The Effects of Lignosulfonic Acid on Fertilization Events of the Sea Urchin, *Strongylocentrotus purpuratus.*

By

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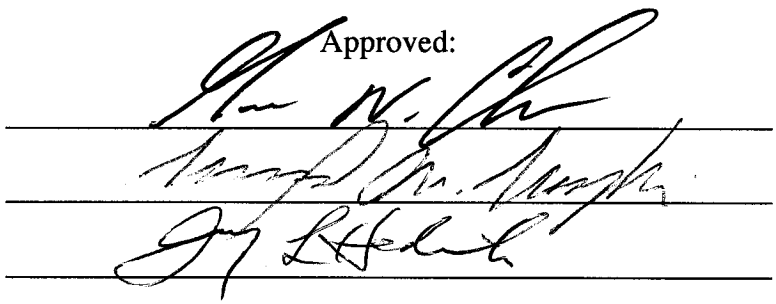

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Dissertation Abstract

Breakdown products of the wood structural polymer lignin are major components in the effluent from commercial pulp and paper manufacturing mills. Polar lignin-derived macromolecules (LDMs) isolated from vastly different pulping processes and from different source wood have surprisingly similar properties and biological activity. Our group has previously reported that a LDM from bleached Kraft mill effluent (BKME) inhibits sea urchin fertilization by binding to sperm cells and blocking the egg jelly induced acrosome reaction. Lignosulfonic acid (LSA), an LDM from sulfite mill effluent, is structurally distinct from BKME-LDM and data presented herein indicate that it was a more potent inhibitor of fertilization. LSA ranged in molecular size from <3K to >300K, with higher molecular size fractions being the more potent inhibitors of fertilization. The mode of action of LSA on sea urchin fertilization was characterized by examining its effects on two critical prerequisite sperm-egg interactions: acrosome reaction induction by egg jelly and sperm-to-egg binding. LSA inhibited both of these processes at low concentrations. Microscopically, a biotinylated LSA derivative (LSA-b) bound to localized domains of the sperm surface, particularly over the head and acrosomal regions. The interaction of LSA with binding sites on the sperm surface was further characterized using a quantitative solid phase whole-cell binding assay and the LSA-b probe. LSA-b behaved in a manner consistent with receptor-ligand binding models in that its interaction with sperm was specific, saturable, kinetically dependent, and reversible. LSA specifically inhibited the binding of antibodies to proteins that entirely co-localize within its binding domain, sea urchin receptor for egg jelly (suREJ3) and bindin. These results support the hypothesis that LSA exerts its biological action by binding to key molecules involved in gamete recognition.

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Abbreviations and Acronyms

AI..... acrosome intact or non-reacted

AR ..... acrosome react or acrosome reacted

ASW-H ..... artificial seawater buffered with 10 mM HEPES

BKME..... bleached Kraft mill effluent

C-9..... Nine carbon

FDN..... fucoidan

FSW..... filtered seawater

HBS ..... HEPES buffered saline

HEPES..... N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HRP ..... horseradish peroxidase

IC_{50}..... 50% inhibition concentration

Kd'..... apparent affinity constant

LDM ..... lignin-derived macromolecule

LSA ..... lignosulfonic acid

LSA-b ..... biotinylated LSA

LSA-NH..... amine LSA

MWCO ..... molecular weight cut off

NFM ..... non-fat milk

NSB ..... non-specific binding

OPD..... o-phenylenediamine

PAGE..... polyacrylamide gel electrophoresis

PASA..... polyanetholesulfonic acid

SME..... sulfite mill effluent

SDS..... sodium n-dodecyl sulfate

suREJ..... sea urchin receptor for egg jelly

UV ..... ultraviolet

Chapter 1. Lignosulfonic Acid and other Polar Lignin-Derived Macromolecules Inhibit Sea Urchin Fertilization.

ABSTRACT

Polar lignin-derived macromolecules (LDMs) isolated from vastly different pulping processes and from different source wood have surprisingly similar properties and biological activity. Lignosulfonic acid (LSA) is a commercially available LDM from the acid sulfite processing of wood. LSA had a similar UV-visible spectra and electrophoretic profile as laboratory-isolated LDM from sulfite mill effluent but was distinct from bleached Kraft mill effluent LDM. Data from centrifuge filtration experiments and electrophoresis suggests that LSA ranges in molecular weight from >300K to <3K. Approximately 1/3 of the mass of whole LSA was <30K, 1/3 >30K to <100K, and 1/3 >100K. Polyacrylamide gel electrophoresis supports the notion that LSA has self-associative behavior, as LSA tends to form a high molecular weight smear and diffuse bands in the presence of the dispersant sodium n-dodecyl sulfate (SDS), but migrates as two tight bands under native (without SDS) conditions. The lower native gel band is associated with the <30K fraction while the upper band is present in fractions >30K. The biological activity of LDM isolated from pulp mill effluents and LSA fractions were compared using a sea urchin fertilization bioassay. LSA was a more potent inhibitor of fertilization than LDMs isolated from pulp mill effluents. LSA's potency as an inhibitor of fertilization was correlated to higher molecular weight fractions similar to results that have been previously reported for bleached Kraft mill effluent LDM. By contrast, the lowest molecular weight fraction of LSA (<3K) had an inverse

concentration response, where the lowest concentration was most toxic and the highest concentration the least toxic.

INTRODUCTION

Lignin is a natural, polymer that is a major component of all woody plants. Indeed, the root of the word lignin is the Latin, *lignum*, meaning wood. Lignin acts as a major structural molecule in plants by binding and stiffening cellulose fibers in and between cell walls, and also protects plant tissues by imparting resistance to microbial attack (Reid, 1995). The hydrophobicity of lignin aids plant physiology by retarding the diffusion of water across cell walls, particularly in xylem and phloem tissue (Lin and Dence, 1992). Lignin is biosynthesized from three types of phenylpropane units (Figure 1.1a) that vary according to species, thus a 9-carbon unit is a common metric among lignin chemists. The specific chemical makeup of a lignin sample varies based on several different factors including wood species, age, tissue type, and morphological region (Hassi, 1985). Despite relatively simple building blocks, the structure of lignin is exceedingly complex and heterogeneous (Figure 1.1b) leading many to believe that it is formed by random polymerization. While lignin has been studied for over a century, most studies of lignin chemistry have focused on lignin derivatives since it has not been possible to separate lignin from its cellulosic companions in an unaltered state (Lin and Dence, 1992; Pearl, 1967).

