *Streptococcus mutans* and that this binding requires a structural determinant in the N-terminal region. In the present study an expression construct, pNMuc7, encoding the N-terminal 144 amino acids of MUC7 was generated, and the recombinant protein rNMUC7 was expressed in *Escherichia coli*. Purified rNMUC7 was characterized and the binding of this protein to oral bacteria was investigated in an established assay. The results showed that the recombinant protein bound to *S. mutans* ATCC 25175 and ATCC 33402, and that alkylation of the two cysteine residues (Cys⁴⁵ and Cys⁵⁰) resulted in the complete loss of bacterial binding. This suggests

that binding of MUC7 to *S. mutans* occurs between the N-terminal region of the mucin molecule and the bacterial surface, and that this interaction is dependent on a cysteine-containing domain within this region of MUC7. In addition, the killing activity of rNMUC7 was compared with that of the candidacidal salivary protein histatin 5 in an established *Candida albicans* (ATCC 44505) blastoconidia killing assay. It was found that the LD₅₀ values of rNMUC7 and histatin 5 were comparable, and that the recombinant protein displayed significant killing activity at the physiological concentration range of MUC7 in whole saliva. This study is the first to show that the N-terminal region of MUC7 contains a structural determinant for bacterial binding and that this region exhibits candidacidal activity.

Key words: anti-microbial effect, apomucin, dimerization, saliva.

INTRODUCTION

Human saliva contains two mucous glycoproteins, referred to as MG1 and MG2, which are found in the secretions of the submandibular and sublingual glands and of minor salivary glands [1]. Salivary mucins are multifunctional molecules that have been implicated in the protection of oral surfaces against desiccation, lubrication of hard and soft tissues to minimize injury from mastication and other mechanical injury, facilitation of speech, and formation of a diffusion barrier between underlying tissues and the external environment [1–4]. MG2 is also considered to be an important component of the non-immune host defence system, because it binds to a large number of oral bacteria [5–8] and to the opportunistic yeast *Candida albicans* [9,10].

MG2 isolated from human salivary secretions has an apparent molecular mass of 150–200 kDa and contains about 30% protein, 68% carbohydrate and 1.6% sulphate [11]. MG2 apomucin has been cloned from a human submandibular gland cDNA library, and the gene encoding this mucin has been named *MUC7* [12,13]. For clarity, MG2 is subsequently referred to as MUC7. The apomucin moiety of secreted MUC7 is composed of 357 amino acids, which are organized into a 144-residue N-terminal region containing the only two cysteines in the molecule, a 138-residue

tandem repeat region containing either five or six nearly identical 23-residue repeats, and a 75-residue C-terminal region. Since MUC7 is believed to occur as a monomer in salivary secretions and whole saliva, the cysteine residues in the N-terminal region do not form intermolecular disulphide bonds.

Analysis of the primary structure of MUC7 apomucin has identified a leucine zipper motif and a histatin-like domain in the N-terminal region [14]. The functional significance of the leucine zipper is not known, although it has recently been reported that a synthetic peptide corresponding to the histatin-like domain, and N- and C-terminally modified derivatives of this peptide, exhibit candidacidal activity [15]. The histatins are a family of small histidine-rich peptides found in parotid and submandibular/sublingual secretions [16–18], and the candidacidal properties of these molecules have been extensively investigated [19–21]. MUC7 has also been reported to agglutinate the AIDS virus HIV-1 [22] and to inhibit HIV infection by displacing the envelope glycoprotein gp120 [23].

Most of the studies on the functional properties of MUC7 have been carried out using salivary secretions or whole saliva, and relatively little is known about the biological properties of the purified mucin. For this reason, we developed a novel method for purification of MUC7 from submandibular and sublingual glands, and found that purified MUC7 was able to bind to four

Abbreviations used: rNMUC7, recombinant protein consisting of the N-terminal 144 residues of MUC7; RM-rNMUC7, reductively methylated rNMUC7; RM/RA-rNMUC7, RM-rNMUC7 that has been reduced and alkylated; RA-rNMUC7, reduced and alkylated rNMUC7; RT-PCR, reverse transcriptase-PCR; IPTG, isopropyl β -D-thiogalactoside; SSB, simulated saliva buffer; RSMG and MSMG, rat and mouse homologues respectively of MUC7 (MG2).

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strains of *Streptococcus mutans* [24]. Reductive methylation of the purified mucin had little effect on bacterial binding, although reduction and alkylation of the reductively methylated molecule resulted in a greater than 10-fold decrease in bacterial binding. These results suggested the possibility that the cysteine residues located in the N-terminal region are components of a domain that binds bacteria. This is consistent with the premise that this region of the mucin molecule contains some type of higher-order structure [24].

In the present investigation, a recombinant protein (rNMUC7) consisting of the N-terminal 144 residues of MUC7 was expressed in *Escherichia coli* and the properties of this recombinant protein were examined. It was found that rNMUC7 bound to oral *Streptococci*, that binding could be abolished by reduction and alkylation, and that this portion of MUC7 apomucin exhibits significant candidacidal activity at the physiological concentration range of MUC7 found in whole saliva.

EXPERIMENTAL

Reverse transcriptase-PCR (RT-PCR)

Human submandibular and sublingual gland tissue was obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA, U.S.A.) and total RNA was isolated by the phenol/guanidinium isothiocyanate method, as described in [25]. RNA (1 µg) was reverse-transcribed in a 20 µl reaction mixture containing 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM MgCl₂, 5 mM dithiothreitol, 1 mM dNTPs, RNasin (20 units; Promega, Madison, WI, U.S.A.), random hexamers (1.25 µM; Promega) and murine Moloney leukaemia virus reverse transcriptase (200 units; Gibco-BRL, Gaithersburg, MD, U.S.A.) at 37 °C for 2 h.

