Physical Characterization of Embryo Hyalin from the Sea Urchin, 

*Strongylocentrotus purpuratus*

A thesis submitted in partial fulfillment of the requirements
For the degree of Master of Science
in Biology

By

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California State University, Northridge
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<td>α</td>
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<td>β</td>
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<td>β-ME</td>
<td>beta-mercaptoethanol</td>
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<td>ConA</td>
<td>Concanavalin A</td>
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<td>column volume</td>
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<td>DBA</td>
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<td>DTT</td>
<td>dithiothreitol</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>IEF</td>
<td>isoelectric focusing</td>
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<tr>
<td>GalNAc</td>
<td>N-acetylgalactosamine</td>
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<td>kDa</td>
<td>kilodaltons</td>
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<td>NaCl</td>
<td>sodium chloride</td>
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<td>NaOH</td>
<td>sodium hydroxide</td>
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<td>NIH</td>
<td>National Institutes of Health</td>
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<td>OD</td>
<td>optical density</td>
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<td>pI</td>
<td>isoelectric point</td>
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<tr>
<td>PNA</td>
<td><em>Arachis hypogaea</em> (peanut) agglutinin</td>
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<td>SEC</td>
<td>size exclusion chromatography</td>
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<td>SBA</td>
<td><em>Glycine max</em> (soybean) agglutinin</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>UEAI</td>
<td><em>Ulex europaeus</em> agglutinin I</td>
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<tr>
<td>RCA120</td>
<td><em>Ricinus communis</em> agglutinin</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>$V_e$</td>
<td>volume of elution</td>
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<td>$V_0$</td>
<td>void volume</td>
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<tr>
<td>Vt</td>
<td>total volume</td>
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<tr>
<td>WGA</td>
<td><em>Triticum vulgaris</em> (wheat germ) agglutinin</td>
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ABSTRACT

Physical Characterization of Embryo Hyalin from the Sea Urchin, *Strongylocentrotus purpuratus*

By Mai T Phan

Masters of Science in Biology

Hyalin is a major glycoprotein of the hyaline layer, the extracellular matrix surrounding sea urchin embryos and is required for normal morphogenesis. It is an extremely large molecule consisting of three species ranging from $2.1 \times 10^5$ to $6.3 \times 10^4$. The aim of this thesis is to use separation and analytical methods to further characterize the components of hyalin with special reference to its carbohydrate moieties. Hyalin was purified according to the procedures of Gray J, Justice R, Nagel GM, Carroll EJ Jr. 1986. Resolution and Characterization of a Major Protein of the Sea Urchin. *Journal of Biological Chemistry*, 261(20), pp. 9282-9288. Three major species of hyalin with sizes of 228kDa, 147kDa, and 63kDa were observed using analytical size exclusion chromatography on a Sephacryl S500 (10/300) column. The pI of the hyalin was also characterized to be pH 6.3, pH 4.9, and pH 4.8 for three components. To examine the nature of the carbohydrate content of hyalin beta-elimination with sodium hydroxide was done and was followed kinetically at 240nm. Based on these results hyalin was determined to contain o-linked carbohydrates. The sugar moieties on hyalin were further
characterized using lectins. Hyalin was found to bind to concanavalin A, soybean agglutinin, wheat germ agglutinin, and Ricinus communis agglutinin. Of the components detected by size exclusion chromatography, only the 63kDa component of hyalin was shown to consist of mannose or glucose moieties that bound to Concanavalin A and suggest the heterogeneity of sugar moieties for hyalin. Further physical characterization of hyalin is important in understanding the structure-function relationships and how its individual components and glycans are involved in embryonic development.
INTRODUCTION

Sea urchins as model organisms for embryogenesis

Embryos of echinoderms, such as the purple sea urchin (*Strongylocentrotus purpuratus*), have long been studied as model organisms. Research on sea urchin embryos have led to many advances in understanding developmental biology. These small, spiny invertebrates develop as simple, optically transparent, free-living embryos that can be conveniently fertilized in salt water and easily manipulated experimentally to perturb their development. They can be grown in large quantities as staged cultures and provide significant materials for biochemical or molecular biology analyses (Kurihara & Amemiya, 2005). As a result, they make a great organism in which to study morphogenetic movements and effects that shape the embryo. Also, the sea urchin embryo was designated by the U. S. National Institutes of Health (NIH) as a model organism because about twenty-five discoveries in physiological mechanism have been found to be also important in higher organisms, including humans (Contreras, et al., 2008).

Embryogenesis is a developmental process by which the embryo forms and develops after the egg has been fertilized. Gastrulation is a key process that occurs early in embryonic development during which the embryo reorganizes from the blastula, a simple spherical ball of cells, into a multi-layered organism. The cells begin to segregate spatially at this stage to form the three germ layers: the ectoderm, the mesoderm, and the endoderm. These layers will eventually give rise to organ differentiation. Highly
coordinated movement of cells inward, at or near the surface of the blastula, bring the endodermal and mesodermal cells into the interior of the embryo, while the ectoderm cells remains at the surface (Hammerschmidt & Wedlich, 2008; Gilbert 2000). Regulated cell adhesion and cellular mobility drive much of early morphogenesis during gastrulation of sea urchins and changes in cell adhesion could be the mechanism that leads to apical constriction (Gustafson and Wolfert 1963; 1967). Johannes Holtfreter first introduced the concept of selective adhesion in germ layer assembly by performing in vitro dissociation and re-association assays and showed that cell adhesion played a central role in tissue and organ formation (Steinberg & Gilbert, 2004). The invagination of the outer cells of the blastula forms the archenteron, the primitive gut, which elongates inwards as a tubular archenteron forms and attaches to a specific anatomically defined region on the blastocoel wall (McClay, 1991).

**Hyalin**

Hyalin is involved as a cell adhesion molecule during the process of archenteron elongation and mediates the attachment to the blastocoel roof (Razinia, et al., 2007; Alvarez, et al., 2008; Contreras, et al., 2008; Carroll, et al., 2008). Hyalin, a glycoprotein exocytosed from cortical granules at fertilization, was shown to localize at tip of the archenteron and on the roof of the blastocoel at the site of the adhesive interaction (Carroll, et al., 2008). It is the major component of the hyaline layer, a clear, gel-like layer that forms around the surface of the egg following insemination and serves as an adhesive substrate during gastrulation (Adelson & Humphreys, 1988). The ingression of certain cells has been correlated to changes in adhesion affinity (Wessel &
McClay, 1985). Cells decrease their binding affinity to neighboring cells and to the hyaline layer and increase their affinity to cells that line the basal lamina of the blastocoel (McClay and Fink, 1982; Fink and McClay, 1985; Gilbert, 2000).

