Preface

The mucins (mucus glycoproteins) have long been a complex corner of glycoprotein biology. While dramatic advances in the separation, structural analysis, biosynthesis, and degradation have marked the progress in general glycoprotein understanding, the mucins have lagged behind. The reasons for this lack of progress have always been clear and are only now being resolved. The mucins are very large molecules; they are difficult to separate from other molecules present in mucosal secretions or membranes; they are often degraded owing to natural protective functions or to isolation methodology and their peptide and oligosaccharide structures are varied and complex. Understanding these molecules has demanded progress in several major areas. Isolation techniques that protect the intact mucins and allow dissociation from other adsorbed but discrete molecules needed to be developed and accepted by all researchers in the field. Improved methods for the study of very large molecules with regard to their aggregation and polymerization were also needed. Structural analysis of the peptide domains and the multitude of oligosaccharide chains was required for smaller sample sizes, for multiple samples, and in shorter time. In view of these problems it is perhaps not surprising that the mucins have remained a dilemma, of obvious biological importance and interest, but very difficult to analyze.

The driving force behind the production of *Glycoprotein Methods and Protocols: The Mucins* has been the accumulation of novel advances in the ability to analyze mucins reliably and the impact of molecular biology and immunology on the general awareness of mucins as important molecules. This volume is overdue as there is no comprehensive compendium of methods for mucin analysis. It is vital to gather together protocols from those groups who have sorted out the fundamental methods in order that others wanting to use these advances have a reference to follow. In this way *Glycoprotein Methods and Protocols: The Mucins* will make a major contribution in eliminating variation between individual labs and enable the mucin field as a whole to make genuine comparative studies. The range of analytical techniques presented here represents the culmination of the recent advances in the mucin field alluded to above. In several cases this is the result of many years' continuous struggle and it is very satisfying to bring together these new methods in one volume.

The initial problems of mucin analysis were related directly to their purification from secretions and tissues. These methods have been refined to include extraction in denaturing solvents, protection with antiproteolytic agents, and combinations of repeated density gradient centrifugation, gel filtration, ion-exchange chromatography, and electrophoresis, especially in agarose gels (Chaps. 1, 2, 7, and 8). Parallel to these developments have been the efforts to detect and quantify mucins in tissues and in extracts during purification (Chaps. 3–6, 29, and 30); this is still a growing area.

Much of the current knowledge of mucin polypeptide structure has been derived from direct peptide analysis and sequencing (Chaps. 10–13). Confirmation of much of these data and considerably more information with regard to molecular organization and tissue-specific expression patterns has been derived from the molecular biological description of mucin genes (Chaps. 24–28). This has led to the identification of mucin domains, variable number tandem repeat sequences, and new proposals for the way in which mucins are assembled and for their tissue-specific function.

In keeping with the high proportion of carbohydrate typically present in mucins, the latest sensitive methods for the total monosaccharide composition and sequence determination of oligosaccharides is covered (Chaps. 14–16). This is often a large undertaking since the number of individual oligosaccharide chains in a purified mucin is often high (i.e., at least 20–50 structures). Further modifications of the oligosaccharide chains are also common, especially sulfation (Chap. 17), and these additions present their own analytical problems.

The biosynthesis of mucins has been studied in a variety of tissue and cell culture systems. The new developments in separation and mucin gene structure have focused the direction of this work on the design of new specific reagents (Chaps. 18–21). In addition, the glycosylation and sulfation reactions and their inhibition have opened new concepts in the approach to mucin carbohydrate biology (Chaps. 22 and 23).

Study of the degradation of mucins has been hampered by the limited availability of suitable mucin-related substrates. This is still an area of development, one that has benefited from the new information appearing on the detailed structure and organization of the mucins. The concept of a whole "mucinase" activity is also addressed in this volume (Chap. 31) and is backed up by a more detailed consideration of the known members of the total mucin degrading activity (Chaps. 32–34).

One of the most exciting and novel aspects of mucin biology to appear in the last few years has been the interaction of mucins with organisms. This volume would not be complete without these novel concepts concerning bacte-

rial interaction in biofilms (Chap. 36) and the general interactions of bacteria with mucins (Chap. 35).

The cellular and humoral responses to mucins (largely MUC1) has proved to be a major item of interest in cancer biology. As a result it is appropriate that representation of this methodology is also part of the volume (Chaps. 37–41).