Many different methods are available to remove lignin from wood and each generates unique lignin-derived macromolecules (LDMs). Lignosulfonic acid (LSA), also known as lignosulfonate (Anonymous, 1999), is a readily available byproduct of the sulfite pulping of wood. In this process, wood is digested in an aqueous solution of sulfur

dioxide and base at high temperature. This results in the hydrolysis of the polysaccharide-lignin matrix, partial lignin depolymerization and creates a soluble, sulfonated, polydisperse lignin derivative. Sulfonation often occurs at the α or γ carbon of the phenylpropane unit by nucleophilic substitution (Glasser et al., 1974; Glasser et al., 1973; Gupta and McCarthy, 1968; Hassi, 1985). The LSA thus produced can be isolated as the salt of the base used, commonly Na^{+}, Ca^{+2}, Mg^{+2}, or NH_4^{+} (Pearl, 1967). Due in part to its convenient water-solubility, LSA has been studied extensively, primarily for the purpose of better understanding the parent compound, lignin (Felicetta et al., 1956). Consequently, a great deal of physicochemical data has been gathered for this polymer.

Commercially available LSA has a broad molecular weight range, from 5K to 400K g/mol (Fredheim et al., 2002; Gardon and Mason, 1958). Ritter et al. (1950) established an average empirical formula for softwood LSA, $C_9H_{7.5}O(OH)(OCH_3)(SO_3H)_{0.5}$. The degree of sulfonation varies from 0.4 to 0.7 SO_3 per C_9 unit in LSA fractions (Buchholz et al., 1992) and is generally lower as average molecular weight increases (Fredheim et al., 2002; Fredheim et al., 2003; Hassi, 1985). Despite its polydispersity, solutions up to 100 mg/ml LSA show very little change in viscosity (Browning, 1957; Fredheim et al., 2002). Since sulfonic acid groups are strong acids, LSA is anionic and soluble throughout the entire pH range (Rezanowich and Goring, 1960), although its charge density may vary due to the presence of some phenolic hydroxyl (Peniston and McCarthy, 1948) and carbonyl groups (Moacanin et al., 1955). LSA's affinity for cations is similar to that of commercial resins (Ernsberger and France, 1948). Associated cations have a low degree of dissociation, but the dissociation is enhanced in the presence of simple electrolytes (Hassi, 1985). Iron is said to be difficult

to remove from LSA and reportedly partly responsible for the residual brown color of the molecule (Collins et al., 1977).

One of the most prominent physicochemical properties of lignin-derived macromolecules is a pronounced tendency to form high molecular weight associated complexes in aqueous solution (Sarkanen et al., 1982). Counter ion concentration, pH, and dispersing agents can affect this association (Watanabe et al., 1993). LSA has been described as a microgel of associated micelles with a charged exterior and a relatively hydrophobic interior (Rezanowich and Goring, 1960). These micelles are compact spherical structures, composed of lightly charged large fragments surrounded by highly charged smaller molecular weight fragments (Fredheim et al., 2003; Hassi, 1985). LSA's anionic outer layer allows it to adsorb to cationic surfaces. Surprisingly, it can also simultaneously adsorb to hydrophobic surfaces by exposing it's non-polar core, suggesting a great degree of molecular flexibility (Hassi, 1985). This dual surface activity makes LSA a useful industrial product as a dispersant (in cement, printing ink, oil well drilling mud, and pesticides) as well as a binder (in animal feed pellets and ceramics).

LSA reaches environmentally relevant concentrations in pulp mill effluent (Kringstad and Lindström, 1984) and as a dispersant in drilling muds (Carls and Rice, 1984). Environmentally, most toxicological concerns are not focused on polar lignin-derived macromolecules, but on the small molecule components of the mixture in which they occur. Generally very low acute toxicity (Luscombe and Nicholls, 1973; Roald, 1977) has been associated with LSA and other lignin derived macromolecules. In fact, lignin-derived macromolecules can be environmentally protective by sequestering some

organic contaminants and limiting their bioavailability (Kosiková et al., 1990; Kukkonen, 1992; Kukkonen and Oikari, 1992). However, these macromolecules do affect some sublethal endpoints (Roald, 1977) and specific biological processes, particularly during reproduction and development (Cherr et al., 1987; Higashi et al., 1992; Pillai et al., 1997). In the sea urchin, a lignin-derived macromolecule isolated from bleached Kraft mill effluent (BKME-LDM) inhibits fertilization without causing cytotoxicity to gametes. BKME-LDM binds to extracellular domains of the sperm cell and inhibits the acrosome reaction, yet pre-exposure to eggs does not inhibit fertilization (Pillai et al., 1997). While LSA is known to affect certain specific biological processes (Loomis and Beyer, 1953; Naess and Sandvik, 1973; Vocac and Alphin, 1969), its effect on sea urchin fertilization is unknown. One account notes that the aqueous fraction of lignosulfonate drilling mud inhibits reproduction in some annelids (Neff et al., 1981), however the causative agent was not identified. BKME-LDM is generated by very different chemistry than LSA and has distinct characteristics (Higashi et al., 1992). The following experiments will compare the physicochemical characteristics and biological activity of commercially available LSA as well as lignin-derived macromolecules isolated from raw sulfite mill effluent and bleached Kraft mill effluent.