PCR was performed using a standard programme in 50 µl reactions containing 20 mM Tris/HCl, pH 8.3, 50 mM NaCl, 2.5 mM MgCl₂, 0.2 mM dNTPs, 1 µl of the reverse transcriptase reaction, and primers (0.2 µM each). The sense primer (5' TCA-CGGTCGGGATCCGGAAGGTCGAGAAAGGGATCAT) contained a *Bam*HI site and nucleotides coding for residues 1–7 of MUC7. The antisense primer (5' ATAATTCTCGAGGTC-TTGTGGAGCTGGGAAT) contained an *Xho*I site and nucleotides coding for residues 138–144 of MUC7. PCR products were electrophoresed on 1% (w/v) agarose TAE (Tris/acetic acid/EDTA, pH 8.5) gels containing 0.1% ethidium bromide, and PCR products were gel-purified and ligated into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA, U.S.A.). The resulting vector (pCMuc7) was used to transform *E. coli* JM 109 (Novagen, Madison, WI, U.S.A.), and plasmid DNA was isolated and the insert was sequenced in both directions by the dideoxy method using universal primers.

Construction of expression vector and production of recombinant protein

The insert in pCMuc7 was released by digestion with *Bam*HI and *Xho*I, gel-purified and ligated into the expression vector pET23b(+) (Novagen) to generate pNMuc7. This plasmid was used to transform *E. coli* JM 109, and the plasmid was isolated and sequenced to verify cloning in the correct reading frame with respect to the T7 promoter.

For expression studies, pNMuc7 was used to transform *E. coli* BL21DE(3) (Novagen), and a colony containing this plasmid was inoculated into 10 ml of LB broth supplemented with 100 µg/ml ampicillin and grown overnight. An aliquot of the overnight culture (200 µl) was used to inoculate each of ten 250

ml Erlenmeyer flasks containing 100 ml of LB/ampicillin, and cultures were incubated at 37 °C with shaking at 225 rev./min. When the D_{600} reached 0.8–1.0, isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1 mM and cultures were incubated for an additional 3.5 h at 37 °C with shaking at 180 rev./min. A 1 ml portion of cell suspension from one flask was centrifuged (10000 g for 5 min), and the pellet was used for gel electrophoresis and Western blotting experiments. The remainder of the cells from the ten flasks were combined, centrifuged (3000 g for 20 min) and used for affinity purification of the recombinant protein rNMUC7. A control experiment was carried out with a 100 ml culture of BL21DE(3) cells containing empty vector pET23b(+), and 1 ml of the cell suspension was processed and examined as described above.

Purification of recombinant protein

Cells from ten 100 ml cultures were collected by centrifugation (3000 g for 20 min), suspended in 40 ml of ice-chilled binding buffer (20 mM Tris/HCl, pH 7.9, containing 500 mM NaCl) containing 5 mM imidazole and disrupted in 10 ml aliquots with the aid of a Branson W185 sonic oscillator (Branson, Danbury, CT, U.S.A.) in six 20 s bursts at a setting of 7. The broken-cell preparations were centrifuged at 39000 g for 30 min at 4 °C and the supernatant (containing rNMUC7) was incubated with 2 ml of nickel affinity resin (Novagen) equilibrated in binding buffer. After incubation overnight at 4 °C on a vertical tube rotator, the slurry was poured into a column and the resin was washed with 12 ml of binding buffer containing 5 mM imidazole and then with 12 ml of binding buffer containing 80 mM imidazole. The recombinant protein was eluted with 12 ml of the same buffer containing 1 M imidazole, and the sample was dialysed against distilled water and lyophilized. The dried residue was dissolved in 1 ml of binding buffer minus imidazole and chromatographed in 200 µl aliquots on a Superose 12 column equilibrated in the same buffer using a Pharmacia FPLC system. Purified recombinant protein (rNMUC7) from five runs was pooled, dialysed, lyophilized and subjected to further analysis.

Analysis of purified recombinant protein

Aliquots of rNMUC7 were hydrolysed in 6 M HCl at 110 °C for 22 h and hydrolysates were examined on a Beckman System 6300 amino acid analyser. Another aliquot was subjected to 20 cycles of automated Edman degradation on an Applied Biosystems 476A protein sequencer. SDS/PAGE was performed on 12% (w/v) acrylamide gels [26], which were stained with Coomassie Brilliant Blue. rNMUC7 in duplicate gels was subjected to Western transfer on to a nitrocellulose membrane, and blots were probed with monoclonal antibodies (diluted 1:3000) against poly-histidine (Sigma, St. Louis, MO, U.S.A.) and rabbit polyclonal antibodies (diluted 1:2000) against a peptide corresponding to amino acids 47–63 in the N-terminal region of MUC7 [24]. The second antibody was either alkaline phosphatase-conjugated goat anti-(mouse IgG) (1:7500; Promega) or alkaline phosphatase-conjugated goat anti-(rabbit IgG) (1:7500; Promega). Colour development was with Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate (Promega) according to the manufacturer's protocols.