The compilation of this practical handbook has been made easier by the trouble taken by the authors to fit their protocols to the format. This volume represents a start in the collection of a reliable and comprehensive collection of methods for the mucin researcher.

Anthony P. Corfield

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Preparation of Membrane Mucin

Kermit L. Carraway

1. Introduction

The first task in this chapter is to define the term *membrane mucin.* In a classical sense, the term is an oxymoron, because mucins were defined as the major glycoproteins of mucous secretions. However, the recognition of the importance of mucinous tumor cell surface glycoproteins and their prominence in the early work on the cloning of mucin has led to a shift in usage ***(1,2)***, in which both secreted and membrane components are recognized as mucins. This usage has led to another complication, in which membrane components with highly *O*-glycosylated mucinlike domains are called *mucins* ***(3,4)***. Such mucinous domains are present in many cell surface molecules, most of which have few of the characteristics of other mucins ***(4)***. For the purpose of this chapter I have assumed a simple definition of membrane mucins. They must exhibit two characteristics: (1) they must be strongly bound to the membrane, and (2) they must have a large, highly *O*-glycosylated domain of mucin. Eventually this definition should evolve to require that membrane mucins contain a defined membrane-binding domain, such as a hydrophobic transmembrane sequence, and mucin repeat sequences. However, application of that requirement at present would exclude epiglycanin, the first membrane mucin to be discovered, for which such information is not available, because it has not been cloned. The present definition still restricts the number of membrane mucins to four examples: epiglycanin, MUC1, sialomucin complex ([SMC], also ASGP-1/ASGP-2 and MUC4), and rat MUC3. Another aspect of these mucins needs to be considered. All three, which have been sufficiently characterized, are found in soluble forms as well as membrane forms. SMC is found in goblet cell secretory granules in the intestine and is secreted via a regulated mechanism ***(5)***. Thus, the term *membrane mucin* is somewhat of a misnomer, although it remains the best descriptor until functional descriptive names become feasible.

Epiglycanin was originally discovered through its implication in the allotransplantability of the Ha subline of the TA3 mouse mammary carcinoma ***(6)***. The allotransplantable Ha subline contained much greater amounts of cell surface sialic acid

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than did the nonallotransplantable St subline. The sialoglycoconjugate was demonstrated to be a glycoprotein by trypsin treatment of the TA3-Ha cells. The released epiglycanin was purified by a single gel filtration step, eluting in the void volume of a Bio-Gel P-100 column ***(7)***. Subsequent analyses of this epiglycanin from metabolically labeled cells by gel filtration on Bio-Gel A-5m showed the presence of two components, which were compositionally similar and appeared to be proteolysis fragments of the same glycoprotein. An extensive series of analyses demonstrated epiglycanin to be a high M_r mucin-type glycoprotein with numerous short oligosaccharides ***(6)***. Proteolysis is frequently used for the identification of cell surface glycoproteins and in some cases can be used for purification. The major advantage is that it eliminates the need for cell lysis or cell fractionation. A major disadvantage is that this method necessarily fragments the protein and results in the loss of the membrane attachment site, a critical feature of membrane proteins. To avoid proteolysis, epiglycanin can also be isolated from ascites fluid ***(8)***, taking advantage of the high-level concentration of the glycoprotein released during cell growth. However, since ascites fluid epiglycanin is a soluble protein, it also does not contain a membrane anchor domain. Two issues have further complicated studies of epiglycanin: the lack of a peptide-specific antibody by which epiglycanin could be unequivocally identified, and the lack of sequence data from either cloning or peptide sequencing by which epiglycanin can be compared with other known mucins. Thus, it is not entirely certain that epiglycanin is not a mouse form of one of the known mucins, as SMC is the rat form of MUC4

MUC1 is one of the two mucins that have been described as components of milk membranes ***(9)***. As such, it was first isolated as the major sialoglycoprotein (GP-2) of bovine milk fat globule membranes (MFGMs) ***(10)***. However, the major interest in MUC1 did not arise until antibodies prepared against defatted human cream fraction from milk were shown to exhibit recognition of human neoplasms ***(11)***. A glycoprotein bearing the antigen, originally called epithelial membrane antigen (EMA), was purified from skim milk by sequential chromatographic methods, with the final step being peanut lectin affinity chromatography ***(12)***. Subsequent studies using human milk, breast tumor cells, or their membranes elicited monoclonal antibodies against the same glycoprotein, which has been variously called DF3 antigen, episialin, epitectin, and polymorphic epithelial mucin ***(9)***. The latter designation arose from its identification with the polymorphic mucin from human urine ***(13)***.

