DISSERTATION

IMMUNOPROTEOMIC IDENTIFICATION OF BOVINE PERICARDIUM XENOANTIGENS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2008

COLORADO STATE UNIVERSITY

December 18, 2007

WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY LEIGH GRIFFITHS ENTITLED IMMUNOPROTEOMIC DISCOVERY OF XENOGENEIC BIOMATERIAL ANTIGENS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

IMMUNOPROTEOMIC IDENTIFICATION OF BOVINE PERICARDIUM XENOANTIGENS

Bovine pericardium (BP) is an important biomaterial used in the production of gluteraldehyde-fixed heart valves and tissue engineering applications. The ability to perform proteomic analysis on BP is potentially useful for several reasons including investigation of immune rejection after implantation. The importance of humoral and cell mediated rejection responses towards such xenogeneic tissues are becoming increasingly apparent. I have applied a novel immunoproteomic approach to survey the antigenic determinants of BP.

Proteomic analysis of fibrous tissues like BP is challenging due to their relative low cellularity and abundance of extracellular matrix. A variety of methods for tissue homogenization, protein extraction, and fractionation were investigated with the aim of producing high quality 2-DE gels for both water- and lipid-soluble BP proteins. MALDI-TOF/TOF MS protein identifications were performed to confirm bovine origin and appropriate subcellular fractionation of resolved proteins. Sixteen unique predominantly cytoplasmic bovine proteins were identified from the water-soluble gels. Twenty-two unique predominantly membrane bovine proteins were identified from the lipid-soluble gels. These results demonstrate that the final 2-DE protocol produced high quality proteomic data from BP for both cytoplasmic and membrane proteins.

Duplicate 2-DE gels were used to generate western blots from both water- and lipid-soluble gels. Western blots were probed with pre- and post-exposure anti-BP rabbit serum, with detection of immune complexes limited to the IgG subtype. Western blots were compared to duplicate 2-DE gels and spots matched using Delta 2D image analysis software. Protein identifications of matched spots were performed using either MALDI-TOF/TOF MS or ESI MS/MS. This approach identified 31 putative antigens, capable of stimulating an IgG humoral rejection response.

To the best of my knowledge, this study was the first to apply an immunoproteomic approach for identification of antigenic targets in xenotransplanted tissues. The results provide important information for understanding and possibly mitigating the immune response to fixed and unfixed BP xenografts.

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Acknowledgements

I would like to thank my mentor Dr E. Christopher Orton for his advice, guidance and friendship throughout my time at CSU; Dr Kenneth Reardon for his strong advice and guidance not only in regard to the project presented here, but also his invaluable advice for my future career; Dr Janice M. Bright for her friendship and mentorship throughout my time at CSU, and for helping me realize that being referred to as a "medical clinician" is not an insult; Dr Steve Dow who's extraordinary career achievements stand as a model which I hope one day to emulate; Carla Lacerda for all of her help, patience and friendship; June Boon for her friendship, patience and unparalleled skill in teaching echocardiography.

I am eternally grateful to Chaplin and Cynthia Nolan. Without their generous support of the cardiac surgery program at CSU, I would never have traveled to America and been able to take advantage of the opportunities this move presented.

Custer King deserves special mention, not only for his friendship and energy, but also for helping me through the grieving process. Special thanks to Debbie and Brian King, for their friendship, support of all aspects of cardiology at CSU and for introducing me to Custer.

Research supported by grant number HL081107 from the National Heart Lung and Blood Institute (NHLBI) at the National Institutes of Health. Contents are solely the responsibility of the author and do not necessarily represent the official views of NIH.

Dedication

To my wife Tiffany,

You complete me in every way.

My parents Terry and Janet, whom we miss terribly.

Chas, thank you for sending Marshal to look after me.

I still miss you every day.

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Chapter I

Introduction and Literature Review

Background and Significance

Scientific understanding of rejection processes for xenogeneic biomaterials and solid organ transplants has historically focused on T-cell mediated processes.[1, 2] This bias developed largely due to the fact that histologic appearance of biopsies from valve tissue undergoing rejection shows cellular invasion, which lead to the assumption that rejection is largely a cell mediated response.[3-5] Until recently, the importance of the humoral immune system in rejection and its interaction with cell mediated immune system has largely been overlooked.[1, 4-6] Abundant evidence now exists for involvement of the humoral immune system in all forms of rejection for both solid organs and xenogeneic biomaterials.[2, 4-15] The research presented here focuses on bovine pericardium (BP), a xenogeneic biomaterial used in the formation of current and future generations of heart valve prostheses, as a model system for investigation of the antigenic targets for the destructive humoral immune response.

Although heart valves were once considered to be an immune privileged site, this assumption has been shown to be invalid. Early attempts to avoid the destructive immune response, through the utilization of formalin fixation, failed.[2, 16, 17] It is clear from this work that xenogeneic biomaterials used for valve replacement are susceptible to aggressive immune mediated rejection. Glutaraldehyde fixation was found to be more successful in

masking antigenicity in the short term, due to its ability to irreversibly cross link proteins[2]. Gluteraldehyde fixed valves were found to be subject to calcification and degeneration with chronic implantation, which were considered to be due to a combination of mechanical stresses placed on the valve and glutaraldehyde within the material.[2, 7, 9, 10] In an attempt to alleviate the adverse effects of glutaraldehyde, low concentrations of glutaraldehyde are currently used.[2, 7, 9, 10] This approach has been successful in reducing but not eliminating calcification and degeneration of chronically implanted valves. Mounting evidence suggests that the chronic calcification and degenerative changes seen in glutaraldehyde-fixed valves may be largely mediated by chronic antibody formation and immune mediated rejection.[2, 7, 9, 18-22] Both humoral and cell mediated responses to glutaraldehyde fixed tissues have been conclusively demonstrated.[2, 7, 9, 18-21] Several groups have shown an increase in graft specific circulating antibodies with low glutaraldehyde concentrations when compared to high concentrations.[2, 7-11, 18-23] These graft specific antibodies have been shown to mediate calcification and low grade rejection, which lead to failure over the lifetime of the implant.[7, 9, 19, 20] Presence of donor specific IgG antibodies, high peak antibody titer and chronic persistence of anti-donor antibodies have all been shown to correlate with progressive structural deterioration of gluteraldehyde fixed or cryopreserved tissue valves.[9, 11] In addition, a macrophage mediated foreign body reaction is implicated in graft degeneration. Although this process is initially antibody independent, macrophages that infiltrate the transplanted tissue are capable of presenting the phagocytosed material on MHC II molecules, thereby ultimately also stimulating a humoral immune response.[2, 7-11, 18-21, 23] Increased understanding of the antigenic stimuli responsible for initiating the rejection

responses seen in implanted valves, has important implications for both current and future heart valve replacement technologies.

Tissue-engineering: Promise and Approaches

Tissue-engineering is an approach whereby a viable tissue construct is created by ex vivo seeding of autogenous cells onto a suitable scaffold material. Two fundamental scaffold types have been investigated: synthetic biodegradable scaffolds and decellularized xenogeneic scaffolds.[24-35] Both approaches have been used in attempts to produce a wide range of tissues including bone, tendon, cornea, vessels and heart valves.[24-39] The advantages, disadvantages and problems encountered with each approach are well illustrated by considering the attempts to produce heart valves, although the same issues exist for almost any tissue generated by a tissue engineering approach.

The advantages of synthetic biodegradable scaffolds are the absence of antigenicity and avoidance of risk for transfer of infectious agents. However, the disadvantages of this approach are numerous and far from being solved at this time. These disadvantages are related to the complexity of natural biomaterials and our current inability to mimic this complexity with synthetic approaches. Thus synthetic tissue engineered materials suffer from lack of mechanical stability, the need for protracted periods of tissue morphogenesis before the construct can be implanted, lack of appropriate matrix architecture produced by the seeded cells and difficulty in stimulating appropriate cell differentiation and orientation within the construct.[25, 29, 31, 34, 36, 38, 40, 41]

The advantages of decellularized xenogeneic scaffolds include a scaffold which is immediately implantable due to its appropriate mechanical properties, appropriate matrix

architecture and provision of a more suitable environment for cell differentiation and orientation to occur.[25, 29, 33, 34, 38, 42-45] The major disadvantage to the xenogeneic biological scaffold approach is that tissue antigenicity must be abolished, or at least significantly ameliorated, by the decellularization process to avoid a destructive immune response.[11, 25, 32, 33, 45-53] Whether or not this is an achievable goal has not yet been demonstrated. Until the questions of where antigenicity resides and whether antigenicity can be removed, modified or avoided (via immune modulation) have been answered, further progress in the field is unlikely.

"Decellularization" vs. Antigen Removal in Xenogeneic Bioscaffolds

The stated goal in production of an ideal xenogeneic scaffold is removal of antigenicity, while leaving the matrix unchanged. However, in the tissue engineering field, decellularization has become synonymous with this goal, with most efforts focusing on removal of cellularity from the scaffold as seen on light microscopy.[25, 26, 28, 29, 32-35, 45, 48-50, 54-58] Several physical and chemical methods for decellularization of cardiovascular tissues including porcine heart valves, porcine small diameter vessels, bovine pericardium, and human allografts have been reported.[25, 26, 28, 29, 32-35, 45, 48-50, 54-58] Physical methods for decellularization have included gamma radiation,[59] and freeze drying.[60, 61] Chemical methods for decellularization have included combinations of hypotonic lysis,[48, 56, 57, 62] detergents (e.g. sodium dodecyl sulfate, Triton X-100, sodium deoxycholate),[35, 49, 50, 54, 58, 62-69] trypsin,[26, 33, 45, 55] and nucleases.[22, 48, 57, 62, 70] Most current methods for chemical decellularization incorporate protease inhibitors (e.g. EDTA, phenylmethyl-sulfonyl floride,

aprotinin) to prevent enzymatic degradation of extracellular matrix proteins.[47, 49, 50, 56, 57, 62, 64-67, 70]

Recently, the assumption that absence of histologic cellularity should constitute the principle endpoint for xenogeneic scaffold generation has been challenged.[46, 52, 66, 70] Kasimir et al. used scanning-electron and laser-scanning microscopy to compare various methods of decellularization (trypsin, detergents, nucleases) and found residual cells within the matrix with all non-detergent based methods.[47] The same group demonstrated that decellularized porcine aortic valves stimulated inflammatory cell migration and platelet activation, even when the decellularization process resulted in apparently complete cell removal as judged by scanning-electron microscopy.[46] Goncalves et al. recently reported that decellularization based on tissue histology does not ensure removal of known xenogeneic cellular antigens including α-gal and major histocompatibility complex (MHC) I based on Western blot analysis and immunohistochemistry.[70]

Few *in vivo* studies of implanted decellularized heart valve xenografts have been performed to evaluate the effectiveness of decellularization on host immune response. Most studies report a variable inflammatory response depending on the method of decellularization, tissue type, implantation site and duration of implantation.[46, 57, 62, 71] Vesely et al. performed chemical decellularization (hypotonic lysis, CHAPS, DNAse & RNAse) on porcine heart valves and implanted them into the subcutaneous tissues of Sprague Dawley rats. They found that decellularization decreased tissue calcification, but apparently increased the inflammatory response compared to untreated fresh tissues.[71] Meyer et al. showed inflammatory cell invasion into decellularized rat aortic valve allografts following infrarenal implantation, regardless of decellularization technique (osmotic lysis, detergent or

enzymatic).[62] The Cryolife group has reported implantation of SynerGraft decellularized (hypotonic lysis, DNAse & RNAse) porcine aortic valve conduits into sheep.[56, 57] Although the histopathologic response to implanted tissues was not detailed, the authors' imply little or no apparent inflammatory response to the graft. However, a recent report of SynerGraft porcine aortic valve in pediatric patients reported catastrophic failure associated with severe inflammation and degeneration.[72] The rapid degeneration of the SynerGraft valve following implantation, combined with the severe inflammatory response indicates a likely immune mediated mechanism of failure. These studies illustrate that the often stated primary goal of "decellularization", elimination of antigenicity, probably has not been achieved by current methods. *Further, at this point, it is not known how many antigens are present in xenogeneic tissues or whether all important antigenic targets are cell-associated, as implied in the term "decellularization".*

Insights from Xenotransplantation of Vascularized Organs

In the xenotransplantation field, rejection is characterized by hyperacute, acute vascular, chronic and cellular rejection phases.[73-76] The first two rejection modes in particular lead to an obvious, aggressive rejection process. In contrast, the rejection process seen with xenogeneic scaffold is more subtle, leading to the temptation to overlook this issue in the tissue engineering field.[2, 10, 77, 78] In both xenotransplants and xenogeneic scaffolds, the immunological processes leading to rejection are mediated by naturally occurring antibodies, acquired antibodies and cell mediated responses. The nature of the implanted tissue (i.e. vascular versus avascular) accounts for the differences seen in the rejection process.

Naturally occurring antibodies: Galactosyl α-1,3-galactose (α-gal) is a carbohydrate epitope that is present on almost all cells from lower mammals and New World Monkeys.[79-82] Old world monkeys and primates, including humans, lack α-gal due to evolutionary loss of a α-1,3-galactosyltransferase enzyme that accounts for its production on cell surfaces.[79, 82, 83] As a result of natural exposure to the α-gal moiety, which is also present on the surface of certain microorganisms, humans and other primates produce natural xenoreactive antibodies. These antibodies can comprise as much as 1 to 2% of circulating immunoglobulins in humans.[79-81, 84, 85] Although α-gal has received the most interest due to its ability to promote hyperacute rejection in vascularized xenotransplants, it is clearly not the only naturally occurring antibody.[2, 79, 81, 84, 85] The antigen targets towards which other naturally occurring antibodies are directed remain unclear.[85]

Natural xenoreactive antibodies are primarily of the IgM class, although some IgG class antibodies are also present.[79, 83, 85] Naturally circulating antibodies have two major modes of action: opsonization of foreign material for subsequent phagocytosis and/or enzymatic destruction, and the direct targeting of cell surface antigens with subsequent complement mediated cell lysis. In decellularized xenogeneic scaffolds the epitopes may no longer be cell associated, but rather part of the cellular debris left behind following the decellularization process. Under these circumstances, IgM and IgG primarily act to promote opsonization of this cellular debris. IgM binding allows macrophage and granulocyte adhesion with subsequent release of matrix metaloproteinases leading to tissue destruction.[2, 82] Complexed IgG mediates direct macrophage and polymorphonuclear neutrophil binding and activation.[2, 82] Overall this activation of the immune response would be expected to

produce an acute immune response leading to inflammation and tissue degeneration in days to weeks (as seen with the SynerGraft implant).[72]

Treatment of porcine endothelial cells with green coffee bean α-galactosidase or endo-β-galactosidase C that cleave the terminal α-gal moiety from cell surface oligosaccharides has been shown to delay the onset of hyperacute rejection in xenotransplantation.[86-88] This strategy was abandoned for xenotransplantation of vascularized organs because living cells eventually restore the α-gal moiety to their cell surfaces. Goncalves et al. recently reported that several standard methods of tissue decellularization do not remove the α-gal moiety from bovine pericardium.[70] They further showed that treatment of bovine pericardium with α-galactosidase removed α-gal from the tissue. In the author's view, such targeted removal of antigens with enzymes is a promising approach to tissue decellularization, once antigens are identified. However, such a strategy for targeted removal, modification or modulation of antigens requires specific knowledge of the antigenic targets or clusters of targets. Other possible approaches to the problem including monoclonal antibody blocking, screening of denaturing agents and xenogeneic antibody depletion prior to implantation could all also benefit from antigen discovery research, as it would allow for both specific targeting and monitoring of the techniques efficacy.

