

Delineation of pulmonary airway fluid protein fractions with HRPO binding-avidity by far-Western ligand blot and mass spectrometry analyses: a model methodology for detecting mannose-binding protein expression profiles

Cody P. Coyne¹, Ann Rashmir-Raven², Toni Jones¹, Cathleen Mocha², Robert L. Linford², Michael Brashier², Alison Eddy²

Department of Basic Sciences¹, and Department of Clinical Sciences², College of Veterinary Medicine at Wise Center, Mississippi State University, Mississippi State, Mississippi 39762. The current address for Dr. Rashmir-Raven is the Department of Clinical Sciences, College of Veterinary Medicine, Michigan State University, East Lansing, Michigan.

Abstract. *Background and aim of work:* Limited research to date has characterized the potential for HRPO to function as a primary molecular probe. *Methods:* Pulmonary airway fluid was developed by non-reducing far-Western (ligand) blot analyses utilizing conjugated HRPO-streptavidin or non-conjugated HRPO without the presence of primary immunoglobulin. Endogenous esterase-like biochemical activity of fractions within pulmonary airway fluid was inactivated to determine if they were capable of biochemically converting HRPO chemiluminescent substrate. Complementary analyses modified pulmonary fluid and HRPO with β -galactosidase and α -mannosidase respectively, in addition to determining the influence of mannose and maltose competitive binding on HRPO far-Western (ligand) blot analyses. Identification of pulmonary fluid fractions detected by HRPO far-Western blot analyses was determined by mass spectrometry. *Results:* Modification of pulmonary fluid with β -galactosidase, and HRPO with α -mannosidase in concert with maltose and mannose competitive binding analyses altered the intensity and spectrum of pulmonary fluid fractions detected by HRPO far-Western blot analysis. Identity of pulmonary airway fluid fractions detected by HRPO far-Western (ligand) blot analysis were transferrin, dynein, albumin precursor, and two 156 kDa equine peptide fragments. *Conclusions:* HRPO can function as a partially-selective primary molecular probe when applied in either a conjugated or non-conjugated form. Some protein fractions can form complexes with HRPO through molecular mechanisms that involve physical interactions at the terminal α -mannose-rich regions of HRPO glycan side-chains. Based on its known molecular composition and structure, HRPO provides an opportunity for the development of diagnostics methodologies relevant to disease biomarkers that possess mannose-binding avidity. (www.actabiomedica.it)

Key words: Pulmonary fluid, horseradish peroxidase, far-Western (ligand) blot, glycoprotein, mannose, maltose

Introduction

Horseradish peroxidase (HRPO) is a biochemically active glycosylated protein of plant-origin (1-4). Although the biochemical properties of HRPO and

the composition of its carbohydrate moieties have been delineated, the interaction of this glycoprotein enzyme with protein fractions of plant and mammalian origin have been infrequently characterized (5-7). Though not nearly as complex in composition as

plasma or whole cell lysates, pulmonary airway fluid was analyzed to determine if it contains fractions that physically interact and possess HRPO binding-avidity. Analysis of pulmonary airway fluid by Western blot analysis for surfactant proteins SP-A and SP-D utilizing HRPO conjugated to strepavidin as a secondary molecular probe produced profiles that were highly analogous to those recognized by non-conjugated HRPO far-Western (ligand) blot analysis.

Several molecular mechanisms for the positive chemiluminescence of pulmonary airway fluid fractions detected by far-Western (ligand) blot analyses utilizing either non-conjugated HRPO or conjugated HRPO-strepavidin were characterized in a series of complementary investigations. The identity of pulmonary airway fluid fractions detected by HRPO far-Western (ligand) blot analysis was then subsequently determined by mass spectrometry analysis.

Materials and methods

Equine pulmonary fluid sample collection

Pulmonary airway fluid was obtained from the lower respiratory tract of horses post-mortem without contamination from the upper respiratory tract or oral cavity by introducing an endotracheal tube through a tracheostomy and advancing its tip to the level of the tracheal bifurcation.

Slight elevation of the middle and rear torso enhanced harvest of between 3 to 5 ml of pulmonary airway fluid from the entire bronchial airway which was collected into a sterile glass beaker chilled on ice that contained a protease inhibitor formulation. Pulmonary airway fluid was then centrifuged at 200 x g for 30 minutes and the resulting cell-free supernatant harvested manually by pipette. Protein concentrations of samples and dilutions of standard reference controls were determined by measuring absorbance at 280 nm (Nanodrop Technologies LLC, Wilmington, DE 19810, USA) followed by transfer of cell-free supernatants into cryovials and preserved frozen at -80°C prior to SDS-PAGE analysis. Horses were only utilized as they became available for harvest of pulmonary fluid immediately post-mortem so research

investigations were officially exempt from review by the university IACUC review board.

Enzyme modification of pulmonary fluid samples to determine if it might influence HRPO binding-avidity involved incubation with β -galactosidase (1 KU in Tris HCl 50 mM, β -mercaptoethanol 5 mM, MgCl₂ 10 mM; Sigma-Aldrich, St. Louis MO, USA) at a final concentration of 25 IU/mg of sample protein. Non-peptide protease inhibitors were simultaneously applied during the 2-hour incubation period at 37°C to suppress proteolytic degradation.

Non-reducing SDS-PAGE development of equine pulmonary fluid proteins

Equine pulmonary fluid samples were adjusted to a standardized protein concentration of 60 μ g/ml before being formulated 50/50 v/v with conventional PAGE sample preparation buffer (Tris/glycerol/bromophenyl blue/SDS) prepared without 2-mercaptoethanol (1-4). Each sample (0.9 μ g/well) was processed without boiling, along with a mixture of reference control pre-stained molecular weight markers (BioRad Laboratories, Hercules CA, USA) which were subsequently developed by non-reducing SDS-PAGE (11% acrylamide; BioRad Laboratories, Hercules CA, USA) performed at 100 V constant voltage (BioRad Laboratories, Hercules CA, USA) and 3°C for 2.5 hours.

Staining of non-reducing SDS-PAGE gels with Coomassie Blue and Ponceau S

Individual acrylamide gels developed by non-reducing SDS-PAGE were initially pre-equilibrated in Tris buffered saline (TBS: Tris HCl 0.1 M, NaCl 150 mM, pH 7.5, 40 ml) and then submerged in either Coomassie Blue (0.1 % in MeOH 40% and acetic acid 1%) or Ponceau-S (0.2% in acetic acid 1%). Coomassie Blue stained non-reducing gels were de-stained in MeOH 50% with acetic acid 1% in combination with gentle horizontal agitation until a clear background was achieved. Similarly, Ponceau-S stained non-reducing SDS-PAGE gels were de-stained in ddH₂O in combination with gentle horizontal agitation until a clear background was achieved.

Lateral transfer of SDS-PAGE size-separated proteins

Non-reducing SDS-PAGE acrylamide gels were equilibrated in tank buffer devoid of methanol. Lateral transfer of SDS-PAGE separated proteins onto sheets of nitrocellulose membrane (BioRad Laboratories, Hercules CA, USA) for Western (immunodetection) and far-Western (ligand) blots was performed at 20 volts constant voltage (BioRad Laboratories, Hercules CA, USA) for 16 hours at 1° to 3°C with the transfer manifold packed in crushed ice.

Western (Immunodetection) and far-Western (ligand) blot analyses

Nitrocellulose membranes with laterally transferred protein fractions for ligand (8-11) and immunodetection (12) methodologies were pre-incubated in Tris buffered saline (TBS: Tris HCl 0.1 M, NaCl 150 mM, pH 7.5, 40 ml) at 4°C for 15 minutes followed by incubation in TBS blocking buffer solution (Tris 0.1 M, pH 7.4, 40 ml) containing bovine serum albumin (BSA 5%, Sigma-Aldrich, St. Louis MO, USA) for 16 hours at 1° to 3°C applied in combination with gentle horizontal agitation. Prior to further processing, nitrocellulose membranes were vigorously rinsed three times in Tris buffered saline (Tris 0.1 M, pH 7.4, 40 ml).

Rinsed BSA-blocked nitrocellulose membranes utilized for Western blot (immunodetection) analyses were developed by incubation in biotinylated rabbit anti-human surfactant protein A IgG (anti-SPA 1:3000 dilution; Millipore-Chemicon, Billerica, MA, USA) or biotinylated rabbit anti-human surfactant protein D IgG (anti-SPD 1:3000 dilution; Millipore-Chemicon, Billerica, MA, USA) at 4°C for 18 hours applied in combination with gentle horizontal agitation. Nitrocellulose membranes were then vigorously rinsed three times in TBS (pH 7.4, 4° C, 50 ml) followed by incubation in blocking buffer (BSA 5% in Tris 0.1 M, pH 7.4, 40 ml). Blocked nitrocellulose membrane were then transferred to TBS (pH 7.4, 4° C, 50 ml) and rinsed three times before incubation with either streptavidin-HRPO (1:100,000 dilution; 10 ng/ml HRPO equivalent, Pierce, Thermo Fisher Scientific, Rockford, IL, USA), or streptavidin-alkaline

phosphatase (1:100,000 dilution in PBS pH 7.4 containing ZnCl₂ 1 mM, MgCl₂ 2 mM, BSA 5%, 50 ml; Millipore-Chemicon, Billerica, MA, USA) at 4°C for 2 hours applied in combination with gentle horizontal agitation. Preparations of HRPO and streptavidin-HRPO were diluted 1:100,000 in buffer in order to eliminate the occurrence of "burn-out" signal images detected by chemiluminescent autoradiography in accord with suggested guidelines of the vendor for streptavidin-HRPO conjugates. Nitrocellulose membranes were then rinsed again three times in Tris buffered saline (Tris 0.1 M, pH 7.4, 40 ml) prior to chemiluminescent autoradiography.