With the development of highly specific antibodies and rapid cloning methods, the strategy for purifying and characterizing mucins has changed substantially, as exemplified for MUC1 ***(14)***. The glycoprotein was purified from human skim milk by immunoaffinity chromatography, deglycosylated with hydrogen fluoride, and used to elicit polyclonal antibodies for screening a cDNA library. Sequencing of these clones initially identified the 20 amino acid repeat, which carries most of the *O*-linked oligosaccharides ***(15,16)***. Subsequent cloning and sequencing characterized the sequence of the remainder of the molecule, including a transmembrane domain and a highly conserved cytoplasmic domain ***(17)***. As the first mucin to be cloned and sequenced, the human gene was assigned the designation *MUC1*. The protein is most frequently called MUC1 or MUC1 protein.

Although cloning methods have greatly enhanced our understanding of mucin structures, they provide an incomplete characterization. Two examples help to illustrate this point. First, biochemical and biophysical studies have shown that the carbohydrate and oligosaccharide composition of MUC1 is tissue and differentiation dependent ***(18)***. Pancreatic tumor MUC1 contains 80% carbohydrate, in contrast to the 50% observed for breast tumors or milk. Changes in MUC1 oligosaccharides result in altered accessibility of the polypeptide to antibodies and differences in antibody recognition of tumor cells compared to their normal counterparts ***(18)***. Second, biosynthesis studies have shown that membrane MUC1 is a heterodimeric glycoprotein, which is cleaved into two subunits during the early part of its transit to the cell surface ***(19)***. Although MUC1 is usually described as a membrane mucin, the purification procedure from skim milk undoubtedly isolated a soluble form. Soluble MUC1 is also released from tumor cells and has been used as a diagnostic serum marker ***(20)***. Two mechanisms have been proposed for the release—alternative splicing and proteolysis ***(2)***; each may be operative in different contexts. An alternative form of MUC1 that is missing the mucin repeats has been described ***(21)***. Obviously, it is not a mucin by my definition.

Ascites sialoglycoprotein-1 (ASGP-1) was first recognized by cell surface and metabolic labeling and proteolysis studies as the major glycoprotein on the cell surface of highly metastatic rat ascites 13762 mammary adenocarcinoma cells ***(22,23)***. It could be rapidly purified from cell lysates or cell membranes by CsCl density-gradient centrifugation in 4 *M* guanidine hydrochloride, and was recognized as a mucin by its large size and high content of *O*-linked oligosaccharides ***(24)***. The membrane association mechanism was identified with a second subunit (ASGP-2) by isolation under nondissociating conditions ***(25)***. Biochemical studies indicated that ASGP-2 is heavily *N*-glycosylated and strongly associated with both ASGP-1 and the ascites cell membranes (**Fig. 1**). Although the two subunits are quite different compositionally, biosynthesis studies indicate that they are derived from a common precursor and single gene ***(26)***. This result was verified by cloning and sequencing, demonstrating the transmembrane domain in ASGP-2 and multiple repeats of ≈125 amino acids in ASGP-1 ***(27,28)***. ASGP-1 overexpression in tumor cells has been demonstrated to reduce their adhesiveness to both cells and the extracellular matrix ***(29)***, providing one explanation for its previous implication in metastasis ***(30)***. Interestingly, ASGP-2 also contains two epidermal growth factor (EGF) domains, both of which have the consensus residues found in such domains exhibiting the ability to act as ligands for ErbB receptor tyrosine kinases ***(27)***. By transfection studies, I and my colleagues have shown that ASGP-2 can form a stable complex with ErbB2, the central member of that family in cellular signaling ***(31)***. In addition, we have proposed that ASGP-2 can modulate ErbB signaling through its interaction with ErbB2, although the mechanism remains uncertain ***(32)***.