Acquired Antibody Response: Acquired antibodies are implicated both in the acute and chronic rejection responses to xenogeneic scaffold material. As with naturally occurring antibodies, acquired antibodies have the ability to induce complement mediated cell lysis and to promote opsonization with subsequent cellular infiltration. The mechanism of acute rejection due to acquired antibodies is the same as that for natural antibodies. Chronic

rejection is thought to occur due to continued production of anti-donor antibodies. These antibodies have been shown to react with extravascular cells and components of the extracellular matrix.[2, 89, 90] When considered in the xenogeneic tissue engineering field, production of acquired xenogeneic antibodies may lead to rejection even under immunosuppressed or antibody depleted conditions, as even low grade damage to critical tissues such as heart valves will lead to failure of the tissue. The findings from glutaraldehyde-fixed tissue valves (a severe antigen modification procedure) suggest that xenoreactive antibody production may be involved in the chronic rejection and calcification processes.[2, 7, 10, 20, 21, 91] Current evidence suggests that mild chronic antibody mediated immune rejection, directed towards both cellular and matrix targets, leads to long term failure. Antigen removal via decellularization may be able to completely remove cellular antigens, however assessment of such removal can only be achieved if the identity of the antigens are known. Matrix antigens may prove more problematic as their removal may lead to loss of tissue integrity. Although some of the components of the extracellular matrix involved in chronic rejection have been identified (e.g. laminin and heparin sulphate proteoglycan) the full range of antigens within the extracellular matrix is unknown.[89, 90] No information is currently available regarding how rapidly and completely autogenously seeded cells replace the antigenic matrix components and thus potentially avoid the chronic rejection process. Recellularization of the scaffold would be expected to lead to extracellular matrix synthesis leading to gradual replacement of the matrix proteins.[92] Such replacement of matrix components may alleviate chronic rejection issues related to the matrix. Thus the situation with regard to matrix antigens may not be as simple as aiming for antigen removal, as antigenic replacement by matrix turnover may with time be able to avoid destructive

immune responses. Clearly, further understanding of this issue requires a through determination of which matrix components are antigenic so that their replacement can be followed.

Cellular Response: Human T cells are capable of recognizing antigens through both the direct (MHC class I) and indirect (MHC class II) pathways.[93, 94] Since a large percentage of the T cell repertoire can recognize a single MHC disparity, MHC antigens represent a major barrier to transplantation. Sequence analysis shows that porcine SLA molecules do not differ significantly from their human HLA homologues.[95] As such, porcine or bovine MHC I or II proteins are likely important xenoantigens capable of being recognized by the direct pathway and must be removed during the decellularization process. Goncalves et al. have shown the presence of MHC I within bovine pericardium, and the inadequate removal of this antigen by all but harsh detergent protocols.[70]

T cell activation via the direct pathway is unlikely to play a major role in rejection of decellularized scaffolds as viable cells are no longer present within the scaffold following the decellularization process. The indirect pathway consists of antigen presenting cells (APC's), which sample the extracellular environment and process any foreign molecules they encounter for presentation on class II MHC molecules. The processed antigen/MHC II complex is then recognized by CD4 T cells (T-helper cells), leading to their activation. Since T-helper cell help is required for B-cell isotype switching and maturation, the indirect pathway is necessary to mount an effective B cell humoral immune response. Thus, monitoring antibody production effectively screens both the CD4 cellular immune response and the humoral response. Although many studies have shown antibody production to a

wide range of cellular and matrix antigens, in most cases, the identity of the target protein is unknown.[2, 73, 89, 90]

All of the above known antigenic barriers to discordant xenotransplantation of vascular organs are also probable barriers to the transplantation of xenogeneic scaffolds for tissue-engineered implants. Goncalves et al. have demonstrated the presence of α-gal and MHC I antigens within the xenogeneic scaffold. What lies beyond these known antigenic barriers to xenotransplantation is largely unknown because so far these barriers have not been overcome for vascularized organs. However, all major antigenic barriers to xenogeneic scaffolds must be identified and targeted for removal, modification or modulation in order for the field to progress beyond its present state. *In order to achieve this goal, the full scope of the antigenic potential of xenogeneic tissues must be defined and characterized. The research presented here outlines the development of a proteomic methodology for antigen detection and identification, and the use of these methods to identify antigenic proteins in BP.*

Hypothesis and Specific Aims

Hypotheses

1: Unknown antigens are present within the cytoplasm, cell membrane and extracellular matrix of BP.

2: Identification of these antigens is possible using an immunoproteomic methodology.

Specific Aims

1: To extract and separate by 2-D gel electrophoresis the water-soluble and lipid-soluble protein fractions of BP.

2: To determine the antigenicity of BP by exposing the proteins separated in aim 1 to acquired (polyclonal rabbit immune serum) antibodies generated against BP.

3: To identify by MALDI-TOF-TOF or ESI MS/MS mass spectrometry, the proteins that are found in specific aim 2 to elicit an IgG specific humoral immune response towards BP.

Chapter II

Extraction and two-dimensional gel electrophoresis of water- and lipid-soluble proteins from bovine pericardium: a low-cellularity tissue

Abstract

Bovine pericardium (BP) is an important biomaterial used in the production of gluteraldehyde-fixed heart valves and tissue engineering applications. The ability to perform proteomic analysis on BP is potentially useful for several reasons including investigation of immune rejection after implantation. However, proteomic analysis of fibrous tissues like BP is challenging due to their relative low cellularity and abundance of extracellular matrix. A variety of methods for tissue homogenization, protein extraction and fractionation were investigated with the aim of producing high quality 2-DE gels for both water- and lipid-soluble BP proteins. MALDI-TOF/TOF MS protein identifications were performed to confirm bovine origin and appropriate subcellular fractionation of resolved proteins. Sixteen unique predominantly cytoplasmic bovine proteins were identified from the water-soluble gels. Twenty-two unique predominantly membrane bovine proteins were identified from the lipid-soluble gels. These results demonstrated that the final protocol produced high quality proteomic data from this important tissue for both cytoplasmic and membrane proteins.

Introduction

Bovine pericardium (BP) is used in the fabrication of glutaraldehyde-fixed bioprosthetic heart valves[10, 56, 96, 97] and may have an emerging application as an unfixed biological scaffold for tissue-engineering applications.[98-101] In the latter application, BP is treated by a process known as "decellularization" in an attempt to prevent immune rejection.[26, 66, 70, 98-102] Several groups have shown that the current generation of decellularized biological scaffolds are subject to immune-mediated rejection.[2, 9, 10, 42, 47, 72] The fundamental concept of decellularization has thus come under scrutiny with the focus shifting away from removal of visible cells and toward identification and removal of antigenic proteins.[46, 70, 102] Any protein that differs in its structure between the donor and recipient species is a potential antigen. As a result the full spectrum of potential antigenic proteins when BP is implanted across species lines is not known.[6, 103, 104] The goal of this study is to survey the large number of BP proteins for antigenic potential using a powerful proteomic approach. A necessary first step is to generate high quality two-dimensional electrophoresis (2-DE) separations from BP.

Development of a 2-DE protocol for BP presents difficulties due to its low cellularity (high matrix-to-cell ratio) and relative low abundance of soluble proteins compared to other tissues or organs.[70, 102, 105] The problem is exacerbated by high abundance structural or matrix proteins that can overwhelm the remainder of the proteome.[106-110] Approaches to these problems in the proteomic analysis of other tissues, have included prefractionation at the protein isolation stage or fractionation of the sample after protein extraction.[106-110] Lipid-soluble proteins in the tissue present a further complication, as lipids in general are particularly difficult to separate via 2-DE gels owing to their relative insolubility.[111, 112]

These difficulties make BP a challenging tissue from which to generate high quality proteomics data.[113]

Since both water- and lipid-soluble proteins from BP may have biologic interest, I was interested in methods for obtaining both fractions. Here, I describe the development and validation of protocols for extraction, prefractionation, and 2-DE separation of water- and lipid-soluble protein fractions from BP. The methods reported here should have application for other low-cellularity high-matrix tissues.

Materials and Methods

Tissue Harvest

BP was harvested aseptically from adult cattle within 8 h of death. Animals showed no signs related to cardiovascular disease prior to death or evidence of cardiothoracic pathology at postmortem. BP was transported in pH 7.4 PBS, 0.1% w/v EDTA, 100 KIU/ml aprotinin, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B. Pericardial fat and connective tissue were removed. Pericardium was sectioned into 1 cm squares (approximately 0.1-0.2 g), placed in separate 5-ml cryogenic vials with 85% DMEM and 15% DMSO, and stored at -70 °C within 1 h of harvest.

Tissue Homogenization

Manual, ultrasonication, and mechanical homogenization protocols were compared using water-soluble protein extraction solutions defined in Protocol A of Table 2.1. For manual homogenization, samples of BP were snap frozen in liquid nitrogen, minced into pieces approximately 0.5-1 mm on a side using sterile surgical instruments, and placed in 1 ml of water-soluble extraction solution. For ultrasonic homogenization, minced samples of

BP were subjected to an additional step of 2 min continuous ultrasonication at 30 kHz on ice (Artek Systems Corporation, Farmingdale, NY, USA). For mechanical homogenization, minced samples of BP were subjected to 2, 10, or 60 s of homogenization on ice at 15,000 rpm using a Powergen 700 homogenizer (Fisher Scientific, Pittsburgh, PA, USA). The remainder of the extraction protocol was performed as described for the water-soluble fraction below.

The potential for nucleic acid contamination was investigated by comparing homogenized samples with and without incubation with 2000 KIU/mL deoxyribonuclease I (Sigma-Aldrich, St. Louis, MO, USA) and 10 μg/mL ribonuclease A (Sigma-Aldrich, St.Louis, MO, USA), prior to 2-DE. DNA and RNA levels in homogenized samples were determined by measuring 260/280 absorption ratio using a GeneQuant II RNA/DNA calculator (Pharmacia Biotech, Buckinghamshire, UK).[114]

Protein Extraction

The standard extraction solution for all protocols contained 10 mM pH 8.0 Tris HCl, 100 KIU/mL aprotinin, 1 mM DTT, 2 mM $MgCl_2$, 10 mM KCl, and 0.5 mM Pefabloc (Sigma-Aldrich, St.Louis, MO, USA) in Nanopure water. Additives to this standard extraction solution were compared and are defined in Table 2.1.

Homogenized BP was placed in 1.5 ml cryogenic vials, containing 1 mL of a water-soluble extraction solution (Table 2.1). Tubes were shaken on ice for 1 h. Samples were centrifuged at 17,000xg, 4 ^{o}C for 25 min. The supernatant was collected and designated the water-soluble protein fraction (Figure 2.1). The pellet was washed twice in 1 mL of water-soluble extraction solution by repeating the above extraction procedure. The supernatant from each wash was discarded. The remaining pellet was resuspended in 0.5 ml of various

lipid-soluble extraction solutions (Tables 2.1 and 2.2) and shaken on ice for 2 h. Samples were centrifuged at 17,000xg, 4 °C for 25 min. The supernatant was designated the lipid-soluble protein fraction (Figure 2.1).

The water-soluble fraction was concentrated with Centricon Ultracel YM-3 (cut off 3000 Da) centrifugal filters (Millipore, Billerica, MA, USA) at 6,500xg, 4 °C for 2 h. The lipid-soluble fraction was concentrated with the same filters at 6,500xg, 4 °C for 90 min. The final concentrated fractions were stored at -80 °C until required (Figure 2.1). Differences in protein yield between extraction protocols were determined by a 1-way ANOVA. Values of $p < 0.05$ were considered significant. Where significant differences where found, post-hoc analysis was performed using Tukey Kramer HSD test.

Protein Precipitation

The effects of two protein precipitation methods were investigated for the lipid-soluble fraction. TCA/acetone precipitation was performed using a modification of the technique described by Song et al. (Table 2.2).[110] Briefly, lipid-soluble extracts were precipitated with three volumes of 10% TCA w/v in acetone. The sample was incubated at -4 °C for 45 min and then centrifuged at 17,000 xg for 25 min at 4 °C. The pellet was washed three times in ice-cold acetone, incubated for 45 min at -4 °C, and centrifuged at 17,000xg for 25 min at 4 °C. The final pellet was air dried at room temperature and resuspended in one of several precipitate resuspension solutions detailed in Table 2.2.

Ethanol precipitation was performed as previously described.[115] Briefly, lipid-soluble extracts were precipitated with nine volumes of ice-cold 100% ethanol. Samples were incubated at -4 °C for 60 min, and then centrifuged at 17,000xg for 25 min at 4 °C. The

supernatant was discarded. The pellet was air dried and resuspended in one of several precipitate resuspension solutions (Figure 2.1 and Table 2.2).

Protein Assays

Total protein concentration was determined using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) was used to make standard solutions. Hydroxyproline content was determined using a modification of the procedure reported by Woessner.[116] Repeatability of both assays was within 1%.

1-D SDS-PAGE

Protein samples were separated in a XCell, SureLock™ electrophoresis system (Invitrogen, Carlsbad, CA, USA) using 4-12% Tris-glycine polyacrylamide gels (Invitrogen, Carlsbad, CA, USA). The 1-D SDS-PAGE protocol recommended by the manufacturer was used. Briefly, 5 µl of Magic Mark XP protein standard (Invitrogen, Carlsbad, CA, USA) was loaded into Lane 1 of the gel. Samples were diluted 1:1 with sample buffer (Invitrogen, Carlsbad, CA, USA), with 2.5% β-mercaptoethanol (Invitrogen, Carlsbad, CA, USA) and boiled for 1 min. Protein extracts were compared by loading 37 µg of water-soluble protein or 11 µg of lipid-soluble protein onto the gel. Non-sample lanes were loaded with the sample buffer. The upper and lower buffer chambers were loaded with SDS running buffer (Invitrogen, Carlsbad, CA, USA). Duplicate gels were electrophoresed at 125 V for 90 min and stained with Sypro® Ruby protein gel stain (Invitrogen, Carlsbad, CA, USA). Digital gel images were recorded using a UVP Bioimaging system (UVP Inc, Upland, CA, USA).

2-D Gel Electrophoresis

IEF was carried out using 18-cm pH 3-10 non-linear IPG ReadyStrips™ (Bio-Rad, Hercules, CA, USA). For all samples, 100 μg of protein was loaded per gel. A minimum of three replicate gels were performed for each protocol outlined in Tables 2.1 and 2.2. Samples were diluted with the rehydration buffer listed for each protocol (Tables 2.1 and 2.2) to a final volume of 408 μl. IEF rehydration was performed overnight using Immobiline™ DryStrip rehydration trays (GE Healthcare, Piscataway, NJ, USA). Protein IEF was performed using a Multiphor II electrophoresis system (GE Healthcare, Piscataway, NJ, USA) at 20 °C, with an initial 1 min linear increase in voltage to 500 V, followed by a linear increase in voltage to 3500 V over 5 h, and then a constant voltage of 3500 V for 17.5 h.

IPG strips were reduced by submersion in 2% DTT w/v, 6 M urea, 30% glycerol v/v and 0.1% SDS w/v for 15 min and then alkylated by submersion in 2.5% iodoacetamide, 6 M urea, 30% glycerol v/v, 0.1% SDS w/v and a trace of bromophenol blue for 5 min. Strips were immediately loaded onto cast 12% polyacrylamide 2-D gels (18 cm x 20 cm x 1 mm) and electrophoresis performed at 3000 V for 3 h in a Protean II XL 2-D Multi-Cell (Bio-Rad, Hercules, CA, USA).[117]

Gels were stained with a silver staining protocol.[118] Digital gel images were created using a UVP Bioimaging system. The number of gel spots was determined with ProteomWeaver software (Version 3.1, Definiens AG, Munich, Germany). Differences in spot number between protocols were compared with one-way ANOVA. Values of $p < 0.05$ were considered significant. Where significant differences where found, post-hoc analysis was performed using Tukey Kramer HSD test.