For bacterial binding assays (see below), 1 mg of rNMUC7 was subjected to reductive methylation [27] under mild conditions to yield RM-rNMUC7, and half of the reductively methylated protein was reduced and alkylated to yield RM/RA-rNMUC7, as described previously [24]. In a separate experiment, 0.5 mg of purified rNMUC7 was reduced and alkylated to generate RA-

rNMUC7. The BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, U.S.A.) was used for routine quantification of covalently modified recombinant protein using known amounts of rNMUC7 as a standard.

Bacterial binding assays

Streptococcus mutans cells (strains ATCC 25175 and ATCC 33402) were grown in 5 ml of Todd–Hewitt broth (Difco, Detroit, MI, U.S.A.) supplemented with 1% (w/v) yeast extract, 1% (w/v) dextrose, sodium carbonate (0.4 g/l), L-cysteine hydrochloride (0.4 g/l), haemin (10 mg/l) and menadione (0.5 mg/l). Cultures were incubated statically at 37 °C for 16 h. Cell cultures were centrifuged at 2000 g for 20 min at 4 °C, pellets were washed ($\times 2$) in 2.1 mM sodium phosphate, pH 7.3, 36 mM NaCl and 1.0 mM CaCl₂ {simulated saliva buffer (SSB); see [28]}, resuspended in SSB and adjusted to an A_{600} of 2.0.

Microcentrifuge tubes were preincubated with SSB containing 0.05% Tween-20 for 1 h at 37 °C to minimize non-specific binding of bacterial cells and test proteins. Binding assays were carried out in treated tubes by mixing cell suspensions (500 μ l) and protein samples (50 μ l, containing 3 μ g of rNMUC7) in SSB and incubating mixtures for 1 h at 37 °C on a vertical tube rotator. After incubation, tubes were centrifuged at 12000 g for 10 min at 4 °C. Cell pellets containing bound material were washed ($\times 3$) with SSB, suspended in 30 μ l of Laemmli sample buffer, heated at 95 °C for 5 min and subjected to SDS/PAGE on 12% (w/v) polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and blots were probed with monoclonal antibodies against polyhistidine (1:3000) and rabbit polyclonal antibodies against MUC7 (1:2000). The second antibody was alkaline phosphatase-conjugated goat anti-(mouse IgG) (1:7500) or alkaline phosphatase-conjugated goat anti-(rabbit IgG) (1:7500) respectively, and colour development was with Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate. Control binding assays were carried out as above, except that cell suspensions (500 μ l) were mixed with BSA (50 μ l of a 1 μ g/ μ l solution). After incubation, cells were centrifuged (10000 g for 10 min) and washed, and the cell pellets and supernatants were examined by SDS/PAGE followed by staining with silver (Bio-Rad, Hercules, CA, U.S.A.).

In binding studies with rNMUC7, RM-rNMUC7, RM/RA-rNMUC7 and RA-rNMUC7, recombinant proteins (3 μ g each) were incubated with 500 μ l of cell suspension and Western blots were prepared as above. Blots were probed with monoclonal antibodies against polyhistidine (1:3000); the second antibody was horseradish peroxidase-conjugated goat anti-(mouse IgG) (1:10000; Pierce). Blots were incubated with enhanced chemiluminescence reagent (NEN-Life Science, Boston, MA, U.S.A.) and exposed to Kodak film for 1 min.

Candida albicans blastoconidia killing assay

Killing assays were performed with *C. albicans* (ATCC strain 44505) blastoconidia according to the standard protocol available in our laboratory [19]. Briefly, 50 μ l aliquots of cell suspension (10^5 cells \cdot ml⁻¹) in 10 mM sodium phosphate buffer, pH 7.0, were combined with 50 μ l of serially diluted (32–0.12 μ M) rNMUC7 or synthetic histatin 5 in the same buffer in a 96-well microtitre plate, and the plate was incubated for 60 min at 37 °C. At the end of the incubation, wells were washed three times with 10 mM sodium phosphate buffer, pH 7.0, then 100 μ l of molten Sabouraud's dextrose broth (Difco) at 45 °C containing 2% (w/v) agarose was added to each well, and the plate was incubated at 30 °C for 5–6 h. Under these conditions, surviving cells divide and form colonies, while dead cells remain as single cells. A

colony was defined as a cluster of more than five contiguous cells. To estimate killing activity, 100 dead cells or live colonies were counted using an inverted microscope, and the results were expressed as the percentage of killed cells per total number of cells counted. Duplicate wells were examined for both rNMUC7 and histatin 5 at each serial dilution, and cells in each well were counted twice.

RESULTS

RT-PCR procedure

The structural organization of the entire MUC7 apomucin is shown in Figure 1(A). Parallel arrows indicate the positions of PCR primers used to amplify the nucleotide sequence coding for the N-terminal 144 amino acids, which are located upstream of the tandem repeat region (grey). Vertical arrows indicate the positions of the two cysteine residues (Cys⁴⁵ and Cys⁵⁰) and the boundary of the synthetic peptide (His¹⁷ to Lys⁶³) used to generate the polyclonal antibody against MUC7. The diagram of rNMUC7 indicates the position of additional residues at the N- and C-terminal regions contributed by the vector pET23b(+). RT-PCR amplified a 0.45 kb fragment from both submandibular and sublingual gland RNA (Figure 1B, lanes 2 and 3). The PCR product from the submandibular gland was cloned into pCR2.1 to generate pCMuc7, and sequence analysis verified that the insert encoded the N-terminal 144 residues of MUC7 (results not shown).