SMC is found in a large group of normal epithelial tissues, including the ependymal epithelium of the brain, lactating mammary gland, trachea, oral cavity, intestine, cornea, and uterus ***(33)***. In contrast to the 13762 ascites cells, which contain predominantly membrane SMC, most of these tissues express both membrane and soluble

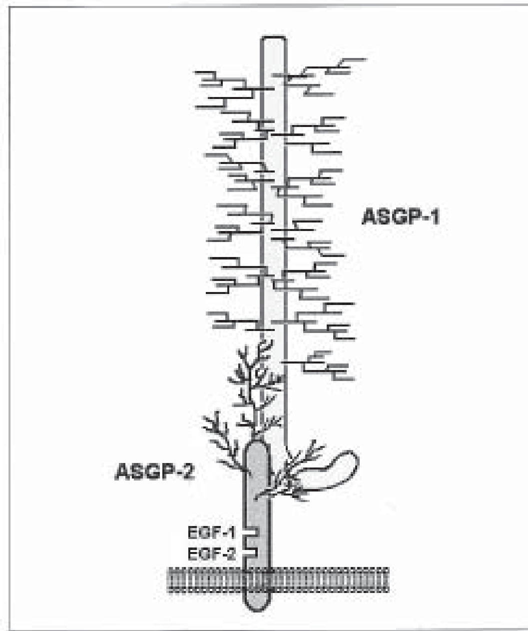


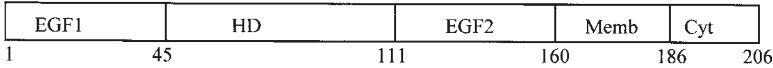
Fig. 1. Model for the structure of SMC. Generically, this model also applies to MUC1, which has both a mucin and a transmembrane subunit synthesized from a precursor encoded by a single gene.

forms ***(33)***. An exception is the intestine, in which SMC is predominantly in a soluble form ***(5)***. SMC in the intestine is intracellular instead of at the apical surface of the epithelium, where it is found in many other tissues ***(33–35)***. These results suggest that SMC provides multiple functions for the protection of the epithelium. Possibilities include acting as a classical secreted mucin, serving as a membrane-blocking (antiadhesive) agent, and modulating cell survival and proliferation in damaged epithelia through its interaction with ErbB2.

Rat Muc3 was recently identified as a potential membrane mucin by cloning and sequencing ***(36)***. The original clones were isolated from a cDNA library screened with antibody prepared against deglycosylated rat mucin isolated from rat mucosa by sequential gel filtration and CsCl gradient centrifugation ***(37)***. Interestingly, Muc3, like ASGP-2, has two EGF-like domains, one of which is a juxtamembrane domain similar to EGF-2 of ASGP-2 (**Fig. 2**). Furthermore, direct sequence comparisons show that the EGF domains from these different mucins have a substantial number of amino acid identities—eight cysteines rather than the usual six for EGF domains—a similar, but not identical spacing of the cysteine residues, and a similar spacing between the last cysteine and the putative transmembrane domain. Now that the C-terminal peptide sequence is known, it will be interesting to see whether a membrane form can be detected on intestinal cells and isolated in intact form. In contrast to rat Muc3, human

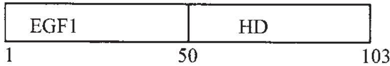
A

ASGP-2



RMuc3

HMUC3



B

EGF2

DGVTCVSPCSEGY----CHNGGQCKHLPDGPQCTCATFSIYTSWGERCEHLSVKLGAFF ASGP-2
NKWYCVTPCSSGYSTSKNCSYGKCQLQRSGPRCLCLSTDTNWYSGENCDWGTQKSLVYG RMuc3

EGF1

CACLPGFSGDRCQLQTR---CQNGGQWDGLKCQCPSTFYGSSCDFAV HMUC3
CVCPNGFSGDRCQNRVPVVDCQNGGTWDGLKCQCTGLFYGPRCEEVM RMuc3
SEFCQNHSCPVNYCYNHGHCDISGPPDCQPTCTCAPAFTGNRCFLAG RASGP-2

Fig. 2. Comparisons of the C-terminal domains of ASGP-2, rat Muc3, and human MUC3, **(A)** domain organizations; **(B)** sequence comparisons between EGF-2 domains of rat ASGP-2 and rat Muc3, and sequence comparisons between the EGF-1 domains of rat ASGP-2, rat Muc3, and human MUC3. Since the full sequences of the MUCs are not known, position 1 for all sequences has been assigned strictly for comparisons. Likewise, the EGF domain sizes are only estimates. Sequence identities are double underlined. All cysteines are shown in bold.

MUC3 appears not to be a membrane protein. The recently published sequence of human MUC3 *(38)* suggests that it is truncated at the C-terminus compared to the rat analog, missing the juxtamembrane EGF, transmembrane, and cytoplasmic domains. Human and rat MUC3 do have one highly similar EGF-like domain (**Fig. 2**), suggesting that it serves some function in the mucins.