Protein Identification

Gel spots were excised from silver-stained 2-D gels, destained overnight with a 1:1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulphate, washed twice for 30 min in 200 mM ammonium bicarbonate (ABC) in 40% acetonitrile (ACN), and dehydrated with 100% ACN for 5 min. The supernatant was removed and gel pieces were dried using a SPD SpeedVac® (Thermo Electron Corporation, Waltham, MA, USA).

Tryptic digestion was accomplished by incubation of the gel spots with 0.4 μg sequencing grade modified trypsin (Promega, Madison, WI, USA) in 40 mM ABC overnight at 37 °C. Peptides were extracted from the gel spots in a stepwise manner by sequential incubation with 40 mM ABC for 15 min at room temperature, 5% formic acid for 15 min at 37 °C and 100% ACN for 15 min at 37 °C. The supernatant from the overnight tryptic digestion and those from all subsequent peptide extraction steps were collected and pooled. The pooled supernatants were dried using the Speedvac to a final volume of 5-10 μl. Peptides were desalted using a ZipTip® C18 pipette tips (Millipore, Billerica, MA, USA).[119]

Desalted digests were spotted onto target plates with 5 mg/mL α-cyano-4-hydroxycinnamic acid and 1 mg/ml ammonium phosphate. MALDI-MS/MS was performed on a 4800 Proteomics Analyzer running v2.0 software (Applied Biosystems, Framingham, MA). MS was performed in positive ion reflector mode over the 800-4000 *m/z* mass range, with 1250 laser shots per spot and internal calibration. Up to four of the most intense peaks, excluding trypsin autolysis peaks, were selected from each MS spectrum for MS/MS analysis. Tandem MS was performed in positive ion mode with 4200 laser shots, 2 kV collision energy, air at $1E^{-6}$ torr as the collision gas, and default calibration. GPS Explorer (v3.6 Applied Biosystems) was used as an interface between the raw data from the mass

spectrometer and a local copy of Mascot search engine (v2.1.04 Matrix Science, London, UK). A combined MS and MS/MS search was performed against a local copy of NCBInr (downloaded 8/15/06). Mascot searches were restricted to mammalian taxonomy with 50 ppm MS and 0.3 Da MS/MS mass tolerances, trypsin specificity allowing for one missed cleavage and the following three variable modifications: methionine oxidation and cysteine modifications by iodoacetamide and acrylamide. The criterion used to determine protein identification was a GPS Explorer protein score confidence interval greater than 99%.

Cellular location (membrane, cytoplasmic or nuclear) of identified proteins was determined by a combination of peer-reviewed literature search, NCBI protein database search,[120] PSORT subcellular localization prediction,[121] and prediction of trans-membrane protein segments using HMMTOP computer server program.[120-122]

Results

Tissue Homogenization

Homogenization protocol had a significant effect on the yield and quality of water-soluble proteins obtained. Manual homogenization and sonication produced similar protein yields. Mechanical homogenization for 10 s increased the protein yield by 62%, but created smearing in the high MW-low pH region of the 2-D gel that worsened with increasing duration of homogenization. Nucleic acid contamination was ruled out as the source of the gel smearing. Hydroxyproline analysis of samples eliminated fragmented collagen as the cause of gel smearing. Although the spot pattern of gels from mechanically homogenized samples was similar to manually homogenized samples, the number and intensity of the spots decreased with increasing duration of mechanical homogenization. For this reason,

mechanical homogenization was abandoned as a viable protocol and the cause of gel smearing was not investigated further. Manual homogenization gave the best combination of yield and protein separation on 2-D gels, and for this reason was used for subsequent comparisons among protein extraction protocols.

Protein Extraction

Protein extraction protocols A through E in Table 2.1 were compared based on protein yield and 1-D SDS-PAGE band resolution. Protocols C (0.01% Triton X-100) and E (134 mM 3-(benzyldimethylammonio)-propanesulfonate (NDSB-256)) produced the highest yield of the water-soluble proteins, while Protocol A (1.25% SDS) produced the highest yield of the lipid-soluble proteins (Figure 2.2). Protocol E (134 mM NDSB-256) produced the best overall water-soluble protein profile, especially in the high MW region, based on distinct bands that were not resolved by other protocols (Figure 2.3A). Protocol E (1% n-dodecyl β-D-maltoside) also produced the best 1-D SDS-PAGE gels for lipid-soluble proteins based on distinct bands in both the high MW (120-200 kDa) and low MW (30-40 kDa) regions that were not resolved by other protocols (Figure 2.3B).

2-D Electrophoresis

For the water-soluble protein extracts, only protocols using 0.1% SDS (Protocols A, F and G) or 134 mM NDSB-256 (Protocol E) produced good quality gels with a large number of well defined spots. Other protocols produced gels with significant horizontal smearing, low spot number (mean < 50 spots/gel), and/or poor spot resolution. The mean number of spots per gel was 294.3 ± 48.3 for 0.1% SDS (Protocols A, F and G) and 305.5 ± 30.4 for 134 mM NDSB-256 (Protocol E) and was not significantly different among protocols ($p = 0.77$).

For the lipid-soluble extracts, only protocols incorporating 1% n-dodecyl β-D-maltoside (Protocols E, F and G) or 1.25% SDS (Protocol A) yielded resolved spots. Other protocols produced horizontal smearing with no discernable spots. Extraction with 1.25% SDS (Protocol A) resulted in a low mean number of resolved spots (153.5 ± 21.9) and substantial horizontal and vertical smearing. By comparison, extraction with 1% n-dodecyl β-D-maltoside (Protocols E, F and G) yielded a higher number of well resolved spots (248.8 ± 47.5) and with minimal smearing. Overall, the number of spots was lower ($p = 0.02$) for the lipid-soluble extracts (Protocols E, F and G) compared to water-soluble extracts even though the protein load was equal. In addition, the combined spot intensity of the gels from lipid-soluble extracts was lower (qualitative assessment) than for gels from water-soluble extracts.

The effects of a precipitation step were assessed for the lipid-soluble fraction. No combination of precipitation solution, resuspension buffer, or running buffer (Protocols A1 - A4 in Table 2.2) improved gels produced by 1.25% SDS lipid-soluble extraction (Protocol A). Both TCA/acetone and ethanol precipitation (Protocols E1, E2, F1 and F2 in Table 2.2) increased spot number and intensity for 1% n-dodecyl β-D-maltoside extractions. For 0.1% SDS followed by 1% n-dodecyl β-D-maltoside extraction (Protocol F), ethanol precipitation increased the spot number from 153.5 ± 30.4 to 430.5 ±2.1 per gel (Protocol F2). Similarly the use of ethanol precipitation with protocol E (134 mM NDSB-256 followed by 1% n-dodecyl β-D-maltoside extraction) increased the spot number from 344 ± 89.1 to 472 ± 52.3 spots per gel (Protocol E2). In addition to producing the highest number of spots, Protocol E2 yielded the best spot resolution with minimal smearing in either dimension (Figures 2.4 and 2.5).

Protein Identification

Protein identifications from gels generated by the Protocol E2 are reported in Tables 2.3 and 2.4. Twenty-five proteins were identified from water-soluble protein gels, yielding 16 unique proteins. The subcellular location was confirmed or predicted to be cytoplasmic in 80% of these proteins.[120, 123-126] Twenty-eight protein identifications, representing 22 unique proteins, were made from lipid-soluble protein gels. The subcellular location was confirmed or predicted to be nuclear or membrane in 71% of the proteins identified from lipid-soluble gels.[120, 123-133]

Discussion

Fibrous tissues like BP have a large extracellular matrix component and low cellularity compared to other tissues. Type 1 collagen alone accounts for as much as 76% of the protein content of BP.[42, 105, 134] Proteomic analysis of fibrous and other tissues with high extracellular matrix content such as bone or cartilage can prove challenging, particularly when the proteins of interest are relatively low abundance cellular proteins.[106-110, 135-137] Because extracellular matrix proteins such as collagen are large relatively insoluble polymers, the problem they present is fundamentally different from tissues such as serum with high abundance soluble proteins (e.g. albumin).[136] The presented approach aims to avoid sample contamination with high abundance extracellular matrix proteins by avoiding homogenization methods that could fragment and solubilize matrix proteins and to exploit differences in protein solubility between matrx and cellular proteins. Protein extraction methods and solutions are known to affect 2-DE gel spot number and resolution in ways that can be difficult to predict.[109, 110, 138-140] I thus adopted a systematic trial-and-error approach to

protein extraction and fractionation with the ultimate endpoints of generating the highest number and best resolution of gel spots for both water- and lipid-soluble protein fractions.

In this study, simple mincing of the tissue prior to protein extraction produced the best 2-DE gels. Both mechanical and ultrasonic homogenization methods introduced significant horizontal and vertical smearing in the high MW, low pH region of the gel. Such smearing has been variously reported to result from high ionic detergent concentration, high salt concentration, nucleic acids, lipids, polysaccharides, or proteoglycans and glycosaminoglycans.[135, 141, 142] I eliminated nucleic acids, lipid, high ionic detergent, high salt concentration, and fragmented collagen as causes of the observed smearing, but ultimately did not determine the cause of the smearing. Because smearing could be prevented by avoiding aggressive methods of tissue homogenization, its cause was not pursued further.

Protein yields were predictably low compared to yields reported for more cellular tissues such as myocardium and skeletal muscle.[136, 138, 139] Yields were comparable to those reported for other relatively low cellularity tissues,[139] and we thus concluded that extraction efficiency was acceptable. Differences in yield for the lipid-soluble extract were identified between SDS- and n-dodecyl β-D-maltoside-based protocols: however, differences were not found in total protein yield. This suggested that the observed differences were due to differences in protein fraction segregation rather than total protein extracted.

Several extraction protocols studied here were shown to be incompatible with subsequent 2-DE despite the fact that they have been used successfully for other tissues.[107, 109, 138, 141, 142] Since the extraction detergent was the only variable among these protocols, incomplete sample solubilization in the extraction buffer or poor solubility in the rehydration

buffer were the most likely explanations for the poor 2-DE results with protocols B, C and D.[142, 143] One extraction protocol (Protocol E, NDSB-256) produced clearly superior overall results for water-soluble proteins based on both 1-D and 2-D SDS-PAGE gels. NDSB-256 is a zwitterionic molecule which has previously been shown to be useful in increasing protein yield during extraction.[142-144] Sixteen unique bovine proteins were identified from gels generated using this protocol. Eighty percent of these proteins were known or predicted to be cytoplasmic in origin suggesting that fractionation to the water-soluble fraction was appropriate.[120, 123-126]

Lipid-soluble proteins are difficult to display on 2-DE gels because their relative insolubility in non-ionic or zwitterionic detergents, and tendency to precipitate at pH values close to their isoelectric point.[111, 141, 143] Only protocols employing n-dodecyl β-D-maltoside in the extraction solution produced resolved gel spots with the lipid-soluble fraction. This nonionic detergent has been useful in other protocols for analysis of lipid-soluble proteins.[111, 112, 142] Contamination of samples with lipid as been shown to cause low spot number and intensity on 2-DE gels by forming protein-lipid complexes or protein precipitation during IEF focusing.[111, 141, 143, 145] This possibility was investigated by adding TCA/acetone or ethanol precipitation delipidation methods to the extraction.[137] These methods significantly increased the number of spots resolved with one protocol (protocol E2) producing gels with the highest number of spots. Twenty-two unique bovine proteins were identified from gels using this protocol. Seventy-one percent of these proteins were known or predicted to be membrane or nuclear in origin.[120, 123-132]

The GRAVY index values for proteins identified from the lipid-soluble fraction were not found to be significantly different from those for the water-soluble fraction. Previous

reports have noted that not all membrane proteins are hydrophobic, and thus may have a negative GRAVY index value.[146, 147] Such proteins may contain only a single transmembrane domain with the remainder of the protein being hydrophilic.[146, 147] The presented findings suggest that GRAVY index may not be a reliable validation of appropriate fractionation of water- and lipid-soluble proteins.

In conclusion, I found that extraction of water- and lipid-soluble protein fractions is possible from relatively low cellularity fibrous tissues such as bovine pericardium. Further I demonstrated that high-quality 2-D gels can be generated and appropriate protein identifications made from both protein fractions. To the author's knowledge, methods developed here are the first to be successfully applied to pericardium for 2-DE gel proteomic analysis. These methods should have application to other fibrous tissues such as tendon, ligament, fascia, and cardiac valves.

Table 2.1. Protein extraction protocols evaluated in this research. The reagents listed in the second and third columns were added to the standard extraction solution (see methods).

Protocol	Water-Soluble Extraction Solution	Lipid-Soluble Extraction Solution	IEF Rehydration Buffer
Protocol A	0.1% SDS	1.25% SDS	8M urea, 2%CHAPS, 0.3% DTT, 2% Ampholyte 3-10NL
Protocol B	0.4% CHAPS	4% CHAPS	8M urea, 2%CHAPS, 0.3% DTT, 2% Ampholyte 3-10NL
Protocol C	0.01% Triton X-100	0.1% Triton X-100	8M urea, 2%CHAPS, 0.3% DTT, 2% Ampholyte 3-10NL
Protocol D	0.005% sodium deoxycholate	0.05% sodium deoxycholate	8M urea, 2%CHAPS, 0.3% DTT, 2% Ampholyte 3-10NL
Protocol E	134mM NDSB-256	1% n-dodecyl β-D-maltoside	8M urea, 1% CHAPS, 0.2% DTT, 1% Triton X-100, 3% NDSB-256, 1.5% ASB 14, 2% Ampholyte 3-10NL
Protocol F	0.1% SDS	1% n-dodecyl β-D-maltoside	8M urea, 2% CHAPS, 0.3% DTT, 2% Ampholyte 3-10NL
Protocol G	0.1% SDS	1% n-dodecyl β-D-maltoside	8M urea, 1% CHAPS, 0.2% DTT, 1% Triton X-100, 3% NDSB-256, 1.5% ASB 14, 2% Ampholyte 3-10NL

Table 2.2. Subprotocols for lipid soluble extraction precipitation experiments. The basic components added to the standard solution (see methods), for each of the water and lipid soluble extraction steps is shown for each protocol. The precipitation method, resuspension solution, and IEF rehydration buffer for each protocol is detailed.

Protocol	Water Soluble Extraction Solution	Lipid Soluble Extraction Solution	Lipid Soluble Fraction Precipitation Method	Precipitate Resuspension Solution	IEF Rehydration Buffer
Protocol A1	0.1% SDS	1.25% SDS	10% w/v TCA in acetone	1.25% SDS in standard solution	8M urea, 2% CHAPS, 0.3% DTT, 2% Ampholyte 3-10NL
Protocol A2	0.1% SDS	1.25% SDS	100% ethanol	1.25% SDS in standard solution	8M urea, 2% CHAPS, 0.3% DTT, 2% Ampholyte 3-10NL
Protocol A3	0.1% SDS	1.25% SDS	10% w/v TCA in acetone	9M urea, 3% CHAPS, 1% DTT, 1% Triton X-100, 1% NDSB-256	8M urea, 1% CHAPS, 0.2% DTT, 1% Triton X-100, 3% NDSB-256, 1.5% ASB 14, 2% Ampholyte 3-10NL
Protocol A4	0.1% SDS	1.25% SDS	100% ethanol	9M urea, 3% CHAPS, 1% DTT, 1% Triton	8M urea, 1% CHAPS, 0.2% DTT, 1% Triton X-100, 3%

				X-100, 1% NDSB-256	NDSB-256, 1.5% ASB 14, 2% Ampholyte 3-10NL
Protocol E1	134mM NDSB-256	1% n-dodecyl β-D-maltoside	10% w/v TCA in acetone	9M urea, 3% CHAPS, 1% DTT, 1% Triton X-100, 1% NDSB-256	8M urea, 1% CHAPS, 0.2% DTT, 1% Triton X-100, 3% NDSB-256, 1.5% ASB 14, 2% Ampholyte 3-10NL
Protocol E2	134mM NDSB-256	1% n-dodecyl β-D-maltoside	100% ethanol	9M urea, 3% CHAPS, 1% DTT, 1% Triton X-100, 1% NDSB-256	8M urea, 1% CHAPS, 0.2% DTT, 1% Triton X-100, 3% NDSB-256, 1.5% ASB 14, 2% Ampholyte 3-10NL
Protocol F1	0.1% SDS	1% n-dodecyl β-D-maltoside	10% w/v TCA in acetone	9M urea, 3% CHAPS, 1% DTT, 1% Triton X-100, 1% NDSB-256	8M urea, 1% CHAPS, 0.2% DTT, 1% Triton X-100, 3% NDSB-256, 1.5% ASB 14, 2% Ampholyte 3-10NL
Protocol F2	0.1% SDS	1% n-dodecyl β-D-maltoside	100% ethanol	9M urea, 3% CHAPS, 1% DTT, 1% Triton	8M urea, 1% CHAPS, 0.2% DTT, 1% Triton X-100, 3%

				X-100, 1% NDSB-256	NDSB-256, 1.5% ASB 14, 2% Ampholyte 3-10NL

Table 2.3. Protein identification, MW, pI and GRAVY index for water-soluble proteins in Figure 2.4. * = Unique Protein ID, *1 = Duplicate Protein ID within gel.