Expression of the recombinant N-terminal region of MUC7

The insert in pCMuc7 was excised with *Bam*HI and *Xho*I and cloned into the same sites in pET23b(+) to generate expression vector pNMuc7, which codes for the N-terminal 144 residues of MUC7 with a hexahistidine tag at the C-terminus. After sequencing to verify in-frame cloning of the MUC7 cDNA fragment, pNMuc7 was used to transform BL21DE(3) host cells. Pilot experiments conducted to determine conditions for adequate expression showed that the best results were obtained when mid-exponential phase cells were induced for 3.5 h at 37 °C with 1 mM IPTG and rotary shaking at 180 rev./min. Control induction experiments were carried out in the same manner using BL21DE(3) transformed with empty pET23b(+) vector.

An aliquot (1 ml) of cell cultures was centrifuged and proteins in the cell pellet were examined by SDS/PAGE (Figure 2). Gels stained with Coomassie Brilliant Blue showed no prominent band in the control (lane 1), whereas a prominent 24 kDa band was observed in cell lysates containing pNMuc7 (lane 4). Proteins on duplicate gels were transferred on to nitrocellulose membranes and the blots were probed with monoclonal antibodies against polyhistidine and polyclonal antibodies against an epitope in the MUC7 N-terminal region. No immunoreactive band was observed on blots of control cells probed with either antibody (Figure 2, lanes 2 and 3), whereas a strong immunoreactive 24 kDa band was observed with both antibodies on blots of lysates from cells containing pNMuc7 (lanes 5 and 6). These results indicate that the 24 kDa immunoreactive band is rNMUC7, corresponding to the N-terminal region of MUC7. In other pilot experiments it was found that rNMUC7 was present almost entirely in the soluble portion of cell extracts (results not shown).

Purification of the recombinant protein

The hexahistidine tag at the C-terminus of rNMUC7 was utilized for affinity purification of the recombinant protein from crude

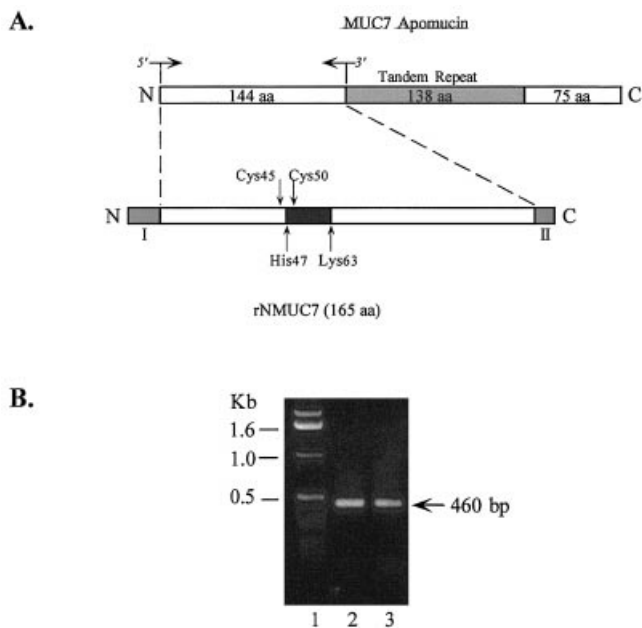


Figure 1 (A) Structural organization of MUC7 apomucin and the recombinant protein rNMUC7, and (B) RT-PCR of the fragment encoding the N-terminal region of MUC7

(A) The locations of the PCR primers used are indicated by horizontal arrows. The locations of cysteine residues and the boundary of the sequence used to prepare the peptide polyclonal antibodies against MUC7 are indicated by vertical arrows. Stippled regions I and II indicate residues contributed by the expression vector pET23b(+). The amino acid sequence of region I is Asp-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg-Asp-Pro, and the amino acid sequence of region II is Leu-Glu-(His)₆. (B) cDNA was synthesized with random hexamers and amplified with MUC7-specific primers. PCR products were electrophoresed on 1% (w/v) agarose/TAE gels and stained with ethidium bromide. Lane 1, 1 kb ladder (Gibco-BRL); lane 2, PCR product from submandibular gland cDNA; lane 3, PCR product from sublingual gland cDNA.

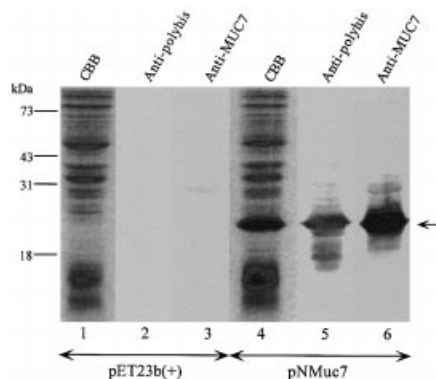


Figure 2 Expression of the recombinant protein

E. coli BL21DE(3) cells containing empty vector pET23b(+) (lanes 1–3) or expression vector pNMuc7 (lanes 4–6) were induced with IPTG for 3.5 h at 37 °C. A 1 ml aliquot of the cell culture was centrifuged, cells were boiled for 5 min in Laemmli sample buffer and the sample was electrophoresed on a 12% gel in the presence of SDS. Gels were stained with Coomassie Brilliant Blue (CBB) or transferred to nitrocellulose, and blots were probed with antibodies against polyhistidine (Anti-polyhis) or antibodies against MUC7 (Anti-MUC7). Lanes 1 and 4, stained gels of cell lysates; lanes 2 and 5, Western blots of cell lysates probed with antibodies against polyhistidine; lanes 3 and 6, Western blots of cell lysates probed with antibodies against MUC7. The arrow on the right indicates the position of the 24 kDa recombinant protein rNMUC7.