Rinsed nitrocellulose membranes blocked with BSA (5%) that were development by far-Western (ligand) blot analyses required incubation with non-conjugated horseradish peroxidase (HRPO 1:100,000 dilution; 10 ng/ml HRPO equivalent, EG/EC 232-668-6), or streptavidin-HRPO conjugate (1:100,000 dilution) at 4°C for 2 hours applied in combination with gentle horizontal agitation. Enzymatic modification of non-conjugated HRPO was performed by incubation with α -mannosidase at a concentration of 15 mU/ μ g and 0.15 U/ μ g of HRPO at 37°C for 24 hours in sodium acetate (50 mM, pH 5.5 with CaCl₂ 5 mM).

In studies designed to determine if positive detection of pulmonary airway fluid fractions by non-conjugated HRPO far-Western (ligand) blot analysis was due to mechanisms of simple binding-avidity involving molecular interactions between carbohydrate moieties, BSA-blocked nitrocellulose membranes were pre-incubated in maltose (40 mM in TBS, pH 7.4) or mannose monosaccharide (40 mM in TBS, pH 7.4) for 2 hours at 4°C followed by three vigorous serial rinses in Tris buffered saline (Tris 0.1 M, pH 7.4, 40 ml).

Chemiluminescent autoradiography

Nitrocellulose membranes processed with non-conjugated HRPO or HRPO-streptavidin or HRPO-streptavidin in combination with biotinylated-IgG conjugates were developed for autoradiography by incubation in HRPO chemiluminescent substrate at 25°C for 5 to 10 mins (Pierce Chemical) (8-11). Au-

toradiography images were acquired by exposing Kodak BioMax XAR radiograph film (Eastman Kodak, Rochester, NY, USA) between 3–10 seconds to nitrocellulose membranes sealed within transparent ultra-clear plastic bags.

In order to determine if pulmonary airway fluid possessed endogenous biochemical activity capable of enzymatically converting HRPO chemiluminescent substrate biological fractions size-separated by SDS-PAGE and transferred laterally onto nitrocellulose membranes were incubated in BSA blocking buffer (BSA 5% in Tris 0.1 M, pH 7.4, 40 ml, 16 hrs), serially rinsed in PBS (0.1 M, NaCl 150 mM, pH 7.4, $n = 3$), and then immersed in peroxidase inhibitor (4 mls) at 25°C for 30 minutes (Pierce, Thermo Fisher Scientific, Rockford, IL, USA). Nitrocellulose membranes were then serially rinsed in PBS (0.1 M, NaCl 150 mM, pH 7.4, $n = 3$), incubated in HRPO chemiluminescent substrate (Pierce, Thermo Fisher Scientific, Rockford, IL, USA), and developed by conventional chemiluminescent autoradiography.

In instances when an alkaline phosphatase conjugate (Prozyme) was applied as a detection modality, pulmonary airway fractions size-separated by SDS-PAGE and transferred laterally onto sheets of nitrocellulose membrane were serially rinsed in assay buffer ($n = 2$ for 2 minutes each) and then incubated with alkaline phosphatase chemiluminescent substrate (Applied Biosystems) at 25°C for 5 minutes. Nitrocellulose membranes were then developed by conventional chemiluminescent autoradiography.

Preparation of HRPO-binding pulmonary fluid protein fractions for LC/MS-MS mass spectrometry analysis

Pulmonary airway fluid fractions size-separated by non-reducing SDS-PAGE (11% acrylamide) for LC/MS-MS mass spectrometry analysis were transferred laterally onto sheets of poly-vinylidene difluoride membrane (PVDF, BioRad Laboratories, Hercules CA, USA) pre-equilibrated in transfer buffer for 5 minutes followed by development at 100 volts constant voltage 4°C for 30 minutes. Sheets of PVDF membrane were then serially rinsed in ddH₂O ($n = 3$) and equilibrated in methanol (MeOH 100%) for 1 minute. Staining of PVDF membranes entailed incu-

bation in Coomassie Blue R-250 (0.1% in MeOH 40% with acetic acid 1%) for 1–2 minutes followed by de-staining in methanol (MeOH 50%) until an unstained background was acquired. De-stained PVDF membranes were then serially rinsed in ddH₂O and allowed to air-dry (4°C). Utilizing nitrocellulose membranes developed by chemiluminescent autoradiography as a reference guide, individual Coomassie Blue-stained protein bands were excised using a sterile size 15 surgical scalpel blade and transferred into sterile conical-tipped snap cap tubes containing ddH₂O (100 µl) followed by storage at 4°C between 1–5 days prior to submission for LC/MS-MS mass spectrometry analysis. Methodologies applied in this segment of the investigation were in accord with the suggested guidelines of the laboratory utilized for LC/MS-MS analyses (Taplin Biological Mass Spectrometry Facility, Harvard School of Medicine) (13).

Mass spectrometry analysis of pulmonary airway fluid protein fractions by LC/MS-MS

Individual stained bands excised from blotted PVDF membranes were initially dehydrated with acetonitrile for 10 minutes followed by removal of the liquid phase by pipette. Membrane sections were dehydrated to complete dryness in a vacuum centrifuge (Speed-Vac) and then rehydrated in ammonium bicarbonate solution (50 mM) containing modified sequencing-grade trypsin (Promega, Madison, Wisconsin, USA) formulated at a concentration of 12.5 ng/µl (4°C, 45 minutes). Residual trypsin solution was removed and replaced with ammonium bicarbonate solution (50 mM) using a volume sufficient to just cover divided membrane fragments. Samples were then incubated at 37°C overnight to enhance recovery. Peptides were subsequently extracted by removal of the ammonium bicarbonate liquid phase followed by a single wash of membrane fragments with a solution of acetonitrile (50%) and acetic acid (5%). The extracts were then dried in a vacuum centrifuge (Speed-Vac) for approximately 1 hour and samples stored at 4°C prior to further analysis.

Establishing the identity of pulmonary airway fluid fractions detected by HRPO far-Western (ligand) blot analyses initially entailed reconstitution of

samples in HPLC solvent A (2.5% acetonitrile, 0.1% formic acid, 5–10 μ l). A nano-scale reverse-phase HPLC capillary column was created by packing C₁₈ spherical silica beads (5 μ m) into a fused silica capillary (100 μ m inner diameter x ~12 cm length) with a flame-drawn tip (Taplin Biological Mass Spectrometry Facility, Harvard University School of Medicine (13–14). Each sample was then loaded onto an equilibrated C₁₈ spherical silica bead column by a Famos auto sampler (LC Packings–Dionex, San Francisco, CA, USA). A concentration gradient was formed and peptide fragments ultimately eluted with increasing concentrations of solvent B (acetonitrile 97.5%, formic acid 0.1%).

Sample peptides eluted from the LC column subsequently entered a coupled in-line electrospray ionization system followed by an LTQ linear ion-trap mass spectrometer (Thermo Scientific, Waltham, MA, USA) with peptides detected, isolated, and fragmented to produce a tandem mass spectrum of peptide fragment ions specific for each peptide from pulmonary airway fluid fractions (Taplin Biological Mass Spectrometry Facility, Harvard University School of Medicine (13–14).

Peptide identification

MS targets with MS/MS spectra were searched against the comprehensive protein databases such as, Homo sapiens sub database created from NCBI “nr” (13–14). Output of the comparisons was the identity of peptides that were subsequently used to compile a protein identification list. The MS spectra for all peptides were analyzed using Turbo SEQUEST (Bioworks Browser 3.1 SRI; Thermofinnigan, Thermo Scientific, Waltham, MA, USA) software. The search parameters were generated without molecular weight restrictions.

The multiple threshold filter applied at the peptide level consisted of: (a) Peptide Sequence Length: 2.5 (b) Charge State: Auto; (c) Minimum Ion Count: 15, (d) Minimum Group Count: 1 as analytical criteria. The AutoSequest algorithm filter used for protein identification was X-Corr-vs-Charge Parameter: +1/1.5, +2/2.0, +3/2.5. Multiple ($n > 2$) different peptides had to be matched before a pulmonary airway

fluid protein fraction could be positively identified within the database. Individual proteins were validated manually as a function of their corresponding scores (Table 1).

Results

Pulmonary airway fluid developed by non-reducing SDS-PAGE and Western blot (immunodetection) analysis utilizing primary biotinylated anti-(SP-A) IgG or primary biotinylated anti-(SP-D) immunoglobulin in combination with a secondary streptavidin-HRPO conjugate detected a spectrum of protein fractions (Fig. 1). Conversely, a more confined array of protein fractions were detected in pulmonary airway fluid that was developed by non-reducing SDS-PAGE and Western (ligand) blot analysis utilizing primary biotinylated anti-(SP-A) IgG or biotinylated anti-(SP-D) IgG immunoglobulin when applied in combination with a secondary streptavidin-alkaline phosphatase conjugate (Fig. 1). Subsequent pulmonary airway fluid evaluation by far-Western (ligand) blot analysis utilizing conjugated HRPO-streptavidin compared to non-conjugated HRPO as primary molecular probes in the absence of biotinylated immunoglobulin detected essentially the same fraction profile (Fig. 2). The optimum dilution for non-conjugated HRPO or HRPO-conjugates in far-Western (ligand) blot analyses was 1:100,000 in contrast to the more conventional 1:10,000 and 1:20,000 dilution schemes (Fig. 3). Although amounts of pulmonary airway fluid added to individual wells could have been reduced, it would have substantially decreased the sensitivity of detecting many individual fractions present at lower concentrations within standardized samples (Fig. 3).

