Two-dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE) Characterization of Decorin

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Abstract

Left-ventricular cardiac hypertrophy is a disorder resulting from uncontrolled chronic hypertension. Extensive remodeling of the cardiac extracellular matrix (ECM) through differential expression of ECM proteins allows for the hypertrophied state of the heart to exist and eventually lead to cardiomyopathy. Increases in the levels of collagen in hypertrophied animal samples were noted in a previous study, it is unclear whether is this a result of decreased collagen degradation or increased collagen synthesis. This study aimed to determine the effect of Small Leucine Rich Proteoglycans (SLRPs) which are believed to have a dramatic effect on the degradation of collagen (decreased degradation). The SLRP decorin was characterized via two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) using recombinant decorin. These results showed that decorin is not a peptide that is well suited for 2D-PAGE analysis, and show that another method of analysis must be used in order to characterize and identify *in-vivo* decorin in animal samples.

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Introduction

Hypertension and Hypertension Induced Cardiac Hypertrophy

A 2008 study by the Centers for Disease Control and Prevention (CDC), states that 32% of non-institutionalized Americans age 20 or older are classified as having hypertension (Office of Analysis and Epidemiology, 2009). This number is likely inaccurate due to the silent nature of hypertension and the low compliance of Americans for preventative medical screenings. Over 23,000 Americans die each year due to complications caused by hypertension (Office of Analysis and Epidemiology, 2009) and the number continues to rise. The current state of the economy will most likely cause an increase in the incidence of hypertension because of the lack of proper medical screenings and preventative care sought by Americans.

Hypertension

Hypertension is defined by a mean arterial pressure greater than 110 mm Hg, or a resting diastolic blood pressure greater than 90 mm Hg and a systolic blood pressure greater than 135 mm Hg (Cuspidi, *et al.*, 2009). Essential hypertension is the diagnosis given to a patient that has hypertension from an unknown cause (Calò, 2009). Essential hypertension is a nearly all-encompassing term that can describe nearly any type of elevated blood pressure; it does not give the underlying cause. Hypertension can also be classified by its suspected origin, such as sodium induced hypertension, atherosclerosis hypertension, and obesity induced hypertension.

Cardiac hypertrophy is in many cases the result of hypertension. The causes for hypertension can include many factors such as dietary (high salt intake), genetic, and excessive weight gain, however, this is not the only cause. Hypertension (a chronic systolic pressure greater than 135 mmHG and a chronic diastolic pressure greater than 90 mmHg) causes a much higher risk of the development of cardiac hypertrophy if not reversed after a significant amount

of time. While cardiac hypertrophy can result from poor health conditions, it also occurs in athletes.

Athletes hearts tend to enlarge in response to the increased stress placed on them by the strenuous physical activity of their day-to-day lives. An athlete's heart will undergo hypertrophy due to increased cardiac wall tension and increase in the myocardial oxygen demand, this condition is diagnosed as physiological hypertrophy (Urhausen; Kindermann, 1999). This is not considered a pathological condition, but rather a necessary adaptation for the lifestyle an athlete endures. The physiological hypertrophied heart presents with different morphological characteristics as well as different system wide physiologic conditions. Physiologically hypertrophied hearts have much large volumes inside the ventricle than pathologically hypertrophied hearts. Physiological hypertrophied hearts also do not occur through chronic hypertension, whereas the pathological condition has a high degree of co-morbid occurrence with hypertension.

Hypertrophy as a result of a pathological condition, such as hypertension, is a condition where the cardiac walls thicken in response to an increase in pressure loading from an increased stress, like that caused by hypertension. While both physiological and pathological cardiac hypertrophy have similar effects on cardiac tissue, physiological hypertrophy does not lead to a higher mortality rate, whereas pathological hypertrophy does. It is hypothesized that the underlying reason for this phenomenon is the duration of the condition that causes hypertrophy (Perrino, *et al.*, 2006). An athlete whose heart enlarges due to exercise places an increased demand for cardiac output on the heart. The demand for a higher cardiac output facilitates the change in cardiac morphology from a normal state to state of hypertrophy. The duration of the hypertrophied cardiac morphology is reversible and generally will return to a normal state after

the demand for increased cardiac output has subsided. A heart that enlarges due to a chronic condition such as hypertension is constantly under an increased demand for cardiac output. In response to this increased demand, the heart hypertrophies. After a prolonged period in this state, the heart begins to lose its ability to return to the normal morphologic state and remains hypertrophied. This is considered a pathological condition because of the non-reversible nature of the chronic state of cardiac hypertrophy.

Cardiac Hypertrophy

A hypertrophied heart has a different morphologic state than a normal heart and is characterized by having an increased cardiac mass to body mass ratio, reduced inner volume of the ventricles, thickened walls, and an increase in collagen deposition. A hypertrophied heart will not only display different physical morphologies but will also display different expressions of proteins and signaling molecules, all of which are associated with cardiac remodeling. A study by Saglam, *et al.*, shows a significant increase in the expression of the extracellualr matrix (ECM) collagen degrading enzymes: matrix metalloproteases 3 and 9 (MMP3, MMP9) when plasma levels of these enzymes were examined in left ventricular hypertrophy (LVH) subjects, these levels were significantly higher than non hypertrophied subjects (Saglam, *et al.*, 2006). Hypertrophied hearts show drastic alterations in their intigrin (adhesion molecule / regulatory) profiles. A study by Tsutomu, Y., *et al.*, shows a significant difference in the presence and amounts of intigrins that control remodeling and cellular motility within a hypertrophied heart, when compared to a non-hypertrophied heart. This study also showed a significant alteration in the expression of autocrine and paracrine agents that are secreted by the heart (when hypertrophied subject was compared to non-hypertrophied subject) (Yamazakia, *et al.*, 1995).

By altering the expression of physiologically active proteins in response to increased cardiac output demand, a hypertrophied heart's metabolism is significantly altered to cope with the demands placed on it. These alterations show promise for understanding underlying mechanisms present in the process of cardiac hypertrophy.

The reduced inner volume of the ventricles results from an enlargement of the myocytes and reduction of the lumen in the ventricles and has a profound effect on cardiac output. The reduced lumen of the ventricles will cause a lower ejection fraction to be pumped out of the ventricle for each beat of the heart (Cuspidi, *et al.*, 2009). The lower cardiac output resulting from the thickening and fibrosis of the cardiac tissue and the increased blood pressure only serve to create havoc on the body's circulatory system in pathologic hypertrophy**.** Clinical observations show a decrease in cardiac output from 9.3 +/- 1.7 L/min in control patients to 7.6 +/- 2.0 L/min in patients with left ventricular hypertrophy ($p = 0.02$).

Animal studies of hypertrophy have often yielded the same results as those seen in clinical human studies. There is a greatly reduced cardiac output from a hypertrophied heart when compared to a normal sized heart. A study using the spontaneously hypertensive rat (SHR) by Chen-Izu, Y., *et al.,* demonstrated a reduction in cardiac output in hypertrophied SHRs when compared to non hypertrophied, normotensive Wistar-Kyoto rat (WKY) hearts (Chen-Izu, *et al.*, 2007).

A hypertrophied left ventricle shows many different morphological changes when compared to a non-hypertrophied left ventricle in both humans and SHRs / WKYs. A left ventricle that has hypertrophied in response to pressure overloading will display a thicker myocardium when compared to a non-hypertrophied left ventricle. The inner lumen of the left ventricle is significantly reduced due to the thickening of the myocardium causing a lower

volume of blood to be pumped out per contraction of the muscle. In addition to ventricular dysfunction, cardiac hypertrophy causes significant alterations in the functioning of the atrium. Clinical studies show a decrease in the amount of blood atria are able to empty into the ventricles upon hypertrophy. These changes in the atrium compound the problem of a lower cardiac output by further reducing the ejection fraction of the heart (Nunez, *et al.*, 1987).

Collagen

Collagen is the most abundant protein in nature, and is found in all vertebrates. Collagen is a fibrous, long protein with amazingly high tensile strength. The general primary structure of collagen is Glycine - Proline - X, or Glycine - X - Hydroxyproline (X = any other amino acid). This allows for collagen fibrils to have a left-handed α-helical conformation. Each collagen fibril is composed of three α-helices that aggregate via hydrogen bonding in the quaternary structure. The surface of a collagen fibril is extremely hydrophilic, allowing for ~~interaction~~ with myocytes in a primarily aqueous environment (Persikov, *et al.*, 2005).

Collagen is a very stable protein with a long half-life at higher temperatures (37°C +). Because of its high tensile strength, stability, and fibrous nature, collagen is an ideal protein to use as a scaffolding element. Collagen has many structural motifs located within its primary sequence that allows for the binding of other specific proteins and cells, allowing for the creation of an organized, stable structure (Persikov, *et al.*, 2005).

Cardiac Extracellular Matrix

The cardiac extracellular matrix (ECM) is comprised mainly of collagen I and collagen III. The basement membrane which surrounds the ECM is composed mainly of collagen IV, lamanin, fibronectin, and other collagen-associated proteins of different molecular mass.

Collagen I and collagen III form the structural component of the ECM and maintain scaffolding for the attachment of cardiac myocytes. Laminin is a basement membrane protein that has influence on cellular metabolism and migration / differentiation. Fibronectin, an ECM glycoprotein binds to intigrins and collagen in the ECM and influences cellular adhesion, growth, migration, and differentiation. Laminin and fibronectin are responsible for controlling the biochemical events of the ECM and cardiac myocytes and play a major role in processes such as remodeling or healing (Macfelda, *et al.*, 2007). The ECM serves not only as a scaffold for attachment of cardiac myocytes, but as a regulatory center for controlling the placement and distribution of the cardiac tissue cells. Cardiac myocytes are anchored to the ECM by intigrins (cell adhesion molecules), which bind the actin filaments in the cardiac myocytes to the fibronectin component of the ECM for attachment. The binding of the intigrin to its ligand (actin filaments and fibronectin) causes a second messenger activation signal activating Focal Adhesion Kinase (FAK), small GTPases Rho and Rac, tyrosine kinases, serine / threonine kinases, and protein kinase C (PKC) (Bullard, *et al.*, 2005).

Cardiac myocytes are anchored to the ECM by intigrins which have a vital role in cellular adhesion and other metabolic processes. Intigrins are integral membrane proteins, they are composed of α and β subunits. Alpha subunits range between 120 to 180 kDa, and β subunits range between 90 to 110 kDa. The intigrin generally has most of its mass located outside the membrane arranged in a sequence dependent manner. In addition to the outer membrane segment, intigrins contain a single transmembrane segment, and a short cytoplasmic tail composed of 20 to 60 amino acids. There are more than 18 α and 8 β subunits that compromise intigrins in mammals. Different combinations of the subunit chains allow for specificity and give a library of more than 24 paired intigrin receptors (24 currently discovered). Intigrins

regulate cellular adhesion, migration and regulation of the myocyte phenotype. Intigrins anchor cells to the ECM and are bound to cytoskeletal elements such as talin and α-actinin by their cytoplasmic tails. This allows intigrins to function not only as mechanical anchors, but also as signal transducers for cellular events. The transduced signal from the outer portion of the intigrin to the cytoplasm of the cell is termed the "outside-in" signaling process. This has major effects on cytoplasm physiology such as gene expression, alteration of cellular morphology, proliferation, and extracellular calcium concentration. Intigrins have no enzymatic activity, but are able to activate focal adhesion kinase (FAK), GTP binding proteins (Rho and Rac), and regulate cytoskeletal components such as talin and Paxallin. Intigrins are detected in clusters near the Z-band of muscle tissue and play a major role in maintaining the physiologic state of the myocytes. Intigrin expression is closely related to the metabolism of the ECM in the adult heart. Intigrins play vital roles in the synthesis and degradation of collagen, fibronectin, osteoponin, and tenascin, and have a major impact on the phenotype of the ECM (Ross, 2002).