Spot Number	Protein Name	Accession N°	Subcellular Location	MW (Da)	pI	Peptides Matched	GRAVY Index
1	triosephosphate isomerase*1	gi|61888856	Cytoplasm	26672.8	6.45	16	-0.116
2	apolipoprotein A-I*1	gi|245563	Cytoplasm/ Membrane	28414.8	5.57	19	-0.777
3	albumin*1	gi|30794280	Cytoplasm	69278.5	5.82	17	-0.443
4	triosesphosphate isomerase*1	gi|61888856	Cytoplasm	26672.8	6.45	16	-0.116
5	haptoglobin*1	gi|83638561	Cytoplasm	44830.6	7.83	11	-0.400
6	albumin*1	gi|30794280	Cytoplasm	69278.5	5.82	17	-0.443
7	alpha enolase*1	gi|4927286	Cytoplasm/ Membrane	47247.3	6.44	13	-0.267
8	beta hemoglobin*	gi|27819608	Cytoplasm	15944.3	7.01	6	-0.004

9	glutathione S-transferase pi[*1]	gi|29135329	Cytoplasm	23598	6.89	4	-0.237
10	apolipoprotein A-IV precursor[*]	gi|79160178	Cytoplasm/ Membrane	42991.4	5.3	18	-0.607
11	haptoglobin[*1]	gi|83638561	Cytoplasm	44830.6	7.83	11	-0.400
12	endoplasmic reticulum protein 29 precursor[*]	gi|109658363	Cytoplasm	28787.9	5.63	6	-0.376
13	GRP78[*]	gi|73968064	Cytoplasm	50923.7	6.03	10	-0.294
14	glutathione S-transferase pi[*1]	gi|29135329	Cytoplasm	23598	6.89	10	-0.237
15	albumin[*1]	gi|162648	Cytoplasm	69278.5	5.82	19	-0.443
16	PARK7 protein[*]	gi|73586598	Cytoplasm/ Nucleus	19598	6.84	8	-0.034
17	apolipoprotein A-I[*1]	gi|245563	Cytoplasm/ Membrane	28414.8	5.57	19	-0.777
18	heat shock 27kDa	gi|71037405	Nuclear	22665.4	5.77	8	-0.597

	protein 1*						
19	enolase 1*1	gi|74354056	Cytoplasm/ Membrane	47296.4	6.37	10	-0.212
20	haptoglobin*1	gi|83638561	Cytoplasm	44830.6	7.83	5	-0.4
21	Serotransferrin*	gi|2501351	Secreted	77753.1	6.75	5	-0.452
22	superoxide dismutase 2 mitochondrial*	gi|86827690	Cytoplasm/ Membrane	24622.6	8.7	7	-0.353
23	IgG Chain C (Membrane Bound)*1	gi|76678688	Membrane	45138.2	5.25	4	-0.211
24	IgG Chain C (Membrane Bound)*1	gi|76678688	Membrane	45138.2	5.25	4	-0.211
25	α-1-acid glycoprotein precursor*	gi|58531035	Secreted	21781.8	5.9	2	-0.361

Table 2.4. Protein identification, MW, pI and GRAVY index for lipid-soluble proteins in Figure 2.5. * = Unique Protein ID, *1 = Duplicate Protein ID within gel.

Spot Number	Protein Name	Accession N°	Subcellular Location	MW (Da)	pI	Peptides Matched	GRAVY Index
1	beta-2-glycoprotein I*	gi|6	Membrane	37901.6	8.56	13	-0.265
2	annexin I*1	gi|74	Membrane	38873.2	6.44	8	-0.436
3	inter-alpha-trypsin-inhibitor (protein HC)*	gi|27806743	Membrane	39209.4	7.81	4	-0.334
4	superoxide dismutase2 mitochondrial*	gi|86827690	Cytoplasm/ Membrane	24622.6	8.7	7	-0.353
5	apolipoprotein A-I*1	gi|245563	Cytoplasm/ Membrane	28414.8	5.57	19	-0.777
6	beta-actin*	gi|73958063	Cytoplasm	31222.5	5.04	7	-0.213
7	apolipoprotein A-IV precursor*	gi|79160178	Cytoplasm/ Membrane	42991.4	5.3	18	-0.607
8	gamma-actin*	gi|809561	Cytoplasm	40992.5	5.56	15	-0.201

9	chain F bovine F1-ATPase covalently inhibited with 4-chloro-7-nitrobenzofurazan*	gi|3660253	Membrane	51448.9	4.97	14	0.008
10	albumin*1	gi|30794280	Cytoplasm	69278.5	5.82	17	-0.443
11	ATP synthase H+ transporting mitochondrial F1 complex, alpha subunit. Isoform 1, cardiac muscle*	gi|94574274	Membrane	59684.7	9.21	9	-0.076
12	HSBP1 protein*1	gi|74354863	Nucleus	22379.3	5.98	14	-0.591
13	alpha-1-B glycoprotein*	gi|86823979	Cytoplasm	53520.3	5.29	17	-0.127
14	HSBP1 protein*1	gi|74354863	Nucleus	22379.3	5.98	14	-0.591
15	AMBP protein*	gi|74354219	Membrane	39206.4	8.07	8	-0.359

16	haptoglobin*1	gi|83638561	Cytoplasm	44830.6	7.83	5	-0.4
17	smooth muscle protein SM22 homolog*	gi|543113	Membrane	19325.6	6.96	7	-0.418
18	alpha enolase*	gi|4927286	Membrane	47247.3	6.44	13	-0.267
19	heat shock 27kDa Protein 1*	gi|71037405	Nucleus	22665.4	5.77	8	-0.597
20	apolipoprotein A-I*1	gi|245563	Membrane	28414.8	5.57	19	-0.777
21	chain B, phosphatidylethanolamine-binding protein*	gi|6729707	Membrane	20841.6	7.38	3	-0.69
22	haptoglobin*1	gi|83638561	Cytoplasm	44830.6	7.83	5	-0.4
23	annexin I*1	gi|74	Membrane	38873.2	6.44	8	-0.436
24	HSBP1*1	gi|74354863	Nucleus	22379.3	5.98	14	-0.591
25	albumin*1	gi|76445989	Cytoplasm	53890.1	6.09	5	-0.390
26	ATP synthase, H+ transporting	gi|27807305	Membrane	18680.7	5.99	6	-0.658

	mitochondrial F0 complex subunit d						
27	triosephosphate isomerase*	gi|61888856	Cytoplasm	26672.8	6.45	16	-0.116
28	voltage-dependant anion channel 1	gi|73586892	Membrane	30820.7	8.82	13	-0.417

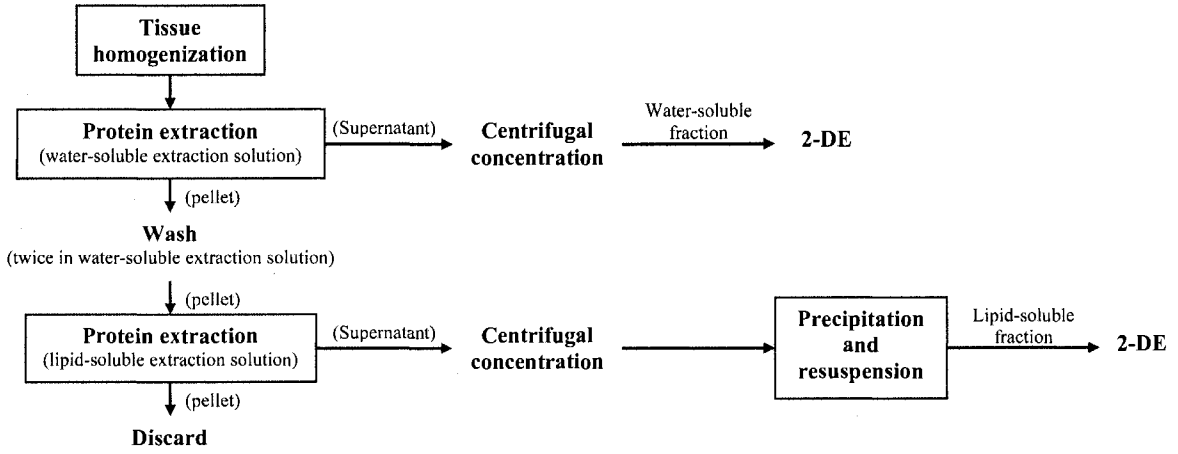

Figure 2.1. Schematic of the workflow involved in testing protocols for Tables 2.1 and 2.2.

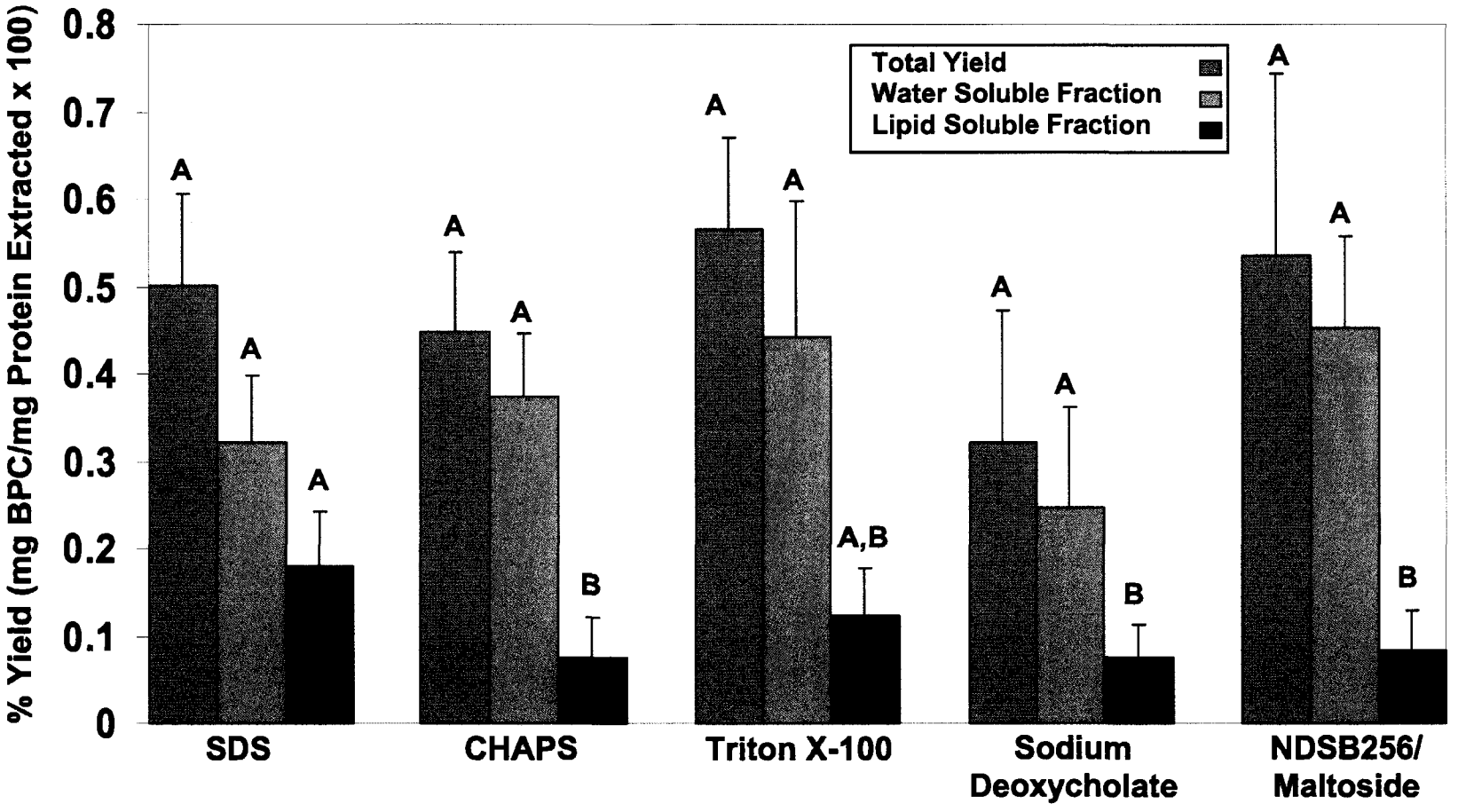

Figure 2.2. Protein yield for water- and lipid-soluble fractions and total protein yield for each extraction protocol. Error bars indicate one standard deviation. A minimum of three replicates per sample were performed. Fractions not connected by the same letter are significantly different.

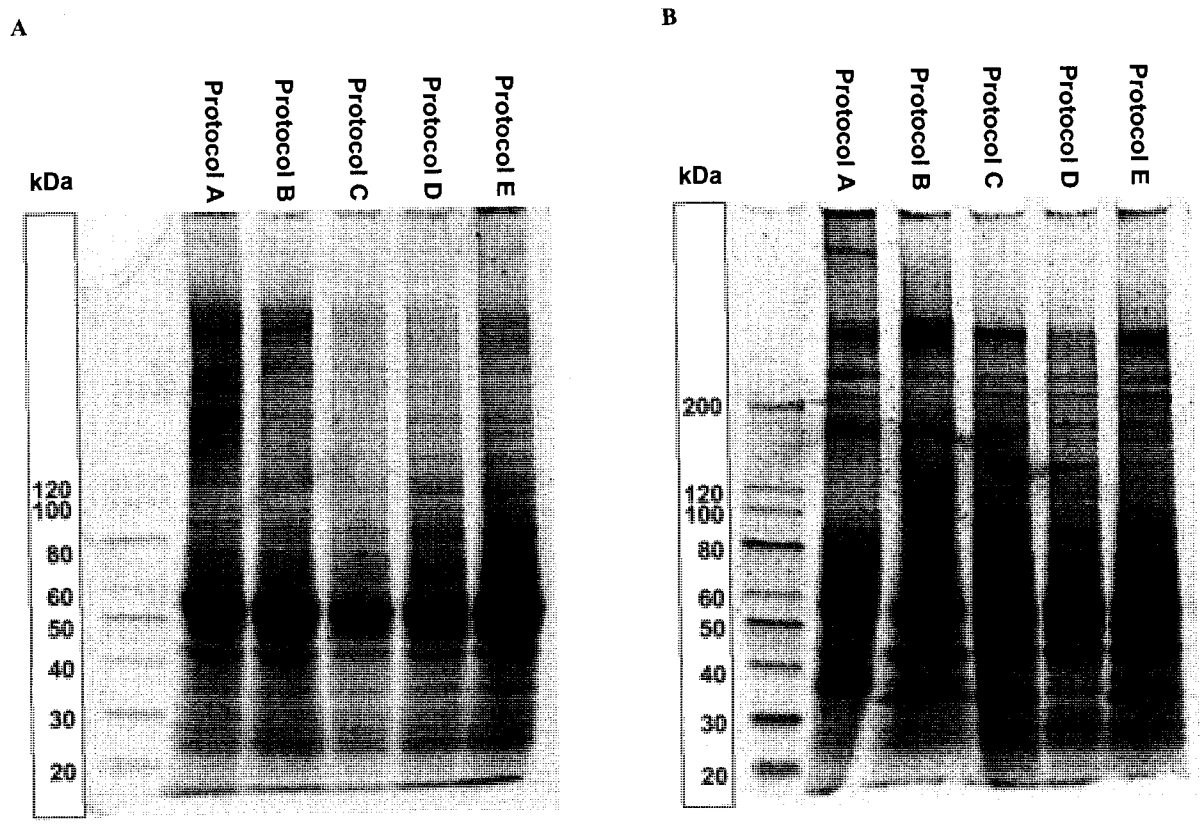

Figure 2.3. 1-D SDS-PAGE gels of water-soluble (A) and lipid-soluble (B) extracts from Protocols A-E.