Membrane mucins can be isolated either by classical biochemical techniques or by immunoaffinity methods. The former most appropriately takes advantage of specific attributes of mucins—their large size and high density—to simplify purification. Regardless of which approach is used, I would argue that membrane mucins should be isolated for characterization from membranes or cells. Furthermore, they should be isolated under the least dissociating conditions feasible. Characterization of soluble forms of these molecules provides useful information but yields an incomplete story and may actually hinder progress. For example, isolation of the membrane form of epiglycanin might have yielded unglycosylated peptide that was more amenable to

producing peptide-specific, instead of carbohydrate-specific, antibodies. Alternatively, it might have provided peptides for sequencing as a step toward cloning. Similarly, isolation and characterization of intact MUC1 should give information about its transmembrane subunit, which is still lacking and might be useful in understanding MUC1 functions, particularly in normal epithelia. After all, it is the functions of these molecules that are most important, yet still inadequately understood.

2. Materials

1. Ascites cells and tissues: TA3-Ha ascites mouse mammary carcinoma cells grown in strain A mice for isolation of epiglycanin; MAT-B1 or MAT-C1 ascites sublines of 13762 mammary adenocarcinoma cells grown in female Fischer 344 rats for isolation of ASGP-1 or SMC; rat tracheal tissue snap-frozen in N_2.
2. Cultured cells for isolation of MUC1: H.Ep.2 cells were maintained as monolayers or in suspension culture in Eagle's minimum essential medium supplemented with glutamine, sodium pyruvate, nonessential amino acids, and 10% (v/v) heat-inactivated fetal calf serum. At confluence the cells were harvested by versene treatment, centrifuged at 1000g, and washed with phosphate-buffered saline (PBS).
3. Bovine milk or human milk for isolation of MUC1.
4. Gel filtration columns: BioGel P-100, G-200, Sepharose 6B, and Sepharose CL-2B (Amersham Pharmacia Biotech, Piscataway, NJ).
5. Immunoaffinity columns: anti-MUC1 HMFG-1 on protein A-Sepharose, anti-MUC1 Ca1 or bovine γ-globulin coupled to Sepharose 4B with cyanogen bromide ***(39)***, anti-ASGP-2 on Immunopure® Protein A IgG Orientation Kit (Pierce, Rockford, IL).
6. Other chromatography columns: hydroxyapatite column (Bio-Gel HT, BioRad, Hercules, CA), carboxymethyl-Sephadex G-25, peanut lectin (Sigma, St. Louis, MO) immobilized on Sepharose 4B.
7. CsCl gradients: Preformed CsCl/guanidine gradients were prepared by gently layering 2 mL each of 4 *M* guanidine hydrochloride in 10 m*M* Tris-HCl, pH 7.4, containing 1.58, 2.0, 2.37, 2.79, and 3.15 *M* CsCl. CsCl/Triton gradients were formed by successively underlaying five CsCl solutions of 1.3, 1.35, 1.40, 1.45, and 1.5 g/mL densities buffered with PBS containing 0.2% Triton X-100.
8. Collodion bags for vacuum dialysis/concentration.
9. Tosylphenylalanine chloromethyl ketone-trypsin (Sigma) for proteolysis.
10. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) apparatus for electrophoretic analyses; Coomassie blue for staining proteins; periodate-Schiff reagent for staining glycoproteins; immunoblot transfer apparatus and chemiluminescence reagents for detection of antigens.
11. RIPA buffer for immunoprecipitation: 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 m*M* Tris-HCl, pH 8.0.
12. Protease inhibitors: 0.5 m*M* EDTA, 1 m*M* phenylmethylsulfonylfluoride, 1 kIU/mL aprotinin, 1 m*M* leupeptin, 1 m*M* pepstatin.

3. Methods

3.1. Purification of Epiglycanin by Proteolytic Release (7)

1. Treat washed TA3-Ha cells (1 to 2×10^9 cells in 20 mL) in balanced salt solution with 18 μg of TPCK-trypsin at 0–4°C for 20 min with rotation. Repeat each treatment five to seven times, combine the batches, centrifuge at 20,000*g* to remove cellular debris, and lyophilize.