The ECM is a dynamic structure that changes in response to different stimuli, such as mechanical overload, reduced cardiac output, hypoxia, and increased peripheral resistance; this makes it a key player in the development of ventricular hypertrophy. The ECM detects mechanical overload caused by hypertension through the altered expression of biochemical molecules such as intigrins, small leucine rich proteoglycans (SLRPs), and laminin. This causes a modulation in the phenotypic expression of collagen, elastin, proteoglycans (PGs), glycosaminoglycans (GAGs), matrix metalloproteinases (MMPs), glycoproteins, and soluble peptide growth factors. Through the alterations of bioactive molecules, the ECM is able to alter itself to better support a heart that has undergone hypertrophy (de Simone; de Divitiis, 2002). The ECM can change its morphology to a more fibrous structure, able to cope with the increased

load of a hypertrophied heart. This aids in the perpetuation of the hypertrophied condition by providing a conducive environment.

Fibroblasts and Cardiomyocytes Role in the Cardiac Extracellular Matrix

The major cell types found in cardiac tissue are cardiac fibroblasts, cardiomyocytes, and endothelial cells. Roughly two thirds of cardiac tissue is composed of fibroblast cells and these cells are primarily responsible for cardiac ECM production. Cardiomyocytes have a contractile role, and make up most of the remaining one third of cells found in cardiac tissue. The cortex of the cardiac tissue is composed of a lining of endothelial cells. This lining of cells forms a semi-permeable membrane that allows for exchange of biological substances. Increases in external stress on cardiac tissue creates a need for the tissue to respond to the new stress, the straining of cardiac tissue cells can occur in a burst of cyclic stress or as a constant stressor. Both types of stress will cause the heart to respond in a situational dependent manner. Variation of stress levels and frequency plays a significant role in regulating the ECM through activation of intracelluar pathways through intigrins attached to the myocytes; affecting cardiac tissues and protein synthesis. The nature of the strain, be it cyclic or static, determines the response of the ECM and cardiac tissue cells. Frequency and amplitude are minor determinants in the stress-induced response and do not alter the metabolism of the ECM and cardiac tissue cells to the same degree as cyclic versus static stress (Gupta; Grande-Allen, 2006).

Fibroblasts are cells typically found in connective tissues that secrete collagen and are of mesenchymal descent. Fibroblasts are generally devoid of a basement membrane and contain surface receptors that confer tissue-dependent fibroblast specificity. The surface receptor, discoidin domain receptor 2 (DDR2), is found only on fibroblasts in cardiac tissue, thus the

presence of DDR2 indicates a cell is a cardiac fibroblast. DDR1, a discoidin domain receptor isotype which is similar to DDR2, mediates important cellular functions such as growth, migration, differentiation, and different morphological stages (such as hypetrophication, apoptosis, etc…). Unlike DDR2, the surface receptor DDR1 does not confer fibroblast specificity; DDR1 is found on many types of somatic cells. DDR2 is a collagen receptor that allows for fibroblast interaction with collagen and regulates collagen synthesis by the fibroblast (Yamazakia, *et al.*, 1995). Fibroblasts are a key cellular component in tissue wound repair. Collagen produced by fibroblasts is integrated into the ECM and further modified by different ECM components. Because of the ever changing nature of the ECM, fibroblasts are constantly being up and down-regulated through increasing the number of cells or inhibiting cellular division in response to different environmental factors, this places fibroblasts as a major regulatory cell involved in tissue wound repair. The close relationship between fibroblasts and the ECM allows for fibroblasts to detect mechano-transduced stresses, like that placed on the heart by hypertension. This allows the fibroblast to change its collagen synthesis rate in response to this increase in stress and could be an underlying mechanism for cardiac hypertrophy. Fibroblasts are a major component of the ECM they allow for the secretion of collagen and the metabolic influence on other nearby cells (Olaso, *et al.*, 2001).

Cardiac myocytes are specialized cells in the heart, responsible for properties such as automaticity, contractility, rhythmnicity, and the overall efficiency of the heart. Cardiac myocytes are generally arrested in the G_1 period of the cell growth cycle. With the cells arrested in the G_1 state, they are unlikely to die by mechanisms of apoptosis or necrosis, unless an environmental stress creates favorable conditions for cell death. Myocytes are attached to the ECM by intigrin molecules and can move on the scaffolding (of the ECM) to respond to different

environmental factors. The ECM in conjunction with fibroblasts and other neuroendocrine signals control the general metabolism and physiological state of the myocyte. Cardiac myocytes undergo numerous different types of stresses, such as increased mechanical load, increased peripheral resistance, ischemic conditions, and reduced pumping efficiency from various hemodynamic factors. The myocyte is charged with compensating for these stresses and sometimes these compensations can cause pathological conditions (Olaso, *et al.*, 2001).

Cardiac hypertrophy is one of these pathological conditions that can result from the over-compensation of the myocytes in response to an increase in stress. Cardiac myocytes hypertrophy in response to an increase in demand for cardiac output, the hypertrophy of the cells allows them to maintain a higher cardiac output for a short period of time. After the hypertrophied cells begin to age, they lose their ability to return to normal size (under normal physiological states, current research suggests it may be possible to return myocytes to normal states with therapeutic drugs) and begin to reduce the cardiac output through the loss of compliance in the myocardium. To support the enlarged hypertrophied myocytes, the ECM must undergo changes to accommodate the enlarged myocytes which aids in the progression of the chronic disease. Together with fibroblasts and the ECM, cardiac myocytes are responsible for the general function of the cardiac tissue (Baudino, *et al.*, 2006).

Mechanical Stress Response of the Cardiac Extracellular Matrix: Role of ECM and non-ECM Proteins

Collagen is the main component of the ECM, it provides tensile strength and scaffolding for cells. Collagen is a fibrous protein secreted by fibroblasts in a vesicularized form. It is then assembled in the extracellular fluid to form ECM scaffolding. Elastin is a protein which is found in conjunction with collagen in the ECM. Elastin provides an elastic substance in the ECM of

cardiac tissue, allowing for recoil of the cardiac tissue. Proteoglycangs (PGs) are composed of glycosoaminoglycans (GAGs), and are formed by branching several GAG chains around a protein center. PGs and GAGs have numerous biological functions in the ECM, one of which is a maintenance role. Hyaluronan (a GAG), and versican (a PG) have properties that allow them to sequester large volumes of water in the spaces of the cardiac tissue. This influx of water into the tissue provides resistance against pressure (compression) generated by an increased load on the cardiac tissue. This resistance can result from a multitude of conditions, such as hypoxia, increased peripheral resistance, clogged circulation system, and hypertension. Evidence shows that the rates of synthesis of PG and GAG are modulated by stresses such as mechanical pressure loading. Studies from Pietila, K., *et al.*, and Grande-Allen K.J., *et al.*, show that pressure loading of cardiac tissue significantly increases the synthesis and proliferation of glycosaminoglycans and alters the metabolism of the ECM (Grande-Allen, *et al.*, 2003), (Gupta and Grande-Allen, 2006), (Pietilä, *et al.*, 1983).

PGs are known to play a role in determing cellular cycles. Cardiac myocytes, fibroblasts, and other ECM components respond differently depending upon what stage of the cell cycle they are in. Thus PGs can play an important role in the determination of the cardiac morphology and physiological state during different stresses. Cell cycle dependent functions such as rates of cellular proliferation and migration show evidence PG dependence. Cellular phenotype determination has a similar relationship with PGs. PG synthesis was shown to be regulated by growth factors and mechanical strain in an investigation by Kinsella M.G., *et al.*, the use of cytokines and growth factors influenced the levels of small leucine rich proteoglycans (SLRPs) – a subset of PGs – such as versican, decorin, and biglycan. These SLRPs then were shown to indirectly influence rates of cellular proliferation, migration, and expression of cell

differentiation phenotype (Kinsella, *et al.*, 2004). The roles of PGs are numerous and the extent of their influence on ECM homeostasis is beginning to grow. Processes that are both directly and indirectly related with the expression of PGs can become potential targets of drugs to reduce the pathogenesis caused by incorrect expression of these peptide molecules.

Cardiac Tissue Response to increased Pressure-loading

Hypertensive patients have an increased load on their circulatory system. This load causes a significant amount of pressure-loading on the heart and alters the physiological state of the cardiac tissue. In response to an increased load from hypertension induced pressure-loading, cardiac tissue will begin to thicken. This is accomplished by the inflammation of cardiac myocytes, and the increased mass / volume of the ECM. The ECM, as previously stated, is a vital control point for the metabolism and physiological state of the cardiac tissue. The dynamic nature of the ECM causes a constant remodeling of the cardiac tissue in response to stress. To better understand the genetic and molecular mechanisms associated with cardiac tissue thickening (in response to cardiac hypertrophy), Heidrun, R., *et al.*, analyzed genetic expression levels of left ventricle (LV) cardiac tissue from hearts that had a left ventricular hypertrophy (LVH) characteristic. Animals in this study developed LVH as a consequence of nephrectomization. Levels of genetic expression were compared to LVH nephrectomized rats and sham-operated control rats. LVH nephrectomized animals showed down-regulation of collagen type VI α1 precursor and procollagen type VII αI, as well as up-regulation of procollagen type II αI, and the anti-adhesive glycoprotein gene *SPARC*. Up-regulation of matrix-metalloproteinase 24 (MMP24) α-actinin 4 (an actin filament structural gene), and β1

intigrin were also noted in LVH nephrectomized rats. Down-regulation of cadherin 2 (CDH2), gelsolin, L-caldesmon, and β3 intigrin were noted.

CDH2 plays an important role in cell-cell interactions in the cardiac ECM linking adjacent cells through its transmembrane projections, and β3 intigrin is an important cell surface protein used for communication. The interactions between these two molecules (CDH2 and β3 intigrin) was demonstrated using *in-vitro* studies by Ridinger, H., *et al.* Increases in PGs and GAGs can be predictors of early hypertrophy, by implementing routine screens into normal health physicals once more substances can be identified as predictors of hypertrophy. This was demonstrated by the use of sham-operated control rats which showed down-regulation of versican (an SLRP), and up-regulation of chondroitin sulfate proteoglycan 6 (CSPG6/SMC3), it was also noted the MMP2 gene expression was significantly lower than the LVH nephrectomized model. These results were obtained by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) comparison of mRNA levels of experimental and control models to basal levels of mRNA expression (Ridinger, *et al.*, 2009).