Hyalin was isolated using its sensitivity to polymerization with calcium by altering calcium-free solubilization with calcium-induced precipitation (Faust, et al., 1959; Vacquier, 1969; Kane, 1970; Citkowitz, 1971). Analysis of isolated hyalin suggest that it is extremely large about 330 kDa in size with approximately 25% acidic residues and only 3.5% basic residues. Hyalin also consists of only 2–3% carbohydrate (Kane, 1970; Citkowitz, 1971).

Hyalin is a set of three to four large components, with native molecular weights vary from 280-920 kDa (Gray, et al, 1986; Justice, et al., 1992, 1988). Velocity sedimentation experiments show the major unit of hyalin to be 11.6 S and molecular weight close to 9.2 X 10^5. Also a minor unit of 8.8 S and molecular weight of 3x10^5 was also seen with some preparations of hyalin. Further investigation found that altering the stringent acid treatment during purification to remove the jelly coat can vary the composition of hyalin. More stringent conditions resulted in the 11.6 S component comprising of >90% of the hyalin preparation and less stringent conditions resulted in the 8.8S and 6.5S components consisting of almost 50% of the preparation (Gray, et al, 1986). A 9.6 S component with a molecular weight of 4.5 ± 0.1 × 10^5 was also observed (Justice, et al., 1992).

Hyalin and anti-hyalin antibody were shown to block the cellular adhesive interaction in sea urchin embryos and that hyalin carbohydrates may be involved in
hyalin’s binding activity (Razinia, et al., 2007; Alvarez, et al., 2008; Contreras, et al., 2008; Carroll, et al., 2008). Hyalin contains carbohydrates that may include mannose and/or glucose residues (Citkowitz, 1971; Carroll, et al., 2008). Hyaline layers were labeled with mannose and/or glucose binding lectins in other studies in echinoderms (Cerra, 1999). Therefore, the carbohydrates of hyalin may be involved in the adhesive interaction, or the hyalin-binding receptor or hyalin-binding ligand may contain mannose/glucose residues (Carroll, et al., 2008).

**Carbohydrates**

In living systems there are four classes of organic compounds: nucleic acids, proteins, lipids, and carbohydrates. Carbohydrates are the most abundant organic molecule in living cells; nearly all plants and animals synthesize and metabolize them. Carbohydrates are ring structured organic compounds that are named “hydrates of carbons” or “carbohydrates” because of their molecular formula \( C_n(H_2O)_m \), suggesting carbons form with water. Monosaccharides are simple sugars that are carbohydrates that cannot be hydrolyzed into further compounds (Wade, 1999). Three types of “single” sugars are glucose, galactose, and fructose. Glucose is known as “blood sugar”, the immediate source for cellular respiration. Galactose is a sugar in milk and dairy, and fructose is found in fruit and honey. Two monosaccharides can be linked together to form a disaccharide. The most common disaccharides are: 1) sucrose (fructose and glucose), also known as common table sugar, 2) lactose (galactose and glucose), a sugar found in milk, and 3) maltose (glucose and glucose), a product of starch digestion. Polymers of carbohydrates that can be hydrolyzed into many monosaccharide units are
polysaccharides. Cells can store excess energy as polysaccharides by linking together many molecules of glucose as starch in plants or glycogen in animals (Wade, 1999).

**Carbohydrate structures**

Carbohydrates have the potential to encode enormous biological information. Information in peptides and oligonucleotides is encoded primarily by the sequence of their monomeric units. Whereas information can be encoded in carbohydrates by the bond formation via different linkage positions and two possible anomeric configuration, alpha (α) or beta (β), of the glycosidic units and the branch points (Sharon & Lis, 1989). Two molecules of glucose, a single monosaccharide, can form 11 different joining disaccharides, but two molecules of amino acids or of nucleotides can only form one dipeptide or dinucleotide, respectively. For hexamer formations, oligosaccharides can theoretically form more possible configurations by several orders of magnitude [4096 (oligonucleotides), 6.4 × 106 (peptides) and 1.44 × 1015 (saccharides)] (Laine, 1997). In addition, the attachments of sulfate, phosphate, and acetyl groups to the sugars further diversify the structures (Schmidt, 1987). In animal cells, carbohydrates may be attached to proteins as a single sugar unit or as a long chain, which may be branched, and can be attached by two types of linkages: N-linked by an asparagine residue or O-linked by a threonine or serine (Goochee, et al., 1991). Glycosylation is the most common post-translational modification involving proteins; nearly 50% of proteins are glycosylated. About 90% of glycoproteins contain only N-linked carbohydrate units or both N-linked and O-linked ones and the rest are only O-glycosylated (Apweiler, et al., 1999). The biological significance of having this structural variability and complexity of glycans on
proteins allows them to function as signaling, recognition, and adhesion molecules (Ghazarian, et al., 2011; Sharon and Lis, 1989, 1993; Ofek, et al., 2003).

**Carbohydrates role in adhesion**

Carbohydrates are ubiquitous cell surface molecules that act as ideal candidates for cell-cell recognition molecules for many reasons. First, the surface of the cell is an optimal place for the carbohydrates to be located in carbohydrate-directed cell adhesion. Most cells are covered with a carbohydrate layer, which consists of glycoproteins, glycolipids, and proteoglycans. Second, they have the structural diversity to carry information. And third, carbohydrates can interact with certain carbohydrate binding proteins called lectins. Cell surface carbohydrates have been shown, across a broad phylogenetic spectrum, to mediate cell-cell adhesion along with the complementary carbohydrate binding proteins on opposing cell surfaces in vitro (Brandley & Schnaar, 1986).

**Lectins**

At the end of the 19th century, protein preparations from caster bean extracts were shown to agglutinate red blood cells (Allen, 1969). Sumner and Howell showed the sugar specificity of haemagglutinin with the inhibition of ConcanavalinA, the first purified plant haemagglutinin, by sucrose and suggested the involvement of glycans on the surface of the cells (Sumner & Howell, 1936). Later, some plant haemagglutininins were found to selectively agglutinate red blood cells from a particular ABO human blood group. The ABO antigens were glycans, and this was the first time that carbohydrates
were shown to be antigenic (Boyd & Reguera, 1949). The term lectin was introduced from the Latin *legere*, “to select”. They are ubiquitous proteins that interpret information encoded by carbohydrate and bind to the specific carbohydrate structures. They are found in plants, animals, and microorganisms and usually contain two or more binding sites for carbohydrates which allow them to function in animals to facilitate cell-cell contact (Berg, et al., 2002).

