Expression and Purification of Recombinant Human Salivary Apo-MG2

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Ⅰ. INTRODUCTION

Salivary mucins are considered to play a major role in the maintenance of oral health by exhibiting multiple host defense functions. Such functions include (1) acting as a permeability barrier against environmental insult at the tissue-environmental interface; (2) lubricating oral surfaces; (3) concentrating antimicrobial molecules onto oral surface; and (4) modulating colonization of oral bacteria, fungi, and viruses.[1] Two mucin components in human submandibular-sublingual saliva, MG1 (high-molecular-weight) and MG2 (low-molecular-weight), have been identified and characterized on the basis of their biochemical and biophysical properties as well as molecular weight.[2-4] Biochemical studies have been determined that low-molecular-weight human salivary mucin, MG2, is a 125 kDa glycoprotein that is composed of 30.4% protein, 68.0% carbohydrate, and 1.6% sulfate.[2,5] Carbohydrate oligosaccharide side chains, which are primarily O-linked glycans, have been reported to be intimately involved in MG2 bacterial binding functions.[5-8] Chemical and/or enzymatic deglycosylation of mucins can be used for studying specific MG2 oligosaccharide structural requirement for bacterial binding, but the difficulty of complete deglycosylation and significant degradation of the peptide core were problems.[9,10] A potential alternative to the biochemical deglycosylation of MG2 is the employment of molecular biologic techniques to produce recombinant apo-MG2 peptide cores as a tool for studying the oligosaccharide and peptide requirements of MG2 bacterial binding. The gene encoding the protein core of MG2 has been cloned and designated MUC7.[11] Tissue specificity of the MUC7 expression was also reported.[11]

Here, the expression of the recombinant apo-MG2 in *Escherichia coli* and the purification of it by a simple and rapid strategy are reported.

Ⅱ. MATERIALS AND METHODS

1. Construction of the expression plasmids

MUC7 *SfiI/NotI* fragment (~2.4 kbp) from

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purified recombinant λDNA subcloned into pGEM11Zf(+) (Promega, Madison, WI, USA) at the *SfiI/NotI* sites was kindly provided by Dr. M. J. Levine at Dept. of Oral Biology, SUNY at Buffalo. Because initial attempts to purify fusion apo-MG2 expressed using pET-14b/MUC7 by His-Tag affinity chromatography were unsuccessful, another plasmid, pET-3a (Novagen, Madison, WI, USA) was selected for the expression of MUC7 non-fusion peptide. Briefly, the pET-14b (Novagen) containing the MUC7 *NdeI/BamHI* 1.5 kbp fragment (pET-14b /MUC7) was constructed by a PCR approach to modify the 5′ end of the MUC7 cDNA to facilitate subcloning in the correct reading frame into the pET-14b plasmid. A 5′ modified MUC7 primer (5′-GCCATGGCGCATATGGAAGGTCGAGAAAG GGATCAT-3′) containing a 5′ *NdeI* site (underlined), with annealing site just 3′ to the MUC7 cDNA sequence encoding for the predicted signal peptide and λ reverse primer were used for the PCR amplification. Following a 2 min denaturation step at 94℃, 30 cycles of three amplification were performed under the following parameters; denaturation at 94℃ for 15 sec, annealing at 52℃ for 30 sec, and extension at 72℃ for 30 sec. This amplification cycle was followed by an extension step of 6 min at 72℃. The insert orientation and correct frame of pET-14b/ MUC7 were confirmed by dideoxynucleotide chain