MATERIALS AND METHODS

Chemicals

All chemicals used were obtained from Sigma or Aldrich unless otherwise specified. BKME and sulfite mill effluent (SME) were all donated from pulp mills processing softwoods. Effluents were collected as 24-hour composite samples just prior to final discharge from the mill. The polar LDM fraction was isolated from these effluents

according to the procedures of Higashi et al. (1992) with minor modification. Briefly, this involved filtration and lyophilization of raw effluent, followed by sequential solvent (methylene chloride and acetonitrile) extraction and dialysis (3.5-kDa cutoff) against distilled water. The final product was lyophilized to dryness and kept desiccated at –20°C. Lignosulfonic acid, sodium salt (CAS 8061-51-6) was purchased from Aldrich Chemical Co. and similarly purified to remove contaminants.

Centrifuge filtration

LSA was fractionated using Centricon centrifuge filtration devices of various molecular weight cut off sizes (Millipore, Billerica, MA) according to manufacturers instructions. Generally, 2 ml of a 5-mg/ml aqueous solution of LSA was filtered through the device to a retentate volume of ~100 μl. In some instances, the retentate was subjected to additional "washes" by bringing the volume back to 2 ml with dH_2O and filtering again. Some filtrate samples were sub-fractionated with a lower molecular weight cut off Centricon device (Figure 1.5 C, lane 3). Retentate and filtrate fractions were lyophilized and kept desiccated at –20°C until needed.

Spectroscopy

UV-visible spectrophotometry was conducted according to Higashi et al. (1992) using a Hewlett-Packard Model 8524A diode-array instrument and quartz cuvettes. The UV-visible absorbance spectra were measured from 190 nm to 800 nm. All solutions were made in deionized water and spectra were measured against a similar blank.

Electrophoresis

Procedures for SDS and native (without SDS) polyacrylamide gel electrophoresis (PAGE) followed the guidelines set forth by Cherr et al. (1993). Electrophoresis was performed using a Bio-Rad Mini-Protean II dual slab cell (Bio-Rad Laboratories, Richmond, CA) and a Bio-Rad Model 1000/500 power supply. Pre-cast 4-15% or 4-20% gradient gels (Bio-Rad Laboratories) were loaded with 5-25 μg of sample per lane and run using standard Tris-glycine buffers. Gels were electrophoresed at a constant 150 V for 45 – 60 min in a 4°C cold box then stained with 0.5% alcian blue 8 GX cationic dye as previously described (Cherr et al., 1993).

Fertilization Bioassay

Gametes from Pacific purple sea urchins (*Strongylocentrotus purpuratus*) were collected by injecting adults with 0.5 to 1.0 ml of 0.5-M KCl into the coelomic cavity via the oral peristomal membrane. Sperm were collected "dry" (undiluted) directly from the gonopores with a pipette and stored on ice in a capped tube. Eggs were spawned into 50-ml beakers filled with 0.7-μm filtered sea water (FSW), carefully decanted to remove large debris and kept at 4°C until used. Sperm motility and egg morphological quality were assessed using standard criteria (Chapman, 1995; Cherr et al., 1987). Fertilization bioassays were preformed according to guidelines described by Chapman (1995) and Pillai et al. (1997). All experiments were conducted in artificial sea water (Cavanaugh, 1978) buffered with 10 mM HEPES pH 8 (ASW-H, 490 mM NaCl, 27 mM $MgCl_2$, 28 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM KCl, 2.5 mM $NaHCO_3$) at 12°C. In order to ensure that sensitivity to LDM was not diminished with excess sperm, a sperm to egg ratio resulting in 70% to $\leq$ 90% fertilization was determined for each batch of gametes

immediately prior to experiments. Sperm concentration was determined using a hemocytometer. Sperm were pre-exposed to an LDM for 10 minutes prior to the addition of eggs. Fertilization was allowed to proceed for 10 minutes then samples were fixed with 1% glutaraldehyde in ASW. As a control, eggs were pre-exposed to the highest LDM concentration for 10 minutes prior to the addition of sperm, given 10 minutes to fertilize then fixed as above. Two to four separate bioassays were performed for each LDM. Each bioassay consisted of one pair of urchins run in 3-4 replicate batches with 100-300 eggs scored per replicate. Concentration-response data was normalized to controls using Abbott's formula as described in Chapman (1995). Each concentration-response data set was analyzed by non-linear regression with Prism software (version 4.0, GraphPad Software Inc., San Deigo, CA) to identify the 50% inhibitory concentrations (IC_{50}), Hill slope, and relevant statistics. Values with a $p<0.05$ were considered significantly different.

RESULTS

Size fractionation of LSA via centrifuge filtration

LSA ranges in molecular weight from <3 K to >300 K based upon the operationally-defined molecular weight cut off (MWCO) values for the Centricon devices used (Figure 1.2). Each of the fractions indicated in Figure 1.2 represents the pooled filtrate from a thrice-washed sample of whole LSA (see Methods). Approximately 1/3 of the mass of whole LSA is permeable to a 30-K MWCO membrane, and 2/3 of the mass is permeable at 100-K MWCO. This distribution ratio is supported when samples are filtered in series as well. When a 100-K MWCO filtrate sample was again subjected to

centrifuge filtration at 30-K MWCO, the mass of the subsequent filtrate and retentate was each approximately1/3 of the mass of the whole LSA equivalent (data not shown).

Spectroscopy of LSA and effluent-LDM

The UV-Visible spectrum of BKME-LDM differs considerably from that of LSA and SME-LDM (Figure 1.3A), whereas the spectra of the latter two are rather similar. As previously reported (Higashi et al., 1992), the spectrum of BKME-LDM has a maximum at 208 nm and no other prominent features. SME-LDM and LSA share the peak in this region (208 nm and 210 nm respectively), but also have a second peak at 280 nm that is indicative of aromaticity. Overall the absorbtivity of BKME-LDM is considerably lower than that of SME-LDM and LSA at similar concentrations. A comparison of the spectra of LSA filtrate fractions at different MWCO is illustrated in Figure 1.3B. While all the spectra share the same general features, an ordered decrease in absorbance is apparent with decreasing molecular size. The spectra of all retentate fractions (up to >300K) overlapped with that of whole LSA. There was no noteworthy absorbance measured in the 400 – 800 nm region for any of the samples.