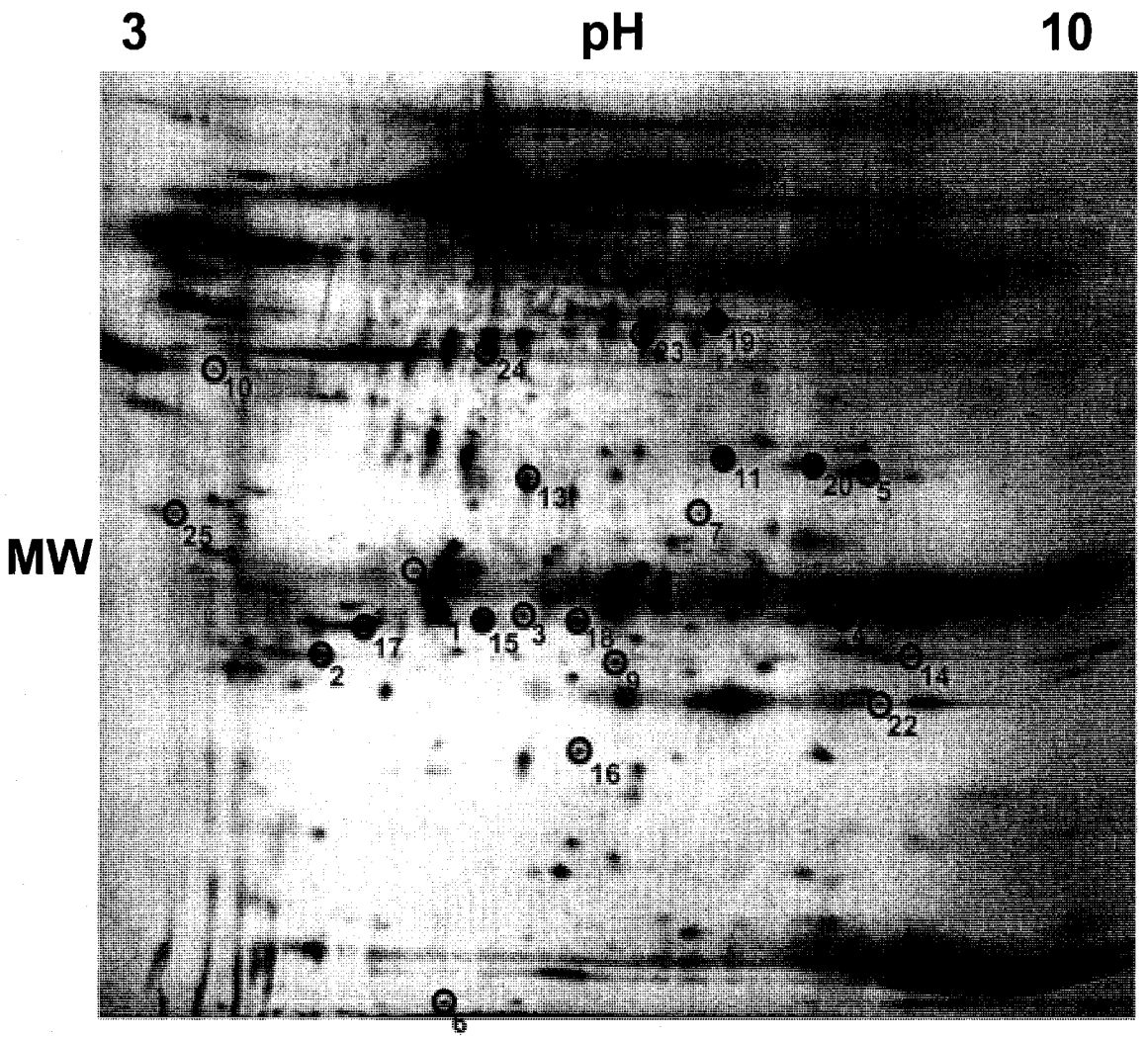

Figure 2.4. 2-DE gel of water-soluble proteins prepared with Protocol E2. Circled spots were excised, subjected to tryptic digestion, MALDI-TOF/TOF MS and database searching for protein identification (Table 2.3).

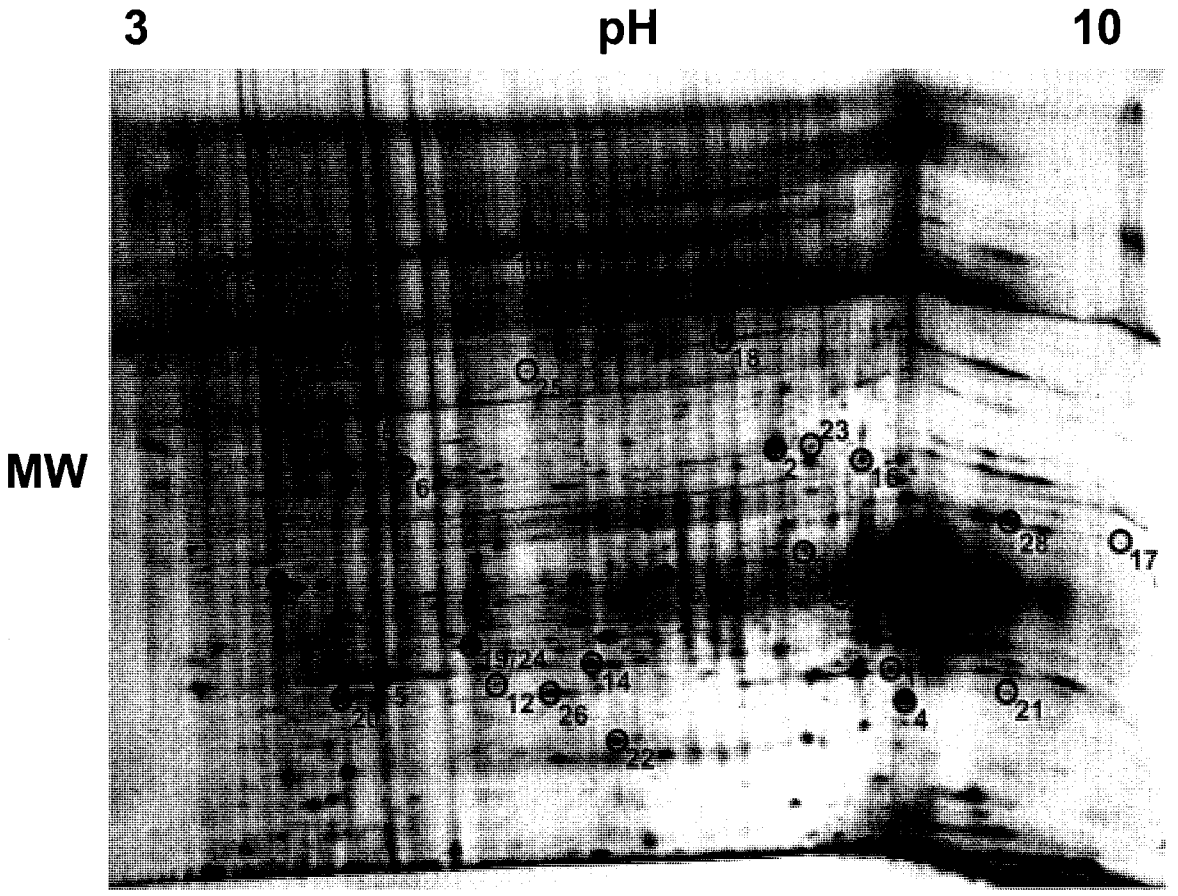

Figure 2.5. 2-DE gel of lipid-soluble proteins prepared with Protocol E2. Circled spots were excised, subjected to tryptic digestion, MALDI-TOF/TOF MS and database searching for protein identification (Table 2.4).

Chapter III

Immunoproteomic identification of bovine pericardium xenoantigens

Abstract

Bovine pericardium (BP) is an important biomaterial with current and potential future application in the production of heart valves. The importance of humoral and cell mediated rejection responses towards such xenogeneic tissues are becoming increasingly apparent. We have applied a novel immunoproteomic approach to survey the antigenic determinants of BP. A two stage protein extraction protocol was used to separate BP proteins into water- and lipid-soluble fractions. Two-dimensional gel electrophoresis (2-DE) was performed to separate proteins from each fraction. Duplicate two-dimensional gels were used to generate western blots from both fractions. Western blots were probed with pre- and post-exposure anti-BP rabbit serum, with detection of immune complexes limited to the IgG subtype. Western blots were compared to 2-DE gels and spots matched using Delta2D image analysis software. Matched spots were excised, subjected to tryptic digestion and the resulting peptides analyzed by either electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI) mass spectrometry (MS). This approach identified 31 putative antigens, capable of stimulating an IgG humoral rejection response. This study is the first to apply an immunoproteomic approach for identification of antigenic targets in xenotransplanted tissues. The results provide important information for understanding and possibly mitigating the immune response to fixed and unfixed BP xenografts.

Introduction

Bovine pericardium is an important biomaterial with current application in glutaraldehyde-fixed bioprosthetic heart valves and possible future application as an unfixed "decellularized" scaffold for tissue-engineering. The importance of the immune response to xenogeneic biomaterials such as BP is becoming increasingly apparent in both applications.

Fixation with glutaraldehyde was once thought to largely mitigate the immune response to connective tissue xenografts by irreversibly cross-linking graft matrix proteins.[2] However it is now clear that both humoral and cell-mediated immune responses to glutaraldehyde-fixed xenografts occur.[2, 7, 9, 18-21] Mounting evidence now implicates chronic antibody formation and immune rejection in bioprosthetic heart valve degeneration and calcification.[9, 18, 19, 21] This raises the possibility that treatments might be devised to reduce antigen driven activation of the immune system and thereby improve bioprosthetic heart valves durability. However, proteins triggering immune rejection in bioprosthetic heart valves remain largely unknown.

Unfixed BP has received attention as a potential xenogeneic biological scaffold for tissue-engineering, including tissue-engineered heart valves.[23, 25, 29, 32, 45, 70] Because tissues are unfixed in this application, it is necessary to remove graft antigenicity prior to implantation. Numerous physical and chemical treatments designed to decrease the immunogenicity of unfixed xenogeneic biomaterials have been investigated.[25, 26, 28, 32-35, 45, 48-50, 54-58] These treatments have generally been characterized as tissue "decellularization" based on an assumption that antigens mediating an immune response to the graft would likely be associated with the cellular component of the tissue. It is now clear that disappearance of cells on light microscopy does not assure adequate removal of xenoantigens or mitigation of

immune rejection.[46, 70-72] As a result, emphasis has shifted from tissue "decellularization" to "antigen clearing".[52, 66] However, the full scope xenogeneic tissue antigenicity remains largely undefined with regard to the number, origin, and identification of potential xenoantigens.

In the study, I applied a novel immunoproteomic approach to survey BP proteins for antigenic potential. The effort identified 31 BP proteins as potential xenoantigens.

Materials and Methods

Tissue Harvest

BP was harvested aseptically from adult cattle shortly after death and transported to the laboratory in pH 7.4 phosphate buffered saline with 0.1% (w/v) ethylenediamine tetraacetic acid, 100 KIU/ml aprotinin, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B. Pericardial fat and loose connective tissue were removed. BP was cut into 1 cm^2 pieces and stored at -80 °C in 85% Dulbecco's modified eagles medium and 15% (v/v) dimethyl sulphoxide.

Antiserum Production

Anti-BP serum was generated in New Zealand White rabbits. Rabbits were used in accordance with the guidelines established by Colorado State University IACUC and the *Guide for the Care and Use of Laboratory Animals*.[148] BP (1 g) was placed in 5 ml of 10 mM pH 8.0 Tris HCl, 100 KIU/mL aprotinin, 1 mM dithiothreitol (DTT), 2 mM $MgCl_2$, 10 mM KCl, and 0.5 mM Pefabloc (Sigma-Aldrich), and mechanically homogenized on ice. One ml of the homogenized BP suspension was injected subcutaneously into rabbits (n = 4)

at days 0 and 14 without adjuvant. Serum was collected from the rabbits at days 0, 7, 14, 21, 42, 56, and 72, and stored at -80 °C.

Protein Extraction

Protein extraction of BP was accomplished with a two stage protocol to optimize collection of water- and lipid-soluble proteins. Water-soluble proteins were extracted in a solution containing 134 mM dimethyl benzyl ammonium propane sulphonate (NDSB-256) and lipid-soluble proteins were extracted in a solution containing 1% n-dodecyl-β-D-maltoside (Figure 3.1). Delipidation of the lipid-soluble extract was achieved by ethanol precipitation.[115] Detailed methodology is provided in Appendix 1.

One-Dimensional SDS-PAGE and Western Blot

One-dimensional gel electrophoresis (1-DE) was performed on a mini-gel electrophoresis system (Xcell II, Surelock Mini-Cell, Invitrogen). Western blotting was to 0.2 μm nitrocellulose membranes. Blots were treated sequentially with primary rabbit anti-BP serum and horseradish peroxidase conjugated monoclonal anti-rabbit IgG light chain specific antibody (Jackson ImmunoResearch). Western blots were stained with chemiluminescence detection reagent (Supersignal Femto, Invitrogen). Digital images were recorded with a charge-coupled device (CCD) camera bioimaging system (UVP Inc.). Detailed methodology is provided in Appendix 1.

Two-Dimensional Gel Electrophoresis and Western Blot

Two-dimensional gel electrophoresis (2-DE) was performed in the first dimension using pH 3-10 non-linear gradients (Bio-Rad) and in the second dimension by 12% SDS-PAGE using pH 8.3 Tris-Glycine/SDS running buffer. Gels were stained with a silver staining protocol.[118] Digital gel images were recorded using a CCD camera bioimaging

system. Western blotting of 2-DE gels was performed using a wet blot electrophoresis transfer system (Trans-Blot, Bio-Rad). Western blotting was performed onto 0.2 μm nitrocellulose membranes with a running buffer of 25 mM Tris HCl, 192 mM glycine at 100 V and 1 A for 1 h. Blots were probed with rabbit anti-BP serum, stained and imaged as for 1-DE western blots. Detailed methodology is provided in Appendix 1.

Image Analysis and Protein Identification

Resolved spots on 2-D western blots were matched to the corresponding 2-DE gels using Delta 2D digital image analysis software (DECODON GmbH) (Figure 3.1). Matched spots were excised from the gel, and subjected to tryptic digestion and desalting. Peptides were analyzed by either electrospray ionization (ESI) mass spectrometry (MS) or matrix assisted laser desorption ionization (MALDI) MS, with the most abundant peaks in the spectra subjected to tandem MS (MS/MS).[149] Mascot searches were restricted to the mammalian taxonomy with mass tolerances of 1.5 Da MS and 0.6 Da MS/MS for ESI, and 75 ppm and 0.3 Da MS/MS for MALDI identifications. Detailed methodology is provided in Appendix 1.