cell lysates on a nickel column. rNMUC7 was first affinity-purified, and the resulting preparation was next subjected to gel filtration on a calibrated Superose 12 column, which yielded purified rNMUC7 devoid of detectable bacterial proteins. The elution profile of rNMUC7 from Superose 12 showed a large peak with an apparent molecular mass in the range 18–25 kDa (Figure 3). Pooled fractions containing this protein were analysed by SDS/PAGE under reducing and non-reducing conditions, and gels were either stained with Coomassie Brilliant Blue or blotted on to nitrocellulose and probed with the monoclonal antibodies against polyhistidine or with polyclonal antibodies against MUC7 (Figure 3, inset). Stained gels showed a prominent band of approx. 24 kDa in the absence (lane 1) or presence (lane 2) of β -mercaptoethanol, as well as a faint band of approx. 48 kDa, which probably represents a dimer of rNMUC7. Western blots probed with the anti-polyhistidine antibodies (lane 3) and the anti-MUC7 antibodies (lane 4) also showed a prominent 24 kDa band and a fainter 48 kDa band which reacted with both antibodies (lanes 3 and 4). This strongly suggests that the 48 kDa band represents a dimer of rNMUC7. Finally, an 18 kDa anti-MUC7 immunoreactive band was observed (lane 4); this band probably represents a minor degradation product of rNMUC7. Quantitative amino acid analysis revealed that the yield of purified rNMUC7 from 1 litre of cells was about 3.5 mg in a typical experiment.

Analysis of rNMUC7

In addition to the N-terminal 144 residues of MUC7, rNMUC7 contains 13 residues at the N-terminus contributed by the pET23b(+) polylinker and six histidine residues at the C-terminal end which comprise the tag for affinity purification. The recombinant protein has a calculated molecular mass of 18 509 Da and a calculated pI of 10.15. Automated Edman degradation of rNMUC7 showed that the first 20 residues were identical to those predicted from the cDNA sequence. Amino acid analysis of rNMUC7 showed that the experimentally determined composition was in good agreement with that predicted from the cDNA sequence (Table 1). rNMUC7 has a calculated molecular mass of 18 509 Da, yet exhibits an apparent molecular mass of 24 kDa when examined by SDS/PAGE. This may be explained in part by the high proline composition (14 mol%) that would tend to make the protein adopt a more extended conformation, resulting in a larger apparent size than predicted.

Bacterial binding

S. mutans ATCC 25175 and ATCC 33402 cell suspensions were incubated with purified rNMUC7; after incubation, the bound protein was extracted, electrophoresed, transferred on to nitrocellulose and examined by Western blotting using both monoclonal antibodies against polyhistidine (Figure 4A) and polyclonal antibodies against MUC7 (Figure 4B). The results showed that purified rNMUC7 (24 kDa) bound to both strains of *S. mutans* with comparable affinity (lanes 1 and 3, and lanes 5 and 7). In addition, a 48 kDa immunoreactive band was also observed in the extracts of cells incubated with rNMUC7 (Figures 4A and 4B). Since this band reacted with both antibodies, it seems likely that it is a dimer of rNMUC7, as mentioned above. In the control assays containing bacteria but lacking rNMUC7, no immunoreactive signal was observed (lanes 2 and 4, and lanes 6 and 8). This provides further evidence that the 24 kDa and 48 kDa bands originated from rNMUC7 added to the binding assay and do not represent proteins from bacterial cells. Finally, in another control assay (Figure 4C), *S. mutans* cells were incubated with BSA, and extracts of cell pellets and aliquots of

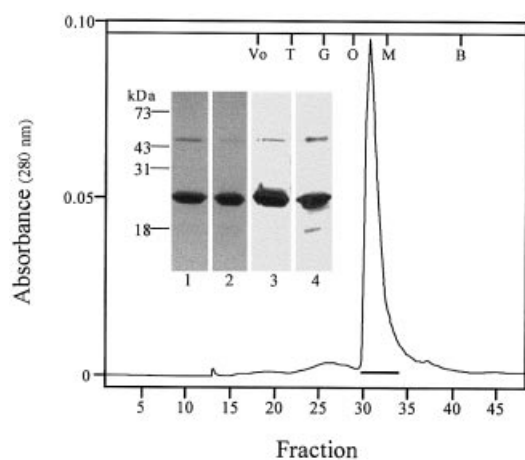


Figure 3 Gel-filtration chromatography of rNMUC7 on Superose 12

The column was calibrated with protein standards of known molecular mass (Da): bovine thyroglobulin (T), 670 000; bovine γ -globulin (G), 158 000; ovalbumin (O), 44 000; horse myoglobin (M), 17 000; vitamin B₁₂ (B), 1350. Cells containing pNMUC7 were induced with IPTG and recombinant protein was isolated on a nickel column. Final purification of affinity-purified rNMUC7 was achieved by gel filtration on a Superose 12 prepacked column. Samples were loaded in 200 μ l aliquots and the column was developed with binding buffer at a flow rate of 1 ml/min, and 0.5 ml fractions were collected. Fractions containing rNMUC7 were pooled as indicated by the bar and subjected to SDS/PAGE (lanes 1 and 2; 5 μ g/lane) and Western blotting (lanes 3 and 4; 1 μ g/lane). Lane 1, protein sample electrophoresed in the absence of β -mercaptoethanol and stained with Coomassie Brilliant Blue; lane 2, sample electrophoresed in the presence of β -mercaptoethanol and stained with Coomassie Brilliant Blue. Lanes 3 and 4 show Western blots of samples electrophoresed in the presence of β -mercaptoethanol and probed with antibodies against polyhistidine and against MUC7 respectively.