The largest family of lectins and best characterized among plant lectins is the Leguminosae (legume) lectin family. ConA, PNA, SBA, DBA, UEAI, RCA are some examples of legume lectins. Solanacease (potato and tomato) and Gramineae (cereals) are two small families of plant lectins (Sharon & Liz, 1990). Two distinct families of animal lectins have been identified: 1) C type (Calcium ion dependent) and 2) S-type (thiol dependent or soluble) (Drickamer, 1988). ConA is a low cost plant lectin that has a high binding affinity with glucose and mannose. PNA binds to preferentially to galactosyl (β1–3) N-acetylgalactosamine (Pereira, et. al, 1976). RCA120 is closely related to Ricinus communis (ricin), which is a highly toxic lectin that is isolated from castor bean. RCA120 binds to galactose and GalNAc. SBA and DBA are legume lectins that recognize GalNAc monosaccharide; however they show subtle differences in the binding of oligosaccharides. SBA shows a broad specificity for αGalNAc and βGalNAc or internal GalNAc structures. DBA recognizes only terminal αGalNAc having a terminal α or βGalNAc linked in different positions to α or βGal, or α or βGalNAc (Piller, et. al, 1990). UEAI also recognizes terminal structures, specifically α-L-fucose (Lis & Sharon, 1977). Wheat germ agglutinin (WGA), in the Gramineae lectin family, has high specificity to N-acetylglucosamine and N-acetylneuraminic acid (Jordan, et. al, 1977).
Many different plant and animal lectins are commercially available and are well characterized for practical use in glycan research. Both lectin and antibodies are used in glycan identification. Although lectins may not be as specific as antibodies, they are more stable and less expensive, which is a major consideration when large quantities of glycan-binding protein is need for purification approaches by affinity chromatography. Also, lectins binding specificity are more characterized (Cummings & Etzler, 2009). Lectins were first classified based on structural and evolutionary sequence similarities. They are also classified based on their affinity to bind with: 1) Glucose/ mannose, 2) Galactose and N-acetyl-D-galactosamine, 3) L-fucose 4) Sialic acid (Kumar, et al., 2012).

A major approach to characterize cell-surface glycoconjugates is to incubate with biotinylated or peroxidase-labeled lectins and then the bound lectins are visualized with streptavidin-peroxidase or labeled secondary antibody. The following controls are important in such studies: 1) use of limiting concentrations of lectins to avoid nonspecific binding; (2) use of appropriate inhibition target or destruction of predicted target glycans to confirm of the specificity of binding; and (3) use of multiple lectins to confirm the conclusions (Cummings & Etzler, 2009).

**Fetuin as a well characterized glycoprotein**

Fetuin is a glycoprotein of fetal calf serum that has been isolated with a high degree of purity and well characterized. This paper will use fetuin as a control to compare with hyalin in characterization studies. Fetuin glycoprotein is 26% carbohydrate in nature, with three N-linked carbohydrate sites (Asp81, Asp138, Asp 158) and four O-linked carbohydrate sites (Ser253, Thr262, Ser264, Ser323), containing galactose,
mannose, glucosamine, and sialic acids (Ding, et.al 2009, Carr, et. al 1993). The velocity sedimentation analysis shows it as 3.47 S at pH 6.5 and a theoretical molecular weight of 48.4kDa. However, fetuin is known to have an apparent molecular weight of 64kDa (Peeters, 1970). Its isoelectric point was pH 3.3 (Spiro, 1960).

Analysis methods for size determination

Some methods that are available to separate biomolecules based on size difference are: 1) ultracentrifugation 2) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and 3) gel filtration chromatography. This paper will use the two latter methods to characterize hyalin components. In addition to size determination, SDS-PAGE can also determine the isoelectric point (pI) of a given protein in which the net charge is zero by separating based on the relative contents of acidic and basic residues.

Molecules can be separated by electrophoresis in an electric field to migrate proteins and other macromolecules through a solid support such as a polyacrylamide gel, formed by the polymerization of acrylamide and cross-linked by methylenebisacrylamide. The velocity of migration of a protein will depend on its net charge. The gel further enhances the separation by acting as a molecular sieve; molecules that are larger than the pores in the gel will be more immobilized at the top of the gel than smaller ones that readily move through the gel. Proteins will give a distinct band that detects as little as 0.1 μg when stained with Coomassie blue and even less when stained with silver stain to detect about 0.02 μg of protein. Proteins are separated by mass under denaturing conditions by dissolving in a solution of sodium dodecyl sulfate.
(SDS), an anionic detergent that disrupts non-covalent interactions in proteins. A SDS-protein complex is formed at a ratio of one SDS for every two amino acid residues to create a large net negative charge that makes the native charge insignificant and is roughly proportional to the mass of the protein. The mobility of proteins are linearly proportional to the logarithmic of the mass and the mass of polypeptides can be determined using calibration curve of known molecular weight standards (Berg, et al., 2002). Samples can also analyzed in reducing condition by addition of beta-mercaptoethanol (β-ME) or dithiothreitol (DTT) to cleave disulfide linkages.

The advantage of gel filtration chromatography (size exclusion chromatography) is that material can be separated under native, non-denaturing conditions with samples recovered that are suitable for downstream processing. The disadvantage is that samples recovered are more dilute than the starting material, the relative lack of resolution, and no more than one sample may be analyzed at a time (Stanton, 2004). In gel filtration chromatography, small porous beads are packed into a column and the sample is applied the top of the column. The set up consists of a mobile phase consisting of the solvent and the molecules being separated, and the stationary phase where small molecules movement is delayed as it diffuses freely into the porous matrix or resin pores. The small molecules travel through the stationary phase at different rates, whereas, the large molecules are unable to enter the matrix and travel a shorter distance around the beads (Stanton, 2004). Therefore larger molecules elute from the column faster than smaller ones. Molecules are separated by decreasing molecular weights. The molecular weight can be estimated using a calibration curve of known molecular standards, assuming the unknown proteins are globular and comparable in shape to the standards (Sheehan, 2009).
There is a linear correlation between the logarithmic of molecular weight of a protein versus the ratio of its elution volume to the void volume ($V_e/V_0$). Dextran blue, a very large dye that cannot enter the gel, is used to determine the exclusion volume or void volume of the column (Whitaker, 1963). Size exclusion assumes that molecules are spherical and do not interact with the resin. Proteins that are spherical will elute later than non-spherical molecules with the same mass and proteins that are more hydrophobic tend to interact with some types of resin materials and are thus may be retained longer (Correia, 2008)

**Analysis by release of glycans**

Chemical or enzymatic procedures have been devised to release oligosaccharide chains from the protein backbone for structural and functional analysis. N-linked glycans are typically released chemically by hydrazinolysis with anhydrous hydrazine (Takasaki, et al., 1982) or by enzyme digestion (Plummer, et al., 1984). Partial acid hydrolysis has been used to release O-linked glycans. However, mild alkaline conditions are more commonly used to chemically release O-linked glycans by beta-elimination (Yu, et al., 2010). This paper uses chemical methods with sodium hydroxide to release O-linked glycans and the beta-elimination reaction is monitored by an increase in absorbance at 240nm. Only carbohydrate chains attached to serine or threonine residues are released, forming the product of dehyroalanyl and β-methyl-dehydroalanyl, respectively. The dehydroalanine product from beta-elimination reaction shows a strong absorbance at 240nm region (Carter & Greenstein, 1945; Mecham & Olcott, 1949; Federici, et al., 1977).
MATERIAL AND METHODS

Purification of hyalin

Embryos from the purple sea urchin, Strongylocentrotus purpuratus, were obtained from Southern California Sea Urchin Co., Corona Del Mar, CA. Hyalin was purified by Keith Segura, Virginia Hutchins-Carroll, and Dr. Edward Carroll following methods in Gray, et al., 1986.