Esterase inactivation of pulmonary fluid laterally transferred onto nitrocellulose membrane after size-separation by non-reducing SDS-PAGE decreased the intensity of fractions detected but did not significantly alter the profile of protein fractions recognized by non-conjugated HRPO far-Western (ligand) blot analysis (Fig. 4). Pulmonary fluid fractions transferred onto nitrocellulose membrane after size-separation by non-reducing SDS-PAGE and then developed with HRPO chemiluminescent substrate alone detected

Table 1. Pulmonary airway fluid protein fractions identified by mass spectroscopy analysis that possess HRPO binding-avidity

GI#	Protein	Peptide Fragments Identified	XCorr Function	Biological	Example Peptide Sequences
136190	Transferrin (80-kDa)	n=27	5.47 5.55 4.83 5.75 4.76	Iron transport Acute-phase response Anti-bacterial properties Innate immunity	K.EDLIWGLLNQAQEHFGTEK.S K.GEADAMSLDGGFIYIAGK.C K.IVKGEADAMSLDGGFIYIAGK.C K.SAGWNIPIGLLYWQLPEPR.E K.SVDGKEDLIWGLLNQAQEHFGTEK.S
149699079	Dynein (74-kDa)	n=9	5.82 6.95 5.99 6.15 5.36	Ciliary motor complex Intermediate subunit Links dynein to cargo	K.DKEVAFWTELLAQK.A K.AISESGVALTAVVADDYMR.A K.LPVMVWIHGGGLVLGGASTYDGR.V K.VLSAHENVVVVTIQYR.L K.TVMWDHGDDELFSLFGAPFLK.E
543794	Albumin Precursor (69-kDa)	n=31	5.46 4.96 4.90 5.60 4.80	Wound healing ARE signaling pathway	K.DAKDVLGTLFLEYEYSR.R K.KAPQVSTPTLVEIGR.T K.HPYFYGPELLFHAEYK.A K.RHPYFYGPELLFHAEYK.A K.DVFLGTLFLEYEYSR.R
149716548	156 kDa Equine Peptide (35-kDa fragment)	n=25	4.90 5.10 5.47 6.04 4.56	Apolipoprotein E Lymphocyte apoptosis Fat transport	K.ASFLAAIDEASK.Q K.DTEGLRQELNK.D K.LREQQLGPVTQDFWDK.L K.LREQQLGPVTQDFWDKLEK.D K.EYVAQFEASALGK.Q
149716548	156 kDa Equine Peptide (28-kDa fragment)	n=25	5.29 5.70 6.30 4.72 5.32	Apolipoprotein AI Lymphocyte apoptosis Fat transport	K.DTEGLRQELNKDLELVK.Q K.LREQQLGPVTQDFWDK.L K.LREQQLGPVTQDFWDKLEK.Q K.EYVAQFEASALGK.Q K.LQALKDGGGASLAEYHSK.A

only trace levels of esterase-like activity, but their profiles were highly analogous to those recognized by non-conjugated HRPO far-Western (ligand) blot analyses (Fig. 5). Inactivation of esterase-like activity in pulmonary airway fluid laterally transferred onto nitrocellulose membranes after size-separation by non-reducing SDS-PAGE followed by development with HRPO chemiluminescent substrate alone resulted in an inability to detect any fractions and in turn further substantiated the perception that some components within pulmonary airway fluid possessed only slight traces of esterase-like activity and chemiluminescent intensity was strongly positive only when far-Western (ligand) blot analyses were developed in the presence of HRPO in either a conjugated or non-conjugated form (Fig. 5).

In the preliminary determination of HRPO binding characteristics, pre-incubation of pulmonary airway fluid with β -galactosidase altered the intensity

but not the profile of fractions detected by far-Western (ligand) blot analysis performed utilizing non-conjugated HRPO reagent (Fig. 6). Most notable in this regard was a decrease in bandwidth and intensity of the 69-kDa fraction (Fig. 6). Similarly, enzymatic modification of non-conjugated HRPO with α -mannosidase only slightly altered the intensity and profile of fractions in pulmonary airway fluid detected by non-conjugated HRPO far-western (ligand) blot analysis which was most prominent for the 35-kDa fraction at the higher enzyme concentration of 0.15 U (Fig. 7). Conversely, pre-incubation of pulmonary airway fluid laterally transferred onto nitrocellulose membranes in either maltose or mannose formulations prior to far-Western (ligand) blot analyses with non-conjugated HRPO noticeably reduced both the intensity and profile of 80-kDa, 69-kDa, 35-kDa and 28-kDa fractions detected by chemiluminescent autoradiography (Fig. 8).

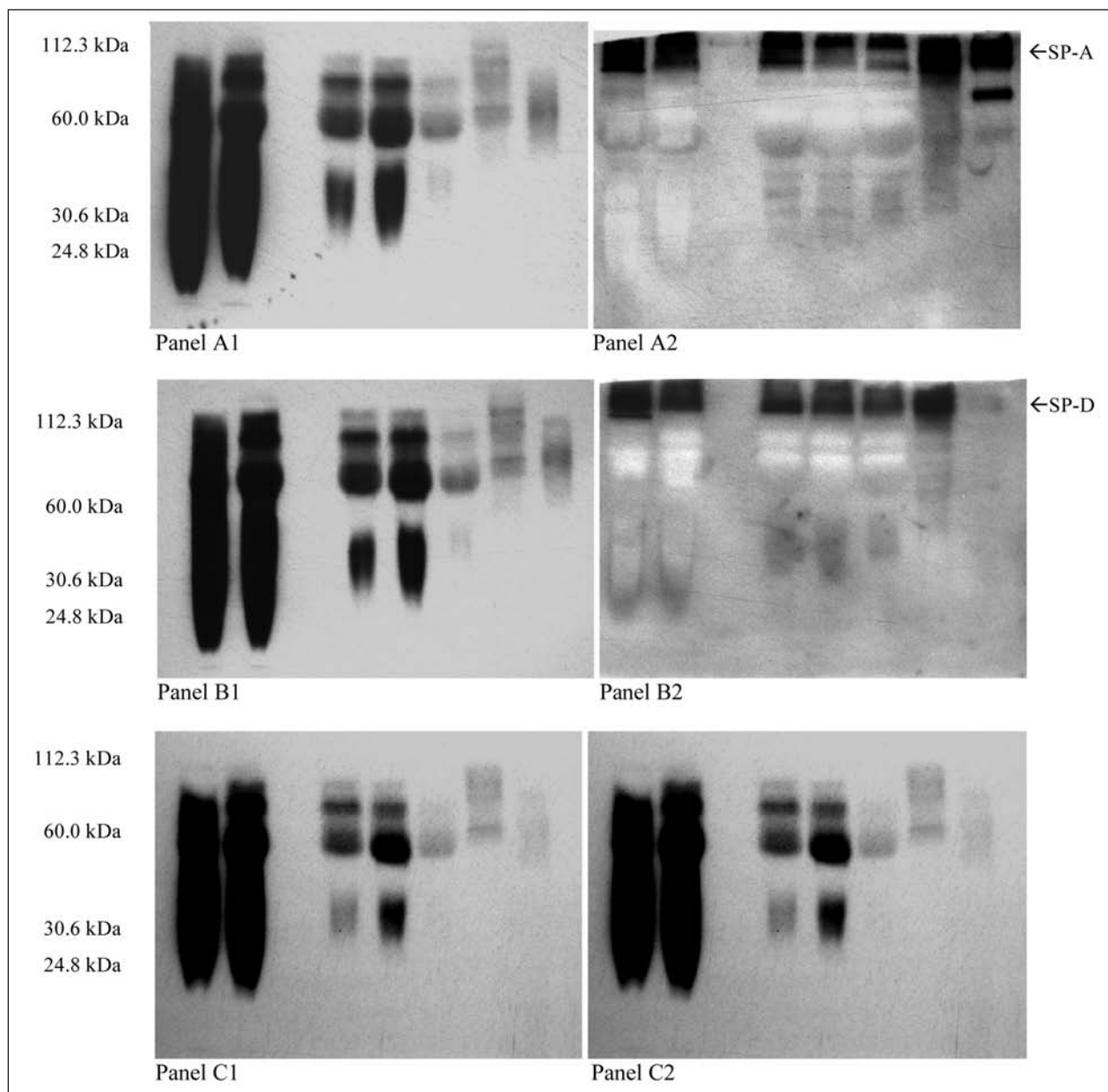


Figure 1. Western blot immunodetection analysis of pulmonary airway fluid (*Lanes 1,2, & 4-8*) for surfactant proteins SP-A and SP-D utilizing biotinylated primary immunoglobulins in combination with secondary enzyme-streptavidin conjugates. (*Panel-A1*) 1° biotinylated anti-SPA with 2° streptavidin-HRPO; (*Panel-A2*) 1° biotinylated anti-SPA with 2° streptavidin-alkaline phosphatase; (*Panel-B1*) 1° biotinylated anti-SPD with 2° streptavidin-HRPO; (*Panel-B2*) 1° biotinylated anti-SPD with 2° streptavidin-alkaline phosphatase; (*Panels-C1 & C2*) HRPO-streptavidin far-Western (ligand) blot analysis. Arrows indicate the position of native high-molecular weight SP-A (←) and SP-D (←) surfactant proteins. Pulmonary airway fluid was initially developed by SDS-PAGE (0.9 µg protein/well) under non-reducing conditions and then transferred laterally onto nitrocellulose membranes. Subsequent development involved application of HRPO chemiluminescent substrate for the acquisition of autoradiography images