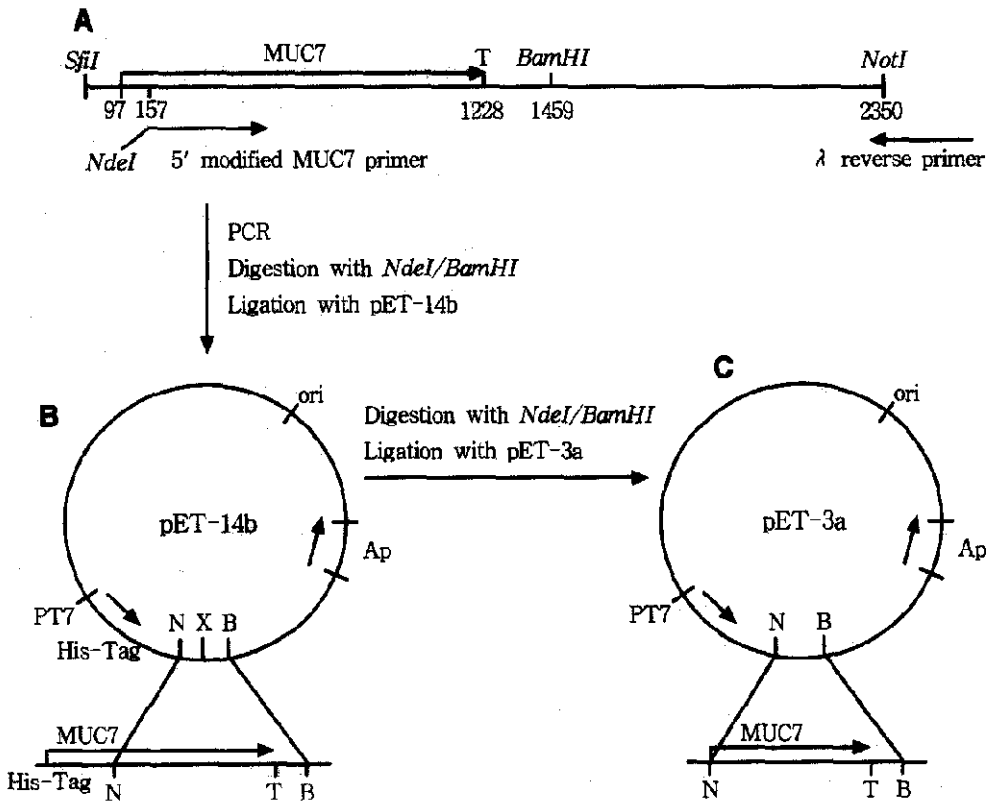

Fig. 1. A: Schematic representation of MUC7 cDNA derived from a MG2 cDNA clone. Numbers below the line indicate the base pair number. B: Schematic representation of construction of pET-14b/MUC7 plasmid. C: Schematic representation of construction of pET-3a/MUC7 plasmid. B: *BamHI*; N: *NdeI*; X: *XhoI*; PT7: T7 promoter; T: termination codon. The figure is not drawn to scale.

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termination sequencing. The pET-3a plasmid was digested with *NdeI* and *BamHI*, and ligated to the *NdeI/BamHI* fragment from pET-14b containing MUC7 gene. The resulting plasmid was named pET-3a/MUC7 (Fig. 1). This construct was used to transform *E. coli* strain BL21(DE3) (Novagen) for expressing the non-fusion protein.

2. Expression of recombinant apo-MG2 in *Escherichia coli*

For expression, 10 ml cultures of strain BL21(DE3) containing pET-3a/MUC7 were grown overnight in liquid Luria-Bertani (LB) medium supplemented with 100 μg/ml of ampicillin at 37℃ in a shaking incubator. The following morning, these starter cultures were added to 1 L cultures (1:100 vol/vol) of LB medium supplemented with ampicillin. These cultures were allowed to grow for about 3 h at 37℃ in a shaking incubator to an OD_{600} of 0.8 - 1.0. IPTG (isopropyl-β-D-thiogalactoside) to a final concentration of 400 μM was added to induce the expression of recombinant apo-MG2 and the cultures were continued at 25℃ in a shaking incubator for additional 2 h. The cells were then harvested by centrifugation and frozen at -70℃. Protein production of the induced and uninduced control culture was analyzed by SDS-PAGE and Western blot transfer using a rabbit antisera to deglycosylated MG2.[10]