Alkaline beta-elimination with sodium hydroxide

Beta elimination was performed in 20mM Tris-HCl pH 8.0 in a disposable UV microcuvette. The reaction was started by addition of a 5M sodium hydroxide (NaOH) stock solution. Dehydroalanine is formed from the beta-elimination of α-linked glycans and reaction was monitored by an increase in OD at 240nm using Beckman Coulter UV-Visible Spectrophotometer DU-730.

Gel filtration (size exclusion) chromatography

An empty GE Healthcare Tricorn 10/300 column (10mmx30mm) was packed with Sephacryl S-500 High Resolution resin (GE Healthcare). This resin has a recommended fractionation range of 4x $10^4$ to 2 x $10^7$. The empty column was connected to an extension reservoir with a connection adapter for 10/300 column to allow for continuous packing. About 40mL of 20% ethanol-resin slurry was slowly poured into column and reservoir and air bubbles were allowed to rise before using pump. Then the resin was packed by a pump running 20% ethanol at a flow rate of 0.5mL/min using GE
Healthcare AKTA Purifier (Unicorn 5.11 Software). After column packing, the column was cleaned with 1CV of 1M sodium hydroxide and rinsed with 4CV of water at 0.5mL/min.

Column was cleaned with 0.5 CV of 1M NaOH and equilibrated with 2CV of buffer prior to each gel filtration runs. Separation in non-reducing condition was run in 50mM Tris-HCl pH 8.0, 150mM NaCl, 1mM EDTA. Separation was performed at a flow rate of 0.3mL/min and protein was monitored at 280nm.

Molecular weight calibration curve for size exclusion column was created by separation of a mixture of known molecular weight standards using Bio-rad Gel Filtration Standard. Each vial contained the following: bovine thyroglobulin (MW 670,000), bovine γ-globulin (158,000), chicken ovalbumin (44,000), horse myoglobin (17,000), Vitamin B12 (1,350). A single vial was resuspended in 1mL deionized water and 75uL of the standard mixture was injected onto column and separated at 0.3mL/min. Protein was monitored at 280nm.

Void volume (Ve) was determined by injection of 2mg/mL of blue dextran from Sigma. Total volume of column was determined by injection of 5% acetone solution. Retention volumes were analyzed using Unicorn built in peak analysis function.

**Electrophoresis**

SDS-PAGE under denaturing reducing conditions was performed using the NuPAGE Novex Gel System from Invitrogen/Thermo Fisher Scientific. Samples were mixed with NuPAGE LDS Sample Buffer and Nu-PAGE Sample Reducing Agent in
order to reduce samples, boiled for 5mins to denature, and loaded onto precast NuPAGE Bis-Tris Protein Gels (1.5mm). NuPAGE Seeblue Plus2 Pre-stained protein standard was loaded in control well to visualize molecular weight separation. Electrophoresis was run using NuPAGE MES SDS Running Buffer. The bis-tris buffered gel along with the MES running buffer has an operating pH about 7.0. In addition, a final concentration of 10mM DTT was added to the NuPAGE running buffer to keep samples reduced during electrophoresis. Cassettes were run at 200V for 1 hour. Residual SDS was washed from gels by incubating in deionized water for 10mins before staining accordingly.

Western Blot

Gels were transferred with NuPAGE transfer buffer to NuPAGE nitrocellulose paper using the NuPAGE Novex Gel System from Invitrogen/Thermo Fisher Scientific. Transfer electrophoresis was run at 40V for 1 hour. Western blot probed with biotinylated lectins was performed after the transfer using the same method as lectin immunodot blot assay.

Gel Staining

Coomassie gels were stained using Thermo Scientific GelCode Blue Stain Reagent, a coomassie G-250 dye-based reagent, for 1 hour at room temperature on a rocker and then washed by incubating in water overnight.

Silver stained gels were stained using Thermo Scientific/Pierce Silver Staining Kit according to manufacture instructions. Gels were washed in 2 x 5min in water and fixed with 2 x 15mins in 10% acetic acid/30%ethanol solution. Then gel was washed
with 2 x 5min in 10% ethanol and then 2 x 5 min in water. Gel was then sensitized for 1min in Sensitizer Working Solution (50ul Sensitizer in 25mL water) and then washed for 2 x 1min in water. Gel was stained for 30mins in Staining Working Solution (0.5mL Enhancer with 25mL Stain) and washed 2x 20secs in water. Then gel was developed for 2-3mins with Developer Working Solution (0.5mL Enhancer with 25mL Developer) until bands appear and stopped with 5% acetic acid for 10mins.

Isoelectro-point focusing (IEF)

IEF was run using Novex pH 3-10 IEF Protein Gels, 1.0mM, 10-well. 1X IEF Anode Buffer was used to fill the outer buffer chamber of Invitrogen XCell SureLock Mini-Cell for electrophoresis. This buffer was prepared from Novex IEF Anode Buffer (50X) diluted in deionized water. 1X IEF Cathode Buffer was used to fill the inner chamber of the electrophoresis system. This buffer was prepared from Novex IEF Cathode Buffer pH 3–10 (10X) diluted in deionized water. To avoid affecting protein PI, samples are prepared for gel without SDS and reducing agents. Samples were mixed 1:1 with Novex IEF Sample Buffer pH 3–10 (2X) and not heated before running. Cassettes were run at 100V for 1 hour, 200V for 1 hour, and then 500V for 30mins.

IEF Analysis Using Software

Gels images were scanned using an Epson Perfection V750 Pro at 600dpi. Scanned gel image was analyzed by CLIQS 1D-Gel/WesternBlot Analysis Software. Lanes were created manually and background was performed using the ‘rolling ball’
subtraction method. Distance of migration of each band was calculated by subtraction Y-coordinate position of wells from Y-coordinate position of band.