2. Purify by gel filtration on Bio-Gel P-100 at 4°C using 0.05 *M* pyridine acetate (pH 5.3) as the eluent.
3. Assay fractions for epiglycanin with peanut lectin blots.

3.2. Purification of Epiglycanin from Ascites Fluid (8)

1. Collect ascites fluid from mice on d 7 after tumor cell injection and centrifuge sequentially at 80 and 37,000*g* to remove cells and cellular debris, respectively.
2. Treat supernatant with perchloric acid by dropwise addition at 0°C to a concentration of 0.25 *M*. After 20 min remove the precipitate by centrifugation at 10,000*g* and neutralize with 2.5 *M* $KHCO_3$. Remove insoluble K_2ClO_4 by centrifugation at 10,000*g*, and concentrate supernatant by vacuum dialysis in collodion bags against 0.05 *M* pyridine acetate, pH 5.3.
3. Fractionate the concentrated material on Sepharose 4B.

3.3. Preparation of GP2 (Bovine MUC1) from Bovine Milk Fat Globule Membranes (10) (see *Note 1*)

1. Prepare MFGMs from cream fraction of bovine milk by a freeze-thaw procedure to release the membranes from lipid globules, followed by centrifugation at 40,000*g* for 1 h.
2. Solubilize MFGMs (10 mg protein/mL) in 1% SDS, 10 m*M* phosphate buffer (pH 6.4), and 1% mercaptoethanol and dialyze overnight against 0.1% SDS in the phosphate buffer with 2 m*M* mercaptoethanol.
3. Fractionate the sample on Bio-Gel HT, eluting with a gradient of phosphate buffer.
4. Purify GP2-containing fractions further by gel filtration on Sephadex G-200 or Sepharose 4B.
5. Assay GP2 by SDS-PAGE and periodic acid-Schiff (PAS) staining.

3.4. Biochemical Purification of EMA (MUC1) from Human Milk (12) (see *Note 2*)

1. Fractionate human skim milk by sequential precipitations with 40 and 80% saturated ammonium sulfate.
2. Dissolve the latter precipitate in distilled water, dialyze against water, bring to 1% Triton X-100, and fractionate on Sepharose 6B.
3. Extract EMA-containing fractions with chloroform-methanol (2:1).
4. Dialyze the aqueous phase against water, concentrate, bring to 0.01 *M* acetate, pH 6.0, and fractionate on carboxymethyl–Sephadex G-25.
5. Purify the eluted fractions containing EMA further by peanut lectin affinity chromatography.

3.5. Immunoaffinity Purification of MUC1 from Human Skim Milk (14) (see *Notes 3 and 4*)

1. Pass human skim milk through an affinity column of anti-MUC1 MAb HMFG-1 on protein A-Sepharose.
2. Wash the column with PBS and elute with 0.1 *M* glycine (pH 2.5).

3.6. Immunoaffinity Purification of Epitectin (MUC1) from H.Ep.2 Cells (39) (see *Notes 4 and 5*)

1. Suspend washed cells (approx 10^8) in 20 mL of 10 m*M* Tris-HCl, pH 8.0, and 0.2% sodium deoxycholate with protease inhibitors and break by passage several times through a 19-gage syringe needle.
2. Stir the lysate for 1 h at 4%C and centrifuge at 60,000*g* for 30 min.

3. Heat the supernatant in boiling water for 10 min, recentrifuge to remove denatured proteins, and apply to tandem affinity columns of bovine γ-globulin and anti-MUC1 (Ca1) antibody.
4. Wash the column with 0.5% deoxycholate-1% Triton X-100 and elute with 3 *M* KSCN.

3.7. Isolation of ASGP-1 from Ascites Cell Membranes (40)

1. Suspend washed ascites cells in 10 vol of 10 m*M* Tris HCl, pH 8.0, and keep at 0ºC for 2 min prior to centrifugation at 600*g* for 2 min.
2. Suspend the pellet of swollen cells in 10 vol of the same buffer and homogenize by four to five strokes of a Dounce homogenizer with a tight pestle.
3. Bring the suspension immediately to a concentration of 3 m*M* in Mg^{2+} by addition of 30 m*M* $MgCl_2$, and 10 m*M* NaCl.
4. Centrifuge the homogenate successively at 1000*g* for 1 min and at 10,000*g* for 10 min.
5. Collect membrane vesicles by centrifugation at 100,000*g* for 90 min.
6. Suspend vesicles in 4 *M* guanidine hydrochloride in 10 m*M* Tris, pH 8.0, by homogenization.
7. Layer 1.0 mL (≈5 mg of protein) onto the CsCl/4 *M* guanidine hydrochloride gradient and centrifuge in a Beckman 75 Ti rotor at 4ºC for 16–24 h at 55,000 rpm (or another rotor at approx. 100,000*g*).
8. Collect fractions and assay for ASGP-1 by SDS-PAGE.
9. Pool fractions containing ASGP-1 and remove CsCl and guanidine hydrochloride by dialysis/concentration with a collodion bag apparatus.