Table 1 Comparison of the amino acid composition of rNMUC7 with that predicted from the cDNA sequence

Predicted values are calculated based on the amino acid sequence of the first 144 residues of secreted MUC7 [12] plus 13 residues at the N-terminal end and eight residues at the C-terminal end of rNMUC7 contributed by the expression vector.

Amino acid	Composition (mol%)	
	Measured	Predicted
Asx	7.5	8.5
Thr	6.9	7.7
Ser	8.7	9.1
Glx	9.1	9.1
Pro	16.1	14.0
Gly	3.6	3.6
Ala	4.9	4.8
Cys	1.0	1.2
Val	4.3	4.8
Met	1.4	1.2
Ile	2.1	2.4
Leu	6.4	6.6
Tyr	1.0	1.2
Phe	3.0	3.0
His	11.0	10.3
Lys	7.6	6.1
Arg	6.0	6.1

the suspending medium were electrophoresed and the gels were stained with silver. A prominent 66 kDa band corresponding to BSA was observed in gels of the suspending medium (lanes 10

and 12), whereas this band was not present in gels of cell extracts (lane, 9 and 11). These results show that, under the assay conditions, the control protein BSA did not bind non-specifically to the surface of *S. mutans* cells.

In our earlier studies, we found that when purified MUC7 was reductively methylated, there was no qualitative alteration in its capacity to bind to *S. mutans*, whereas when methylated MUC7 was reduced and alkylated the binding was almost abolished [24]. This suggested that the two cysteine residues in the N-terminal region of MUC7 may be contained within a structural determinant that is necessary for binding. This question was addressed in the present investigation using rNMUC7. An aliquot of rNMUC7 was reductively methylated, and the resulting RM-rNMUC7 was reduced and alkylated to generate RM/RA-rNMUC7. In addition, another aliquot of rNMUC7 was reduced and alkylated to yield RA-rNMUC7. The protein concentration of each sample was determined by BCA assay (Pierce), and the same amounts of rNMUC7, RM-rNMUC7, RM/RA-rNMUC7 and RA-rNMUC7 were incubated with the two strains of *S. mutans*. Bound protein was extracted and analysed in Western blotting experiments. The results showed that rNMUC7 had the greatest affinity for both strains of *S. mutans* (Figure 5, lanes 1 and 5) and that RM-rNMUC7 displayed somewhat decreased, but still appreciable, binding (lanes 2 and 6). However, in assays containing RM/RA-rNMUC7 (lanes 3 and 7) or RA-rNMUC7 (lanes 4 and 8), there was no detectable immuno-signal on Western blots probed with monoclonal antibody against polyhistidine and developed with the highly sensitive enhanced chemiluminescence method, indicating the complete absence of binding. Collectively, these results strongly suggest that MUC7 binding to *S. mutans* occurs via interactions between the bacterial surface and a cysteine-containing binding domain in the N-terminal region of the mucin.

Killing assays

In order to assess the possible role of MUC7 as an anti-fungal agent in the oral cavity, we compared the killing activity of rNMUC7 with that of histatin 5 in a *C. albicans* blastoconidia killing assay. The dose-response curves for rNMUC7 and histatin 5 killing of blastoconidia are shown in Figure 6. rNMUC7 displayed an LD₅₀ of approx. 16 μ M, while histatin 5 displayed an LD₅₀ of approx. 6 μ M, consistent with previous reports from this laboratory [19,20]. However, rNMUC7 showed a greater killing activity than histatin 5 below 2 μ M. Remarkably, rNMUC7 killed 30% of the blastoconidia at a concentration as low as 0.5 μ M. In negative control assays containing phosphate buffer only, the number of live cells was 97–99 per 100 cells counted. These results are the first to show that the N-terminal region of MUC7 possesses *C. albicans* killing activity, and suggest that MUC7 in the oral cavity may serve as an anti-fungal component in the non-immune defence system.

DISCUSSION

Previous studies have reported that MUC7 in salivary secretions could interact with a variety of micro-organisms, such as oral *Streptococci* [29,30], *Pseudomonas aeruginosa* and *Staphylococcus aureus* [5], *Actinobacillus actinomycetemcomitans* [8] and *Eikenella corrodens* [31]. Although MUC7 in salivary secretions binds to different species of bacteria, after purification involving methylation and alkylation highly purified MUC7 loses the ability to bind to these same bacteria [4]. Recently we described a new method for purification of MUC7 from human submandibular and sublingual glands that does not involve covalent modification, and found that the purified mucin retained the