**Lectin immuno-dot blot assay**

Two and one half mm strips were uniformly cut from nitrocellulose paper from Biorad using a cassette strip cutter. 0.5ul volume for each sample was dot blotted using a micropipette and immediately absorbed onto nitrocellulose strips. Dotted samples were allowed to dry for 30mins. Strips were then placed into slotted tray for immunoassay and each step was performed at room temperature on a tabletop see-saw rocker. Samples were blocked for 30 mins with a Carbo-Free Blocking Solution purchased from Vector Labs. Standard blocking solution using non-fat milk was avoided because it contained carbohydrates that may bind to lectins. After blocking, strips were washed three times for 3 mins each with Wash Buffer (Phosphate Saline Buffer + 0.2% Tween 20). 1:500 dilution of biotinylated lectins were made in Wash Buffer (with exception of a 1:1000 dilution for ConA lectin-biotin) and 1mL was added to incubate with strips for 1 hour. After incubation, strips were washed three times for 3 mins each with Wash Buffer. A 1:1000 dilution of streptavidin-alkaline phosphate was prepared in conjugate diluent (2:1 dilution of alkaline phosphatase conjugate stabilizer from SurModics in water) and 1mL was added to each strip to incubate for 30mins. After incubation, strips were washed three times for 3 mins each with Wash Buffer. Strips were developed with Alkaline Phosphatase Substrate Solution (Moss, Inc). Reaction was stopped by rinsing strips and incubating for 5 minutes with water. Strips were dried and scanned using Epson Perfection V750 Pro at 600dpi.
The molecular weights of hyalin components were measured by size exclusion chromatography (SEC). The elution volume of blue dextran, a high-molecular weight dextran polymer of about 2000 kDa, was used to determine the void volume (Vo). The elution volume of acetone was used to determine the total column volume (Vt), which is equivalent to the volume of the packed bed (also known as CV). Figure 1 shows the overlay of the chromatograms for the two runs that were used to determine the Vo and Vt of the column. Vo was determined to be 9.29 mL and Vt was determined to be 22.89mL. The volume of elution (Ve) over the void volume is the retention ratio (Ve/Vo) of a molecule or protein. This ratio has a linear relationship to the log of molecular weight and the molecular weight of an unknown protein can be calculated by calibrating the column with known standards.

The following molecular weight standards were separated on the S-500 (10/300) column: thyroglobulin (bovine), γ-globulin (bovine), ovalbumin (chicken), myoglobin (horse). Vitamin B12 was present in the standard mixture that was injected; however, it was not included because it was not detected as a peak in the chromatogram (Figure 2). It was verified by its pink color in solution to have eluted in the tail end of the last peak. Figure 2 is a representative chromatogram for the three separation runs of the molecular weight standards. The molecular weights of the standard corresponding to the peaks are listed with the volume of elution for the three runs (Table 1). Thyroglobulin (670kDa), γ-globulin (158kDa), ovalbumin (44kDa), myoglobin (17kDa) was eluted at average volumes of 9.51mL, 16.97mL, 19.51mL, and 23.03mL, respectively. The retention ratios
were calculated for each of the standard by dividing the average $V_e$ with $V_o$ of 9.29mL. This ratio was plotted with the log of molecular weight (Figure 3). The linear calibration curve ($y = -1.1039 + 4.0251$) was used to estimate the molecular weight of hyalin components.

The molecular weight of a well-known glycoprotein, fetuin, was determined by SEC to verify the validity of the calibration curve for estimating unknown proteins. Figure 4 shows the chromatogram for 0.2µg of fetuin on the S-500 column with the peak having an elution volume of 18.70mL. The molecular weight of fetuin was extrapolated from the curve to be 64kDa (Table 2). This corresponds to the documented apparent molecular weight of 64kDa for fetuin.

The molecular weight of hyalin components were determined by the separation of 0.10µg, 0.15µg, 0.20 µg of purified hyalin on Sephacryl S-500 column (Figure 5, 6, 7, respectively). Five peaks were detected and the elution volumes are listed on the chromatogram. The molecular weights for each of the peaks are calculated for each run (Table 3, 4, 5). The three runs are summarized in Table 6. The peaks from purified hyalin separates were calculated and averaged for the runs and were determined to be 883kDa, 228kDa, 147kDa, 63kDa, and 34kDa.

The $\alpha$-linked glycans were characterized from purified hyalin by alkaline beta elimination (Figure 8 – Figure 19). 0.5mg/mL of hyalin was treated with 0.4M sodium hydroxide (NaOH) and compared to 0.5mg/mL of fetuin treated also with 0.4M NaOH. Three runs for each of the proteins were monitored at 280nm by the increase in the dehydroalanine (DHA) with time as the beta elimination reaction cleaved $\alpha$-linked
glycans. Figure 8- Figure13 show the kinetics for hyalin reaction and Figure 14-Figure 19 show the kinetics for the fetuin reaction. The maximum absorbance, at which the reaction plateaus, was converted to the concentration dehydroalanine produced by using the extinction coefficient for dehydroalaine (\(\varepsilon=4000\text{M}^{-1}\text{cm}^{-1}\)) and beer’s Law \(A=\varepsilon cl\). The initial rates in the linear range of the reaction was analyzed by creating a trendline for both hyalin and fetuin beta-elimination reactions (Figure 9, 11, 13 and Figure 15, 17, 19, respectively) The total amount of DHA produced by 0.5mg/mL hyalin with 0.4M NaOH was 8.4 x10^{-5} \pm 0.3 x10^{-5}M and this was similar to the amount produced by 0.5mg/mL fetuin with 0.4M NaOH with the amount being 8.7 x10^{-5} \pm 0.1 x10^{-5} M (Figure 20). On the other hand, the rate of beta elimination of 0.5mg/mL of hyalin with 0.4M NaOH was much faster than 0.5mg/mL of fetuin with 0.4M NaOH, with rates of 0.0161 +1.15x10^{4} (1/m) and 0.0136 +1.28x10^{3} (1/m), respectively (Figure 21).

The beta elimination of hyalin was also shown to be concentration dependent for both the concentration of hyalin starting material and for the concentration of NaOH. Figure 22 shows the rates increased linearly as initial concentration for hyalin starting materials were increased from 0.3mg/mL to 0.4mg/mL to 0.5mg/mL. This linear trend was seen when using 0.2M, 0.3M, and 0.4M NaOH. The rates also increase linearly as concentration of NaOH was increased from 0.2M to 0.3M to 0.4M NaOH (Figure 23). This trend was seen in all the reactions using 0.3mg/mL, 0.4mg/mL, and 0.5mg/mL of hyalin starting material.

Biotinylated lectins were used in immuno dot blots to screen for the types of sugars present on hyalin along with fetuin protein (Figure 24). Hyalin (2.8m/mL) and
fetuin (2.0mg/mL) were dotted with serial 1:2 dilutions on nitrocellulose strips. Serial dilutions were used to rule out non-specific binding. Immunostrips were probed with 1:500 dilution of each biotinylated lectin (stock of 1mg/mL) and 1:1000 dilution of strepavidin-alkaline-phosphatase (stock of 1 mg/mL). Hyalin was shown to have high affinity to ConA, with dark dots appearing similar in all serial dilutions of hyalin at the primary biotinylated lectin antibody and secondary alkaline phosphate conjugate concentrations used. Hyalin also is shown to bind to SBA, WGA, and RCA120 (Figure 24). This is not from non-specific binding as seen from lower intensity of more dilute hyalin dots on each positive strip. Fetuin was shown to bind with high affinity ConA, WGA, and RCA120. It also showed weaker affinity to DBA (Figure 24).