Localization of proteins in the heart and its associated tissues plays an important role in determining its physiologic state. Immunohistochemistry studies by Heidrun, *et al.,* have localized many of the PGs and other proteins that influence pressure-loading cardiac hypertrophy. Antibodies to different ECM PGs, MMPs, and cytoskeletal elements were used in order to mark the localized position of the proteins. These studies confirmed that increases in mRNA production were correlated with higher concentrations of protein being present at localized points in the cardiac tissue. Thus, not only is there an increase in mRNA production, but there is a subsequent increase in protein synthesis.

Secreted protein acidic and rich in cysteine, (*SPARC*) is a peptide that is expressed as a result of tissue injury. *SPARC* has a profound effect on endothelial permeability and controls export of biochemical molecules. It was found that there was a significantly higher amount of *SPARC* in the cardiac muscles of LVH nephrectomized rats than in sham-operated control cardiac muscle tissue. Versican, another PG, concentration was found to be at a significantly lower level in LVH nephrectomized samples than in the sham-operated controls. MMP2 and MMP7 concentrations were significantly higher in sham-operated control cardiac muscle than LVH nephrectomized subjects. The localization of biochemical molecules in the cardiac myocytes and cardiac tissue is a means of controlling the interaction of molecules such as intigrins, SLRPs, fibronectin, and very importantly the MMPs. This is not a surprising result, as MMPs have a collagen cleaving function. A hypertrophied heart would not have as much degradation of collagen as a non-hypertrophied sample, so an increase in the MMPs in non-hypertrophied tissue is logical. β-Catenin, a cell contact linker, was found to be in lower concentration upon immunohistochemical analysis of LVH nephrectomized subjects. There was a high concentration of β-Catenin localized near the intercalated discs of the cardiac muscle tissue of hypertrophic subjects. Alpha-actinin concentration was increased in LVH nephrectomized rats and was found to have a nuclear translocation (Ridinger, *et al.*, 2009).

The Role of the Matrix Metalloproteinase on the Cardiac Extracellular Matrix

The ECM is primarily degraded by matrix metalloproteinases (MMPs), which are specific enzymatic proteins that digest a vast array of ECM proteins such as collagen. An inherent characteristic of the MMP is the ability to degrade at least one component of the ECM at a physiological pH (neutral). MMPs require two Zn^{++} ions to become activated and maintain their activity, they also require one Ca^{++} ion to maintain a stable structure. Inhibition of MMPs

can occur through deactivation with a metal chelating agent that removes the ionic cofactors, regulation at the transcriptional level by decreasing the mRNA production, or enzyme inhibition via tissue inhibitors of matrix metalloproteinases (TIMPs). MMPs are secreted into the extracellular fluid in a zymogenic form and require serine protease cleavage for activation (D'Armiento, 2002).

MMPs have different subclasses defined by their digestive role. The collagenase family of MMPs will be examined in particular because of its significant role in the etiology of cardiac hypertrophy. As of 2010, there are over 25 known members of collagenase MMPs: amongst the most important to this study are MMP1, MMP8, MMP13, two newly identified mouse proteins Mcol-A, and Mcol-B (murine collagenase-like A/B). Each collagenase MMP has different affinities for different collagens and they have different specificities for the portion of collagen they cleave (through amino acid sequence recognition). It can be assumed that all 5 members of the collagenase MMP family have an extremely high affinity for both collagen type I and collagen type III (major forms found in the cardiac tissue). MMP1 cleaves the collagen triple helix at a Gly-Leu or Gly-Ile site, located at a carboxy terminal quarter of the collagen fibril. This results in the dissociation of the 3 α-helices and allows for other non-specific proteases to degrade the fibril (D'Armiento, 2002), (Devy, J., *et al.*). The specificity of collagenase MMPs allows for a greater range of control by the heart to selectively degrade different forms of collagen independently.

Matrix Metalloproteinase Action in the event of Cardiac Damage / Stress

MMP expression and concentration in the ECM is significantly altered by different physiological states of the heart. Studies by Montfort, *et al.*, show that levels of MMPs are modulated during cardiac injury and ischemia, both significant physiologic states of the heart.

Typically, in response to stress the initial levels of MMPs are increased in order to degrade collagen in the cardiac tissue. In the Montfort, *et al.,* studies, a significant loss of cardiac collagen occurred during an ischemic attack which was noted during the first few minutes of the attack. Significant collagen deterioration was obvious within the first hour. It was hypothesized that this rapid deterioration of collagen was caused by the increased activity of the MMPs (Takahashi, *et al.*, 1990). After the initial loss of collagen following a stressful event such as an ischemic attack, the levels of collagen type I and collagen type III are shown to increase due to increased levels of collagen type I and collagen type III gene expression. It was thought that this increase in collagen production caused fibrosis which better served the needs of the heart by reinforcing cardiac walls and thus allowing more force generation. However, as the fibrotic condition of the heart continues, stiffness and less compliance result, thus reducing the cardiac output. Other complications caused by this fibrotic remodeling include less cross-linking of ECM collagen as seen in fibrotic hearts when compared to non-fibrotic hearts. This is detrimental to the structural function of the ECM and is an underlying mechanism which allows for cardiac hypertrophy (Janicki, 1995). Alterations in the expression of MMPs can lead to many other conditions that are pathological to the heart. With a more encompassing database of normal levels (during different physiologic states) MMP blood concentrations may lead to an early means of detection of a possible physiologic state that can lead to cardiac hypertrophy.

Alterations in the activation and production of MMPs can lead to a variety of cardiac dysfunctions. Over activation of MMPs can cause a high degree of collagen degradation which in turn causes a condition known as dilated cardiomyopathy (DCM). DCM manifests itself with cardiomegaly, an enlargement of the heart, and can be either hypertrophic or dilatory (Kato, R., *et al.,* 2005). Tissue inhibitors of Metalloproteinases (TIMPs) can be found in complex with

MMPs. The presence of a TIMP on an MMP causes inactivation of the MMP. It is suggested that there is normal production of TIMPs by the cardiac tissue in order to maintain homeostasis of ECM degradation. Studies performed by Thomas, *et al.*, show that an increased amount of TIMPs were present at the end stage of DCM. This study provides further evidence to show that the inactivity of MMPs is a means of fibrosis to occur (Thomas, *et al.*, 2008).

MMPs are necessary for proper turnover of collagen in the ECM. Their dysregulation can cause over-remodeling of the ECM to occur and can lead to conditions such as DCM, left ventricular hypertrophy (LVH), and other hemodynamic problems, such as atherosclerosis. It is crucial that MMPs be kept in proper regulation and allowed to function as they are intended for the proper maintenance of the cardiac ECM.

Regulation of Matrix Metalloproteases in the Cardiac Extracellular Matrix

The MMPs primary role in the cardiac ECM is the degradation of collagen, gelatin, elastin, and fibronectin (Li-Saw-Hee, *et al.*, 2000). There is a heavy degree of control on MMPs from the TIMPs. MMP-2 and MMP-9 are known as gelatinases because of their gelatin degrading properties. These enzymes have been shown to substantially alter the left ventricular function and properties by allowing increased vascular permeability resulting in fluid and protein transfer to cardiac myocytes, regulation of molecule import / export in the myocytes, alteration of the basement membrane, and left ventricular hydration (Tyagi, 1998). Spinale showed that the gelatinases (MMP-2 and MMP-9) had a significant role in altering the collagen fibers that anchor cardiac myocytes to other nearby myocytes, this action appears to prevent cellular slippage (Spinale, 2002). These gelatinases, like many MMPs, are controlled by the TIMPs. TIMP coupled with an MMP can disable the MMP from degrading any ECM components. TIMP production is regulated in accordance with external stress on the cardiac tissue, when

stress increases from stimuli such as pressure overload from hypertension, TIMP levels increase (Tozzi, *et al.*, 2007). MMP production is independent of TIMP production, thus a balance in the relationship between the MMP and TIMP must exist in order to maintain homeostasis.

There are two distinct phases of response by the heart when exposed to stress: the compensation stage and the decompensation stage. Experimentation by Rossana, T., *et al.*, examined the levels of MMP-1, MMP-, MMP-9 (collagenase), and MMP-3 (stromalysin). Levels of these enzymes were measured on animals that had LVH induced hypertension, animals that had general heart failure, and sham-operated control animals. Findings from this study showed there was a significant increase in the amount of MMP-2 in the LVH animals when compared to sham-operated control, and general heart failure animals during the compensatory stage. There was no detectable activity levels of MMP-1, MMP-3, or MMP-9. This suggests that MMP-2 had a significant role in the hypertrophy process during the initial phase of cardiac remodeling. Rosanna, T., *et al.*, extended their study by examining the coresponding amount of TIMP-2, an inhibitor to MMP-2. There was an increased level of TIMP-2 found relative to the increased level of MMP-2 in sham-operated control animals and general heart failure animals. When the TIMP-2 level was examined in the LVH animal group, however, there was no increase. This points to a possible mechanism of ventricular hypertrophy by the over-activity of MMP-2 (Tozzi, *et al.*, 2007).

Local synthesis of TIMPs presents a clear, direct control point for MMP activity by the local synthesis of TIMPs (local to the MMP site of activity). Local TIMP synthesis allows for the increased concentration of TIMP enzymes to bind and inhibit the MMPs. Over-activation of MMPs is the first stage in cardiac damage, and is considered to be the compensatory stage where the heart reacts to the increased stress by remodeling. Unfortunately, the remodeling activity can

become over-regulated and can cause damage to the ECM by over-degradation of important ECM structural proteins. The concentration of TIMPS in the ECM is a major regulator of the degradation activity by MMPs; thus another control point that could be targeted by drug or gene therapy to treat cardiac hypertrophy.

Small Leucine Rich Proteoglycans Effects of MMPs in the Cardiac ECM

Small Leucine Rich Proteoglycans (SLRPs) are low molecular weight (~40KDa) peptides that are extremely rich in leucine repeating segments. These leucine rich segments are flanked by two cysteine residues on either side. SLRPs specifically interact with different types of collagen with different affinities, and appear to have a protective function on the maintenance of collagen fibrils. SLRPs protect collagen fibrils by wrapping themselves around the fibril at specific sites and causing steric interference between the fibril and the MMP, protecting the fibril from cleavage by the MMP. The protection given to collagen from the SLRPs appears to be sterically related due to the increased diameter of the fibril at the site of association between the fibril and SLRP. SLRP binding specificity puts the bound SLRP over sites of potential cleavage by the MMPs (which show a sequence dependent cleavage specificity) and blocks the cleaving action of the MMP allowing for the fibril to not be degraded. SLRPs interact with cells and growth factors causing differential regulation of SLRP synthesis. Decorin is shown to antagonize the action of transforming growth factor – β (TGF-β), a cytokine that plays important function in the regulation of immune system phagocytes (Geng, *et al.*, 2006). SLRPs have many known interactions that control cardiac metabolism. There are many undiscovered interactions that SLRPs have with other homeostatic functions, and they are becoming an increasingly more important area of study for the regulation of connective tissue metabolism.