Mass spectrometry analysis facilitated identification of transferrin glycoprotein, dynein glycoprotein, albumin precursor, and two fragment of 156 kDa

equine peptide as protein fractions contained within pulmonary airway fluid that were positively detected by HRPO far-Western (ligand) blot analyses (Fig. 9,

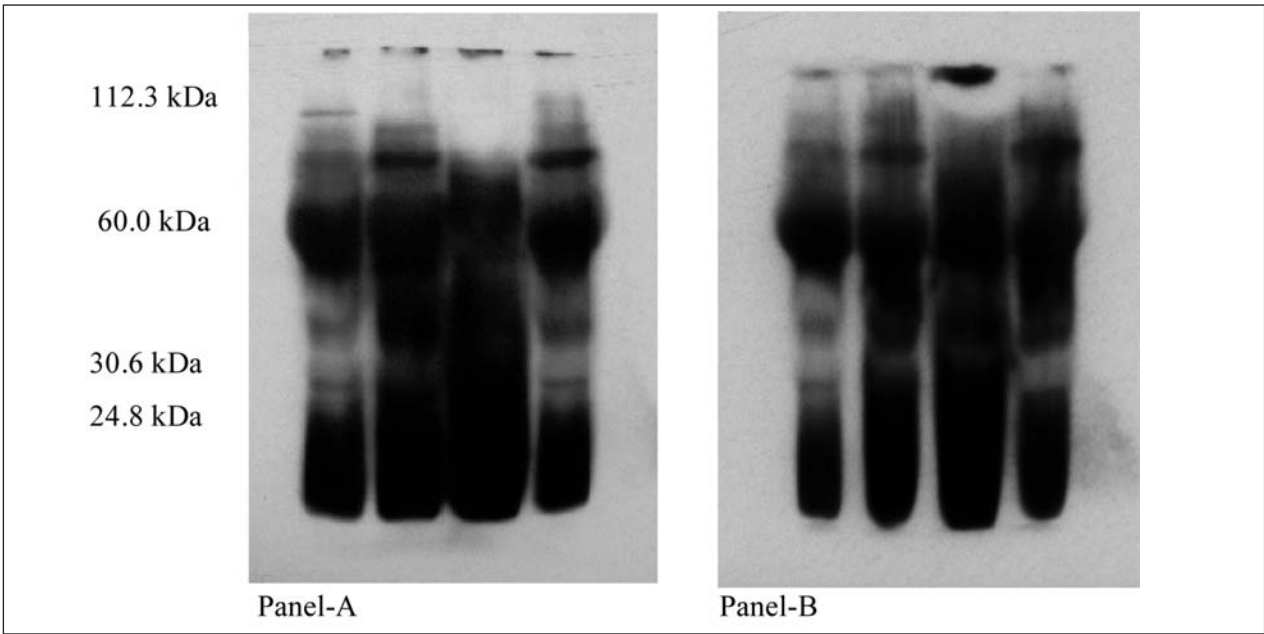


Figure 2. Detection of fractions contained in pulmonary airway fluid (*Lanes 1–4*) positively recognized by far-Western (ligand) blot analyses utilizing either non-conjugated or conjugated HRPO as a primary molecular probe. (*Panel-A*) non-conjugated HRPO; and (*Panel-B*) streptavidin-HRPO conjugate. Pulmonary airway fluid was initially developed by SDS-PAGE (0.9 µg protein/well) under non-reducing conditions and then transferred laterally onto nitrocellulose membranes. Subsequent development involved application of HRPO chemiluminescent substrate for the acquisition of autoradiography images

Table 1). Total positive peptide fragment matches for each of these protein fractions when cross referenced with national mass spectrometry databases were $n = 27$ (transferrin glycoprotein), $n = 29$ (dynein glycoprotein), $n = 31$ (albumin precursor), $n = 25$ (156-kDa equine peptide fragment) and $n = 30$ (156-kDa equine peptide fragment). The top five X-Corr values for each protein fraction identified ranged between 4.76-to-5.75, 5.36-to-6.95, 4.80-to-5.60, 4.56-to-6.04 and 4.77-to-6.30 respectively for transferrin glycoprotein, dynein glycoprotein, albumin precursor, and two fragments of the 156-kDa equine peptide fractions (Table 1). The number of peptide fragments with X-Corr values above 2.5 was $n = 24/27$ (transferrin glycoprotein), $n = 26/29$ (dynein glycoprotein), $n = 24/31$ (albumin precursor), $n = 18/25$ (156-kDa equine peptide) and $n = 23/30$ (156-kDa equine peptide).

Discussion

Western (immunoblot) analysis revealed that 2° HRPO-streptavidin in combination with 1° mono-

clonal antibody produced markedly different protein profiles than was detected with 2° alkaline phosphatase-streptavidin applied in combination with the same 1° monoclonal antibody. The molecular basis for differences in autoradiographic images was largely attributed to individual variations in chemical composition and molecular configuration between HRPO and alkaline phosphatase. Horseradish peroxidase (HRPO) is a 40 kDa glycoprotein enzyme functionally classified as a ferri-heme peroxidase because its biochemically active site is a chelation complex composed of cationic iron (Fe^{+4}) and a heme prosthetic group (Fig. 10) (1-4). The glycosylated segments of HRPO (40-kDa) represent 18% of its total mass weight and consist of eight *N*-linked oligosaccharide side-chains (15). Some HRPO fractions are glycosylated with *N*-glycosylated side-chains at nine different sites including asparagine Asn₈₇ (16), Asn₁₈₈ (16), Asn₂₈₅ (17), Asn₂₉₈ (17), and Asn₃₁₆ (17-18), amino acid residues. Most of the *N*-glycosylated sites of HRPO can alternatively be expressed as a Fuc(1-3)GlcNAc-disaccharide (18). The disaccharide *N*-acetylhexosamine-1-

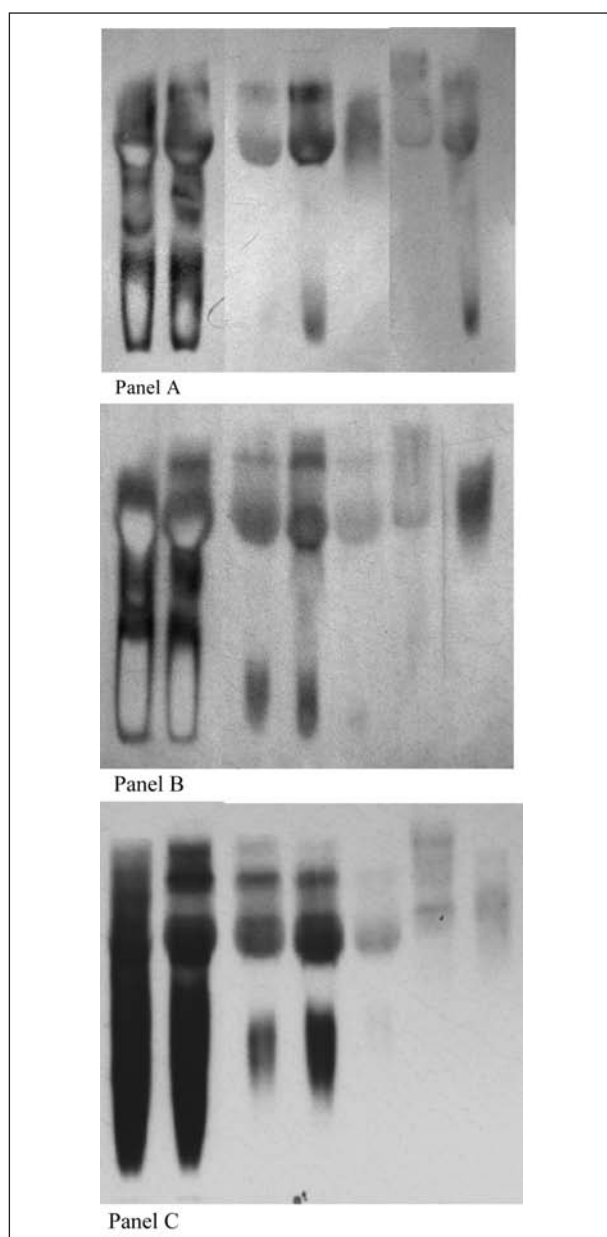


Figure 3. Detection of fractions contained within pulmonary airway fluid (Lanes 1-7) by far-Western (ligand) blot analysis utilizing non-conjugated and conjugated HRPO as primary molecular probes formulated at dilute concentrations. (Panel A) non-conjugated HRPO formulated at a 1:20,000 dilution; (Panel B) streptavidin-HRPO conjugate formulated at a 1:20,000 dilution; and (Panel C) non-conjugated HRPO formulated at a 1:100,000 dilution. Pulmonary airway fluid was initially developed by SDS-PAGE (0.9 μ g protein/well) under non-reducing conditions and then transferred laterally onto nitrocellulose membranes. Subsequent development involved application of HRPO chemiluminescent substrate for the acquisition of autoradiography images

deoxyhexose-1 attached at *N*-glycosylation sites Asn₂₈₅ and Asn₂₉₈ may represent a Fuc(α 1-3)GlcNAc-moiety arising from the processing of *N*-glycans by a horseradish endoglycosidase during HRPO biosynthesis (17). Preparations of HRPO can consist of mixtures of two main variants. One predominant type (approximately 60%) has a total of eight glycosylation sites linked to core(1-3)fucosylated, xylosylated, trimannosyl *N*-glycans, with another ninth potential *N*-glycosylation site located at Asn₃₁₆ that is devoid of any side-chains (18). A second HRPO form (approximately 35%) possesses seven core (1-3)fucosylated, xylosylated, trimannosyl *N*-glycans, with an eighth *N*-glycosylation site alternatively linked to a Fuc(1-3)GlcNAc-moiety (18). Less prominent HRPO sub-fractions contain a xylosylated, trimannosyl *N*-glycan lacking core-fucosylation at a ninth *N*-glycan side-chain located at the Asn₃₁₆ position previously presumed to not be glycosylated (17-18). The glycan structure of Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-asparagine is the prevailing moiety at 8-of-9 and 7-of-8 *N*-glycosylated sites of the predominant and lesser HRPO sub-fractions respectively (Fig. 10) (16-19).