The affinity of hyalin to specific lectins was confirmed by running immunodot blots again along with negative controls using sugars that compete with lectin in binding to hyalin. Dot blots again show hyalin binding to ConA, SBA, WGA, and RCA120 (Figure 25). Biotinylated ConA primary antibody was diluted 1:20,000 to match intensity of blots that used only 1:500 dilution of primary antibody (Figure 25). At this lower dilution of biotinylated ConA, serial dilution of hyalin dotted show a decrease in intensity with more diluted dots. This shows that ConA is binding to hyalin and the positive results are not from non-specific interactions. Hyalin did not show binding to DBA, UEAI, PNA (Figure 25). For each of the positive lectin strips, a duplicate strip was run in the presence of inhibiting sugars as negative controls. Methyl-α-D-mannopyranoside inhibited ConA, sialic acid inhibited WGA, N-acetylgalactosamine inhibited SBA, and galactose inhibited RCA120 (Figure 25).
Finally individual fractions from size exclusion chromatography were tested specifically with ConA. Immunoblot strips were dotted with original hyalin before injection (H) and hyalin components separated in peaks (H1-H5) on Sephacryl S-500 column. Figure 26 shows the representative chromatogram from the separation labeled with peaks H1-H5. The inset shows the results of immunoblot probed with ConA. The dots corresponding to strong binding of ConA to peak H4. Further investigation show that the ConA binding is a result of hyalin components separated in peak H3. Hyalin peaks were run on SDS-PAGE and silver stained (Figure 27). H1 and H5 did not show presence of protein on gel. Hyalin is made of at least three components separated in H2, H3, and H4. The higher molecular weight structures seen in silver stained gel did not show binding to ConA in immunoblot in Figure 27 b. However, a band corresponding to lower molecular weight hyalin component seen in peak H4 showed strong binding to ConA, as seen by the arrow.

Isoelectric focusing (IEF) electrophoresis was used to characterize the pI for hyalin components. Figure 28a shows the original Coomassie stained IEF gel stained on the left and the gel with bands detected using 1-D imaging software on the right. Hyalin was loaded in Lane C. The pixel Y-position from the top of the well was located from peak of band intensity for the gel in Figure 28b. This position was used by software to extrapolate the PI of each of the bands using the migration of pH calibration standards in lane A. The software calculated the pI of the three hyalin species as pH 6.3, pH 4.9, and pH 4.4 (Figure 28c).
DISCUSSION

This study supports previous findings that hyalin is comprised of several molecular weight components (Kane, 1970; Gray, et al, 1986; Justice, et al., 1992, 1988). Size exclusion chromatography on Sephacryl S-500 (10/300) column separated purified hyalin into five peaks (H1-H5) with the following sizes: H1: 883kDa, H2: 228kDa, H3: 147kDa, H4: 63kDa, and H5: 34kDa. Separation was performed in the presence of 1mM EDTA to prevent hyalin aggregation and to characterize the protein by its basic assembly units. The glycoprotein, fetuin, eluted with a MW of 64kDa. This control confirmed the validity of the molecular weight calibration of the column with this calculated size matching the known apparent molecular weight of 64kDa for fetuin (Peeters, 1970). The major hyalin protein in the absence of divalent cations had been characterized by Gray, et al, to have a molecular weight of $9.2 \pm 0.5 \times 10^5$ and coefficient of 11.6S by sedimentation analysis. Peak H1 (883kDa) is within the error of this reported molecular weight. Gray, et al, also found with different preparations of hyalin purifications that smaller 8.8S component with molecular weight of about $3 \times 10^5$ and even a 6.6S component exist. Silver stained SDS-PAGE confirmed protein in peaks H2-H4 for hyalin. Peak H1 and H5 were not detected in SDS-PAGE possibly due to lower concentrations. Peak H5 eluted close to the total volume of the 10/300 size exclusion column and the observance of a peak may be residual optical density from buffer components of purified hyalin at injection. This study did not find the major component to be the higher molecular peak H1 (883kDa) but instead lower molecular weight components of H2 (228kDa), H3 (147kDa), and H4 (63kDa) appear to be the dominant species. The average molecular
weight species of hyalin from \textit{S. purpuratus} varies in different solvents. Previous studies found average hyalin molecular weight to be $3.55 \times 10^5$ in 0.1M NaCl, pH 7 and to be $1.02 \times 10^5$ in 0.1M NaCl, pH 9 (Kane & Hersh, 1959; Stephen & Kane, 1970). Molecular weights of hyalin components are lower in higher pH solvents. This study detected hyalin components with molecular weights between those ranges at 228kDa and 147kDa. Separation of hyalin was performed at pH 8 in this study, which is midway between pH 7 and pH 9 that had been used in the reported weights. Characterizing exact molecular weights for hyalin components may be difficult due to aggregation properties of hyalin in various buffers or to differences in purified hyalin preparations.

Hyalin had been reported to contain 25\% acidic residues and only 3.5\% basic residues. IEF gels supports that hyalin components are mostly acidic, with the presence of at least three components of pI 6.6, 4.4, 4.3.

In addition to characterizing the molecular weight of hyalin components, the carbohydrate moieties of the protein were analyzed. Alkaline beta elimination reaction of hyalin is dependent on the concentration of the starting material and on concentration of sodium hydroxide. Dehydroalanine was produced by the cleavage of O-linked glycans on hyalin and show that hyalin contains glycans linked by serine and threonine residues. The final concentration of dehydroalanine released by 0.5mg/mL of hyalin was $8.3 \pm 0.3 \times 10^{-5}$M and is comparable to the $8.7 \pm 0.1 \times 10^{-5}$M that was released by 0.5mg/mL of fetuin. However, the rates of the beta elimination reaction for the proteins differ with the rate for the release of o-linked glycan for hyalin significantly faster than the rate for fetuin. Beta elimination from glycoserine and glycothreonine residues generally occurs
at the same rate (Tanaka, 1964). However, faster and slower relative rates for glycoserine residues in relation to glycothreonine residues have been reported (Wakayashi & Pigman, 1974; Planter & Carlson, 1975). Since fetuin has O-linked glycans that are mostly attached to serine residues, the differences in rates may suggest that hyalin o-linked glyans may be mostly attached to threonine residues. Another possibility is that o-linked glycans on hyalin may be more accessible than those on fetuin.