MMPs are not only regulated by TIMPs, they are passively inactivated by certain small proteoglycans. One class of proteoglycans that can modulate the function of MMPs are the SLRPs. SLRPs such as decorin, lumican, and fibromodulin have shown to be highly effective in controlling the activity of collagen degradation when bound to collagen fibrils and exposed to MMPs. Studies from Geng, Y., *et al.*, examine the degratory effect of MMP-1 and MMP-13 (collagenases) on collagens type I and III, and collagens type I and III bound by either decorin, lumican, or fibromodulin. Samples of collagen were purified and incubated with different combinations of MMP-1 and MMP-13. Collagens not bound by SLRPs showed a higher degree of degradation when exposed to MMPs than control collagens. When collagen was bound by SLRPs and exposed to MMP degradation and then compared to SLRP bound collagen controls, there was little to no degradation of collagen or the SLRP. This points to the SLRP having a protective effect on the collagen fibril when in the presence of MMPs (Geng, *et al.*, 2006).

Pathologies of collagen deposition exist when there is a dysregulation of SLRPs. Danielson, *et al.*, showed there was a non-uniformity of collagen in cardiac tissue when he examined decorin null mice. When exposed to a myocardial infarction conditions, decorin null mice showed inferior mechanical cardiac function, heart walls that were much thinner than normal, and disruption of the collagen fibrils. The subsequent weakening of the cardiac tissue allows for the onset of ventricular dilation, which in turn can cause many different complications such as myocyte slippage and decreased cardiac output (Danielson, *et al.*, 2004). SLRPs have been shown to impact many aspects of the metabolism and function of the ECM environment. Cytokine and growth hormone alterations, as well as steric hindrance mechanisms are intrinsic properties of the SLRPs and their dysregulation can cause cardiac dysfunction such as left ventricular hypertrophy.

An In-depth Examination of the Function of Decorin in Cardiac Tissue / Cardiac ECM

Decorin is the best characterized of the SLRP proteins. Decorin plays an important role in the proper formation and organization of collagen fibrils in the cardiac ECM as well as in the metabolism in the ECM. Decorin has been shown to modulate cardiac myocyte adhesion. Studies by Winnemöller have shown that decorin interacts with both collagen type I and fibronectin, and increases the affinity of decorin binding these ECM components. It was also noted through this study that decorin had an equally high affinity for thrombospondin, a molecule involved in cellular adhesion to the ECM. In the presence of decorin, cells were not immobilized through attachment to the ECM. The affinity of decorin for thrombospondin aids in the disassociation of cells from the ECM. This anti-adhesive property of decorin allows for conditions such as DCM to occur, thus promoting the initial phase hypertrophy (McEwan *et al.*, 2006).

While decorin has many unique features that separate it from the other SLRPs, it has a degree of overlap with the other SLRPs in that it shares similar functions. This overlap was shown by Ameye and Young with their experimental research of the SLRPs biglycan, decorin, fibromodulin, and lumican (Geng, *et al.,* 2006). Deficiencies in these SLRPs resulted in abnormal collagen fibril formation when SLRP deficient mice tissues were examined for collagen content and collagen assembly formation (Ameye and Young, 2002). Through compensatory mechanisms in response to stress, SLRP production and content of cardiac tissue can be altered. These alterations in SLRPs can result in the improper formation of the newly remodeled ECM, thus making a pathway for disease production possible (McEwan, *et al.*, 2006).

The ability of decorin to disrupt the cleavage of collagen types I and III by MMPs was demonstrated by Geng, *et al.* It was demonstrated that the presence of decorin inhibited the

degradation of collagen I and collagen III. This was completed by incubating decorin with collagen I and collagen III; the peptides were subjected to incubation and degradation by MMP1 (primary collagenase MMP). There was no significant amount of degradation that occurred on collagen peptides bound by decorin and other SLRPs. Likewise when collagen I and collagen III were subjected to MMP1 without decorin or SLRP incubation, there was a significant amount of collagen degradation. This was shown by running protein incubations with one-dimensional SDS polyacrylamide gel electrophoresis (1D-SDS-PAGE) and comparing banding patterns from control collagen profiles to MMP subjected collagen profiles. Also control decorin bound collagen profiles were compared to decorin bound collagen profiles that were incubated with MMP. This finding indicates that decorin and other SLRPs have an inhibitory role when it comes to degradation of the collagen component of the ECM by MMPs (Geng, *et al.*, 2006).

Because PGs, GAGs, and SLRPs play such an important role in regulating the ECM, they are carefully regulated by enzymatic modulators that control the presence and concentration of these important ECM modulators. MMP13 is known to have the ability to degrade SLRPs, this was shown by Monfort *et al.*, through his study using the SLRPs decorin, biglycan, fibromodulin and lumican. The SLRP peptides were run as a standard without being subjected to MMP13, their 1D-SDS-PAGE profiles were obtained. These profiles were compared with the SLRPs after incubation with MMP13. Decorin showed that it was cleaved one time as indicated by the presence of two decorin fragments (30 and 28 kDa). Decorin that was not incubated with MMP13 showed only one fragment at about 45 kDa. It is important to note that decorin took 16 hours of incubation with MMP13 before it was completely degraded, when compared to biglycan (a close relative to decorin) which took 2 hours for complete degradation to occur. To determine the cleavage site off decorin by MMP13, amino acid sequencing was done and showed that

decorin is cleaved somewhere between amino acid positions 240 – 241. These results give more insight as to the potential for decorin to support an ECM that is conducive to a hypertrophied heart. Decorin shows the most resilience in terms of protecting collagen fibrils from degradation by MMP1, and when protecting itself from degradation by MMP13 (Monfort, *et al.*, 2006).

Purpose of this Study

Previous studies done in this laboratory show a significant increase in the amount of collagen found in male spontaneously hypertensive rats (SHRs) compared to females (corrected for wet tissue weight). This suggests that there will be a lower amount of decorin found in female SHR when compared to male SHRs of similar age.

To investigate this question, this study will characterize the two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2D-PAGE) profiles of intact decorin, and cyanogen bromide (CNBr) digested decorin. In future studies these profiles will be compared to CNBr digested collagen I and collagen III and used to analyze the interaction of decorin with collagen I and III in male and female SHRs. These investigations will hopefully lead to a better understanding of the role of SLRPs in cardiomyopathies.

Materials and Methods

Overview Protocol

Purified decorin was obtained from Santa Cruz Biotechnologies and was subjected to digestion by cyanogens bromide (CNBr). CNBr digested samples were analyzed by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) to determine a 2D profile of the digested peptide. Undigested decorin was used to validate the theoretical molecular weight (MW) and isoelectric point (pI) of the undigested peptide. Gels were analyzed for the migration of peptide fragments through the use of SYPRO (Bio-Rad) staining and ultraviolet light detection. R_f and R_i were then calculated to determine protein MW and pI in comparison with a 2D-PAGE standard.

Protein Digestion:

Purified decorin was suspended in 4.0 mL 70% Formic Acid and digested with (CNBr) (20 mg CNBr/mL) for 16 – 18 hours with gentle agitation. Digestion mixture was then centrifuged for 30 minutes at 15,000 rpm, the supernatant was collected and stored at -80°C. The acid soluble supernatant from digestion was dialyzed against water in SnakeSkin® Pleated Dialysis Tubing (3,500 Da molecular weight cut off) until the pH of the water was 7.0. Supernatant was then lyophilized and stored at -80°C.

Protein content of the lyophilate was determined by Bradford assay.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Lyophilates were rehydrated at a concentration of 50 μg/μL in rehydration buffer (8 M Urea, 50 mM DTT, 4% CHAPS, 0.0002% Bromophenol Blue, 25% Type I H_20). Two hundred micrograms of lyophilate protein was combined with 3 μL Bromophenol blue and brought to a

final volume of 125 μL using Rehydration Buffer. The protein mixture was then absorbed to a Bio-Rad Isoelectric Focusing (IEF) strip (Bio-Rad, 7 cm, pH range: 3 – 10, cat. #163-2000) in a preparation tray overnight. Incubated IEF strips were focused for 18 hours, using a Bio-Rad Protean IEF Cell (20,000 Vhrs, 15 μA per strip, 500 V hold). Focused strips were equilibrated with equilibration buffer I (EQ-I) (6 M Urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 104 mM DTT) for 10 minutes, and washed with equilibration buffer II (EQ-II) (6 M Urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 134 mM iodoacetamide) for 10 minutes.

Polyacrylamide gels were made to 12% and 15% polyacrylamide content, depending upon application to be used. Samples were electrophoresed at constant current of 20mA for approximately 1 hr in 1x TGS buffer.

In some cases the gel was stained in Coomassie Blue stain (45% MeOH, 10% glacial AcOH, 2.5% Brilliant Blue 250-R) for 2 – 18 hours and then destained in destain solution (40% MeOH, 10% glacial AcOH) until the background was clear enough to see individual protein spots. In other cases, gel was stained with SYPRO stain (1x SYPRO, Bio-Rad Cat.#: 170-3125) overnight for 18 hours, and destained with SYPRO destain (35% MeOH, 7% glacial AcOH) for 30 minutes.

Imaging and Analysis of Polyacrylamide Gels

Gels were imaged by Bio-Rad gel imager under UV-Trans light for SYPRO stained gels, or EPI-White light for coomassie blue stained gels. Image was enhanced to give maximum visibility of peptide spots through PDQuest software (Bio-Rad). The Image was imported and analyzed in PDQuest software. The isoelectric point (pI), the molecular weight (MW), and the

mass of the peptides were determined by calibrating unknowns to commercial standards (R & D Systems, cat. #1060-DE).

Results

2D-PAGE Profile for Undigested Decorin

Previous studies have characterized the collagen components of the acid soluble cyanogen bromide fraction of the heart homogenates using 2D PAGE. The goal of this project was to create 2D gel PAGE profile of intact and cyanogen bromide digested decorin standards that could be used to examine/analyze2D gels of heart preparations for the presence of decorin. A commercially prepared 2D-PAGE standard was run on a 15% gel (Fig 1) and the MW and pI of component proteins determined. The R_f and R_{pI} of the standard peptides were calculated (Table 1) and these values used to characterizes peptides and peptide fragments visualized on 2D gels of decorin.

Preliminary studies investigated the optimal amount of decorin protein applied to the gel in order to visualize the peptides using SYPRO stain (Bio-Rad). Four protein quantities were tested on the gels: 10 μg, 15 μg, 25 μg, and 50 μg. These gels had significantly different visible profiles. At 10 ug protein, two visible spots appeared on the gel (Fig 2). Both peptides had the same molecular weight (~32.38 KDa) but different pIs (A = 4.21 and B = 5.61). The spot with the pI of 5.61 had a higher concentration of peptide as shown by the higher signal strength. At 15 μg sample protein (Figure 3), no distinct spots were visible and instead a long streak with a MW of approximately 58.38 KDa, extending from a pI of approximately 3.0 to 7.4.