Polyacrylamide gel electrophoresis

All LDM samples migrated as an intrinsically brown band that was visible without the aid of tracking dye in both native and SDS PAGE. This observation is identical to what has been previously reported for BKME-LDM (Cherr et al., 1993). Upon staining with alcian blue dye, however, there were notable differences and similarities (Figure 1.4). All samples shared a doublet-banding pattern in the low molecular weight region of the gel. These bands co-migrated among the different LDM samples. Unlike BKME-LDM, however, SME-LDM and LSA also ran as a broad smear

in the region above the two bands. The staining intensity of this smear increased as it approached the doublet bands with an abrupt decrease in stain intensity immediately proximal to the upper band (labeled "b" in Figure 1.4). The gels in Figure 1.4 were loaded with 25 μg/lane of sample. Often a smear in PAGE gels is an indication of overloading. In order to test this hypothesis and gain better band separation, subsequent PAGE experiments were run on 4-20% gradient gels with 5 μg/lane of sample (Figure 1.5). When a lower mass of LSA was loaded onto gels with a wider gradient, a better-defined electrophoretic pattern emerged. The smear seen in Figure 1.4, while still present was much less pronounced and has a defined band at its leading front (Figure 1.5 A) lane 1, band "c"). Two additional bands are evident below band "c", bands "b" and "a", each of which has a characteristic proximal region of lowered staining intensity. No staining was seen below band "a".

SDS-PAGE of different fractions of LSA, isolated by centrifuge filtration, are also represented in Figure 1.5 A (lanes 2-5). Lanes 2 and 3 are respective retentate and filtrate fractions from a 100-K MWCO filter, and lanes 4 and 5 are respective retentate and filtrate fractions from a 30-K MWCO filter. These fractions are the result of a single pass through the filter. Each fraction gave a unique pattern of staining. In the filtrate lanes, 3 and 5, there is little to no smear staining in the region above band "c". While the smear is present in both retentate lanes (2 and 4) with a staining intensity equivalent to whole LSA (lane 1), staining of the bands in the lower molecular weight region was less intense in retentate fractions. Among the LSA fractions, the staining of band "c" was strongest in the 100-K filtrate (lane 3, <100K LSA) and 30-K retentate (lane 4, >30K LSA) suggesting that it is of intermediate size.

These LSA fractions were also electrophoresed under native conditions (Figure 1.5 B). In the absence of the dispersant, SDS, a very different electrophoretic pattern appears. Under these conditions, LSA migrates as two closely associated bands with very little to no smear staining. The native bands are much more compact and have less staining intensity than their SDS counterparts. This pattern is also seen in the LSA fractions (lanes 2-5); however, the 30-K filtrate (lane 5, <30K LSA) appears to be a single band.

Figure 1.5 C shows the results of SDS-PAGE on thrice-washed LSA fractions (see Methods). These fractions have a more distinctive pattern of staining compared to their single-washed counterparts in Figure 1.5 A, suggesting better separation. Band "c" is absent from all of the fractions. There is an ordered change in alcian blue staining among the different fractions as MWCO decreases. The lowest migrating band, band "a", seems to be associated with filtrate fractions (lanes 2, 4, 5), but excluded from retentate fractions (lanes 1 and 3). All fractions except for the lowest, the 3-K filtrate (lane 5, <3K LSA), have a lightly staining smear in the region between bands "a" and "b", as seen in other SDS-PAGE gels. Low molecular weight bands are absent from the 100-K retentate fraction (lane 1); alcian blue stain appears as a smear only in the upper portion of the gel (bracket) and again in the low MW region. Two bands are evident in the 100-K filtrate fraction. Band "a" is absent in the fraction of 100-K filtrate that is retained by a 30-K MWCO filter (lane 3, <100K > 30K LSA). In this fraction, alcian blue stains a smear in the middle portion of the gel (bracket) characterized by increased intensity in the lower part of the smear, then an abrupt clear area proximal to the band "b".

Effect on sea urchin fertilization

Whole LSA and effluent-LDM samples all inhibited sea urchin fertilization when sperm were pre-exposed and the highest concentrations did not affect fertilization when eggs were pre-exposed. Concentrations of LSA up to 10 mg/ml had no noticeable effect on sperm motility and thus it was not deemed to be cytotoxic. LSA and SME-LDM were more potent inhibitors of fertilization than BKME-LDM (Figure 1.6 A) and had significantly different regression slopes. The toxicity of LSA fractions increased as average molecular size increased, yet the slopes of the regression lines were parallel (Figure 1.6B). The molecular weights indicated are reflective of the MWCO size of the filtration device used. Fractions greater than a given size are retentate fractions and those less than the stated size are filtrate fractions. The IC_{50} for the 30K filtrate sample (<30 K) was estimated to be 34 μg/ml (based on regression extrapolation), since the highest concentration assayed for that fraction, 30 μg/ml, inhibited fertilization by 46.9%.

While most concentration-response curves for the LSA fractions behaved in a predictable sigmoidal manner, the smallest fraction (<3K) did not, and seemed to have an inverse response. With this fraction, the lowest concentration (0.5 μg/ml) was more toxic than the highest (50 μg/ml). This pattern was partially reflected in two other filtrate fractions, <100 K and <30 K (Figure 1.6C); partially reflected because the concentration-response only deviated from sigmoidal at the lowest concentrations. In the filtrate fraction <100 K, 0.5 μg/ml was more toxic than 1 μg/ml, but subsequent concentrations fit a sigmoidal dose response model. For the <30 K fraction, concentrations above 3 μg/ml fit a sigmoidal model, but an inverse concentration response was observed below 3 μg/ml. In order to see if the data fit to sigmoidal models, these anomalous data points

(0.5 μg/ml and 1 μg/ml for <30 K; 0.5 μg/ml for <100 K) were omitted in order to obtain non-linear regressions (solid lines in Figure 1.6 B and C), however, the full data set is represented by the dashed lines in Figure 1.6 C.