Calf intestine alkaline phosphatase is a 140-kDa glycoprotein enzyme that catalyzes the hydrolysis of phosphate groups (Figs. 10, 11). The cyclic catalytic domain of human placental alkaline phosphatase consists of; [i] amino acid Asp₉₁, Ser₉₂, Gly₉₃ residues, [ii] a cationic metal triplet ($n = \text{Zn}^{+2} \times 2$ and $n = \text{Mg}^{+2} \times 1$), and an [iii] amino acid Arg₁₆₆ residue located in the immediate vicinity (20). Glycosylation of both human placental alkaline phosphatase and human germ cell alkaline phosphatase is present at asparagine amino acid residues Asn₁₂₂ and Asn₂₄₉ (21) while calf intestine alkaline phosphatase is glycosylated at Asn₂₄₉ (21) and Asn₄₁₀ amino acid residues (21). Majority of the glycans associated with calf intestine alkaline phosphatase are capped with α -galactose at their non-reducing termini in contrast to glycans linked to other alkaline phosphatase isoenzymes since no degree of sialylation exists (Figs. 10, 11) (21). Glycans linked to calf intestine alkaline phosphatase at Asn₂₄₉ can exist in at least eight different forms (mainly non-fucosylated, biantennary or triantennary) with a bisecting *N*-acetylglucosamine (Fig. 10) (21). Glycans linked to

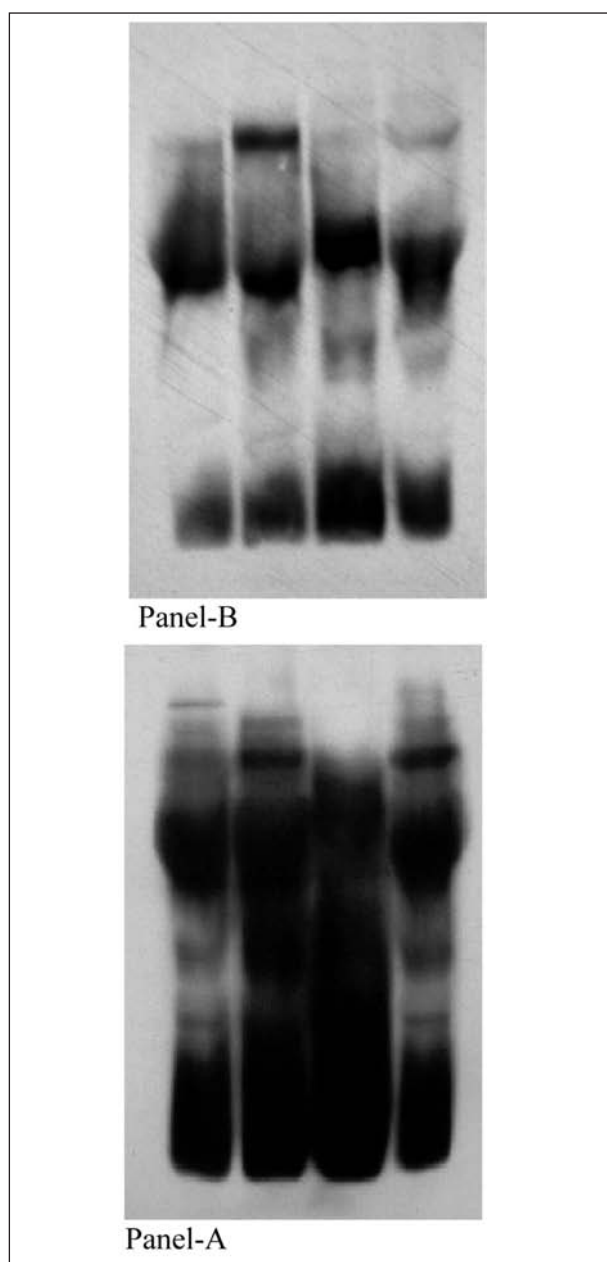


Figure 4. Influence of inhibiting esterase-like biochemical activity within pulmonary airway fluid (*Lanes 1–4*) on the intensity and profile of fractions detected by non-conjugated HRPO far-Western (ligand) blot analyses. (*Panel-A*) non-conjugated HRPO; (*Panel-B*) non-conjugated HRPO following inactivation of esterase-like activity in pulmonary airway fluid fractions. Pulmonary airway fluid was initially developed by SDS-PAGE (0.9 μ g protein/well) under non-reducing conditions and then transferred laterally onto nitrocellulose membranes. Subsequent development involved incubation with an esterase inhibitor followed by the application of HRPO chemiluminescent substrate for the acquisition of autoradiography images

Asn₄₁₀ are a mixture of at least nine, mainly tetraantennary, fucosylated forms with a bisecting *N*-acetylglucosamine (Fig. 11) (21).

Pulmonary airway fluid contains multiple fractions that were positively detected by far-Western (ligand) blot analyses utilizing either non-conjugated or conjugated forms of HRPO as a primary molecular probe that was not inhibited by BSA (5%) blocking buffer (Figs. 1, 2). Initial neutralization of pulmonary fluid esterase activity reduced the relative intensity but did not alter the profile of fractions detected by non-conjugated HRPO far-Western (ligand) blot analysis (Fig. 4). Some of these same pulmonary airway fluid fractions possess only very minor levels of esterase-like biochemical activity based on their ability to convert only trace amounts of HRPO chemiluminescent substrate to its “light-emitting” form in the absence of either conjugated or non-conjugated HRPO (Fig. 5). Intense detection of fractions contained in pulmonary airway fluid was only recognized when far-Western (ligand) blot analyses were performed utilizing either conjugated or non-conjugated HRPO as a primary molecular probe and convincingly suggests that in some fashion HRPO physically forms a molecular complex with fractions contained within pulmonary airway fluid (Fig. 5).

Enzyme modification of pulmonary airway fluid with β -galactosidase detectably altered the intensity of the 69-kDa fraction but not the actual profile recognized by chemiluminescent autoradiography following non-conjugated HRPO far-Western (ligand) blot analyses (Fig. 6). Similarly, alterations in intensity and profile of pulmonary airway fluid fractions detected by chemiluminescent autoradiography following far-Western (ligand) blot analysis utilizing α -mannosidase modified non-conjugated HRPO as a primary molecular probe suggests that terminal α -mannose residues of HRPO glycan side-chains participate in complex formation with some fractions contained within pulmonary airway fluid (Fig. 7). Despite the capacity of α -mannosidase to biochemically cleave terminal α -mannose residues it is incapable of efficiently reacting with more internally located Man β 1-4GlcNAc β 1-4 residues thereby leaving them largely intact (Fig. 10). Complementing results observed with α -mannosidase modified non-conjugated HRPO, the

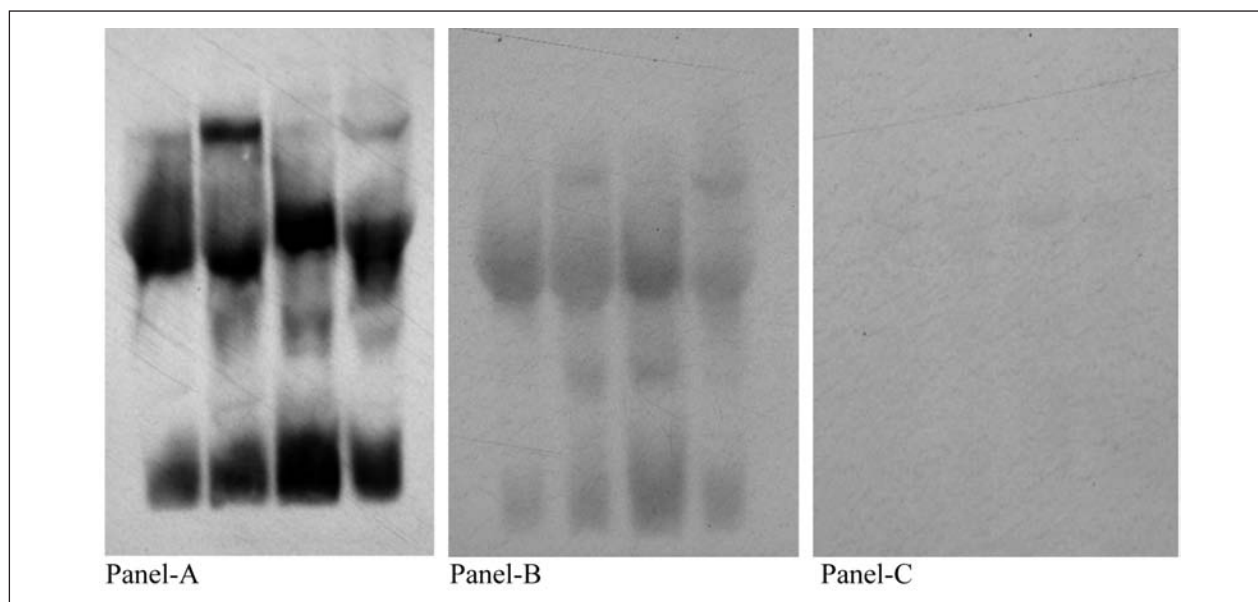


Figure 5. Influence of the presence or absence of HRPO on the intensity and profile (←) of fractions contained within pulmonary airway fluid (*Lanes 1–4*) positively detected by far-Western (ligand) blot analysis utilizing non-conjugated HRPO as a primary molecular probe. (*Panel-A*) non-conjugated HRPO far-Western (ligand) blot analysis performed after initial inactivation of pulmonary airway fluid esterase-like activity; (*Panel-B*) HRPO chemiluminescent substrate development in the absence of either conjugated or non-conjugated HRPO; and (*Panel-C*) HRPO chemiluminescent substrate development of pulmonary airway fluid following inactivation of esterase-like activity and in the absence of either conjugated or non-conjugated HRPO preparations. Pulmonary airway fluid was initially developed by SDS-PAGE (0.9 μ g protein/well) under non-reducing conditions and then laterally transferred onto nitrocellulose membranes. Subsequent development involved acquisition of chemiluminescent autoradiography images