3.8. Purification of SMC and ASGP-2 from Ascites Cell Microvilli or Membranes (41)

1. Extract ascites cell membranes (500 µL, ≈5 mg protein) in 3.2 mL of 0.2% Triton X-100, 5 m*M* glycine, 2 m*M* EDTA, pH 9.5, for 15 min at room temperature.
2. Load the extract onto a two-phase gradient in which the upper phase consists of 1.0 mL of 0.2% Triton X-100/PBS, pH 7.4, in 4% sucrose, and the lower phase consists of 0.5 mL of 2% SDS/30 m*M* imidazole, pH 7.4, in 10% sucrose.
3. Centrifuge the gradient at 100,000*g* for 1 h in an SW50.1 rotor.
4. Dialyze/concentrate the upper layer, which is enriched in detergent-soluble membrane proteins and SMC, and apply to a discontinuous CsCl/Triton density gradient.
5. Centrifuge the gradients in an SW28 rotor at 100,000*g* for at least 40 h at 4˚C.
6. Analyze the gradient fractions for protein and SMC by SDS-PAGE.
7. Pool fraction(s) containing SMC free of lower molecular weight protein contaminants from the CsCl density gradient centrifugation step and dialyze/concentrate against 6 *M* guanidine-HCl, 5 m*M* dithiothreitol, 0.2% Triton X-100, 10 m*M* Tris-HCl, pH 8.0 (complex dissociating buffer).
8. Concentrate to 0.5–1.0 mL, layer onto a column (1.0 × 40 cm) of Sepharose CL-2B, and elute with complex dissociating buffer.
9. Collect fractions and analyze for protein and ASGP-2 by SDS-PAGE.

3.9. Immunoaffinity Purification of Membrane and Soluble Forms of SMC from Rat Trachea (34) *(see Note 6)*

1. Collect rat tracheas from adult Fischer 344 female rats, snap-freeze in liquid N_2, and pulverize with a mortar and pestle.
2. Solubilize powdered tissues in RIPA buffer/proteinase inhibitors and homogenize with a probe sonicator.

3. Centrifuge the lysate at 2000*g* and use the supernatant for affinity purification.
4. Prepare affinity columns with anti-ASGP-2 or anti-C-pep polyclonal antisera (antipeptide Ab elicited with cytoplasmic domain of rat ASGP-2) and goat antimouse IgG1 (heavy chain specific)-agarose or with anti-ASGP-2 MAb 13C4, following the supplied protocols.
5. Fractionate first on the anti-C-pep column, collecting the soluble form in the flowthrough.
6. Elute column with acid (0.1 *M* glycine, pH 2.8) to obtain the membrane form of SMC.
7. Purify the soluble form further on the anti-ASGP-2 column and elute with acid.
8. Add Triton X-100 to both eluents to a final concentration of 0.05%.
9. Neutralize eluents with 1 *M* Tris (pH 9.5), dialyze against water, and analyze by SDS-PAGE and immunoblotting.

4. Notes

1. This method yields the mucin subunit of MUC1, which is the major glycoprotein detected in the bovine MFGM by PAS staining.
2. This procedure will give purified human MUC1, presumably containing both the mucin and transmembrane subunits, although this has not been established.
3. This procedure yields purified MUC1 mucin subunit. Whether part of the transmembrane subunit is included in this soluble material is unclear, because the form of MUC1 in the skim milk fraction has not been characterized.
4. Other anti-MUC1 antibodies can be used but must be tested for reactivity to the source material, e.g., by immunoprecipitation or immunoblotting.
5. This procedure may yield both mucin and transmembrane subunits of MUC1, although this has not been determined. The stringency of the sample preparation, including a heat step in deoxycholate, may cause subunit dissociation.
6. Anti-SMC antibodies are available from K. L. Carraway for appropriate projects.

Acknowledgments

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