At 25 μg decorin protein (Figure 4), two distinct spots were visible, both at a molecular weight similar to that seen in the 10 ug gel (32.69 KDa).. The pIs of these spots were ~4.34 and 7.07. This profile closely matches the theoretical value of decorin's molecular weight and pI (39 kDa, 8.9). Finally at 50 μg decorin protein (Figure 5) 2 spots were visualized, each with different MWs and similar pIs. One spot (MW 57.67 KDa , pI ~5.12) was extended over

several pIs and had a shape that suggested that it might represent more than one peptide. The second spot was more discreet and had a molecular weight of 32.54 KDa and a pI of 4.22. Thus no reproducible profile of decorin was observed at these protein concentrations using Sypro stain.

Undigested decorin has shown to have a high concentration dependant 2D-PAGE profile as shown by the samples of 10 μg, 15 μg, 25 μg, and 50 profiles. To continue the 2D-PAGE profile comparison of the digested and undigested decorin, samples were digested with CNBr and analyzed in the same fashion as undigested samples.

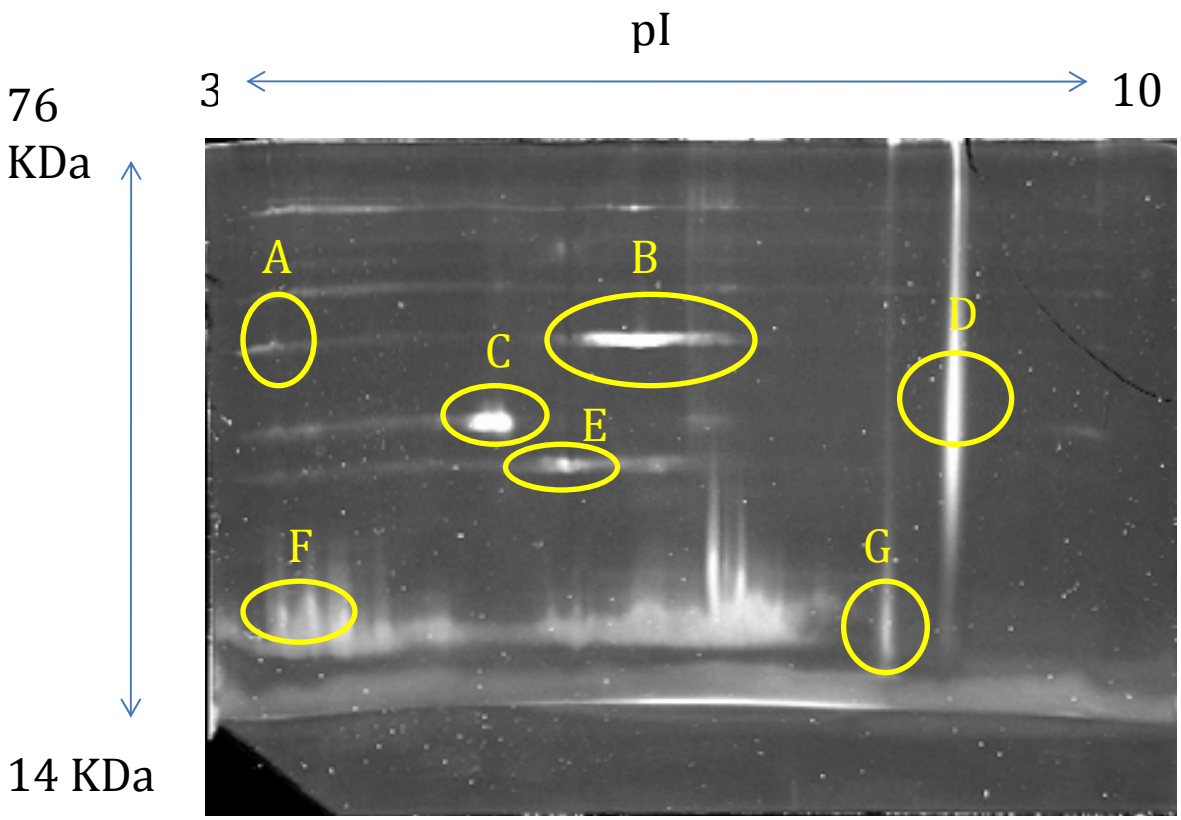

FIGURE 1
25µg 2D-PAGE Standard, 15% Polyacrylamide Gel

Table 1		
Protein	MW	pI
BSA (A)	66.2 kDa	5.5
Conalbumin (B)	76 kDa	5.9 – 6.6
Actin (C)	43 kDa	5.1
GAPDH (D)	36 kDa	8.5
Carbonic anhydrase (E)	31 kDa	6.1
Trypsin inhibitor (F)	21.5 kDa	4.5
Myoglobin (G)	17.5 kDa	7.0