DISCUSSION

Lignin-derived macromolecules comprise the majority of high molecular mass material in pulp mill effluent (Kringstad and Lindström, 1984). The different chemistries used in Kraft and sulfite pulping produces water-soluble LDM with different characteristics. LDM from bleached Kraft mill effluent (BKME-LDM) has been characterized according to physicochemical properties and biological activity (Higashi et al., 1992). This LDM is a potent inhibitor of sea urchin fertilization, yet is not cytotoxic (Pillai et al., 1997). A wide range of biological activity has been attributed to LDM isolated from the sulfite process (i.e. LSA), however there is no definitive information regarding its effect on the reproduction of marine invertebrates. This study compared some of the physicochemical properties of the aforementioned LDMs as well as their ability to inhibit sea urchin fertilization.

The UV-visible spectra of SME-LDM and LSA are characteristic of most lignin preparations (Pearl, 1967) and bear considerable resemblance to high molecular weight water-soluble humic and fulvic acids (Duarte et al., 2003). The similarities in the spectra of SME-LDM and LSA are to be expected since LSA is a product of the sulfite pulping process. Most of the absorbance of whole SME is attributed to LSA (Wildish et al., 1976) or more accurately chlorolignosulfonic acids (van Loon et al., 1993). The prominent features of these spectra include a highly absorptive peak in the 208-210 nm region and another at 280 nm. The peak at 280 nm is indicative of aromaticity and is

likely the result of residual aromatic alcohols from the native lignin structure. BKME-LDM lacks this peak at 280 nm as previously reported (Higashi et al., 1992). While UV-visible spectra do not provide much in the way of structural details, they are reflective of molecular structure. Thus, one can say that BKME-LDM has key structural differences compared to SME-LDM and LSA. These differences are further apparent when the samples are electrophoresed by SDS-PAGE (Figure 1.4). BKME-LDM runs primarily as two bands of relatively low molecular weight. SME-LDM and LSA share this banding pattern, but also consist of much higher molecular weight components. Softwood lignosulfonates have a higher degree of polydispersity compared to softwood Kraft lignin (Hassi, 1985), the origin of BKME-LDM. The observed differences in electrophoretic separation are consistent with the reported differences in polydispersity. Given the differences in the pulping processes it is remarkable that there are two bands that co-migrate among different LDMs. This may reflect some common aspect of the original lignin molecule. Lignosulfonic acids have been used as surrogates for the study of native lignin because relatively little structural degradation occurs during their solublization (Felicetta et al., 1956). Their high degree of polydispersity and absorbance at 280 nm is reflective of their lignin origin. Since these indicators are much less prominent in BKME-LDM it can be though of as a more degraded lignin derivative.

Whole LSA and SME-LDM inhibited sea urchin fertilization in a sperm specific manner as has been previously reported for BKME-LDM. This inhibition occurred without noticeable cytotoxicity to the sperm cells (as determined by motility). Even though LSA and the effluent-LDMs act on sperm cells and without apparent cytotoxicity, there are differences in the response. The concentration of LSA required to inhibit

fertilization by 50% (IC_{50}) was nearly 3 and 5 times less than SME-LDM and BKME-LDM respectively (Figure 1.6). While the slopes of the regression curves were not significantly different between LSA and SME-LDM, the slope of the BKME-LDM curve was different. LSA is a product of the sulfite process, but is isolated prior to chlorine bleaching. SME-LDM and BKME-LDM have been subject to bleaching processes that may contribute to their decreased toxicity. Since the IC_{50} and regression slope of LSA and SME-LDM are significantly different than that of BKME-LDM, the mechanism of inhibition may be altered for these derivatives. BKME-LDM is known to act by specifically blocking the acrosome reaction in sperm, a critical prerequisite for fertilization. LSA may block different precursory events or act on different cell receptors. Since aromatic activity is absent in BKME-LDM, charge or some other structural aspect is likely responsible for the basic biological activity of the LDMs.

Lignin-derived macromolecules have a marked tendency to form high molecular weight associated complexes in solution (Sarkanen et al., 1982). Water-soluble sulfonated lignins associate in aqueous solution to form a "pseudo-macromolecule" microgel (Rezanowich and Goring, 1960) that has been described as compact spherical structure with an anionic surface and a hydrophobic interior (Fredheim et al., 2003). Less sulfonated molecules are likely surrounded by more sulfonated molecules and associated via hydrophobic interaction of their interior regions (Hassi, 1985). Smaller LSA fractions have been shown to be more highly sulfonated than larger fractions (Ernsberger and France, 1948; Fredheim et al., 2002) and would therefore occur on the outer surfaces of these micelles. Even though LSA forms aggregates consisting of heterogeneously sized fragments in solution, these can be separated relatively easily by centrifuge filtration.

Whole LSA was fractionated into different molecular size fractions using centrifuge filtration. Approximately 1/3 of the mass of whole LSA was permeable to a 30-K MWCO membrane, 1/3 was permeable to a 100-K MWCO membrane yet retained by the 30-K, and 1/3 retained by the 100-K membrane (Figure 1.2). These distributions agree with the results of Gardon and Mason (1958), who reported that 35% of LSA is in the range of 10 K – 25 K. Fredheim et al. (2003) have recently reported very similar results using size exclusion chromatography. Despite differences in separation techniques, softwood species, the chemical structure of the parent lignin, and probable inter-facility differences in the pulping process the reported molecular weight distributions of LSA are fairly consistent (Glasser et al., 1974), although it has been reported that LSA generated from hardwood species has a much narrower molecular weight distribution. The general shape of the UV-visible spectra is fairly consistent among different LSA size fractions (Figure 1.3B), however there is an ordered decrease in overall absorbance with decreasing molecular size. These patterns have been reported in the literature as well and have been attributed to decreasing aromatic content with decreasing size (Felicetta et al., 1956; Markham et al., 1949).