lower intensity and reduced profile for some pulmonary airway fluid fractions detected by chemiluminescent HRPO far-Western (ligand) blot analyses after pre-incubation with maltose (monosaccharide) or mannose (disaccharide) formulations at least in part reflects their apparent binding avidity for HRPO glycan side-chains (Figs. 8, 10). Alterations in the profile of pulmonary airway fluid detected by non-conjugated HRPO far-Western (ligand) blot analysis were most prominent for fractions with molecular masses in the 80, 69, 35 and 28-kDa range (Fig. 8). Such properties are consistent with the known binding avidity of mannose-binding proteins for HRPO and other glycoproteins with mannose-rich glycan side-chains (5). Analogous molecular glycoprotein-protein binding-avidity interactions have been recognized between HRPO and several plant lectin (7) including concanavalin-A which preferentially forms complexes with both internal and non-reducing terminal α -mannosyl residues (6).

The reason why there was not a greater effect on the profile and intensity of pulmonary airway fluid fractions detected by far-Western (ligand) blot analysis using α -mannosidase modified non-conjugated HRPO is not entirely certain (Fig. 7). However, α -mannosidase modified non-conjugated HRPO would still contain a number of internally located mannose residues that would be available for complex formation with pulmonary airway fluid fractions (Figs. 10, 11). Alternatively, the absence of the prevailing $\text{Man}\alpha 1-6(\text{Man}\alpha 1-3)(\text{Xyl}\beta 1-2)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4(\text{Fuc}\alpha 1-3)\text{GlcNAc}$ -asparagine glycan moieties at 1-of-9 and 1-of-8 *N*-glycosylation sites within the major and minor HRPO subtypes respectively (16–19) may also account to some degree for an incomplete suppression pulmonary airway fluid fractions detected with α -mannosidase modified HRPO (Figs. 7, 8). Presumably, substantially lower mannose content along with terminal α -galactose “capping” of glycan side-chains found in calf intestine alkaline phos-

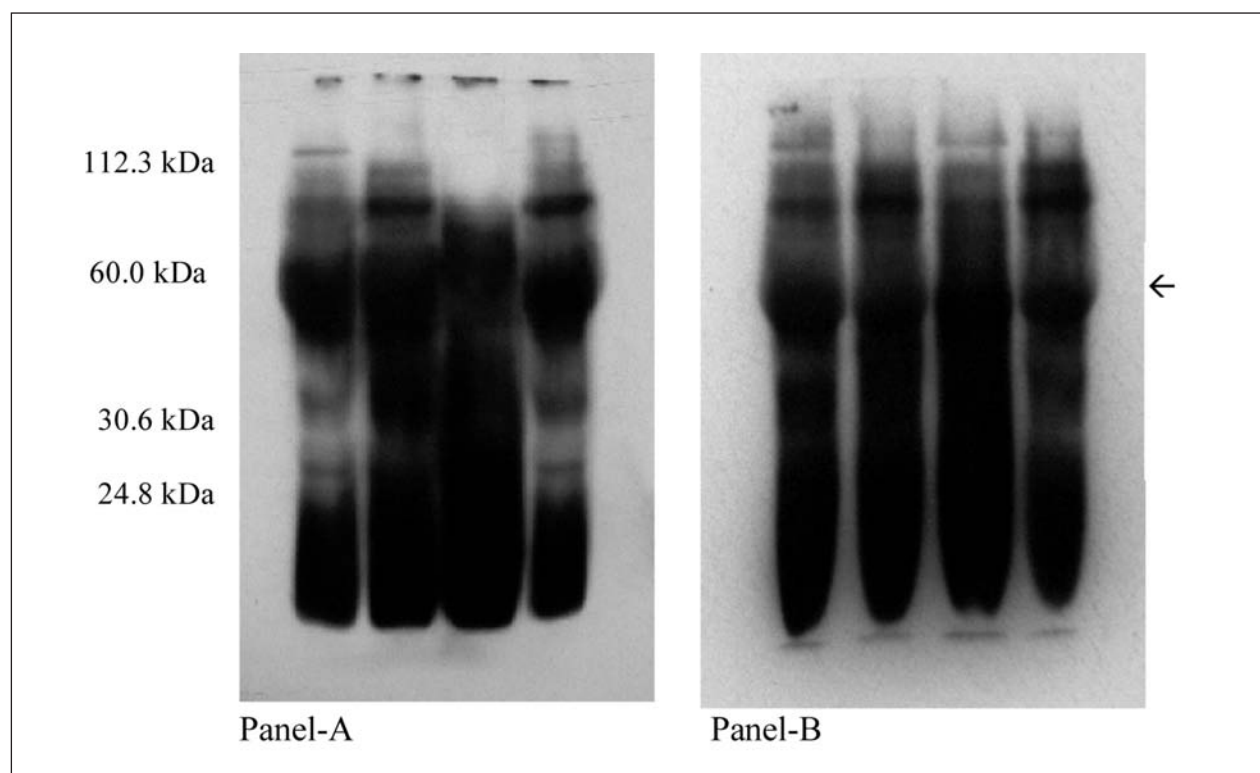


Figure 6. Influence of enzymatic modification of pulmonary airway fluid (*Lanes 1–4*) with β -galactosidase on the intensity and profile of fractions detected by non-conjugated HRPO far-Western (ligand) blot analysis. (*Panel-A*) pulmonary fluid only, and (*Panel-B*) pulmonary fluid pre-incubated with β -galactosidase prior to separation by non-reducing SDS-PAGE. Nitrocellulose membranes were subsequently processed applying a HRPO chemiluminescent substrate for the acquisition of autoradiography images. A detectable decrease in width and intensity (\leftarrow) can be appreciated for the 69-kDa pulmonary airway fluid fraction. Pulmonary airway fluid was initially developed by SDS-PAGE (0.9 μ g protein/well) under non-reducing conditions and then transferred laterally onto nitrocellulose membranes. Subsequent development involved the application of HRPO chemiluminescent substrate for acquisition of autoradiography images

phatase account for differences in the profile of fractions detected in pulmonary airway fluid by Western blot analyses that utilized alkaline phosphatase-streptavidin compared to HRPO-streptavidin as secondary molecular probes (Figs. 1, 10, 11).

Based on mass spectrometry analysis, the identity of pulmonary airway fluid fractions detected by non-conjugated HRPO far-Western (ligand) blot analysis included transferrin glycoprotein, dynein glycoprotein, albumin precursor, and two separate fragments of 156-kDa equine peptide glycoprotein (Figs. 2, 4, 5, 6, 7, 8 & 9) (22–23). Despite their rather varied and diverse biological properties, each individual protein fraction detected in pulmonary airway fluid by non-conjugated HRPO far-Western (ligand) blot analysis is by classification a glycoprotein.

Transferrin is an 80-kDa glycoprotein (24) that functions as an acute-phase protein and participates in molecular processes related to innate immunity (24–25). Anatomically it is found associated with mucosa tissues where it binds iron so extensively that it creates an environment deficient in free iron that is not conducive to the survival of many species of bacteria. Clinical conditions that characteristically have as a feature sub-optimal transferrin concentrations frequently are associated with a high recurrence rate of septic infections.