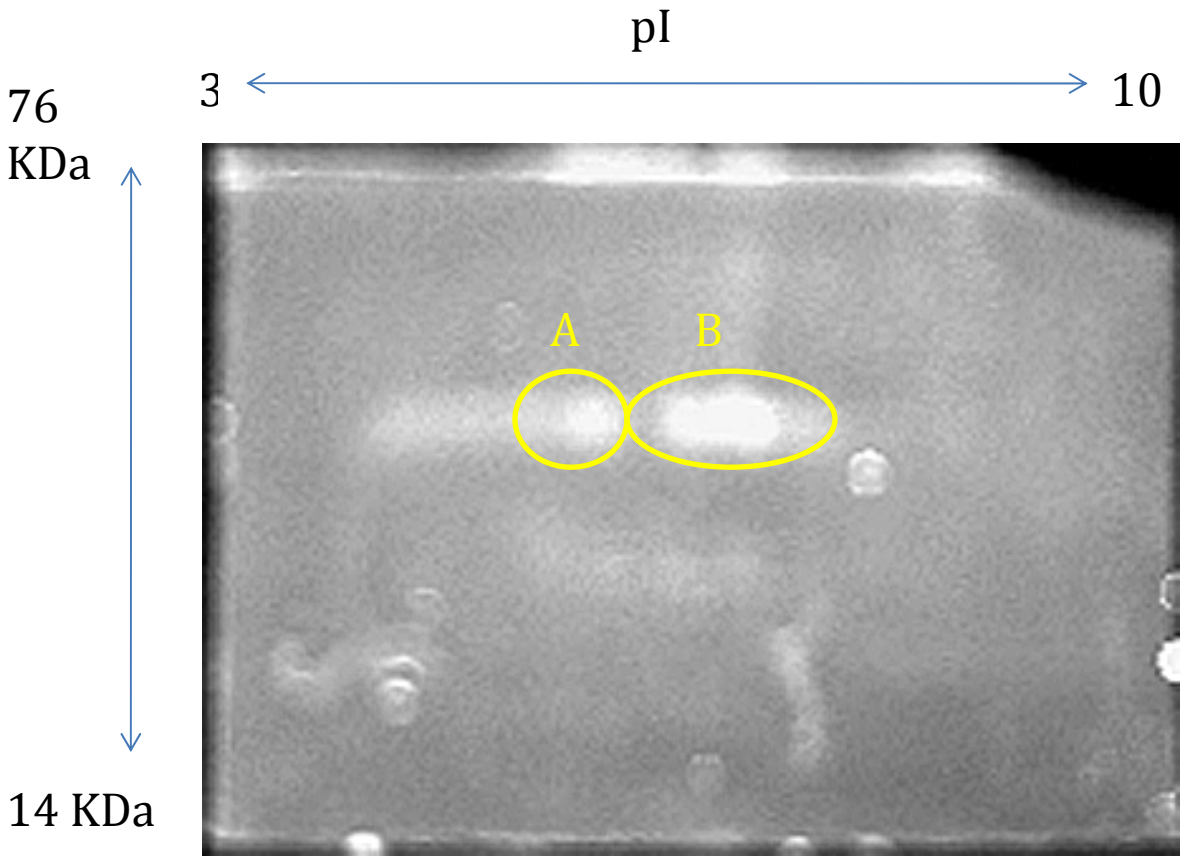

FIGURE 2
10µg Undigested Decorin, 12% Polyacrylamide Gel

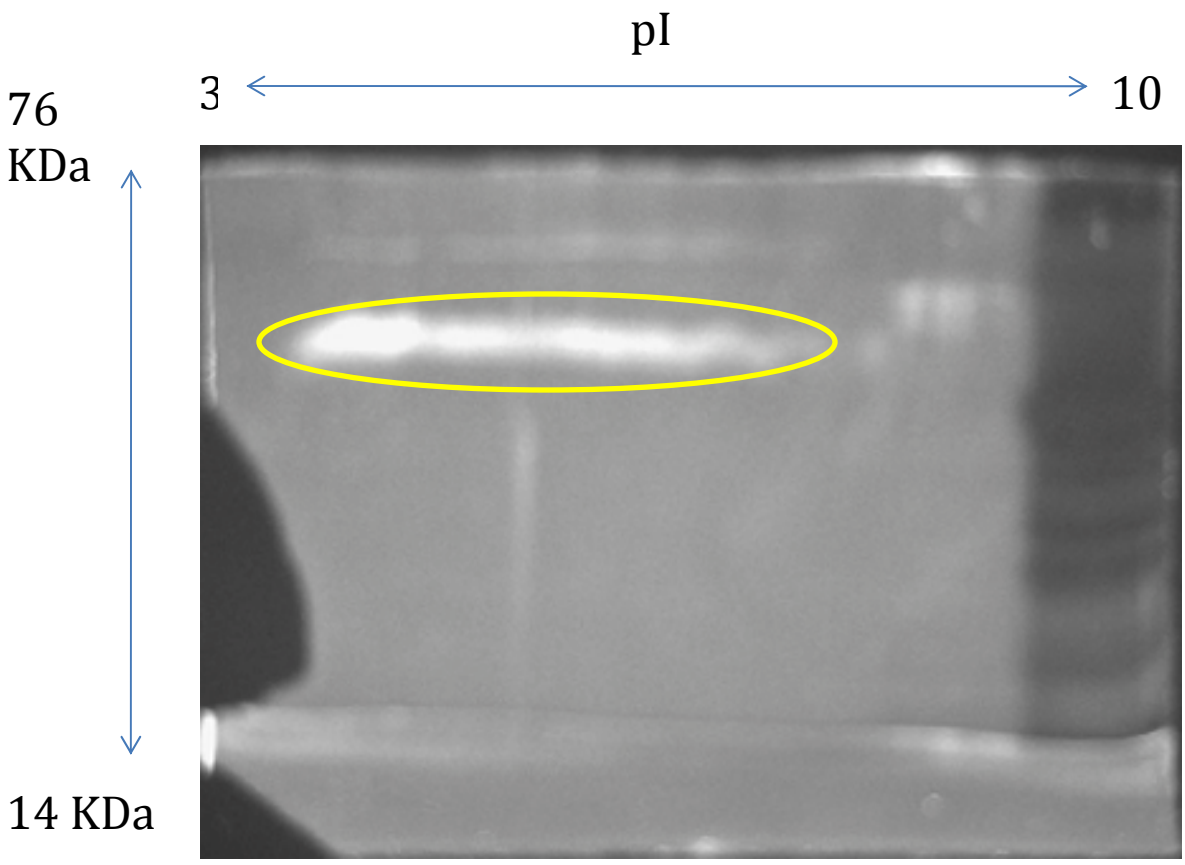

FIGURE 3
15µg Undigested Decorin, 12% Acrylamide Gel

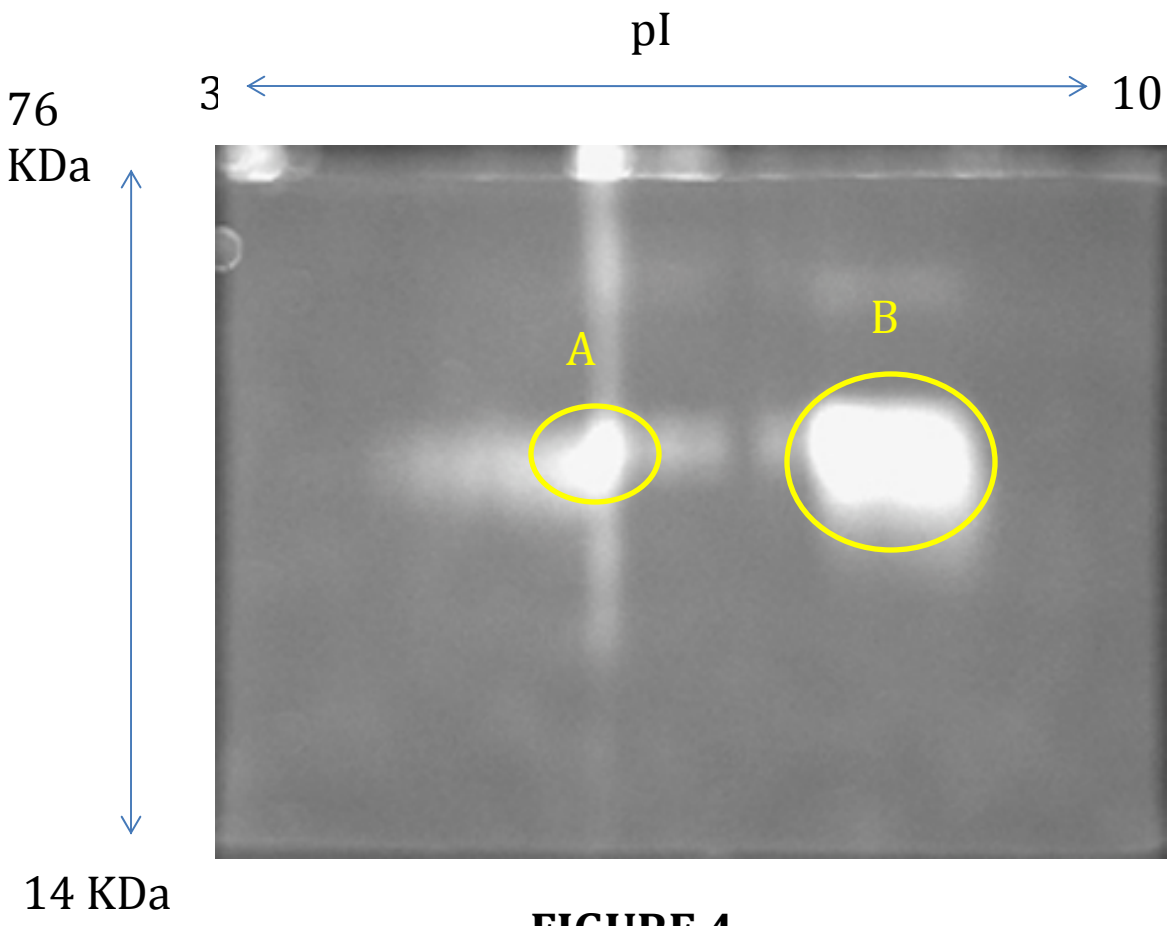

FIGURE 4

25µg Undigested Decorin, 12% Polyacrylamide Gel

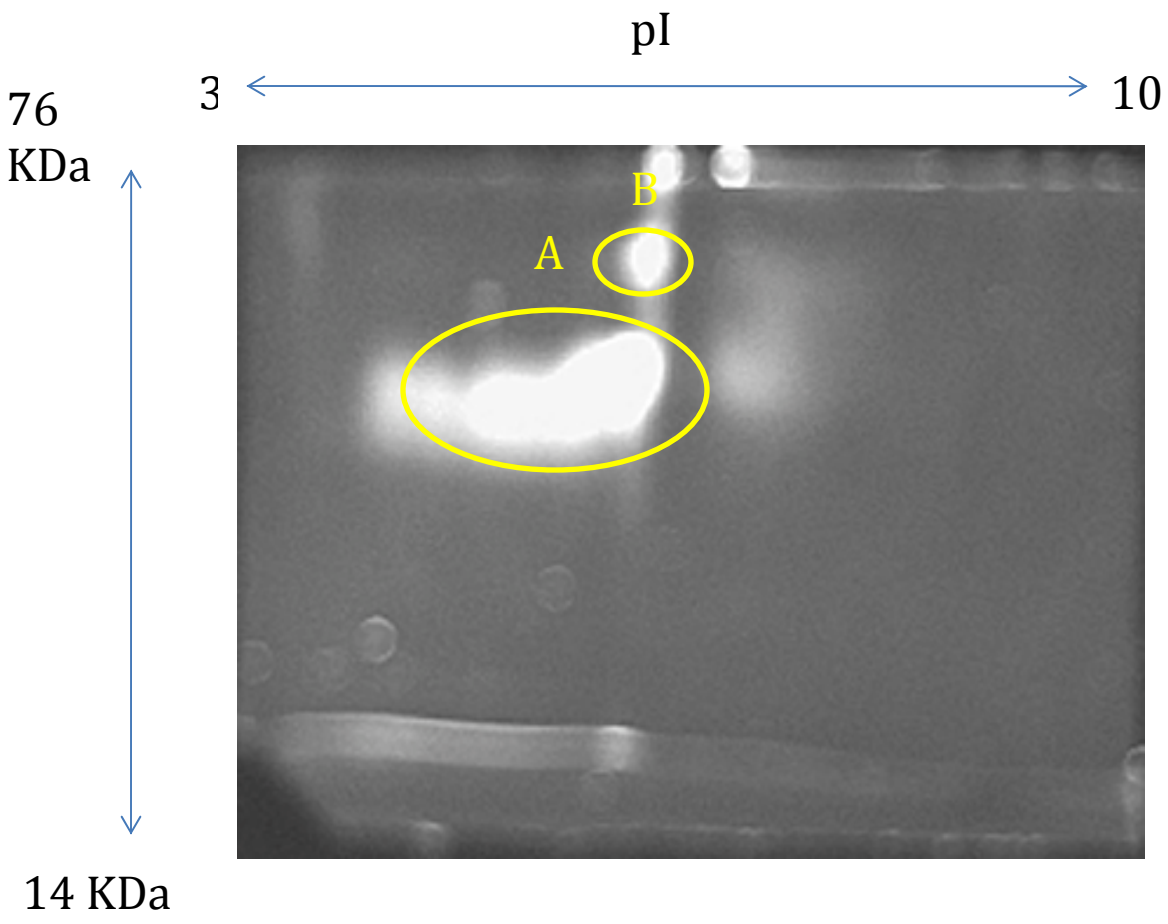

FIGURE 5

50µg Undigested Decorin, 12% Polyacrylamide Gel

2D-PAGE Results From Cyanogen Bromide Digested Decorin

To continue the 2D-PAGE profile comparison of the digested and undigested decorin, decorin standards were digested with CNBr and analyzed in the same manner as undigested samples.

Since the cyanogen bromide reaction cleaves proteins at methionine residues, one can predict decorin fragments produced by the digestion. A pI/MW profile created for these fragments using ExPasy.ca is shown in Table 2. However, the digestion reaction mixture was dialyzed using a dialysis membrane with a MW cutoff of 3.5 KDa. As a result, only 3 of the digested decorin fragments should theoretically appear on the 2D-PAGE profile, and no significant quantity of fragments with MWs below or near 3.5 KDa should remain in the purified sample. The three fragments of CNBr digested decorin present on the decorin preparation should have molecular weights of 14.3 KDa, 4.7 KDa, and 13.1 KDa and pIs of 9.2, 8.4, and 9.6. At 25 μg protein, the CNBr digested decorin yielded no visible spots when applied to a 12% polyacrylamide gel (Figure 6). A slight spot density appeared at the dye front near the location of the largest theoretical digestion product (14.3 KDa). In an effort to slow the migration of small peptides and improve differentiation of the peptides close to the dye front, the digested decorin was reanalyzed at a concentration of 50 ug on a 15% polyacrilymide gel. At 50 μg, the CNBr digested decorin yielded 3 spots visible, with approximate pIs of A = 3.00, B = 3.23, and C = 5.28 and approximate MWs of 14 Kda (A), 16 Kda (B), and 16 Kda (C) respectively (Figure 7). These results were reproducible and visible spots with similar pIs and MWs were obtained upon a second trial (data not shown).