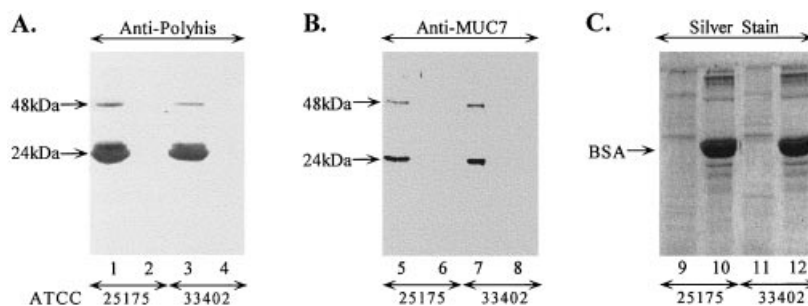


Figure 4 Binding of rNMUC7 or BSA to *S. mutans* ATCC 25175 and ATCC 33402

(A) Cell suspensions were incubated with rNMUC7 (3 μ g) or SSB, and cells were extracted with Laemmli sample buffer, electrophoresed and Western blots were probed with antibodies against polyhistidine (Anti-Polyhis). Lanes 1 and 3, extract of cells incubated with rNMUC7; lanes 2 and 4, extract of cells incubated with SSB. (B) Cell suspensions were treated as in (A) and the blot was probed with antibodies against MUC7. Lanes 5 and 7, extract of cells incubated with rNMUC7; lanes 6 and 8, extract of cells incubated with SSB. (C) Control assay in which cell suspensions of ATCC 25175 and ATCC 33402 were incubated with BSA and centrifuged. Cell pellets were extracted with Laemmli sample buffer and examined by SDS/PAGE, and the gel was stained with silver (lanes 9 and 11). An aliquot of the supernatant was processed as described above, electrophoresed and the gel was stained with silver (lanes 10 and 12).

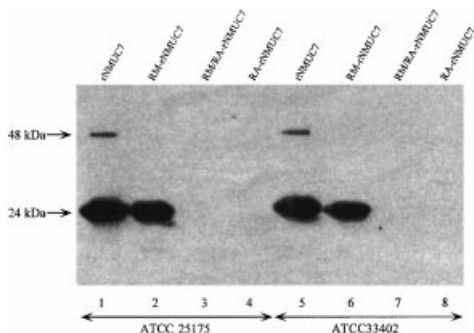


Figure 5 Effects of reductive methylation (RM) followed by reduction and alkylation (RM/RA), or reduction and alkylation alone (RA), on rNMUC7 binding to *S. mutans* ATCC 25175 (lanes 1–4) and ATCC 33402 (lanes 5–8)

Bacterial cell suspensions (500 μ l) were incubated with rNMUC7, RM-rNMUC7, RM/RA-rNMUC7 or RA-rNMUC7 (3 μ g of each in 50 μ l of SSB). After incubation, cells were centrifuged, cell pellets were extracted with Laemmli sample buffer and the samples were electrophoresed and transferred to nitrocellulose. Blots were probed with antibodies against polyhistidine and images were visualized using enhanced chemiluminescence substrate. Lanes 1 and 5, extracts of cells incubated with rNMUC7; lanes 2 and 6, extracts of cells incubated with RM-rNMUC7; lanes 3 and 7, extracts of cells incubated with RM/RA-rNMUC7; lanes 4 and 8, extracts of cells incubated with RA-rNMUC7.

ability to bind to four strains of *S. mutans* [24]. However, when MUC7 purified by this procedure was reduced and alkylated, bacterial binding was significantly decreased, suggesting that the two cysteine residues in MUC7 apomucin, which are located in the N-terminal region, are important for this process. In the present investigation a recombinant protein, rNMUC7, comprising the N-terminal 144 residues of MUC7 was generated, and the intact and covalently modified recombinant protein was used to examine mucin–bacterial interactions. In addition, the antimicrobial properties of rNMUC7 were investigated using a *C. albicaria* blastoconidia killing assay.

The recombinant protein contains a stretch of six histidines at the C-terminal end that facilitates efficient purification by affinity chromatography. The affinity-purified rNMUC7 preparation contained relatively few *E. coli* proteins, which were removed by gel filtration on Superose 12. Since the recombinant protein was contained in the soluble fraction of cell lysates, the affinity purification and gel filtration steps could be performed in the

same buffer without the use of denaturing agents. Although the yield of rNMUC7 (3.5 mg/litre) was relatively low, the occurrence of this protein in the soluble fraction of cell lysates allowed rapid purification under non-denaturing conditions, which yielded sufficient material to investigate the bacterial binding and the anti-candidal properties of this portion of the mucin molecule.

Western blots of cell lysates probed with antibodies against polyhistidine or against MUC7 showed a single immunoreactive band with a molecular mass of 24 kDa. However, stained gels and Western blots of purified rNMUC7 showed a strong immunoreactive band of 24 kDa and a less intense band of 48 kDa. This suggests that rNMUC7 formed a dimer during the purification process, and the dimer was observed after gel electrophoresis under reducing and non-reducing conditions. Although the mechanism of dimer formation is not known, it is clear from the electrophoretic behaviour of rNMUC7 that dimerization does not involve an intermolecular disulphide linkage. Interestingly, a leucine zipper motif with the sequence Leu-Pro-His-Tyr-Pro-Gly-Leu-Leu (residues 25–32) was predicted in the N-terminal region of MUC7 apomucin [14], and this motif could possibly promote aggregation of monomers as the concentration of rNMUC7 increases during purification. In addition, electron microscopic studies indicated that purified MUC7 formed dimers and tetramers by end-to-end self-association [32], and the behaviour of rNMUC7 observed in the present investigation suggests that self-association may involve the leucine zipper motif in the N-terminal region of the molecule.