Dyneins are microtubule-associated ATP motor glycoproteins (ciliary dyneins) that functionally promote sliding of microtubules in the axonemes of cilia in cell populations such as the ciliated respiratory epithelium lining major airways. The AAA ATPase mo-

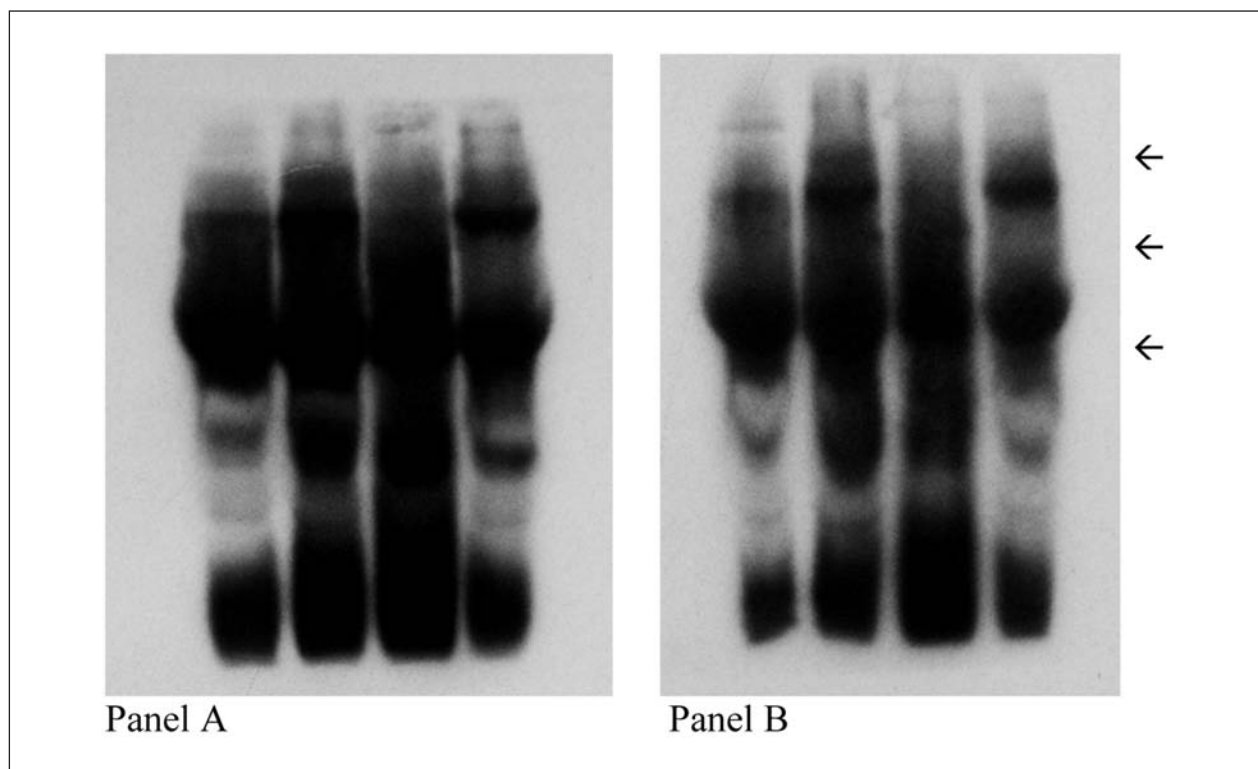


Figure 7. Influence of enzymatic modification of non-conjugated HRPO with α -mannosidase on the intensity and profile of fractions detected in pulmonary airway fluid (*Lanes 1–4*) by non-conjugated HRPO far-Western (ligand) blot analysis. (*Panel-A*) pulmonary airway fluid probed with non-conjugated HRPO; (*Panel-B*) pulmonary airway fluid fractions probed with α -mannosidase modified non-conjugated HRPO (15 mU/ μ g HRPO). A detectable decrease in width and intensity (\leftarrow) can be appreciated for 80-kDa, 69-kDa, and 35-kDa pulmonary airway fluid fractions. Under non-reducing conditions, pulmonary airway fluid were initially developed by SDS-PAGE (0.9 μ g protein/well) and then transferred laterally onto nitrocellulose membranes. Subsequent development involved the application of HRPO chemiluminescent substrate for acquisition of autoradiography images

tor domain of the dynein complex undergoes a conformational change during the “power stroke” phase of movement with the resulting microtubule sliding motion producing the bending configuration required for cilia to beat and propel in a retrograde direction viral agents (e.g. adenoviruses), bacteria and debris. Primary ciliary dyskinesia is an inherited disorder characterized by chronic respiratory infections. Cytoplasmic dynein has a molecular mass of 1500 kDa and consists of multiple ($n = 12$) polypeptide units. The two identical 520-kDa heavy-chains express ATPase activity that facilitate movement along microtubules. In addition, dynein contains two 74-kDa intermediate-chains believed to be anchored to a “cargo” complex consisting of four 53–59 kDa intermediate-chains and multiple light-chains. The dynein glycoprotein detect-

ed by non-conjugated HRPO far-Western (ligand) blot analyses was apparently the 74-kDa intermediate-chain that exists as 2 identical polypeptide strands within each dynein multi-subunit complex (Fig. 9).

Albumin precursor (26) is a 60 to 69-kDa acute-phase protein. In conditions characterized by elevations in systemic lipopolysaccharide concentrations the transcription of albumin precursor mRNA becomes measurably inhibited. Functionally, albumin precursor reportedly facilitates active phases of cell migration, it interacts with fibronectin to aid wound-healing processes (27), and serves as a component of the antioxidant response element (ARE) signaling pathway (28).

Non-reducing HRPO far-Western (ligand) blot analysis also detected two apparent fragments of 156-

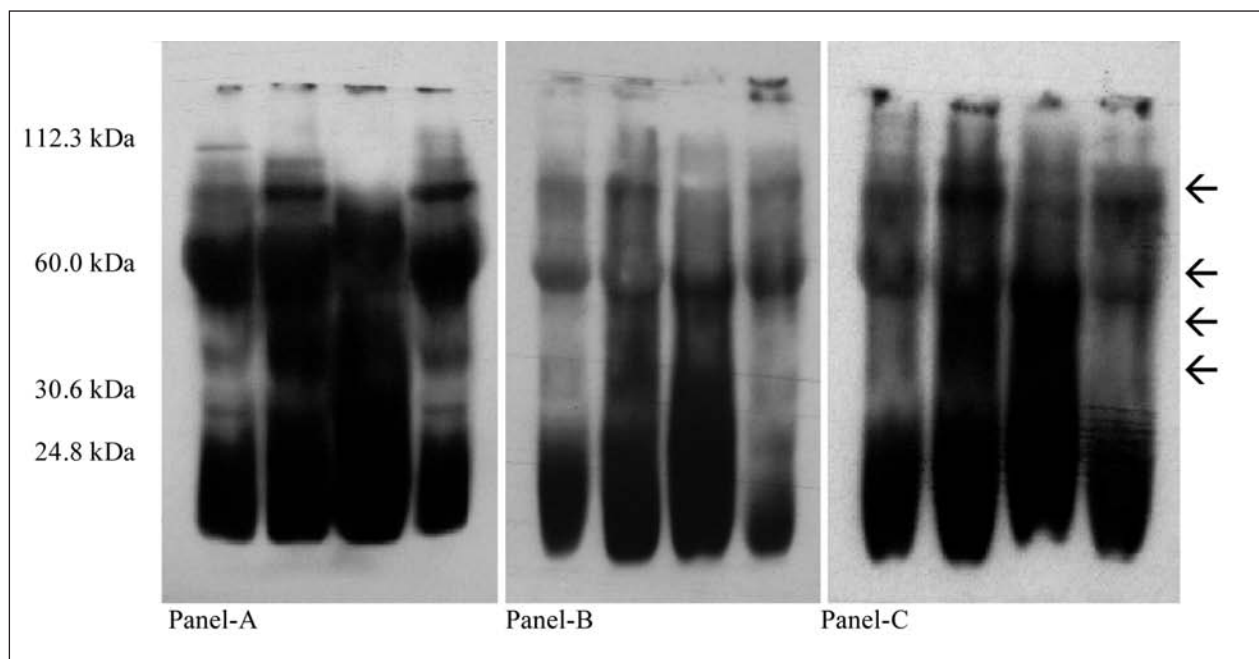


Figure 8. Influence of maltose and mannose on the intensity and profile of fractions contained in pulmonary airway fluid (*Lanes 1–4*) detected by non-conjugated HRPO far-Western (ligand) blot analysis. (*Panel-A*) non-conjugated HRPO, (*Panel-B*) non-conjugated HRPO following pre-incubation of pulmonary airway fluid fractions in maltose; (*Panel-C*) non-conjugated HRPO following pre-incubation of pulmonary airway fluid fractions in mannose. A detectable decrease in width and intensity (←) can be appreciated for the 80-kDa, 69-kDa, 35-kDa and 28-kDa pulmonary airway fluid fractions. Pulmonary airway fluid was initially developed by SDS-PAGE (0.9 µg protein/well) under non-reducing conditions and then transferred laterally onto nitrocellulose membranes. Subsequent development involved the application of HRPO chemiluminescent substrate for acquisition of autoradiography images

kDa equine protein (Figs. 2, 6–9, Table 1). The 156-kDa equine protein identified by mass spectrometry analysis (23) contains an apolipoprotein A1/A4/E like domain (22). Several repeat segments that are 22 amino acid residues in length are also found within the 156-kDa equine protein sequence that corresponds to the presences of a pair of alpha helix structures (22–23). The family of proteins that express pairs of alpha helices include not only 156-kDa equine protein but also apolipoprotein A1 (28.3 kDa), apolipoprotein A4 (44.3–46-kDa), and apolipoprotein E (34-kDa) (22–23). While apolipoprotein E is analogous to 156-kDa equine protein, homology most highly correlates with apolipoprotein A1 (22–29) and apolipoprotein A4 (22–23). A high degree of correlation also exists between the genetic sequences for 156-kDa equine protein and equine apolipoprotein A1 (29). Collectively, apolipoproteins are classified as *N*- and *O*-glycosylated lipoproteins (30). Apolipoprotein

A4 is synthesized by intestinal enterocytes while apolipoprotein A1 is primarily produced by the liver and intestine (31) but expression is also found in tracheal environments (32) and their concentrations within pulmonary tissues are relatively high (32). Apolipoproteins become depleted within the lung during certain disease states (23) while levels retrieved in bronchoalveolar lavage fluid (BAL) increases during the course of inflammatory conditions (34). Based on collective interpretation of mass spectrometry analysis and observed molecular mass, it is highly probable that the 35-kDa and 28-kDa bands represent the equivalent of equine apolipoprotein E and apolipoprotein A1 respectively (Table 1).