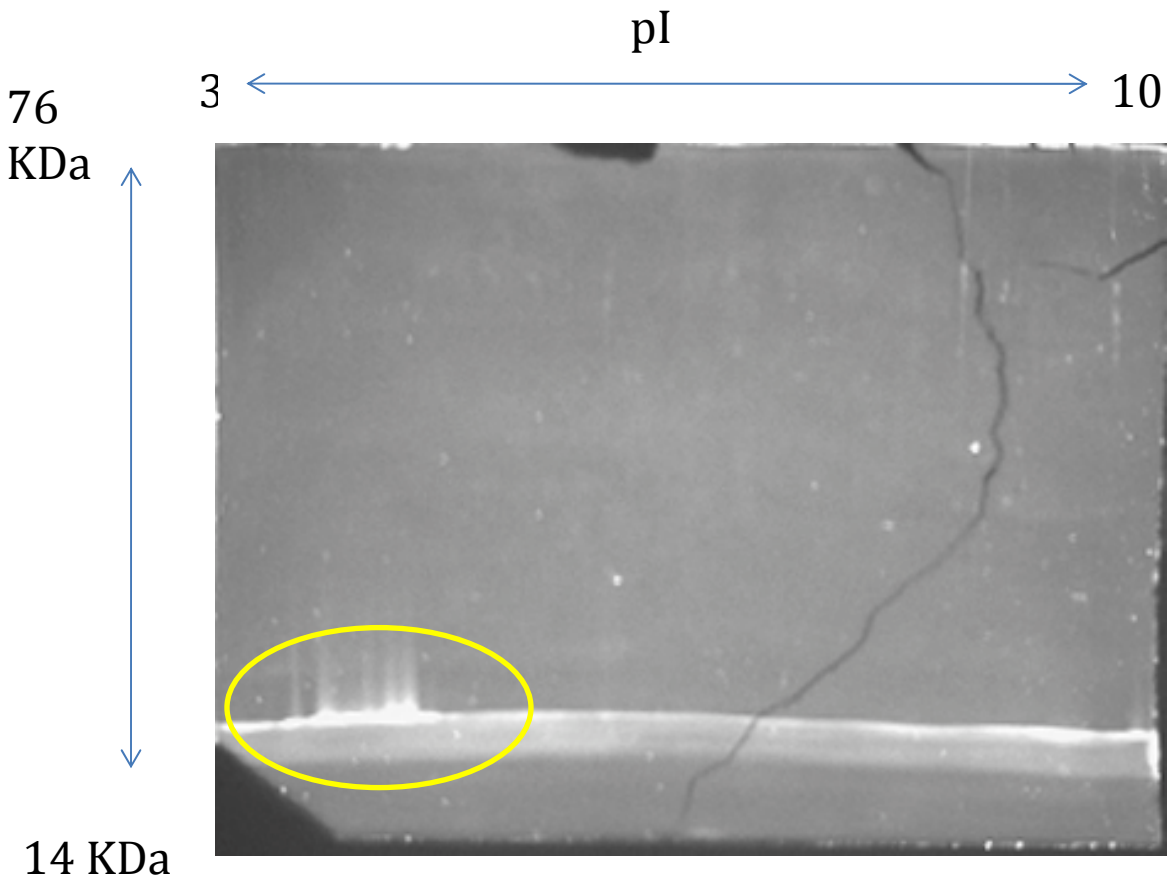

FIGURE 6

25µg CNBr Digested Decorin, 12% Polyacrylamide Gel

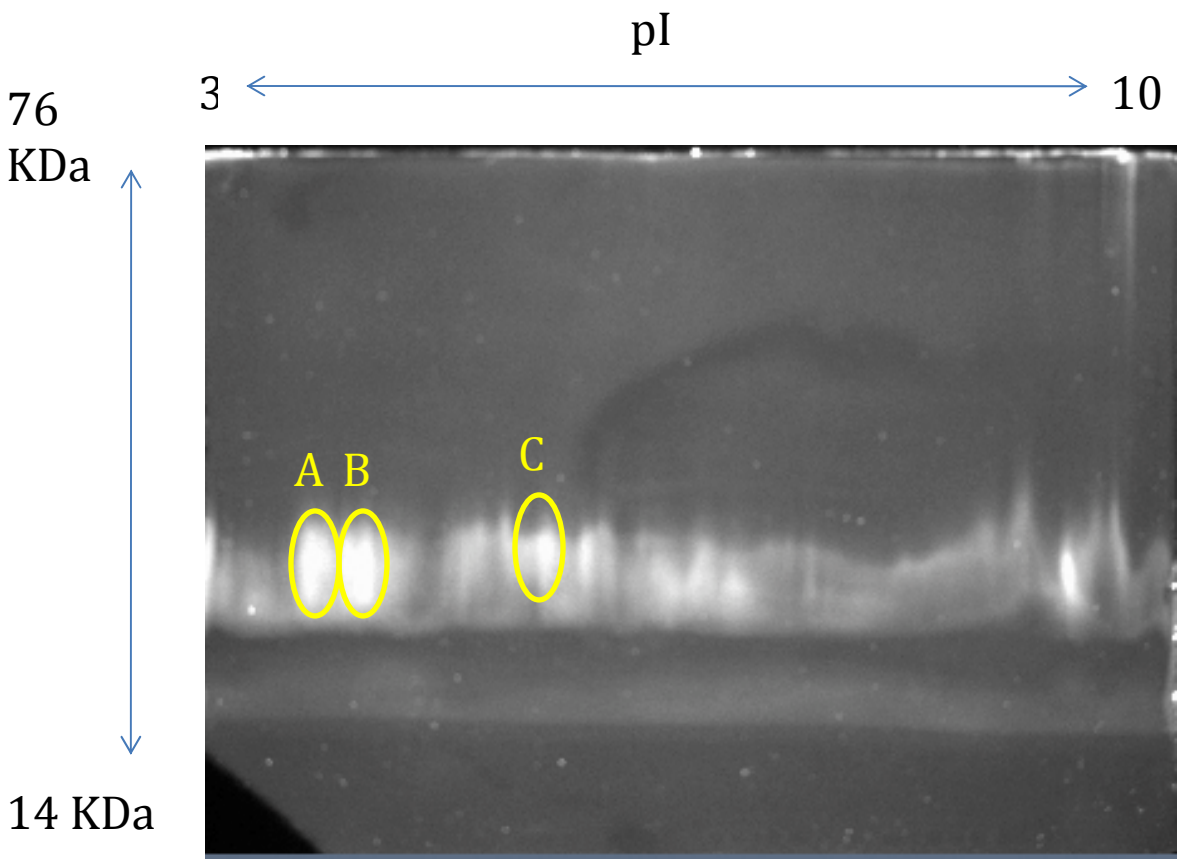

FIGURE 7
50µg CNBr Digested Decorin, 15% Polyacrylamide Gel

10

Fragment	Residue #'s	MW (Da)	pI
CB_1	1 – 18	2086	4.37
CB_2	19 – 39	2189	3.33
CB_3	40 – 162	14331	9.21
CB_4	163 – 185	2325	4.53
CB_5	186 – 230	4708	8.39
CB_6	231 – 345	13125	9.59

Western Blot Results From Cyanogen Bromide Digested Decorin

Two alternative methods were investigated to see if they could provide a more sensitive or more reproducible technique for generating a 2D gel profile of decorin. The first alternative explored was the classic western blot technique used an HRP-coupled anti-decorin antibody to visualize protein. No protein was visualized on 2D gels for either undigested or digested decorin peptide. The second alternative investigated was a modified western blot technique using amido black to visualize the protein. In this protocol decorin was run on 12% polyacrylamide gel, transferred to polyvinylidene fluoride membrane and stained with 0.1% amido black. At 100 μg of CNBr digested decorin, amido black stain yielded one well-defined spot on the polyvinylidene fluoride membrane (Spot F, Figure 8). However these results were not reproducible at 90 ug protein (Figure 9) suggesting that this method is not preferable to the Sypro stain protocol. The only similarity between Figure 8 (100μg) and Figure 9 (90μg) was spot F, the other fragments that are visible do not correlate to one another on different gels. Spot F appears to lie on the focusing electrode (the cathode, pI 10), and points to the incomplete digestion or aggregation of digestion products of decorin.