The bacterial binding studies showed that rNMUC7 bound to *S. mutans* strains ATCC 25175 and ATCC 33402; when rNMUC7 was reductively methylated, binding was little affected. However, when methylated rNMUC7 was reduced and alkylated, binding was completely abolished. These results confirm and extend our earlier observation that cysteine residues are contained in a structural determinant for binding located in the N-terminal region of MUC7. Reduction and alkylation greatly decreased binding of purified MUC7 to bacterial cells [24], but this modification completely abolished binding of rNMUC7. This suggests that, in the native mucin molecule, the glycans may make a minor contribution to bacterial binding, but that the major determinant is a cysteine-containing domain in the N-terminal region. It has also been reported that binding of MUC7 to a strain of *A. actinomycetemcomitans* was dependent upon a peptide domain, rather than a carbohydrate determinant [8], and

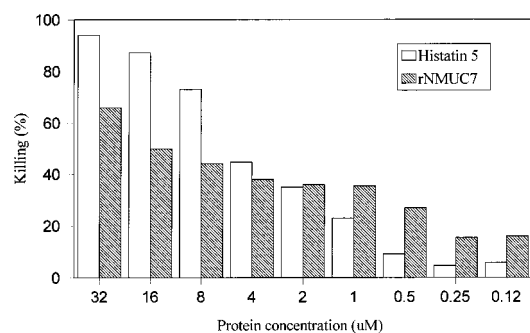


Figure 6 Killing of *C. albicans* (ATCC 44505) blastoconidia by rNMUC7 and synthetic histatin 5

Yeast cells were suspended in 10 mM phosphate buffer, pH 7.4, diluted to 1×10^5 cells/ml and 50 μ l aliquots were added to wells of a 96-well culture plate. Test proteins were dissolved in the same buffer and 50 μ l of each protein solution was added to the wells. Control wells contained 50 μ l of cells and 50 μ l of buffer. Plates were processed as described in the Experimental section, and a total of 100 cells or colonies were counted under an inverted microscope at $\times 400$ magnification. Candidacidal activity was calculated according to the formula: Killing (%) = $[1 - (\text{colonies in treated sample})/(\text{colonies in control})] \times 100$

this is in complete agreement with the results of the present investigation. It is generally accepted that MUC7 in salivary secretions exists as a monomer, whereas other mucins coating epithelial surfaces of the gastrointestinal tract, respiratory tree and urogenital system are large disulphide-linked multimers with apparent molecular masses of $(20-40) \times 10^6$ Da [33]. However, in the present study we observed that both monomeric and dimeric species were present in rNMUC7 preparations after purification, and that both the monomer and the dimer bound to *S. mutans*. The formation of dimers and tetramers of MUC7 has been described [32], and this could be a mechanism for protection against proteolytic digestion in the harsh environment of the oral cavity. Finally, it is not known whether MUC7 in salivary secretions and whole saliva actually forms dimers, tetramers or other multimers, and thus the biological significance of this process remains to be elucidated.

Previous studies have shown that the N-terminal region of MUC7 apomucin contains a 15-residue sequence enriched with arginine and histidine residues (Arg-Glu-Arg-Asp-His-Glu-Leu-Arg-His-Arg-Arg-His-His-His-Gln) which displays 53% sequence identity with histatin 5 [14], and that a synthetic peptide corresponding to this domain exhibited killing activity, albeit less than that of histatin 5 [15]. This suggested that MUC7 may possess anti-candidal activity, and in the present study it was shown that rNMUC7 did indeed exhibit killing activity in an *in vitro* *C. albicans* blastoconidia killing assay. Significantly, rNMUC7 at 0.5 μ M killed 30% of blastoconidia and displayed a 2.5 times higher killing activity than that of histatin 5 at the same concentration. In one of our ongoing studies we have determined that the concentration of MUC7 in whole saliva is in the range 0.5–1.0 μ M (S. A. Rayment, B. Liu, F. G. Oppenheim, G. D. Offner and R. F. Troxler, unpublished work), and this suggests that MUC7 may be an important candidacidal agent in the oral cavity. The anti-candidal properties of MUC7 in the blastoconidia killing assay were not determined in the present work, and this will be addressed in an independent investigation.

Rat (RSMG) and mouse (MSMG) homologues of MUC7 have been characterized and found to display a similar structural organization to that of their human counterpart [34,35]. The apomucin moieties of MUC7, RSMG and MSMG contain 357, 300 and 273 amino acids respectively. Although MUC7 has no

sequence identity with RSMG and MSMG apoprotein, inspection of the N-terminal region of all three mucins reveals some common features. The N-terminal regions of MUC7, RSMG and MSMG possess 144, 104 and 102 residues respectively, and contain the only two cysteines in the molecule. Interestingly, the N-terminal regions of all three mucins have a pI greater than 10.0 and have a similar composition of basic amino acids, although the significance of this basic domain is not known [35]. A recent study using synthetic peptides corresponding to the histatin-like domain in the N-terminal region of MUC7 suggested that candidacidal activity is dependent on electrostatic interactions between the peptide and negatively charged head-groups of phospholipids in the bacterial cell membrane [15]. It should be mentioned in this regard that rNMUC7 contains six histidine residues at the C-terminal end, and our studies did not rule out an involvement of these histidines in the *C. albicans* blastoconidia killing assay. However, the presence of the six histidines decreases the pI of rNMUC7, and on this basis it seems unlikely that these residues play a significant role in the killing assays. The studies described here using recombinant rNMUC7 suggest that the N-terminal regions of RSMG and MSMG may also exhibit similar anti-bacterial and anti-fungal activities.

Human salivary mucin MUC7 has received increasing attention in the past few years as an antimicrobial agent in the oral cavity. In addition, MUC7 has been shown to inhibit infection by the AIDS virus [23], to be expressed at the onset of malignant transformation of the bladder epithelium [36], to be differentially expressed in serous cells of the bronchial airways [37], and to display a unique temporal expression pattern in the fetal respiratory tract [38]. Collectively, these studies demonstrate the functional diversity of MUC7 and point to the importance of this mucin in a broad range of biological contexts.

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