Counter ion concentration, pH, and dispersing agents can affect the association of LSA in solution. Very different gel filtration chromatograms have been observed in the presence and absence of the dispersing detergent, SDS (Watanabe et al., 1993). Similarly, results presented herein show very different electrophoretic patterns in the presence and absence of SDS. As seen in the native PAGE gels (Figure 1.5B), LSA migrates as two very tight bands, whereas under SDS-PAGE three bands are apparent along with a high molecular weight smear. SDS likely disrupts the microgel associations

among LSA fractions allowing for better separation. The cationic dye alcian blue stained the bands on SDS-PAGE darker and with greater intensity than in the native gel. This observation suggests that some of LSA's anionic groups may be unavailable for staining in the native gel, and may reside on the interior of the compact LSA structure as has been noted elsewhere (Fredheim et al., 2003). Each LSA fraction isolated by centrifuge filtration resulted in a different electrophoretic pattern consistent with MWCO values, indicating that the isolated fractions are indeed of a narrower molecular size range than whole LSA. The fractions in Figure 1.5 A and B are the result of a single pass through the centrifuge filter. A clearer distinction between fractions is achieved in thrice-washed LSA fractions (Figure 1.5 C). In the thrice-washed 100-K retentate (>100K) fraction of LSA most of the alcian blue staining occurs in the high molecular weight region of the gel (lane 1, bracket). Some alcian blue staining is evident in the low molecular weight region of the gel as well, however bands are absent. Since the centrifuge filtration occurs in distilled water, some of the highly charged low molecular weight material is probably associated with the high molecular weight LSA in the retentate fraction despite multiple washes. The amount of this material is likely low since no distinct bands are formed, as is clearly evident in filtrate fractions. A more homogenous size fractionation would likely be achieved by the addition of a small amount of a dispersant, like SDS, in the LSA solution during the centrifuge filtration process.

Generally, higher molecular size fractions of LSA were better inhibitors of sea urchin fertilization (Figure 1.6 B). Fractions retained by the 30-K MWCO membrane were more potent than whole LSA and seem to account for the majority of biological activity. Each fraction responded in a sigmoidal manner and there was no significant

difference in the slopes of the regression curves. Interestingly, other studies on the biological effects of LSA have also noted a graded response according to molecular weight. Higher molecular weight fractions of LSA have more anticoagulant and anti-proteolytic activity than lower molecular weight fractions (Loomis and Beyer, 1953; Naess et al., 1973).

An atypical response was observed in the smallest LSA fraction (<3K, Figure 1.6 C) and at low concentrations of the other filtrate fractions. In the 3-K MWCO filtrate fraction (<3K) the lowest concentration tested was more toxic than the highest. In the two other filtrate fractions, this pattern was partially reflected as only the lowest concentrations deviated from the sigmoidal response. This atypical concentration response seems to be driven by the smaller molecular weight fraction of LSA since the effect is more pronounced as MWCO decreases. Each filtrate fraction with an anomalous response includes all LSA sizes below the MWCO. These fractions can be considered "enriched" by mass for the smaller LSA fractions. This effect is not seen in a 100-K filtrate fraction that was retained by a 30-K MWCO filter (the <100 K >30 K fraction, Figure 1.6 B). Therefore it is likely due to these smaller LSA fractions. Smaller LSA size fractions are more heavily sulfonated than larger fractions. These fractions will likely be aggregated on the outer surface of a hypothetical LSA micelle, with the larger and less polar fractions on the interior. The way these small LSA fragments behave by themselves in solution at low concentrations is unknown and may be very different than in the presence of larger fractions. This behavior could affect their observed biological response. For example, at low concentrations in seawater, counter cations could act to "stabilize" smaller and more polar LSA fragments and inhibit their aggregation. It seems

that at low concentrations these highly sulfonated smaller fractions are more bioavailable and as concentrations increase bioavailability changes, perhaps due to the aggregation phenomenon. There may be other mechanisms of fertilization inhibition involved at these low concentrations. This too could change the concentration response. For example, sperm cytotoxicity and impacts to the egg were not specifically evaluated for those fractions, only whole LSA. It should be noted, however, that this effect is not observed at low concentrations of whole LSA.

Polar lignin derived macromolecules from different sources and pulping processes seem to act similarly to inhibit sea urchin fertilization. The mode of inhibition may be a function of the unique physicochemical properties of these compounds. Despite that fact that many metric tons of LDM can be released into marine ecosystems in the vicinity of pulp mill effluent outfall and during drilling operations, it is thought that the environmental impacts of these compounds is minimal (Landner et al., 1994; Neff et al., 1981). Even if the biological effect of LDM is solely on fertilization events, the ecological consequences on populations of free spawning invertebrates in the vicinity of LDM releases is difficult to predict since their larval stages are pelagic and the result of multiple adult spawning populations. However, it is unclear to what extent LDM affects larval settlement or other biological processes. Unpublished work in our laboratory has indicated that these compounds can affect specific events of embryonic development and fertilization in several marine species. LDMs also have known inhibitory effects on microorganisms (Naess and Sandvik, 1973; Sierra-Alvarez and Lettinga, 1991) that could extend to some which may play key environmental roles. Given the diverse biological

activities of LDMs, it is by no means certain that their presence in the environment is inconsequential.