3. Purification of recombinant apo-MG2

Frozen induced bacterial cells were resuspended in 50 mM Tris-HCl (pH 8.0), 0.5 M NaCl containing the protease inhibitor cocktail (Sigma Chemical Co., St. Louis, MO, USA). Following suspension, the cells were lysed by adding lysozyme (1.2 mg/ml) and incubating the suspensions at 4℃ for 15 min. The cell suspensions were then subjected to 5 cycles of repeated sonication on ice for 30 sec each with a pulsed microtip until suspensions were no longer viscous and centrifuged at 20,000 xg for 20 min at 4℃. The resulting supernatant which contained solubilized apo-MG2 was harvested. Fractionation by ammonium sulfate precipitation was used as a first step of purification. Solid ammonium sulfate was added to the cell lysate on ice to a final concentration of 25% saturation, equilibrated for 1 h with gentle stirring, and then centrifugated at 22,000 xg for 30 min. The precipitate which contains recombinant apo-MG2, was resuspended with 50 mM Tris-HCl (pH 8.0), dialyzed against deionized distilled water, and lyophilized. This fraction was further purified by reverse-phase high-performance liquid chromatography (RP-HPLC). Briefly, the samples were dissolved in 20% acetonitrile in water, and applied to a Rainin Dynamax-60Å reverse phase C-18 column (4.6 mm × 25 cm) coupled to a guard column (4.6 mm × 5 cm), and eluted with acetonitrile and water (both containing 0.1% trifluoroacetic acid) at a flow rate of 1.5 ml/min. Fractions containing pure samples were pooled and lyophilized.

4. Amino acid composition analysis

The sample was hydrolyzed with 6 N HCl for 24 h at 110℃ in evacuated sealed tubes. Hydrolysates were analyzed with a Beckman model 6300 amino acid analyzer, with norleucine as an internal standard.

III. RESULTS

1. Expression of recombinant apo-MG2

As a first step of obtaining recombinant apo-MG2, we have demonstrated that it can be expressed in *E. coli*. Fig. 2 shows that the expression level of this recombinant form of apo-MG2 is very low, since it can be rarely detected in Coomassie brilliant blue stained gels. But, the result of immunoblot transfer clearly shows that upon induction by IPTG, a protein of apparent molecular weight of about 85 kDa is expressed in *E. coli* strain BL21(DE3) harboring the plasmid pET-3a/MUC7. Apo-MG2 was not detected in uninduced cultures of *E. coli* strain BL21(DE3) transformed with the pET-3a/MUC7 recombinant plasmid.

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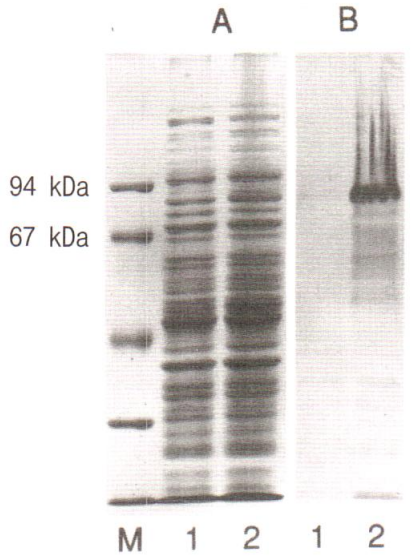

Fig. 2. Panel A) SDS-7.5%PAGE of lysates of *E. coli* BL21 (DE3) cells transformed with recombinant pET-3a/MUC7. The gel was stained with Coomassie brilliant blue. Lane M: molecular weight markers; Lane 1: uninduced cultures of *E. coli* BL21(DE3) cells transformed with recombinant pET-3a/MUC7; Lane 2: IPTG induced cultures of *E. coli* BL21 (DE3) cells transformed with recombinant pET-3a/MUC7. Panel B) Duplicate SDS-7.5% PAGE/Western transfer probed with rabbit antisera to deglycosylated MG2 (1:5,000 dilution).