In analytical practice, HRPO conjugates represent a collection of invaluable biological reagents that are commonly utilized as secondary molecular probes that benefit conduction of both investigative research and the design of molecular diagnostic techniques.

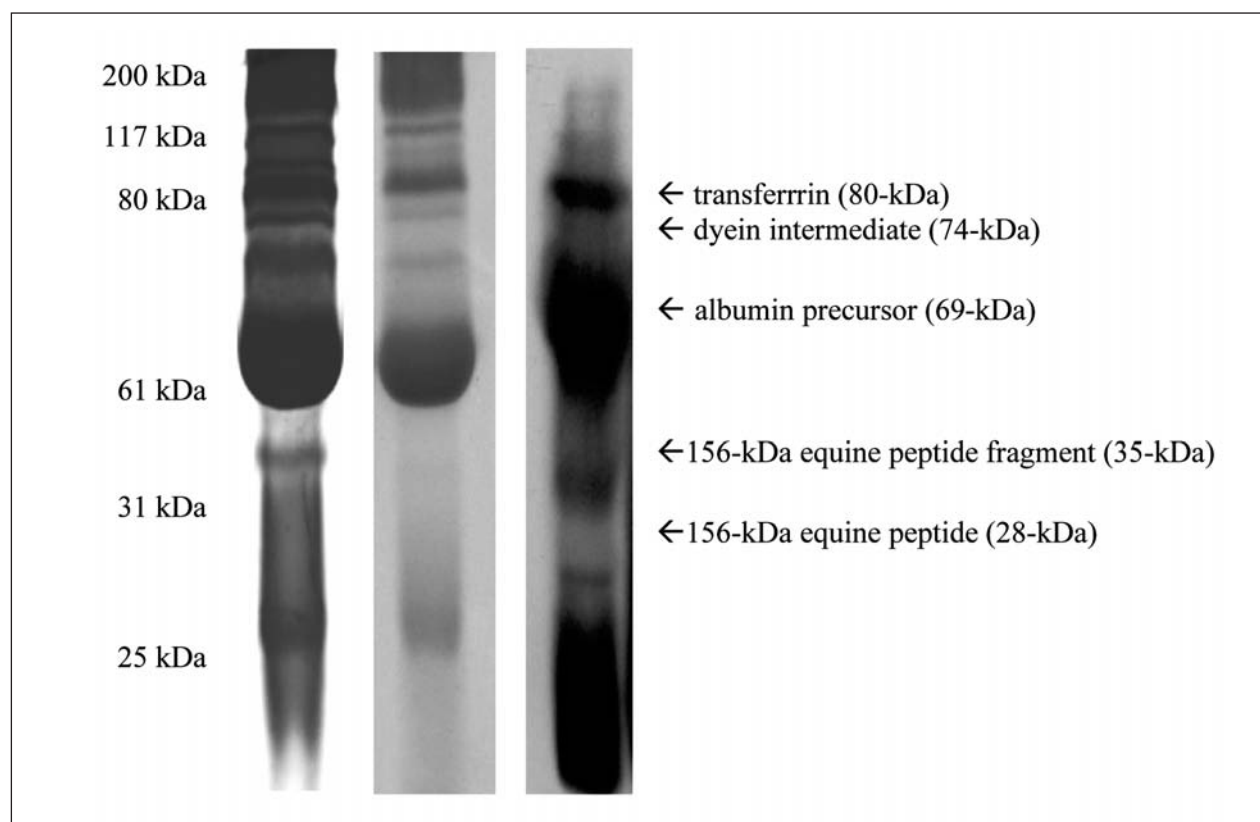
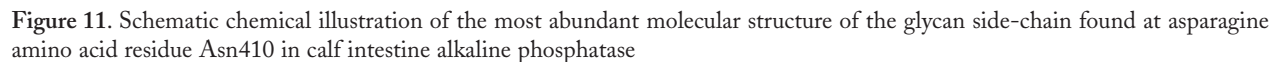
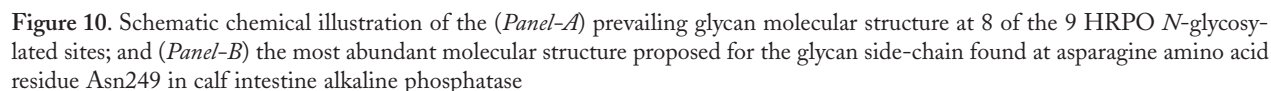


Figure 9. Correlation of fractions contained within pulmonary airway fluid development by non-reducing SDS-PAGE with results from mass spectrometry analysis. (*Lane-1*) Coomassie Brilliant Blue stain; (*Lane-2*) Ponceau S stain; and (*Lane-3*) non-conjugated HRPO far-Western (ligand) blot analysis performed in concert with the application of HRPO chemiluminescent substrate and autoradiography

However, the apparent ligand binding-avidity some biological proteins fractions have for HRPO-conjugates presents several implications related to accuracy and sensitivity. Such considerations are relevant to the design of ELISA, cell-ELISA, far-Western (ligand) blot, and Western blot (immunodetection) methodologies that are dependent on the application HRPO-conjugated reagents that have been initially developed in the absence of evaluating a comprehensive set of positive and negative controls. The ligand-binding avidity of certain biological proteins for HRPO may not always be inhibited by conventional blocking buffer formulations so experimentation to identify the most ideal types may be necessary. In such instances false positive results may be obtained and more accurate analysis of experimental samples could potentially be achieved with other enzyme-signal indicator systems including

alkaline phosphatase and β -galactosidase. Based on this perspective, it is important to pre-screen representative biological samples by non-reducing far-Western (ligand) blot analysis with non-conjugated or conjugated-HRPO reagents during development of new ELISA, cell-ELISA, Western blot, or far-Western (ligand) blot methodologies that intend to apply conjugated-HRPO as a secondary molecular probe.

In clinical practice, diagnostic analysis of pulmonary fluid is of value not only for identifying primary respiratory conditions (33-35), but also for detecting systemic disorders that either secondarily affect the lung (36-38), or produce biomarkers originating from other diseased tissues and organ systems that subsequently diffuse or become transported into the lung parenchyma or bronchial airways (38). In recent years, the number of clinically relevant diseases associ-



nose-rich composition at multiple terminal positions within HRPO carbohydrate moieties (Fig. 10), both the conjugated and non-conjugated forms of this glycoprotein enzyme would chemically make it applicable as a primary molecular probe for detecting mannose-binding protein fractions relevant to both the

development of clinical diagnostics and conduction of investigative research. Directly in accord with results from the described pulmonary airway fluid analysis model, one of the most effective diagnostic applications of HRPO in this capacity would be its utilization as a primary molecular probe for detecting and characterizing alterations in expression profiles for mannose-binding protein fractions found in biological fluids or cell extracts following size-separation by SDS-PAGE in concert with HRPO far-Western blot analysis. Such a diagnostic strategy is highly analogous to the characterization of coagulation factor VIII/vWF by Western-blot analysis where variations in expression profiles for high and low molecular weight fractions strongly correlate with variations in hemophilia type A and von Willebrand disease clinical severity. The terminal α -mannose-rich segments of glycan side-chains therefore allow the application of HRPO as a diagnostic molecular probe for detecting an array of biological fractions with mannose-binding avidity. One example is plasma mannose-binding lectin (MBL: mannan-binding lectin) which activates the complement cascade after binding to carbohydrate functional groups. In this role MBL is an active participant in innate immune defense mechanisms including those effective against pathogens where its function is dependent upon binding to non-self surface oligosaccharide groups. Mannose-binding lectin (MBL), and other members of the defense collagen family of proteins are pattern recognition molecules that enable phagocytosis. Deficiencies of MBL are the most common congenital immunodeficiencies in humans and affected individuals are predisposed to infections, particularly children or clinical conditions with a superimposed state of compromised immune function. Somewhat analogous clinical conditions where HRPO is a candidate as a diagnostic probe based on its mannose composition and molecular configuration include; [i] Guillian Barré syndrome (elevated mannose-binding lectin concentrations); [ii] primary congenital immunodeficiency mannose-binding lectin (characteristically represents a high risk factor for meningococcal infections); [iii] *P. falciparum* susceptibility and manifestation of severe malaria, (particularly young children with weak or absent specific immune responses); [iv] familial clusters of both

cardiovascular disease and diabetes-related vascular complications; [v] diabetic renal complications related to complement-mediated inflammation and cytolysis; [vi] primary biliary cirrhosis mannose-binding protein hyper-synthesis (e.g. high-risk individuals and familial clusters); [vii] systemic lupus erythematosus (SLE); [viii] bronchial asthma mannose-binding lectins (genetic polymorphism that significantly correlates with serum concentration and disease severity); [ix] phagocytic deficiencies (pathogens, cellular debris, apoptotic cells); [x] autoimmune disease susceptibility (post apoptotic cell clearance); [xi] vulnerability to severe LPS inflammatory responses or possible endotoxin-tolerance phenomenon; [xii] coagulation factor V and VIII related bleeding disorders and LMAN1 expression profiles (correlation with autosomal recessive deficiency); and [xiii] autoimmune/rheumatological disorders (risk secondary to conditions of chronic granulomatous disease). Other potential HRPO applications realistically include the identification of cases at high-risk of developing AIDs and autoimmune/rheumatological disorders in conditions of chronic granulomatous disease.

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Correspondence: Cody P. Coyne

Departments of Basic Sciences

College of Veterinary Medicine

Mississippi State University, Mississippi State

Mississippi 39762. 662 325-1120

Fax 662 325-1031

E-mail: coyne@cvm.msstate.edu