Data from immunoblots with lectins further characterized the glycans on hyalin. Purified hyalin showed strong binding to ConA and lesser binding to WGA, SBA, and RCA120. Primary biotinylated-ConA lectin probe was greatly diluted 1:20000 to obtain reactivity similar to the other positive biotinylated-lectin probes that were diluted only 1:500. Positive reactivity with ConA provides evidence that hyalin contains α-D-mannose and/or α-D-glucose residues. Hyalin binding to SBA suggests that the protein contains α or β GalNAc or Gal moieties. Evidence that these moieties are not terminally structured is supported by the negative binding results to DBA, which binds specifically to terminal αGalNAc or Gal structures. These results are opposite of those for fetuin, which binds to DBA and not to SBA. SBA and DBA have a similar carbohydrate binding specificity, but DBA shows a clear preference for α-linkage-D-GalNAc in contrast with SBA which has no anomeric specificity for GalNAc. Positive reactivity to RCA120 suggests hyalin contains β-D-galactose and/or β-N-acetyl-D-galactosamine. Hyalin binding to WGA suggests that it contains GlcNac or sialic acid glycans. Negative reactivity with UEA-I indicates that hyalin does not contain α-L-fucose structures. WGA reacts with GlcNac residues situated in internal and terminal positions (Allen et al., 1973; Parillo et al., 1998b) Future studies should be done to probe hyalin with GSAII lectin to distinguish the
location more specifically since GSAII would only recognize terminal GlcNAc residues. WGA can also bind sialic acids, although much less than to GlcNAc (Monsigny et al., 1980).

Investigation of glycan moieties of individual hyalin components was accomplished by using the strong binding of hyalin to ConA to probe on Western blots. Fractions that separated from the size exclusion chromatography are more dilute than the purified hyalin stock. Since the weaker binding was barely detected in the original sample of hyalin for the other positive biotinylated lectin probes, they were not able to pick up individual fractions (data not shown). Fractions H1-H5 dotted on immunblots showed ConA binding to H4 and minimal binding in H3 peak. The presence of ConA binding to sample from H3 peak maybe due to the presence of H4 peak component not completely separated from the H3 peak during size exclusion chromatography. Western blot by SDS-PAGE was run to confirm binding of ConA specifically to H4 hyalin peak corresponding to the 63kDa component. The western blot on SDS-PAGE detects ConA binding to hyalin component with a molecular weight between 65-43kDa of the molecular weight ladder in the H4 peak lane. This result is significant in showing that individual components of hyalin are heterogeneous in their glycan composition and have different binding affinities towards various lectins.
REFERENCES


Figure 1: SEC void volume (Vo) and total volume (Vt). Chromatogram shows overlay of two size exclusion runs to determine Vo and Vt for the S-500 10/300 column. The peak on the left is blue dextran eluted at the void volume and the peak on the right is acetone eluted at the total volume.
Figure 2: *Representative chromatogram for molecular weight standards calibration.* The chromatogram show the elution profile of the standard and the detected peaks are labeled with letters to represent the following standard: (A) thyroglobulin, (B) γ-globulin, (C) ovalbumin, (D) myoglobin.
Table 1: Elution volume for each peak of molecular weight standard for three calibration runs. The Ve for each standard are listed for three runs and the average Ve was calculated.

<table>
<thead>
<tr>
<th>Label</th>
<th>MW (kDa)</th>
<th>Log MW</th>
<th>Standard Sample</th>
<th>Ve (mL) Elution Volume</th>
<th>Retention Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Run1</td>
<td>Run2</td>
</tr>
<tr>
<td>A</td>
<td>670</td>
<td>2.83</td>
<td>Thyroglobulin (bovine)</td>
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<td>9.54</td>
</tr>
<tr>
<td>B</td>
<td>158</td>
<td>2.20</td>
<td>γ-globulin (bovine)</td>
<td>16.95</td>
<td>16.82</td>
</tr>
<tr>
<td>C</td>
<td>44</td>
<td>1.64</td>
<td>Ovalbumin (chicken)</td>
<td>19.57</td>
<td>19.57</td>
</tr>
<tr>
<td>D</td>
<td>17</td>
<td>1.23</td>
<td>Myoglobin (horse)</td>
<td>23.08</td>
<td>23.11</td>
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</table>
Figure 3: Molecular weight standard calibration curve for S-500 (10/300) column. The log of molecular weight was plotted against the retention ratio (Ve/Vo) for each of the standard protein separated by SEC to create an equation for the linear curve that can be used to estimate unknown proteins.
Figure 4: SEC chromatogram for fetuin. Chromatogram of control glycoprotein, fetuin, eluted from Sephacryl S-500 10/300 column.

Table 2: Molecular weight calculation for fetuin. The molecular weight of fetuin was calculated from the elution volume of fetuin peak.

<table>
<thead>
<tr>
<th>Fetuin</th>
<th>Peak</th>
<th>Ve (mL)</th>
<th>Ve/Vo</th>
<th>Log (MW)</th>
<th>MW (kDa)</th>
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<tbody>
<tr>
<td>F1</td>
<td>18.7</td>
<td>2.01</td>
<td>1.8</td>
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<td>64</td>
</tr>
</tbody>
</table>
Figure 5: SEC run 1 for hyalin on sephacryl S-500 10/300 column.

Table 3: Molecular weight calculation for hyalin peak for SEC run 1.

<table>
<thead>
<tr>
<th>Hyalin</th>
<th>Peak</th>
<th>Ve (mL)</th>
<th>Ve/Vo</th>
<th>Log (MW)</th>
<th>MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>9.09</td>
<td>0.98</td>
<td>2.94</td>
<td></td>
<td>881</td>
</tr>
<tr>
<td>H2</td>
<td>14.06</td>
<td>1.51</td>
<td>2.35</td>
<td></td>
<td>226</td>
</tr>
<tr>
<td>H3</td>
<td>15.69</td>
<td>1.69</td>
<td>2.16</td>
<td></td>
<td>145</td>
</tr>
<tr>
<td>H4</td>
<td>18.79</td>
<td>2.02</td>
<td>1.79</td>
<td></td>
<td>62</td>
</tr>
<tr>
<td>H5</td>
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<td>2.27</td>
<td>1.52</td>
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<td>33</td>
</tr>
</tbody>
</table>
Figure 6: SEC run 2 for hyalin on sephacryl S-500 10/300 column.

Table 4: Molecular weight calculation for hyalin peak for SEC run 2.

<table>
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<tr>
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<th>Ve/Vo</th>
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<tbody>
<tr>
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<tr>
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<td>13.99</td>
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<td>H3</td>
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<td>1.68</td>
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<tr>
<td>H4</td>
<td>18.64</td>
<td>2.01</td>
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<td>65</td>
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<tr>
<td>H5</td>
<td>20.68</td>
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<td>37</td>
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</table>
Figure 7: SEC run 3 for hyalin on sephacryl S-500 10/300 column.

Table 5: Molecular weight calculation for hyalin peak for SEC run 3.