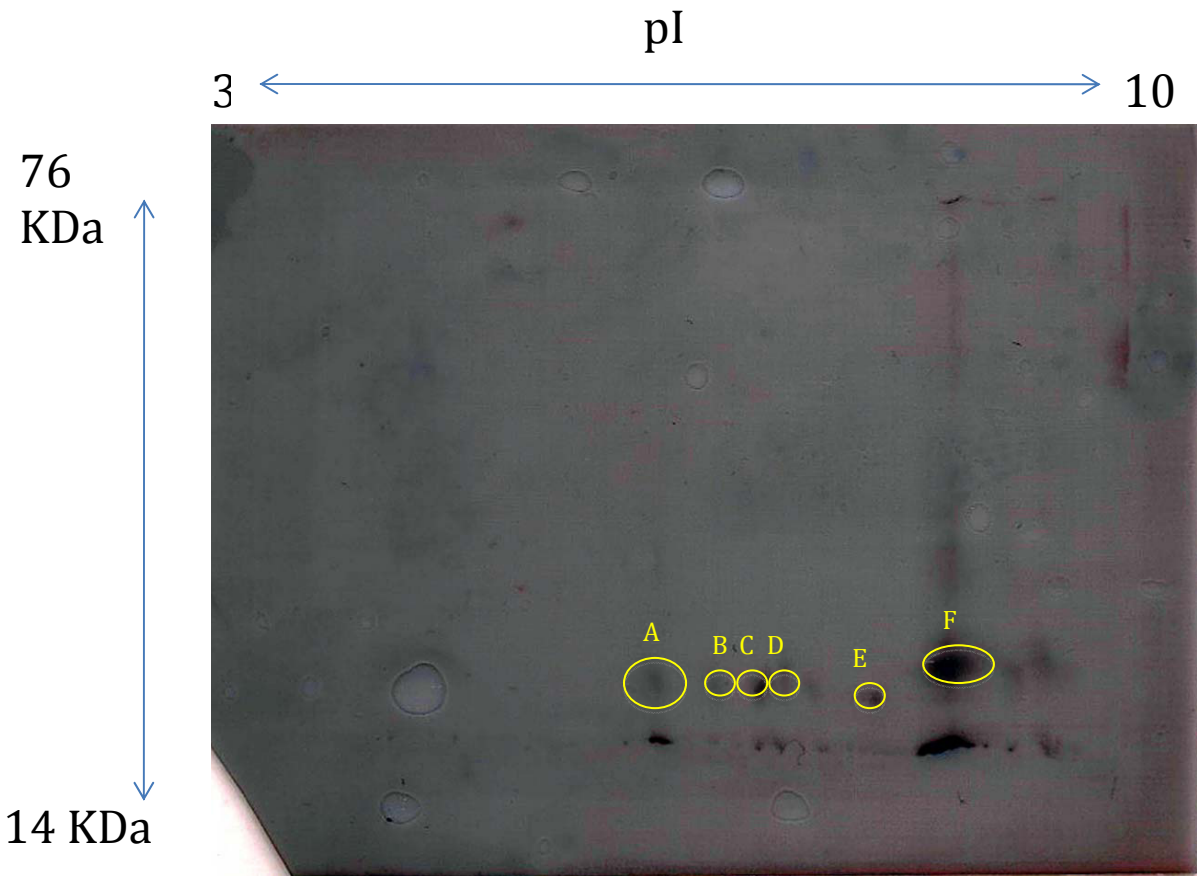

FIGURE 8

100µg CNBr Digested Decorin, Polyvinylidene Fluoride Membrane

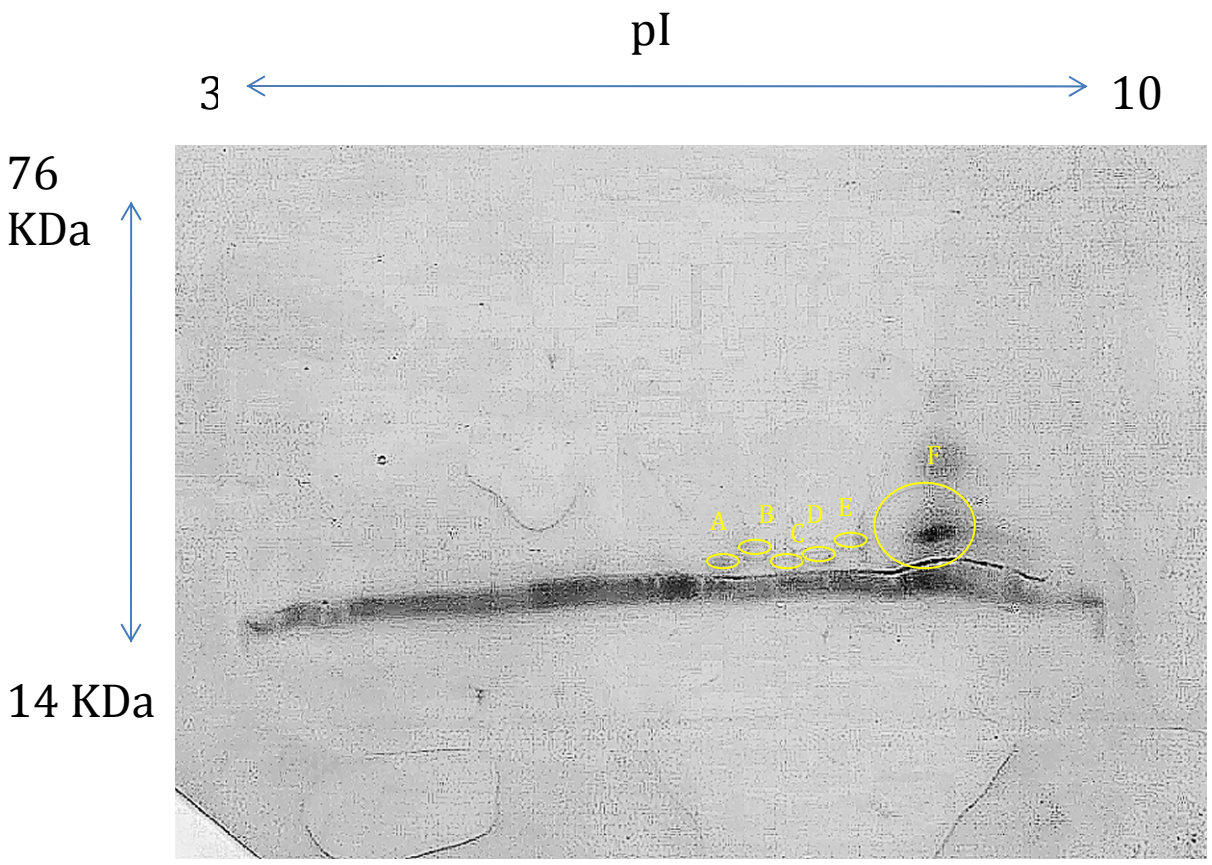

FIGURE 9
90µg CNBr Digested Decorin, Polyvinylidene Fluoride Membrane

Discussion

The present work demonstrates that decorin is a peptide that is prone to degradation and when used *in-vitro,* does not behave as it does *in-vivo.* This is likely due to the supraphysiological concentrations of decorin and the absence of the regulatory extracellular matrix and intigrins when the peptide is analyzed *in-vitro* (Takamiya, *et al.,* 2006). Corresponding Author Information. This is shown by the dissimilar 2D-PAGE spot patterns with replicate samples, the data which most closely relembles theoretical pI and MW of decorin is the 25 μg undigested decorin gel (figure 4, spot B) (theoretical decorin value: 39 kDa, 8.9 pI). Undigested decorin samples varied from one another by horizontal banding, different electromobilities and different pIs. Conditions that may have caused these observations could include overloading of the focusing strip, incomplete focusing of the peptide, and the staining (SYPRO) used may have been to sensitive.

Results from the digested decorin samples more closely matched the theoretical parameters for decorin. There were only 3 spots visible, which may be explained by the loss of CNBr digestion fragments during the purification process. The MWs were not significantly altered from the theoretical values, but the pIs were. The lower pI values could have been caused by issues with the buffers used to treat the digested peptide, formic acid treatment and long dialysis times Since formic acid is used to perform the CNBr digestion, no neutralization to pH is done prior to dialysis and the digestion time is significant enough to cause proteolysis / side chain modification through a much lower pH than the peptide is normally exposed. The analytical techniques could be altered in a manner that reduced possible degradation or modification of the protein.

Decorin is a molecule that has a regulatory effect on collagen degradation, and it is modulated heavily by the extracellular matrix proteins such as fibromodulin and the intigrins (Geng, *et, al*, 2006.). The in-vitro analysis techniques were performed under conditions that did not allow the peptide to be in it's normal physiologic environment of the extracellular matrix and this environment may cause the peptide to migrate as multiple bands viewed on undigested decorin sample gels. The lack of regulatory factors such as intigrins, TIMPS, and substrates such as collagen may have allowed decorin to form aggregates and creates dimers and other aggregated complexes as evidenced by the multiple molecular weights.

The undigested decorin analyzed had profiles that were not consistent with changes in the protein concentration as sample concentration increased. Molecular weights of the peptides shifted and pIs changed resulting in a different peptide profile. These results could have occurred because of the binding site on decorin that contacts to collagen peptides being exposed. In normal physiological conditions, decorin is a molecule that is bound to collagen, not a free peptide in the ECM (Geng, *et al.*, 2006). Since the decorin used in this project was purchased as a pure sample, the binding sites were exposed. Protein View predicted that decorin would have a hydrophilic binding site that interacts with the hydrophilic exterior of collagen. Since there is no collagen substrate in the analytical system, decorin aggregation might occur at the concentration increased and more hydrophilic binding sites were left open.

The work of Scott, *et al.*, suggests that dimerization and other aggregation of the decorin peptide is possible (Scott, *et al.,* 2004). This is consistent with the different molecular masses found on 2D-PAGE runs of undigested decorin. Our study suggests that the polymerization of decorin peptide is highly dependent upon the overall protein load prepared for analysis. The theoretical pI for decorin is 8.9, since decorin samples are loaded onto an isoelectric focusing

(IEF) strip with a continously varying pH, it is possible to generate charges on the individual peptides that would cause electrostatic interactions to promote the formation of decorin aggregates. These aggregates would not have the same electromobility as single peptides and may not move through the pH gradient gel matrix due to steric size immobilization.

The conditions used to prepare decorin for its analysis through 2D-PAGE could have caused alteration in the pI of the molecule through alteration of the amino acid side chains by the use of chemicals such as formic acid, CNBr, dithiothreitol, and iodoacetamide. Iodoacetamide, and dithiothreitol have a high reduction potential and should be able to destroy any quaternary structure or aggregation between decorin aggregates (Léon, J., *et al.,* 1998). Quaternary structure can be eliminated through use of these chemicals if the structure is held together by disulfide bonds or other oxidative interactions. The results of this study would suggest that the quaternary structure of the decorin aggregates were not held together by an oxidative binding structure as these interactions would be reduced through the use of highly reductive chemicals. A possible way to overcome these interactions could be through the use of heat as this method is less specific for disrupting quaternary structures and nearly guarantees the total breakup of any aggregates.

The change from 12% polyacrylamide gel to 15% polyacrylamide gel was necessary for the analysis of the digested decorin samples. The dialysis used to desalt the protein solutions resulted in thee loss of 3 of the theoretically predicted 6 peptide fragments due to their low MW (dialysis membrane MW cutoff is 3.5 kDa). The remaining 3 fragments had MWs of approximately 14Kda, 13Kda, and 4.7Kda. These small increases in MW would not resolve well on a 12% gel, so gel concentration was increased in order to improve resolution of small fragments.

Figure 7 depicts 3 peptide fragments with 2 of the 3 fragments having MWs that closely match the predicted MW (16Kda, and14Kda), however, 1 of the 3 fragments is significantly altered from its predicted MW (4.7Kda). None of these fragments came within an acceptable range for pIs, predicted pIs placed the fragments in a range of 8.4 to 9.6, while the observed fragments had pIs ranging from 3 to 4.

Figure 8 depicts an amido black stained western blot PVDF membrane. There appear to be 6 distinctive spots, however determination of their exact MW and pI is not possible due to the inability to run a 2D-PAGE standard along with peptides for a western blot. These spots seem to have the correct pI (they are in the 7 to 9 pI range) but their MW is inconsistent with with the predicted values in Table 2. In addition, there should have been a loss of 3 fragments from the dialysis of the digestion products. This suggests that there is alteration in fragments to give the different 2D pattern.

Future studies could incorporate whole tissue extract in order to analyze the decorin peptide, this would more closely mimic the physiological conditions that decorin is subjected to while in the ECM. Since decorin closely associates with collagen, it would be a logical next step to analyze decorin while it bound to collagen and attempt to characterize the peptides through 2D-PAGE in this manner. Decorin closely associates with collagen as demonstrated by Geng, *et al.*, this could play a role in protecting a binding site from destruction by preparative methods used for decorin analysis (Geng, *et al.,* 2006).

As shown by this study, *in-vitro* analysis of decorin is not a feasible study to determine its effect on collagen deposition. The lack of companies producing purified decorin and the low quality of the antibody used in this study raises the need to find a more suitable antibody that is able to detect endogenous decorin from in-vivo tissue samples. The anti-decorin antibody

(Santa-Cruz Biotech) was unable to detect decorin from both in-vivo tissue samples and purified recombinant decorin. An antibody that recognized the N-terminus of decorin (Santa-Cruz Biotech #sc-22613) was unable to develop a signal; a second antibody that recognized internal residues #136 – 216 (Santa-Cruz Biotech #sc-22753) was also unable to develop a signal. A suitable antibody must be raised that will detect endogenous decorin and is compatible with the methods used to prepare the tissue sample.

This study can be used in future work for determining the biological significance of decorin in relation to cardiac hypertrophy by lessons learned from the low quality of commercially available antibodies and the sensitivity shown by the molecule. The long term aim of this study is to determine what events take place to allow a hypertrophied heart to become fibrotic and have an over abundance of collagen deposition, to get an accurate mechanism it will be necessary to look at multiple aspects of decorin production both in-vivo and in-vitro. Levels of decorin mRNA need to be determined, fibroblasts should be attached to a synthetic extracellular matrix and have decorin mRNA overexpressed and have a decorin knockout. MMP can then be added and the effects can better be seen on cellular motility, morphology, and attachment to the ECM. What is learned from these studies can be incorporated into animal models and replicated (i.e. decorin overexpression vs. decorin knockout). A review by Kalamasjski and Oldberd revealed that decorin knockout mice have a fragile skin, weak tendon, and slower wound healing phenotype (Kalamajski and Oldberg, 2007). No mechanism biological mechanism has been elucidated to show how this phenotype is controlled. Evidence from Kalamajski, *et al.,* suggests that SLRPs have a regulatory effect on the crosslinking and overall strength and morophology of the collagen associated with the ECM (Kalamajski, *et al.,* 2009). This was shown through selective SLRP knockouts and the screening of the reulting

phenotype. These results will give the biological significance of decorin and can be used to elucidate a molecular mechanism for the trigger of dysregulation of decorin ultimately leading to the over deposition of collagen.

The importance of understanding a process of cardiac hypertrophy at the molecular level will allow for more effective treatment of the disease. To date, there is no information if decorin is controlled at the transcriptional or translational level or both. This is an important fact to know as it plays a key role in production of this molecule that can cause cardiac dysfunction and lead to a chronic condition such as cardiac myopathy. There is an increasing amount of evidence that microRNA (non-protein coding, ~22 nucleotide sequences) play a major role in the expression and destruction of key biologic regulator molecules such as decorin. microRNA has been shown to have both the ability to degrade mRNA as well as play a decoy function on protein targets (keep the protein from binding it's target substrate) (Eiring, *et al.*, 2010). As more of these significant microRNA sequences are uncovered, it may be shown that a microRNA plays a major role in regulating the expression and function of decorin.

Cardiac hypertrophy is a condition that has many factors that can lead to its chronic and acute phase. Literature-based evidence suggests that the overespression of decorin will cause an over deposition of collagen, leading to cardiomyopathies. As this may be a mechanism to trigger this condition, it must be understood that many other factors and mechanisms can lead to the same physiological effects and consequences. Discovering the mechanism of decorin and other SLRP molecule expression is a critical piece in controlling cardiac hypertrophy and eventually cardiac myopathy.

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