Results

One-Dimensional Western Blot

Pre-exposure rabbit serum produced discrete and indiscrete bands on blots of water-soluble fraction BP proteins suggesting that preexisting natural antibodies to BP proteins were present in rabbits (Figure 3.2). New bands appeared on blots of both water- and lipid-soluble BP proteins treated with post-exposure rabbit serum. Band number and intensity

increased progressively on treatment of blots with 21-, 42-, and 72-d post-exposure rabbit serum suggesting IgG antibody production toward several BP proteins.

Two-Dimensional Gels, Western Blots and Antigen Identifications

Two-dimensional gels of water- and lipid-soluble fractions of BP proteins were well resolved with minimal smearing in either direction (Figure 3.3 & 3.4). Sequential treatment of corresponding 2-D western blots with pre-exposure and 21-, 56-, and 72-day post-exposure rabbit serum demonstrated a few pre-exposure and many new post-exposure spots. Similar to results obtained on 1-D blots, spot number and intensity increased with increasing day post-exposure serum on blots of both BP protein fractions (Figure 3.5). Resolved spots on 2-D western blots matched well with corresponding silver-stained gels with minimal warping between the blots and gels (Figure 3.1).

Protein Identification

Proteins identifications were made from gel spots corresponding to resolved spots on western blots of both water- and lipid-soluble fraction of BP. Proteins identified are reported in Tables 3.1 and 3.2. Location of gel spots from which protein identifications were made are shown in Figure 3.3 and 3.4. A total of 63 protein identifications were made (37 from water-soluble and 26 from lipid-soluble gels), representing 31 unique antigens (20 from water-soluble and 11 from lipid-soluble gels).

Discussion

Abundant evidence demonstrates that both glutaraldehyde-fixed and unfixed BP xenografts elicit an immune response.[2, 7, 9, 18-21, 46, 52, 66, 70-72] In this study, I utilized an immunoproteomic approach to screen BP proteins for their ability to generate an IgG

humoral immune response. Two-dimensional western blots of water- and lipid-soluble BP protein fractions were probed with pre- and post-exposure anti-BP rabbit serum. Detection of immune complexes was limited to the IgG subtype. This approach identified 31 putative antigenic proteins capable of eliciting a humoral and T-helper cell immune response, the later being necessary for IgG production.[150, 151] A similar approach has been used to identify antigenic proteins in the disciplines of oncology, autoimmune, and infectious disease.[152-158] To the author's knowledge, this study is the first to apply an immunoproteomic approach to identify antigenic targets in xenotransplanted tissues.

Interestingly, eight of the putative antigens identified in this study have been identified previously as targets of autogenous immune responses in either neoplasia or autoimmune diseases.[126, 155, 159-164] Two additional putative antigens (peroxiredoxin II and III) have closely related isotypes previously identified as antigens.[153, 154, 165, 166] Twenty-three putative BP antigens have not been previously identified as antigenic. Five of these were hypothetical proteins of unknown function, having not previously been described at the protein level. Most BP proteins identified as an antigens demonstrated progressive staining intensity with increasing days of post-exposure serum, lending further support to a conclusion that antigen identifications in this study were the result of an active humoral immune response. Four putative antigens were identified by pre-exposure serum suggesting the presence of natural rabbit antibodies toward these antigens. Staining intensity for these antigens also increased progressively with post-exposure serum.

Several of the putative antigens identified in this study exhibit shared structural or functional properties, which may provide insight into the determinants of antigenicity. A number of the putative antigens identified were isotypes from the same protein family (eg

peroxiredoxin II and III, or glutathione S-transferase M1 and pi). This may indicate presence of a conserved antigen determining region within each family. Additionally, several putative BP antigens were antioxidant proteins. A similarly disproportionate number of antioxidant proteins have been identified as antigens in autoimmune disease.[165] The reason for the high prevalence of antigenicity of antioxidant proteins warrants further investigation.

A diverse functional range of antigenic BP proteins were identified, with cytoplasmic, mitochondrial, nuclear, membrane, secreted and extracellular matrix proteins all represented. The identification of the extracellular matrix protein osteoglycin as a putative BP antigen has important implications for tissue-engineering applications of BP. Osteoglycin is a keratin sulphate proteoglycan, involved in collagen fibrillogenesis.[167, 168] It is becoming increasingly clear that emphasis in preparing unfixed xenogeneic tissues for tissue-engineering applications needs to shift from a concept of tissue "decellularization" to one of "antigen removal".[25, 26, 28, 29, 32-35, 45, 48-50, 54-58] The identification of an extracellular matrix protein as antigenic further supports the need for this shift in paradigm. It remains to be determined whether current or future antigen removal treatments will be sufficient to remove extracellular matrix proteins or what effects this might have on the tissue.

Post-translational modification (PTM) is an important consideration when assigning antigenicity to the proteins identified, as it is possible that in selected cases antigenicity is a result of the PTM rather than the protein itself.[85, 169-171] PTM's clearly do not account for the antigenic domain in all cases, as some of the identified antigens have previously had their antigenic domains mapped to protein sites.[126, 155, 159, 160] Fifteen of the putative antigens were identified from multiple spots, suggesting that these proteins undergo post-translational

modification.[152, 153, 155, 157] However, in these cases it is unlikely that the PTM represents the antigenic domain, as the protein remains antigenic regardless of its PTM's.

The following study limitations are recognized. The 2-DE gel-based proteomics is capable of separations of proteins with molecular masses of approximately 100 kDa and below.[172] This understanding, combined with the 1-DE western blot results in which several antigenic bands are present above this molecular mass range, indicates that additional high molecular weight protein antigens may be present within the tissues. Other proteomic approaches might be useful for detection of additional antigens.[172, 173] A further limitation is the finding that some of the antigenic spots yielded two protein identifications. In all cases, these identifications were made from disparate peptide mass fingerprints, suggesting that both proteins were indeed present within a single spot. It is not possible to determine which protein is antigenic, or if both are indeed antigens using the presented approach.

In summary, the immunoproteomic approach employed in this study successfully identified 31 BP proteins as putative xenogeneic antigens. Identified antigenic proteins represented a wide variety of functional and structural protein types, including extracellular matrix proteins. The results of this study should prove valuable for understanding and possibly mitigating the immune response to fixed and unfixed BP xenografts.

Table 3.1. Bovine pericardium (BP) water-soluble fraction proteins identified by two-dimensional western blotting with rabbit serum collected before or after exposure to BP.

Spot Number	Protein Name	Accession N°	Exposure Time Antibody First Detected	Protein Localization	MW	pI	Peptides Matched	Literature Evidence of Antigenicity
1	beta hemoglobin	gi|27819608	0 d	Cytoplasm	15944.3	7.01	45	[159]
2	UBFD1 protein	gi|151554567	0 d		33143.6	5.55	1	None
3	albumin	gi|162648	0 d	Cytoplasm	69278.5	5.82	19	[160]
	hemoglobin beta-A chain	gi|122539	0 d	Cytoplasm	15964.3	6.36	3	None
4	albumin	gi|162648	0 d	Cytoplasm	69278.5	5.82	19	[160]
	hemoglobin beta-A chain	gi|122539	0 d	Cytoplasm	15964.3	6.36	3	None
5	albumin	gi|162648	0 d	Cytoplasm	69278.5	5.82	19	[160]
	hemoglobin beta-A chain	gi|122539	0 d	Cytoplasm	15964.3	6.36	3	None

6	hypothetical protein LOC540335	gi|134085635	21 d	Unknown	20765.6	5.09	3	None
7	apolipoprotein A-1	gi|245563	21 d	Cytoplasm or Membrane	28414.8	5.57	16	None
8	apolipoprotein A-1	gi|245563	21 d	Cytoplasm or Membrane	28414.8	5.57	9	None
9	glutathione S-transferase M1	gi|28461273	21 d	Nuclear or Cytoplasm	25634.8	6.91	10	None
10	peroxiredoxin 2	gi|27807469	56 d	Cytoplasm	21946.0	5.37	2	None
11	alpha-1-acid glycoprotein	gi|94966811	56 d	Secreted	23182.4	5.62	1	None
12	triosephosphate isomerase	gi|61888856	56 d	Cytoplasm	26672.8	6.45	2	[155]
13	hemoglobin chain A	gi|116812902	56 d	Cytoplasm	15174.9	8.07	35	[159]

14	proline synthase homolog	gi|77735663	56 d	Cytoplasm	29943.3	6.72	8	None
15	peroxiredoxin 3	gi|75948233	56 d	Mitochondrial	31256.2	7.10	11	None
16	Ig gamma-2 chain C region (clone 32.2)	gi|89611	56 d	Membrane	36019.8	8.04	11	None
17	hemoglobin beta-A chain	gi|122539	56 d	Cytoplasm	15964.3	6.36	5	None
18	Enolase 1	gi|87196501	56 d	Cytoplasm or Membrane	47296.4	6.37	9	126, 161
19	Enolase 1	gi|87196501	56 d	Cytoplasm or Membrane	47296.4	6.37	21	126, 161
20	Enolase 1	gi|87196501	56 d	Cytoplasm or	47296.4	6.37	37	126, 161

				Membrane				
21	allergen bos d 6	gi|1351907	56 d	Secreted	69248.4	5.82	43	[160]
	hypothetical LOC534509	gi|77736171	56 d	Unknown	52209.2	7.9	6	None
22	alpha-1-acid glycoprotein	gi|94966811	56 d	Secreted	23182.4	5.62	1	None
23	haptoglobin	gi|83638561	56 d	Cytoplasm	44830.6	7.83	7	None
24	parkinson disease 7	gi|62751849	56 d	Unknown	20035.3	6.84	5	None
25	allergen bos d 6	gi|1351907	56 d	Secreted	69248.4	5.82	18	[160]
26	allergen bos d 6	gi|1351907	56 d	Secreted	69248.4	5.82	18	[160]
27	hypothetical LOC532481	gi|129277510	56 d	Unknown	26176.5	6.59	10	None
28	hypothetical LOC532481	gi|129277510	56 d	Unknown	26176.5	6.59	5	None
29	hypothetical LOC532481	gi|129277510	56 d	Unknown	26176.5	6.59	17	None

30	hypothetical LOC532481	gi|129277510	56 d	Unknown	26176.5	6.59	15	None
31	hypothetical LOC532481	gi|129277510	56 d	Unknown	26176.5	6.59	3	None
32	beta hemoglobin	gi|27819608	56 d	Cytoplasm	15944.3	7.01	7	[159]
33	haptoglobin	gi|83638561	56 d	Cytoplasm	44830.6	7.83	7	None

Table 3.2. Bovine pericardium (BP) lipid-soluble fraction proteins identified by two-dimensional western blotting with rabbit serum collected after exposure to BP.

Spot Number	Protein Name	Accession N°	Exposure Time Antibody First Detected	Protein Localization	MW	pI	Peptides Matched	Literature Evidence of Antigenicity
1	beta hemoglobin	gi|27819608	21 d	Cytoplasm	15944.3	7.01	22	[159]
2	beta hemoglobin	gi|27819608	21 d	Cytoplasm	15944.3	7.01	11	[159]
3	apolipoprotein A-I	gi|245563	21 d	Cytoplasm or Membrane	28414.8	5.57	21	None
4	alpha-1-B glycoprotein	gi|114053019	21 d	Secreted	53553.5	5.3	5	None
5	alpha-1-B glycoprotein	gi|114053019	21 d	Secreted	53553.5	5.3	5	None
6	hemoglobin chain A	gi|116812902	72 d	Cytoplasm	15174.9	8.07	16	[159]
7	hemoglobin chain A	gi|116812902	72 d	Cytoplasm	15174.9	8.07	8	[159]
8	glutathione S-	gi|29135329	72 d	Nuclear or	23613.1	6.89	2	None

	transferase pi			Cytoplasm				
9	hypothetical LOC614712	gi|77736475	72 d	Unknown	18011.9	9.3	1	None
10	hypothetical LOC614712	gi|77736475	72 d	Unknown	18011.9	9.3	1	None
11	hypothetical LOC614712	gi|77736475	72 d	Unknown	18011.9	9.3	3	None
12	hypothetical LOC514335	gi|77735877	72 d	Unknown	28699.1	8.76	4	None
13	hypothetical LOC532481	gi|129277510	72 d	Unknown	26176.5	6.59	3	None
14	apolipoprotein A-I precursor	gi|162678	72 d	Cytoplasm or Membrane	30271.3	5.8	3	None
15	apolipoprotein E	gi|312893	72 d	Secreted	35869.7	5.44	3	None
16	annexin V	gi|260137	72 d	Nucleus or Membrane	35942.7	4.94	6	[162, 163]

17	osteoglycin protein	gi|74354996	72 d	Matrix	34197.4	5.43	5	None
18	osteoglycin precursor	gi|129077	72 d	Matrix	34209.4	5.43	7	None
19	osteoglycin precursor	gi|129077	72 d	Matrix	34209.4	5.43	8	None
20	osteoglycin precursor	gi|129077	72 d	Matrix	34209.4	5.43	9	None
21	chain A dimethylarginine dimethylamino-hydrolase I	gi|109157267	72 d	Cytoplasm	31157.6	5.67	5	164
22	chain D bovine mitochondrial F-1 ATPase	gi|1827812	72 d	Membrane	51705.1	5.00	12	None
23	chain D bovine mitochondrial F-1	gi|1827812	72 d	Membrane	51705.1	5.00	20	None

	ATPase							
24	hypothetical LOC535804	gi|115497132	72 d	Unknown	56453.1	8.57	19	None
25	hypothetical LOC535804	gi|115497132	72 d	Unknown	56453.1	8.57	19	None
26	allergen bos d 6	gi|1351907	72 d	Secreted	69248.4	5.82	8	[160]

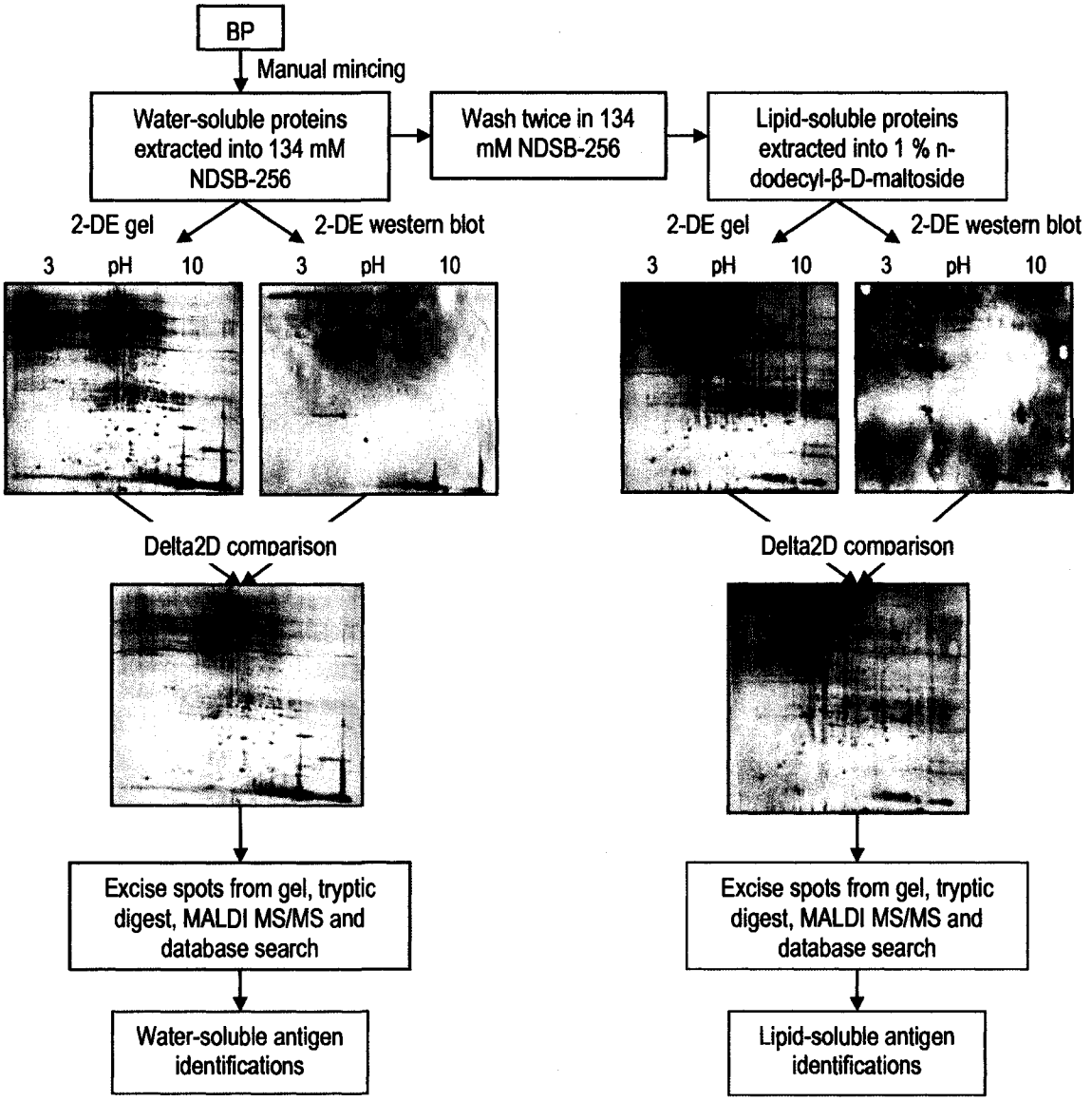

Figure 3.1. Workflow scheme for bovine pericardium (BP) xenoantigen identification.