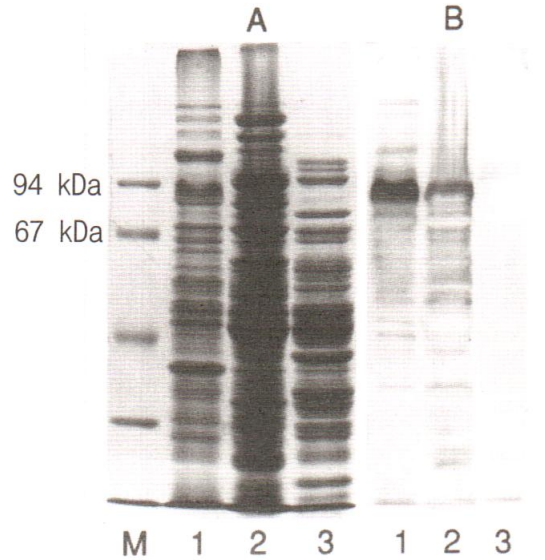

Fig. 3. Fractionation by ammonium sulfate precipitation of lysates of *E. coli* BL21 (DE3) cells transformed with recombinant pET-3a/MUC7. Panel A) SDS-7.5%PAGE stained with Coomassie brilliant blue. Lane M: molecular weight markers; Lane 1: 25% saturation fraction; Lane 2: 25-50% saturation fraction; Lane 3: 50-80% saturation fraction. Panel B) Duplicate SDS-7.5%PAGE/Western transfer probed with rabbit antisera to deglycosylated MG2 (1:5,000 dilution).

2. Purification of recombinant apo-MG2

For purifying non-fusion apo-MG2, fractionation by ammonium sulfate precipitation was tried to remove other bacterial protein before further refined purification steps. Sequential ammonium sulfate fractionation of *E. coli* cell lysates was tried to find the proper range of % saturation which provides maximum fractionation. While most bacterial proteins were precipitated about 50% saturation of ammonium sulfate, almost all apo-MG2 was precipitated with 25% saturation (Fig. 3). Consequently, about 70% of other bacterial proteins were removed and a substantial purification was achieved. RP-HPLC was used as a next purification step. The recombinant apo-MG2, purified by ammonium sulfate precipitation directly followed by RP-HPLC, gave a single peak (Fig. 4) and single band on SDS-PAGE and it was

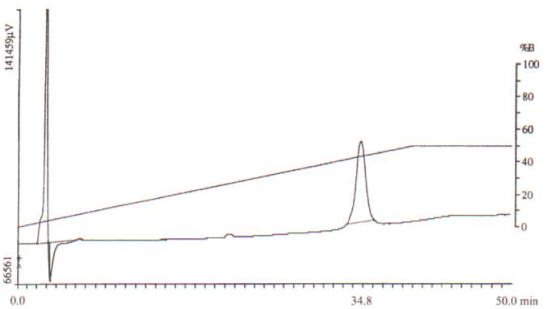

Fig. 4. RP-HPLC profiles

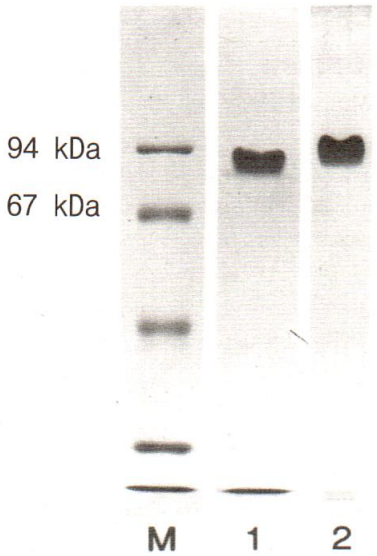

Fig. 5. SDS-7.5%PAGE of RP-HPLC purified recombinant apo-MG2. Lane M: molecular weight markers; Lane 1: Coomassie brilliant blue staining; Lane 2: Duplicate SDS-7.5%PAGE/Western transfer probed with rabbit antisera to deglycosylated MG2 (1:5000 dilution).

highly immunoreactive with rabbit antisera to deglycosylated MG2 (1:5,000 dilution) in Western blot experiment (Fig. 5).

3. Amino acid composition analysis

The apo-MG2 had an amino acid composition that was virtually identical to that of native MG2 and the predicted amino acid composition by the deduced cDNA sequence (Table 1). Considering the low level of expression, the amount of purified protein was satisfactory. Approximately 500 μg recombinant apo-MG2 could be purified from a 1 L of induced *E. coli* culture.

IV. DISCUSSION

The purification of fusion apo-MG2 which has a N-terminal His-Tag fusion area, expressed from *E. coli* containing pET-14b/MUC7 was unsuccessful. Low expression level, low affinity of the fusion protein to His-Tag affinity column, and nonspecific binding of other bacterial proteins were difficulties. For overcoming the problem of very low expression level of apo-MG2, the experiments using several kinds of *E. coli* hosts transformed with plasmids with different promoters were tried, but it was unsuccessful. According to the results of preliminary experiments, it was likely that the low level of expression was mainly because of tandem repeat sequence of MUC7. The toxic effect of expressed apo-MUC7 on *E. coli* growth was observed and this could be regarded as another factor. Fortunately, almost all nonfusion type of apo-MG2 expressed