<table>
<thead>
<tr>
<th>Hyalin</th>
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<th>Ve (mL)</th>
<th>Ve/Vo</th>
<th>Log (MW)</th>
<th>MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>9.09</td>
<td>0.98</td>
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<td>881</td>
</tr>
<tr>
<td>H2</td>
<td>14.06</td>
<td>1.51</td>
<td>2.35</td>
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<tr>
<td>H3</td>
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<td>33</td>
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</table>
Table 6: *Summary of molecular weight calculation for three SEC separation runs of hyalin.* The average molecular weights are summarized for each of the peaks.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Run 1 MW (kDa)</th>
<th>Run 2 MW (kDa)</th>
<th>Run 3 MW (kDa)</th>
<th>Average MW (kDa)</th>
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</thead>
<tbody>
<tr>
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<td>881</td>
<td><strong>883</strong></td>
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<tr>
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<td><strong>147</strong></td>
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<td>H4</td>
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<td><strong>63</strong></td>
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<tr>
<td>H5</td>
<td>33</td>
<td>37</td>
<td>33</td>
<td><strong>34</strong></td>
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</table>
Figure 8: Beta-elimination of Hyalin with 0.4M NaOH – Run 1

Figure 9: Rate for beta-elimination of Hyalin with 0.4M NaOH – Run 1
Figure 10: Beta-elimination of Hyalin with 0.4M NaOH – Run 2

\[
\begin{align*}
\text{Absorbance at 240nm (A.U.)} \\
\text{Time (min)}
\end{align*}
\]

Figure 11: Rate for beta-elimination of Hyalin with 0.4M NaOH – Run 2

\[
\begin{align*}
\text{Absorbance at 240nm (A.U.)} \\
\text{Time (min)}
\end{align*}
\]

\[y = 0.0161x + 0.0146 \quad R^2 = 0.9941\]
Figure 12: Beta-elimination of Hyalin with 0.4M NaOH – Run 3

Figure 13: Rate for beta-elimination of Hyalin with 0.4M NaOH – Run 3
Figure 14: Beta-elimination of Fetuin with 0.4M NaOH – Run 1

Figure 15: Rate for beta-elimination of Fetuin with 0.4M NaOH – Run 1
Figure 16: Beta-elimination of Fetuin with 0.4M NaOH – Run 2

Figure 17: Rate for beta-elimination of Fetuin with 0.4M NaOH – Run 2
Figure 18: Rate for beta-elimination of Fetuin with 0.4M NaOH – Run 3

![Graph showing rate of beta-elimination with 0.4M NaOH](image1)

Figure 19: Rate for beta-elimination of Fetuin with 0.4M NaOH – Run 3

![Graph showing rate of beta-elimination with 0.4M NaOH](image2)
Table 7: Summary of DHA for hyalin and fetuin.

<table>
<thead>
<tr>
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<th>Hyalin</th>
<th></th>
<th>Fetuin</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>A240nm</td>
<td>[DHA]</td>
<td>A240nm</td>
<td>[DHA]</td>
</tr>
<tr>
<td></td>
<td>A.U.</td>
<td>Mx10^3</td>
<td>A.U.</td>
<td>Mx10^3</td>
</tr>
<tr>
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<td>0.326</td>
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<td>0.342</td>
<td>8.6</td>
</tr>
<tr>
<td>2</td>
<td>0.321</td>
<td>8.0</td>
<td>0.339</td>
<td>8.5</td>
</tr>
<tr>
<td>3</td>
<td>0.356</td>
<td>8.9</td>
<td>0.357</td>
<td>8.9</td>
</tr>
<tr>
<td>Mean</td>
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<td>8.4</td>
<td>0.346</td>
<td>8.7</td>
</tr>
<tr>
<td>SE</td>
<td>0.011</td>
<td>0.3</td>
<td>0.006</td>
<td>0.1</td>
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</table>

Figure 20: Graph comparing DHA for hyalin and fetuin.
Table 8: *Summary of reaction rates for hyalin and fetuin*

<table>
<thead>
<tr>
<th>Reaction Rate</th>
<th>Hyalin</th>
<th>Fetuin</th>
</tr>
</thead>
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<td>l/m</td>
<td>l/m</td>
<td></td>
</tr>
<tr>
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<td>0.0139</td>
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<td>2</td>
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<td>0.0156</td>
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<td>Mean</td>
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<td><strong>0.0136</strong></td>
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<tr>
<td>SE</td>
<td>1.15x10^-4</td>
<td>1.28x10^-3</td>
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Figure 21: *Graph comparing reaction rates for hyalin and fetuin*
Figure 22: Beta Elimination reaction rate varying NaOH.

Figure 23 Beta Elimination reaction rate varying Hyalin
Figure 24 *Immuno dot-blot of hyalin and fetuin screened with various lectins.* Serial dilutions of hyalin (2.8mg/mL) and fetuin (2mg/mL) were dotted on nitrocellulose strips. Strips were probed with the following biotinylated lectins (1mg/ml) diluted 1:1000: Con A (Concanavalin A), SBA (*Glycine max* (soybean) agglutinin), WGA (*Triticum vulgaris* (wheat germ) agglutinin), DBA (*Dolichos biflorus* agglutinin), UEA I (*Ulex europaeus* agglutinin I), PNA (*Arachis hypogaea* (peanut) agglutinin), RCA120 (*Ricinus communis* agglutinin).
Figure 25: Immuno dot blot of hyalin probed with biotinylated lectins.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Lectin</th>
<th>Binding</th>
<th>Inhibition</th>
<th>Sugar</th>
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<tr>
<td>1:20,000</td>
<td>ConA</td>
<td></td>
<td></td>
<td>Methyl-α-D-Mannopyranoside (5 mg/mL)</td>
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<tr>
<td>1:500</td>
<td>SBA</td>
<td></td>
<td></td>
<td>GalNAc (2mg/mL)</td>
</tr>
<tr>
<td>1:500</td>
<td>WGA</td>
<td></td>
<td></td>
<td>Sialic Acid (2mg/mL)</td>
</tr>
<tr>
<td>1:500</td>
<td>DBA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:500</td>
<td>UEAI</td>
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<td></td>
</tr>
<tr>
<td>1:500</td>
<td>PNA</td>
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<td></td>
</tr>
<tr>
<td>1:500</td>
<td>RCAI</td>
<td></td>
<td></td>
<td>Galactose (2mg/mL)</td>
</tr>
</tbody>
</table>

Dot Dilution: 1:12, 1:14, 1:16, 1:32, 1:64, 1:128
Figure 26 *Immunoblot of peaks eluted for hyalin on S-500 column.* A representative chromatogram for hyalin is labeled with the eluted peaks (H1-H5). The inset shows the strip dot-blotted with each of the peaks and probed with ConcanavalinA lectin. Peaks H4 shows binding to ConA.
Figure 27 *Hyalin fractions from SEC probed with ConA*

a) Silver stained SDS-PAGE  
b) Western blot with biotinylated-ConA
Figure 28 *IEF for Hyalin*

a) Gel and bands detected with imaging software
b) Peaks detected from Imaging Software

![Graph showing peaks detected from Imaging Software]

<table>
<thead>
<tr>
<th>Band No</th>
<th>PI</th>
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<tr>
<td>1</td>
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<td>2</td>
<td>4.9</td>
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c) PI of hylin components

<table>
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<tr>
<th></th>
<th>PI</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>6.3</td>
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<tr>
<td>2</td>
<td>4.9</td>
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