FIGURES

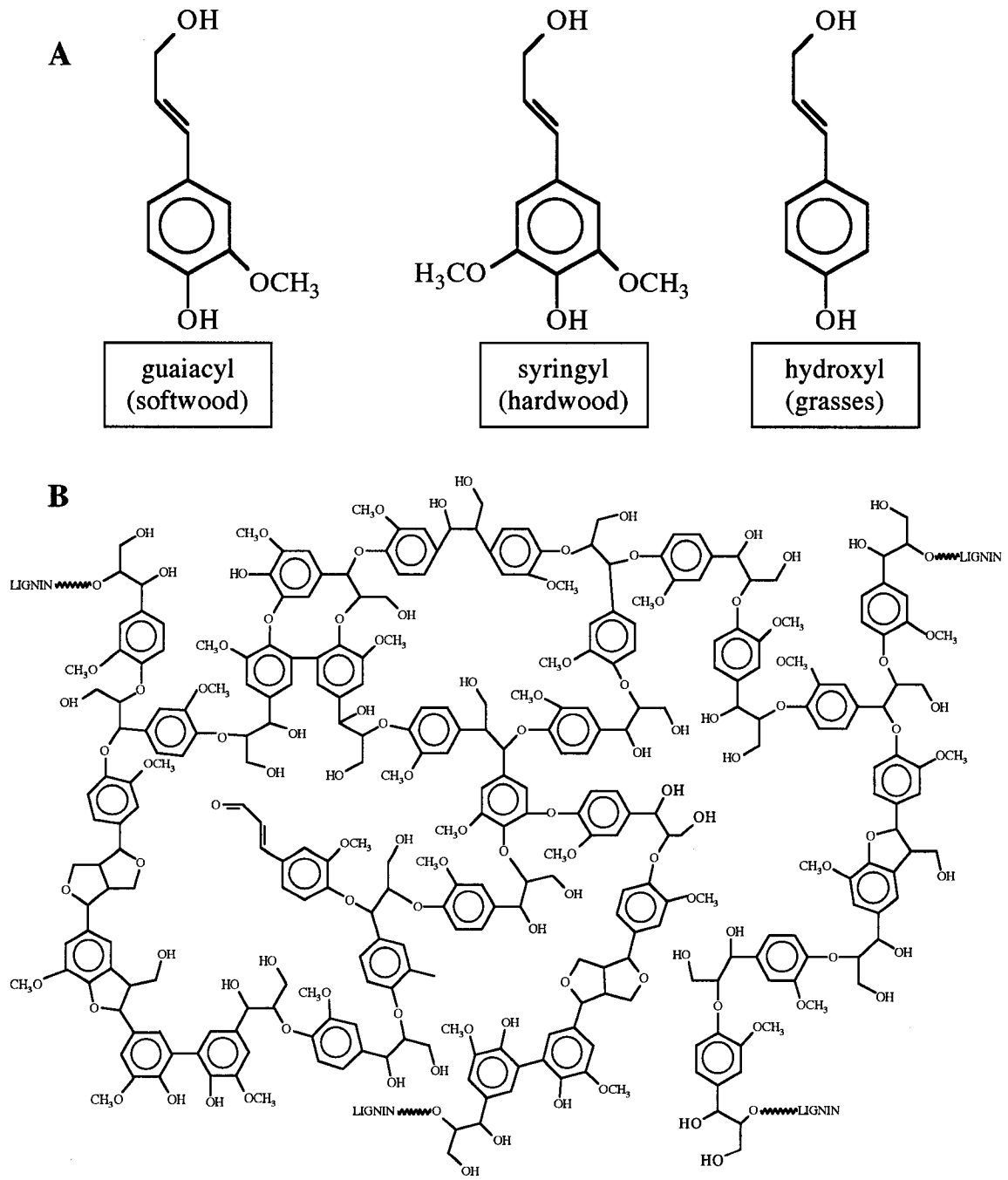


FIGURE 1.1: Lignin monomers and model structure. (A) Nine carbon (C_9) phenylpropane units of lignin. (B) Simplified representation of a portion of a guaiacyl phenylpropane (softwood) lignin polymer (Reid, 1995). The fragment shown has a molecular weight of approximately 5700.