Table 1. Amino acid composition of recombinant apo-MG2

Residue	Native MG2[a]	cDNA clone[b]	Purified apo-MG2
Asp + Asn	61	56(20)	69(25)
Thr	186	171(61)	166(59)
Ser	105	123(44)	112(40)
Glu + Gln	71	73(26)	68(24)
Pro	230	210(75)	212(76)
Gly	10	8(3)	22(8)
Ala	133	118(42)	137(49)
1/2 Cys	14	6(2)	0(0)
Val	34	31(11)	41(15)
Met	1	6(2)	0(0)
Ile	21	22(8)	22(8)
Leu	46	48(17)	47(17)
Tyr	5	8(3)	0(0)
Phe	17	17(6)	16(6)
His	21	34(12)	17(6)
Lys	23	39(14)	36(13)
Arg	22	31(11)	35(12)
Pro+Thr+Ser+Ala	65.4%	62.2%	62.7%
Total	1000	1000(357)	1000(357)

[a]Amino acid composition of isoform MG2a isolated from saliva[4]

[b]Amino acid composition of apo-MG2 deduced from the nucleotide sequences of cDNA clone[11]

Values in parenthesis are actual numbers of residues.

using pET-3a was precipitated with 25% ammonium sulfate saturation. Consequently, about 70% of other bacterial proteins were removed and a substantial purification could be achieved with the simple single step.

The predicted molecular weight of recombinant apo-MG2 was calculated to be about 37 kDa. However, it migrates in SDS-PAGE as a protein of about 85 kDa, more than twice its predicted size. This dissimilarity between experimental and theoretical molecular weight could be explained based on its amino acid composition. The aberrant apo-MG2 mobility on SDS-PAGE analysis can be explained by its high proline content (21%). Proline residues which determine a rigid conformation in the protein structure, can affect protein unfolding necessary for SDS binding and provide a certain rigidity of the polypeptide structure even in the denatured state.[12,13] Other proline-rich molecules, such as salivary proline-rich proteins (PRPs) and collagen peptides show similar aberrant slow mobilities when separated by SDS-PAGE analysis.[12,14]

The MUC7 gene product has biochemical and structural characteristics of other mucin monomers and can an attractive candidate for answering structure-function questions pertaining to mucins. The apo-MG2 purified by this simple and rapid strategy can be used for that purpose.

V. CONCLUSIONS

We have expressed human salivary low-molecular-weight mucin peptide core, apo-MG2, as an non-fusion form and purified it by a simple and rapid strategy. A MUC7 cDNA containing a complete open reading frame encoding for apo-MG2 was used to express apo-MG2 in a pET-3a/*Escherichia coli* expression system. Fractionation by ammonium sulfate precipitation and reverse phase HPLC were used for purification. The expression level was low and the recombinant apo-MG2 showed an aberrant slow mobility in SDS-PAGE. This recombinant protein provides a useful material for further structure-functional studies pertaining to salivary mucins.

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국문초록

재조합 인체 타액 apo-MG2의 발현과 정제

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고 홍 섭

인체 타액 mucin 중 저분자량 mucin의 peptide core 인 apo-MG2 를 non-fusion 형태로 발현시키고 신속하게 정제할 수 있는 간편한 방법을 제시하였다. MUC7 cDNA와 pET-3a/*Escherichia coli* expression system을 이용하여 apo-MG2를 발현한 다음, ammonium sulfate 침전법을 이용하여 분획을 시행하였으며 reverse phase HPLC로 최종 정제를 실시하였다. Apo-MG2의 발현 수준은 매우 낮았으며 재조합 apo-MG2는 SDS-PAGE 상에서 이동이 느리게 나타나 실제 분자량 보다 큰 분자량을 가진 물질처럼 이동하였다. 인체 타액 MG2가 작은 분자량에도 불구하고 mucin의 특성을 보유하고 있음을 고려해 볼 때, 이러한 방법에 의해 발현, 정제된 apo-MG2는 향후 mucin에 대한 구조적-기능적 연구에 활용될 수 있는 의미있는 물질이 될 수 있을 것이다.

주요어 : 타액, mucin, MG2 (MUC7)

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