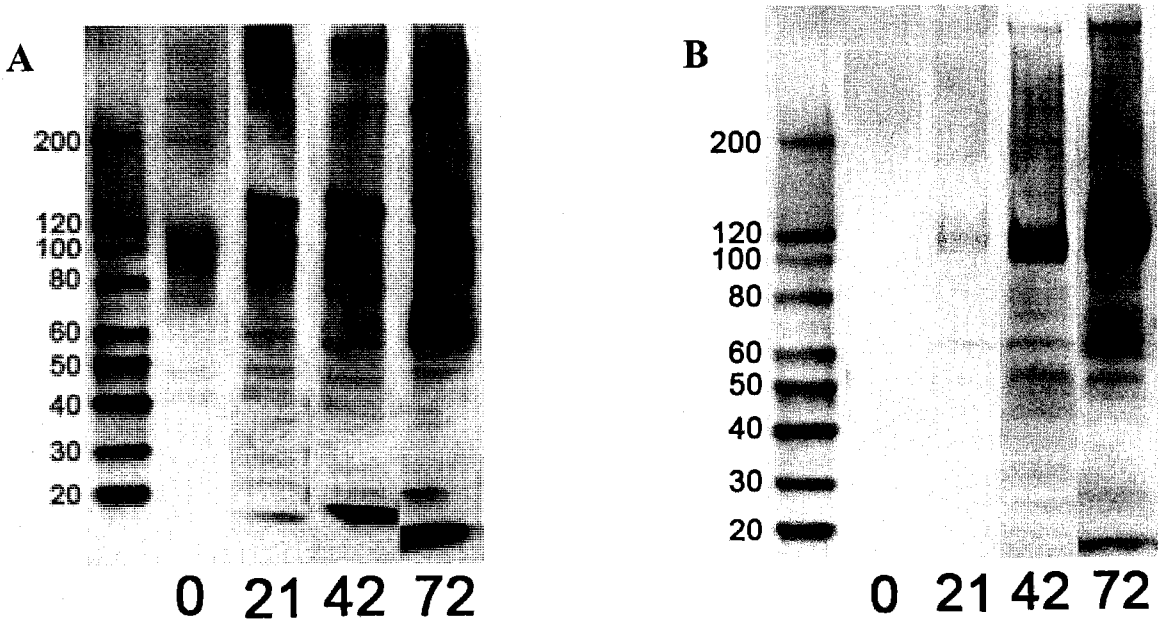

Figure 3.2. One-dimensional western blots of water-soluble (A) and lipid-soluble (B) bovine pericardium protein fractions treated with pre-exposure (0) and 21-, 42-, and 72-d post-exposure rabbit serum.

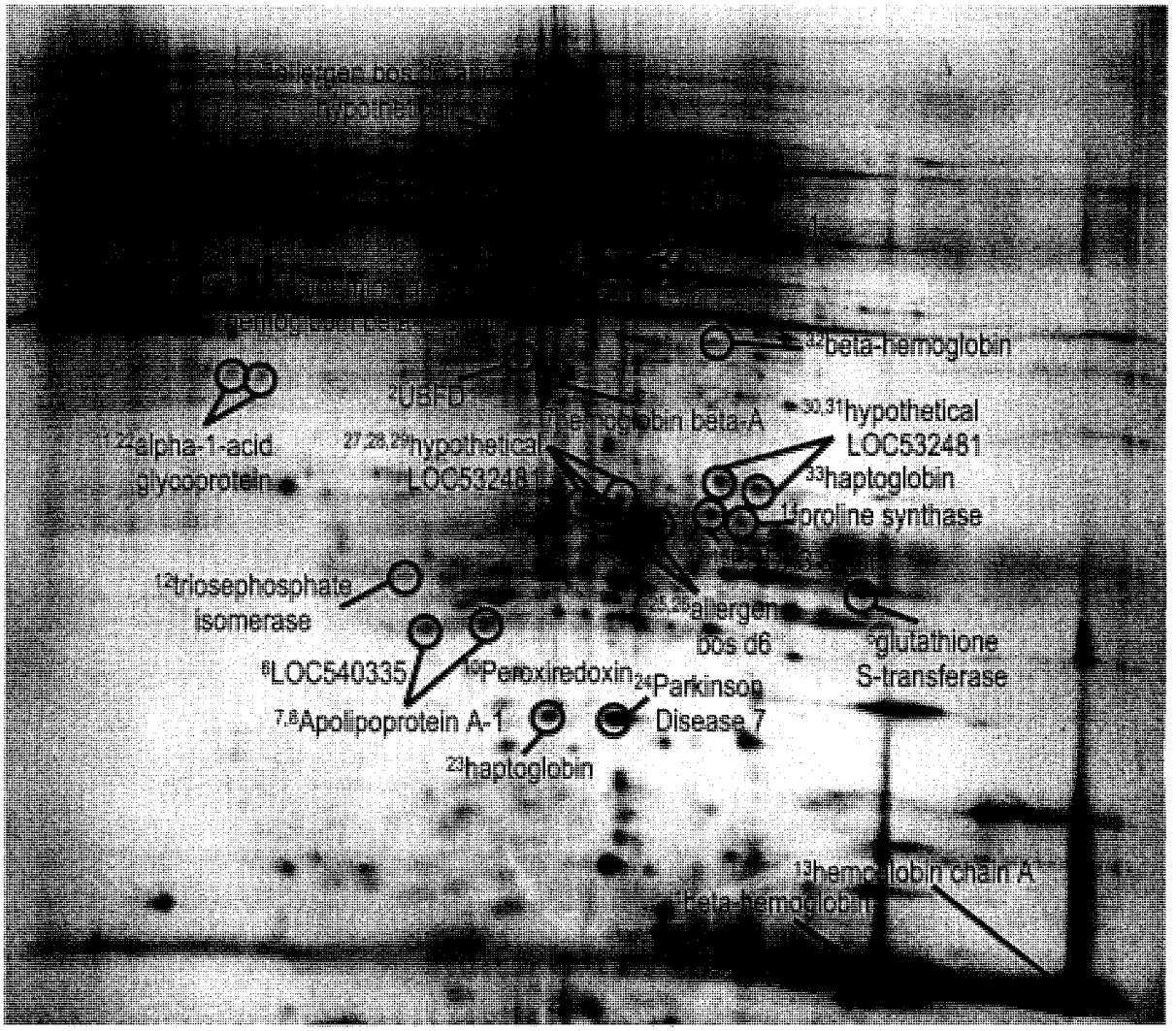

Figure 3.3. Two-dimensional gel of bovine pericardium water-soluble fraction proteins identified as antigens.

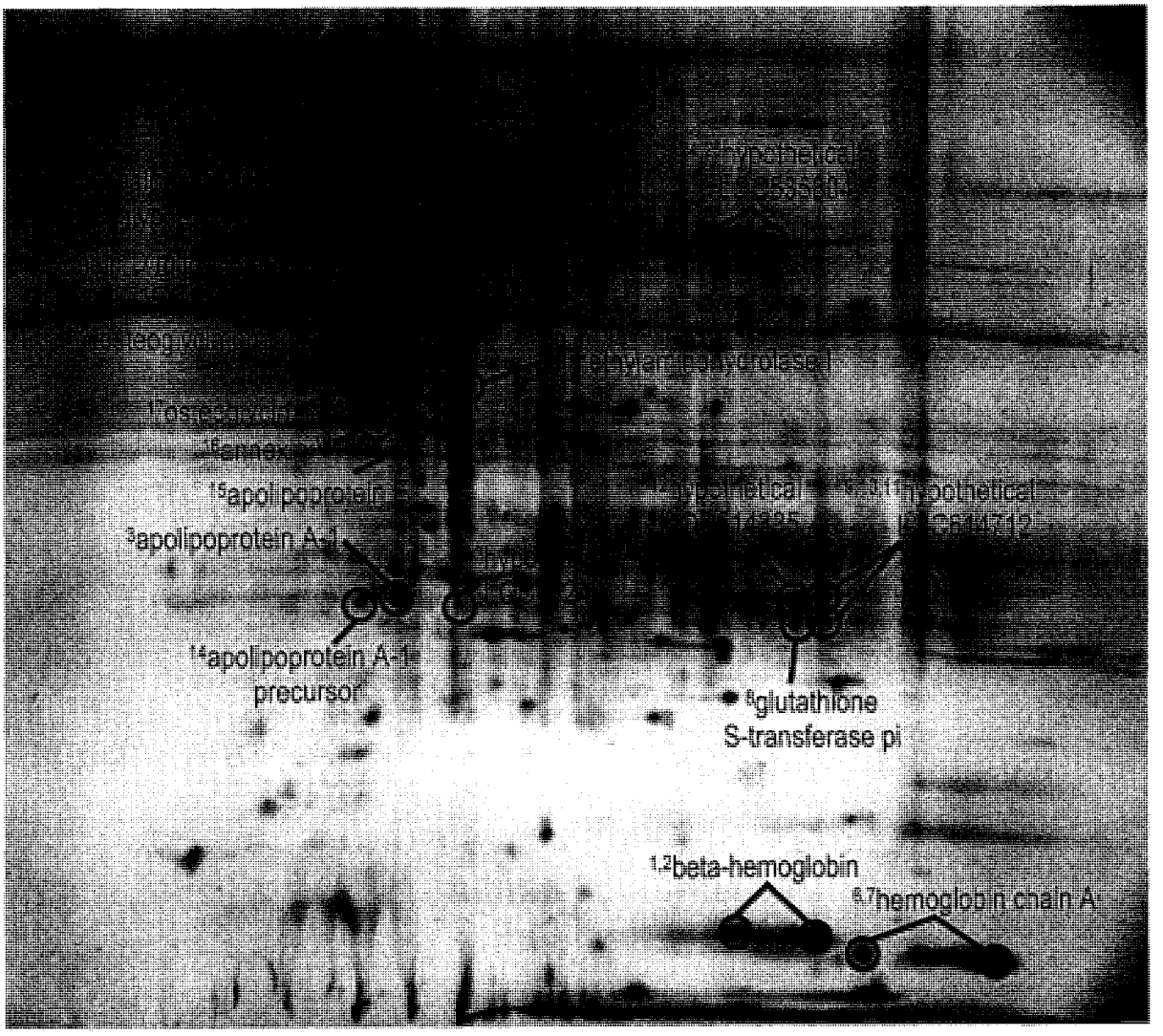

Figure 3.4. Two-dimensional gel of bovine pericardium lipid-soluble fraction proteins identified as antigens.

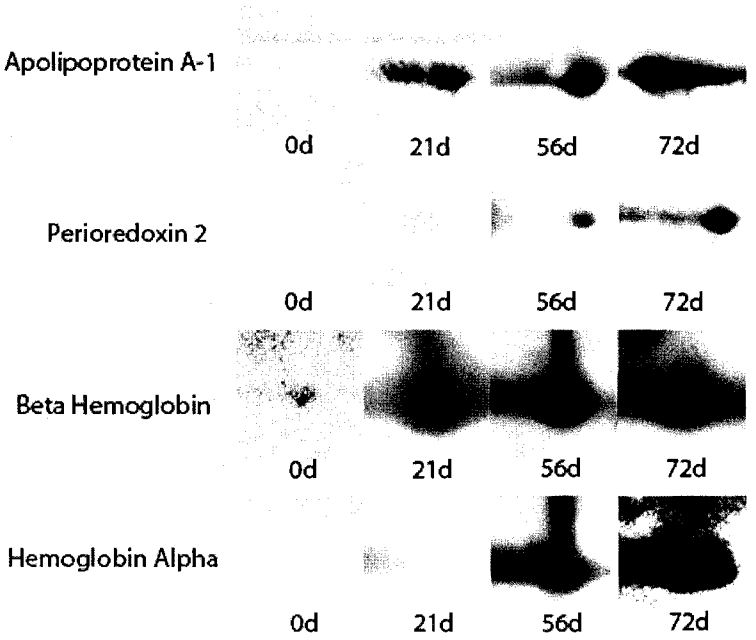

Figure 3.5. Sequential two-dimensional western blot spots of 4 bovine pericardium proteins resolved with pre-exposure (0d) and 21 day, 56 day, and 72 day post-exposure rabbit serum.

Chapter IV

Summary

Bovine pericardium (BP) is a challenging tissue on which to perform high quality proteomic analysis, due to its low cellularity and high matrix content.[70, 105] Indeed, type 1 collagen alone accounts for as much as 76% of the protein content of BP.[42, 105, 134] Extraction and fractionation methods are critical in avoiding sample contamination with such high abundance structural or matrix proteins, which would otherwise overwhelm the remainder of the proteome.[106-110] Lipid-soluble proteins within the tissue present an additional complication, as such proteins are notoriously difficult to separate using 2-DE gels due to their low solubility.[111, 112] Despite these challenges, two fractions of BP proteins were extracted using the principles of differential solubility. Both fractions produced high quality 2-DE gels with minimal smearing in the horizontal or vertical directions. Prefractionation was confirmed to be appropriate, with sixteen unique predominantly cytoplasmic bovine proteins identified from the water-soluble fraction and twenty-two unique predominantly membrane bovine proteins identified from the lipid-soluble fraction.

Immunoproteomics is an emerging field of investigation which targets identification of antigenic determinants in a variety of diseases. This approach involves western blotting of high quality 2-DE gels to either nitrocellulose or polyvinylidene fluoride (PVDF) membranes, which are then probed with patient serum to identify antigenic proteins of interest.[152-158] Prior to this work, immunoproteomic approaches had only been reported for

the discovery of auto-antigens in the oncology, rheumatology, autoimmune and infectious disease disciplines.[152-158] The presented work is successful in expanding the field of immunoproteomics to identification of xenoantigens, using a rabbit model. A total of 31 unique antigenic proteins were identified which elicited an IgG specific humoral immune response. The results are validated by the fact that eight of the identified proteins were known to be antigenic from previous work.[126, 155, 159-164] Additionally, several of the remaining identified antigens exhibit shared characteristics, which may provide insight into the determinants of antigenicity,[85, 169-171] although future work is required to further investigate this hypothesis.

The antigens identified here provide new insight into the field of heart valve bioprostheses. The antibody response was restricted to the IgG subclass, indicating a humoral T-helper cell component to the immune response.[150, 151] Thus, the antigens identified are capable of inducing both a cell mediated and humoral immune response, which would be expected to induce a significant rejection response.[150, 151] Removal of these antigens must be accomplished if the stated goal in production of a xenogeneic scaffold material for tissue engineering applications is to be achieved.[11, 25, 26, 32, 33, 45-55, 174]

Future Directions

Xenoantigens in Humans

One critical future direction for the presented research is the detection of xeno-antigens in human patients following gluteraldehyde fixed valve implantation. Preliminary 1-DE western blot data (not shown), suggest that, as with the rabbit model, humans have pre-

existing xenoantibodies directed towards antigens within BP. However, the interpretation of this finding is complicated due to the fact natural anti-α-gal (a carbohydrate moiety present on all bovine cells) antibodies are known to be present in human serum.[73, 79-81, 83, 85, 89, 175] 2-DE western blots with protein identification would be necessary to determine if the pre-existing antibodies seen in this 1-DE work are solely anti-alpha gal, or directed towards other xenoantigens. Alternatively, both 1-DE and 2-DE western blots using samples subjected to alpha-galactosidase digestion may provide further insight. Ultimately, performing 2-DE western blots with serum from humans, following heart valve replacement with a gluteraldehyde-fixed BP valve, would allow identification of the antigenic determinants of gluteraldehyde-fixed BP in humans. Such information could be used to develop screening test for monitoring rejection, or in improving the manufacturing process for gluteraldehyde-fixed valves to further reduce antigenicity.

Increasing the Number of Antigens Detected

The author recognizes that, as with any proteomic experiment, only a percentage of the entire proteome has been surveyed using the approach described here. The antigens identified in chapter III clearly do not represent an exhaustive list of all antigens in the tissue. To address this issue, the use of other proteomic methodologies is envisioned to broaden the percentage of the proteome surveyed. Other proteomic approaches which should be considered would include 2-DE agarose gels to increase visualization of high molecular mass proteins,[172] IEF using benzyldimethyl-n-hexadecylammonium chloride (16-BAC) to increase resolution of insoluble proteins (both membrane and matrix),[146] and shotgun proteomic approaches to express a protein profile which is complementary to gel based approaches.[173]

Confirmation of Antigenicity and Identification of Antigenic Domain

These results are strongly supported by the fact that eight of the putative antigens identified are known to be antigenic from previous studies.[126, 155, 159-164] Since this finding validates the technique, then it is likely that the remaining antigens are correctly identified. However, in specific cases, it would still be advantageous to consider additional studies to further define the antigen in question. A good example would be osteoglycin, which is a keratin sulphate proteoglycan involved in collagen fibrillogenesis and binding of growth factors. Confirmation of the antigenic determinants of osteoglycin would be prudent, due to the implications that removal of such an integral matrix protein would have on structural integrity and future "decellularization" efforts. Numerous approaches to confirm antigenicity in such cases, and to further define the antigenic domain are envisioned. The remaining questions which may be addressed using such an approach would include:-

Can antigenicity of the protein be confirmed? This question is critical to ensuring the accuracy of the antigen identifications. Previous studies have employed affinity purification or recombinant protein expression in order to obtain the putative antigen in a pure form.[152-155, 165, 166] Antigenicity could be confirmed by 1-DE western blots of the purified protein stained with rabbit serum post-exposure to BP or rabbit serum post-exposure to the purified protein only.

Is the antigenic domain a part of the protein or a post-translational modification (PTM)? This question is relevant, as previous studies have shown the importance of non-protein antigenic determinants such as carbohydrates moieties (eg α-gal, annexin I and II).[85, 152] This question could be investigated by the use of enzymatic digestion to cleave PTM moieties from the protein (eg endoglycosadases, phosphatase treatment),[152] special stains for

2-DE gels (eg periodic acid-Schiff staining, lectin based stains, immunochemical stains),[176] or special mass spectrometry techniques (eg phosphoproteomics, glycoproteomics).[176, 177]

Which domain of the protein is antigenic? Knowledge of the antigenic domain, or domains of the putative antigens described here, may prove beneficial in findings similarities between antigenic determinants, which may ultimately allow for prediction of antigen potential based on protein structure. This question has been approached by the use of limited proteolysis of the antigenic protein,[160] or expression of variously truncated recombinant proteins.[155] In both cases, fragments of the native protein are produced which can be probed by 1-DE western blotting to isolate the minimal antigenic region.

Conclusions

The work presented here has significant impact on the use of BP as a scaffold material for tissue engineering applications. A wide spread antigen distribution within the tissue has been demonstrated, which would be expected to lead to a significant immune response upon implantation. Removal of these antigens is the stated goal in production of a xenogeneic scaffold material. This work provides a foundation for the objective assessment of this goal and provides a basis for rational development of protocols to accomplish antigen removal from BP.

A broader application of immunoproteomic technology in both tissue engineering and transplantation fields is envisioned. Knowledge of the antigenic determinants of transplanted xenogeneic material has potential application in development of screening tests for monitoring of rejection,[178, 179] development of transgenic pig organs or tissues,[175, 180] targeted

immunosuppression using antibody modified drug delivery systems,[181, 182] masking of antigenic determinants and inducing tolerance towards the antigenic determinants.[183, 184]

This work has demonstrated immunoproteomics to be a powerful technique for investigating antigenic determinants of xenogeneic materials. The information gained by this technique has numerous and far reaching implications for a variety of medical settings.

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Appendix 1

Protein Extraction

Samples of BP were snap frozen in liquid nitrogen and minced into pieces approximately 0.5-1 mm on a side using sterile surgical instruments. Minced BP was placed in 1.5 ml cryogenic vials, containing 1 ml of 134 mM 3-(benzyldimethylammonio)-propanesulfonate (NDSB-256), 10 mM pH 8.0 Tris HCl, 100 KIU/mL aprotinin, 1 mM dithiothreitol (DTT), 2 mM $MgCl_2$, 10 mM KCl, and 0.5 mM Pefabloc in nanopure water (Barnstead^{TM}). Vials were shaken on ice for 1 h and then centrifuged at 17,000xg, 4 ^{o}C for 25 min. The supernatant was collected and designated the water-soluble protein fraction. The pellet was washed twice in 0.5 ml of the same extraction solution by repeating the procedure described above. The supernatant from each wash was discarded. The pellet was resuspended in 0.5 ml of 1% n-dodecyl-β-D-maltoside, 134 mM NDSB-256, 10 mM pH 8.0 Tris HCl, 100 KIU/mL aprotinin, 1 mM DTT, 2 mM $MgCl_2$, 10 mM KCl, and 0.5 mM Pefabloc (Sigma-Aldrich, St.Louis, MO, USA) in nanopure water and shaken on ice for 1 h. Samples were centrifuged at 17,000xg, 4 ^{o}C for 25 min, the supernatant collected and designated the lipid-soluble protein fraction.

The water-soluble fraction was concentrated using Centricon Ultracel YM-3 (cut off 3000 Da) centrifugal filters (Millipore, Billerica, MA, USA) at 6,500xg, 4 ^{o}C for 3 h and stored at -80 ^{o}C until required. The lipid-soluble fraction was concentrated with the same filters at 6,500xg, 4 ^{o}C for 90 min. Delipidation of the concentrated lipid-soluble fraction was achieved by ethanol precipitation. Lipid-soluble extracts were precipitated with nine volumes of ice-cold 100% ethanol. Samples were incubated at -4 ^{o}C for 60 min, and then

centrifuged at 17,000xg for 25 min at 4 °C. The supernatant was discarded, the pellet air dried and resuspended in 9 M Urea, 3% CHAPS, 1% DTT, 1% Triton X-100, 1% NDSB-256 in nanopure water and stored at -80 °C until required.[115]

Total protein concentrations were determined using a DC protein assay kit (Biorad, Hercules, CA, USA) with bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA) as the standard.

One Dimensional SDS Page and Western Blot

One dimensional gel electrophoresis (1-DE) was performed on a mini-gel electrophoresis system with 4-12% tris-glycine polyacrylamide gels (Xcell II, Surelock Mini-Cell, Invitrogen).[185] In all cases, 37 μg of water-soluble protein and 15 μg of lipid-soluble protein were loaded in separate lanes on the gel. One dimensional (1-DE) western blotting onto a 0.2 μm pore size nitrocellulose membrane, was performed using a mini-gel blot module in accordance with the manufacturer's recommendations (Xcell II, blot module, Invitrogen). Blots were blocked for 30 min with 5% milk in 10 mM pH 8.8 tris buffered saline plus 0.05% Tween 20 (TBST). Blots were stained overnight at 4 °C, with a 1:10 dilution of rabbit serum either pre (day 0) or post (day 14, 21, 28, 42, 56 or 72) exposure to BP, in 5% milk in TBST. Blots were washed 3 times for 10 min in 5% milk in TBST. Secondary antibody was a 1:5000 dilution of horseradish peroxidase conjugated monoclonal anti-rabbit IgG light chain specific antibody (Jackson ImmunoResearch Laboratories Inc), in 10 mM pH 8.8 tris buffered saline (TBS). Blots were washed 5 times for 10 min in TBST. Digital images of blots were created using chemiluminescent reagent (Supersignal Femto, Invitrogen) and digital images recorded using a CCD bioimaging system (UVP bioimaging system, UVP Inc).

Two Dimensional Gel Electrophoresis

Two-dimensional gel electrophoresis (2-DE) was performed in the first dimension using pH 3-10 non-linear immobilized pH gradient (IPG) (ReadyStrips™, BioRad). IPG rehydration of water-soluble extracts was performed overnight, with 500 μg of protein diluted in 8 M urea, 2% CHAPS, 0.3% DTT, 2% Ampholyte 3-10NL. For overnight IPG rehydration of lipid-soluble extracts, 200 μg of protein was diluted in 8 M Urea, 1% CHAPS, 0.2% DTT, 1% Triton X-100, 3% NDSB-256, 1.5% ASB 14, 2% Ampholyte 3-10NL. Protein isoelectric focusing (IEF) was performed (Multiphor II electrophoresis system, GE Healthcare) at 20 °C, with an initial 1 min linear increase in voltage to 500 V, followed by a linear increase in voltage to 3500 V over 5 h and then a constant voltage of 3500 V for 17.5 h.

Following IEF, IPG strips were reduced by submersion in 2% DTT w/v, 6 M urea, 30% glycerol v/v and 0.1% SDS w/v for 15 min and then alkylated by submersion in 2.5% iodoacetamide (IAA), 6 M urea, 30% glycerol v/v, 0.1% SDS w/v and a trace of bromophenol blue for 5 min. Strips were immediately loaded onto cast 12% polyacrylamide gels (20 cm x 20.5 cm x 1mm) and electrophoresis performed at 200 V for 6 h in a Dodeca cell (BioRad, Hercules, CA, USA) with 0.1% SDS in 25 mM pH 8.3 Tris-HCl, 192mM glycine running buffer.[117]

In-gel Protein Digestion

Tryptic digestion was accomplished by incubation of the gel spots with 100 ng sequencing grade modified trypsin (Promega, Madison, WI, USA) in 50 mM ABC overnight at 37 °C. Peptides were extracted from the gel spots using 30 μL of 50% acetonitrile with 5% formic acid (45 min) and 30 μL of 90% acetonitrile with 5% formic acid (45 min). The

supernatant from the overnight tryptic digestion and those from all subsequent peptide extraction steps were collected and pooled. The pooled supernatants were dried using a Speedvac. Resultant peptides were analyzed either by electrospray ionization (ESI) or matrix assisted laser desorption ionization (MALDI) mass spectrometry.

Analysis by ESI

The extracted peptide samples were reconstituted in 10 µl of 0.5% formic acid with 2% acetonitrile. Nanoflow liquid chromatography (nanoLC) was carried out by an LC Packings Ultimate integrated capillary high performance liquid chromatography system equipped with a Switchos valve switching unit (Dionex, Sunnyvale, CA). For each sample, 6.4 µl were injected using a Famos auto sampler onto a PepMap C18 trap column (5 µm, 300 µm × 5 mm, LC Packings) for on-line desalting and then separated on a PepMap 100 C18 reverse phase column (3 µm, 75 µm × 15 cm, 100 Å, LC Packings). Peptides eluted in a 15-minute gradient of 5% to 40% acetonitrile in 0.1% formic acid at 250 nl/min into a 4000 Q Trap (ABI/MDS Sciex, Framingham, MA), a hybrid triple quadrupole linear ion trap mass spectrometer, that was equipped with a Micro Ion Spray Head II ion source. MS data acquisition was performed using Analyst 1.4.1 software (Applied Biosystems) in positive ion mode for information dependant acquisition (IDA) analysis. The nanospray voltage was set to 2.0 kV for all experiments. Nitrogen was used as the curtain gas, set to 10, and as the collision gas, set to high, with a heated interface at 175 °C. The declustering potential was set to 50 eV and Gas1 was set at 15 psi. After each survey scan between 400 *m/z* to 1600 *m/z* and an enhanced resolution scan, the three highest intensity ions with multiple charge states were selected for tandem MS (MS/MS) with rolling collision energy applied.

MS/MS spectra generated from nanoLC/ESI were interrogated using Mascot 2.2 (Matrix Science, London, UK) and searched against the mammal taxonomy of the NCBI database (downloaded July 2007). The search parameters were set to allow for one missed cleavage, two variable modifications (methionine oxidation and cysteine carboxyamidomethylation), a peptide tolerance of 1.2 Da, and a MS/MS tolerance of 0.6 Da. Only peptides defined by a Mascot probability analysis (www.matrixscience.com/help/scoring_help.html#PBM) to be better than "identity" were considered and used for protein identifications. For protein identifications based on a single peptide match, the identity score criterion was made more stringent by also requiring a confidence interval of $\geq$ 95%.

Analysis by MALDI

The extracted peptide samples were reconstituted in 30 µl of 50% acetonitrile with 0.1% TFA and desalted using ZipTips® C18 (Millipore, Billerica, MA, USA) and then run as reported previously [LH Choe, K Aggarwal, Z Franck and KH Lee (2005), Electrophoresis, 26: 2437-2449] on a 4700 Proteomics Analyzer (Applied Biosystems). Briefly, desalted digests were spotted onto target plates with 5 mg/mL alpha-cyano-4-hydroxycinnamic acid. MS was performed in positive ion reflector mode over a mass range of 700-4500 *m/z,* with 1200 laser shots per spot and internal calibration. Up to eight of the most intense peaks, excluding trypsin autolysis peaks, were selected from each MS spectrum for MS/MS. Tandem MS was performed in positive ion mode with 2700 laser shots, 1kV collision energy and default calibration. GPS Explorer (v2.0 Applied Biosystems) was used as an interface between the raw data from the mass spectrometer and a local copy of Mascot search engine (v1.9 Matrix Science). A combined MS and MS/MS search was performed against a local

copy of NCBInr (downloaded May 2007). Mascot searches were restricted to mammal taxonomy with 75ppm MS mass tolerance, 0.3Da MS/MS mass tolerance, trypsin specificity, two maximum missed cleavages, and the following three variable modifications: methionine oxidation and cysteine modifications by iodoacetamide and acrylamide. The criterion used to determine protein identification was a GPS Explorer confidence interval greater than 95%.