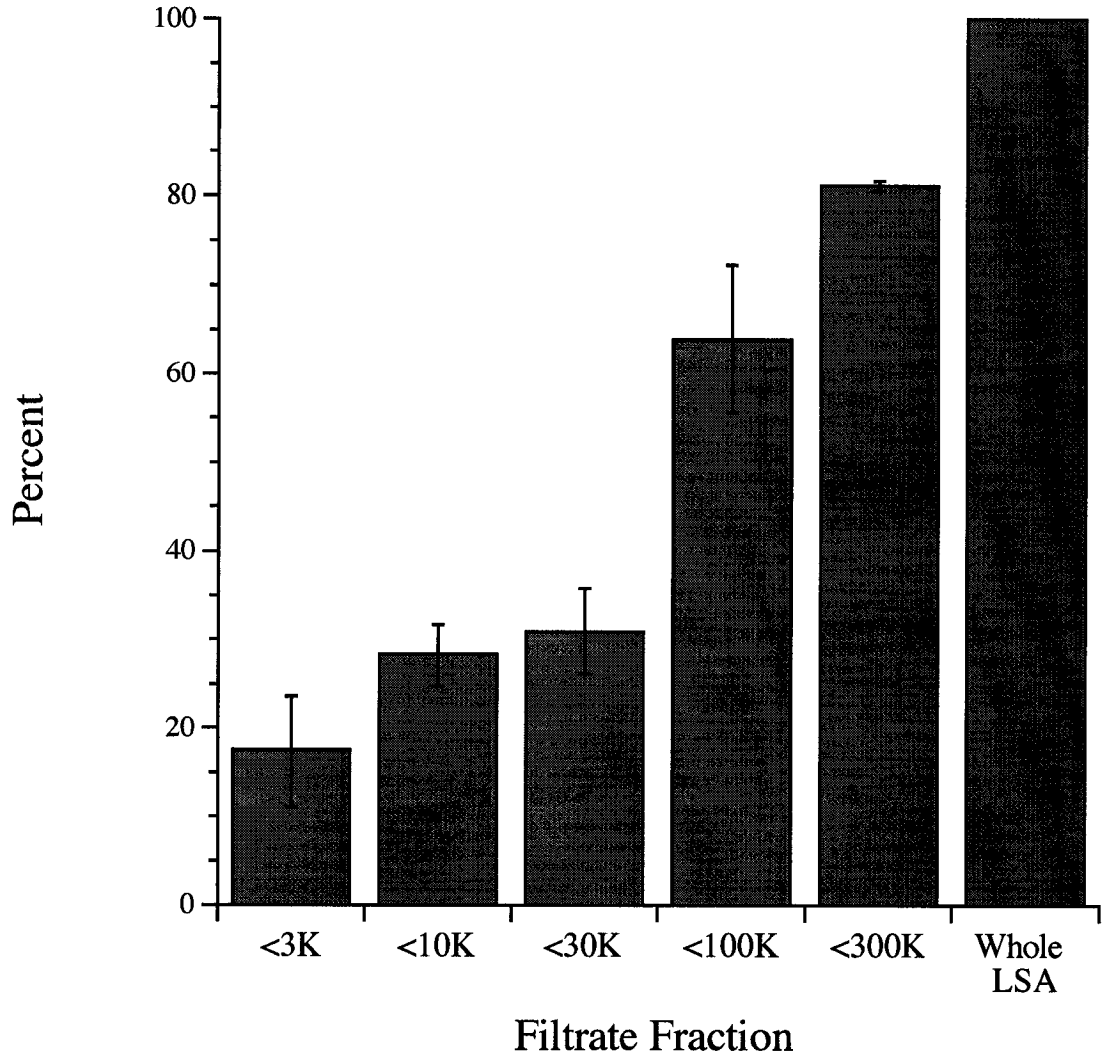


FIGURE 1.2: Molecular size distribution of LSA. Samples were lyophilized after separation and the fraction of whole LSA represented was based on mass. Each size fraction is labeled according to the nominal molecular weight cut off of the device used. Based on these results, one third of the mass of whole LSA is below 30K, one third between 30K and 100 K, and one third above 100K

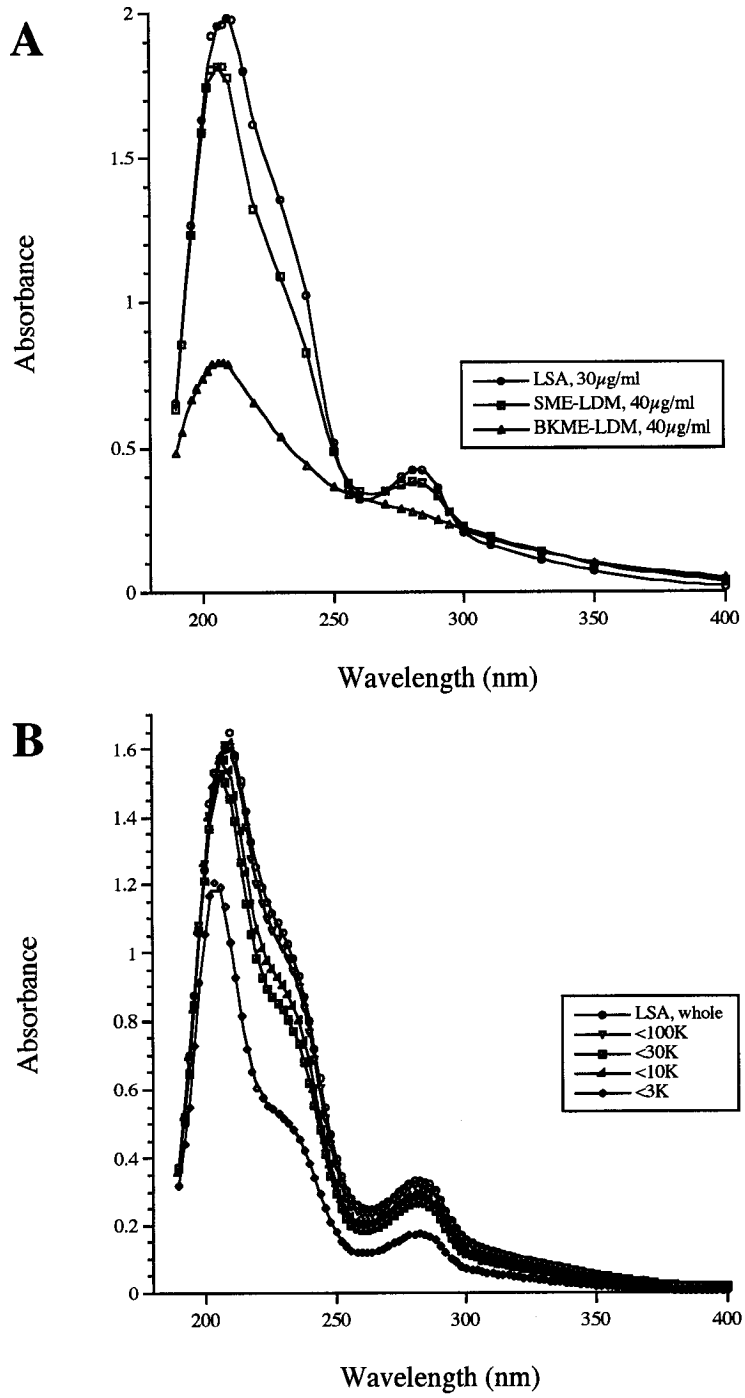


FIGURE 1.3: UV-Visible spectra (A) LSA (max. 210nm, 280nm) and LDM isolated from sulfite mill effluent (SME; max. 208nm, 280nm) and bleached kraft mill effluent (BKME; max. 208nm). (B) Whole LSA compared to filtrate fractions (30 μg/ml each). Retentate spectra overlapped that of whole LSA. In both A and B no prominent absorbance was observed between 400 and 800 nm.

FIGURE 1.4: SDS polyacrylamide gel electrophoresis of lignin-derived macromolecules stained with Alcian Blue. Gel was 4-15% with 25 μg loaded per lane. Lanes are: **1**, BKME-LDM; **2**, SME-LDM; **3**, LSA. BKME runs in doublet bands as previously reported. LSA and SME-LDM run as a high molecular weight smear and doublet bands corresponding to the electrophoretic mobility of the BKME bands.

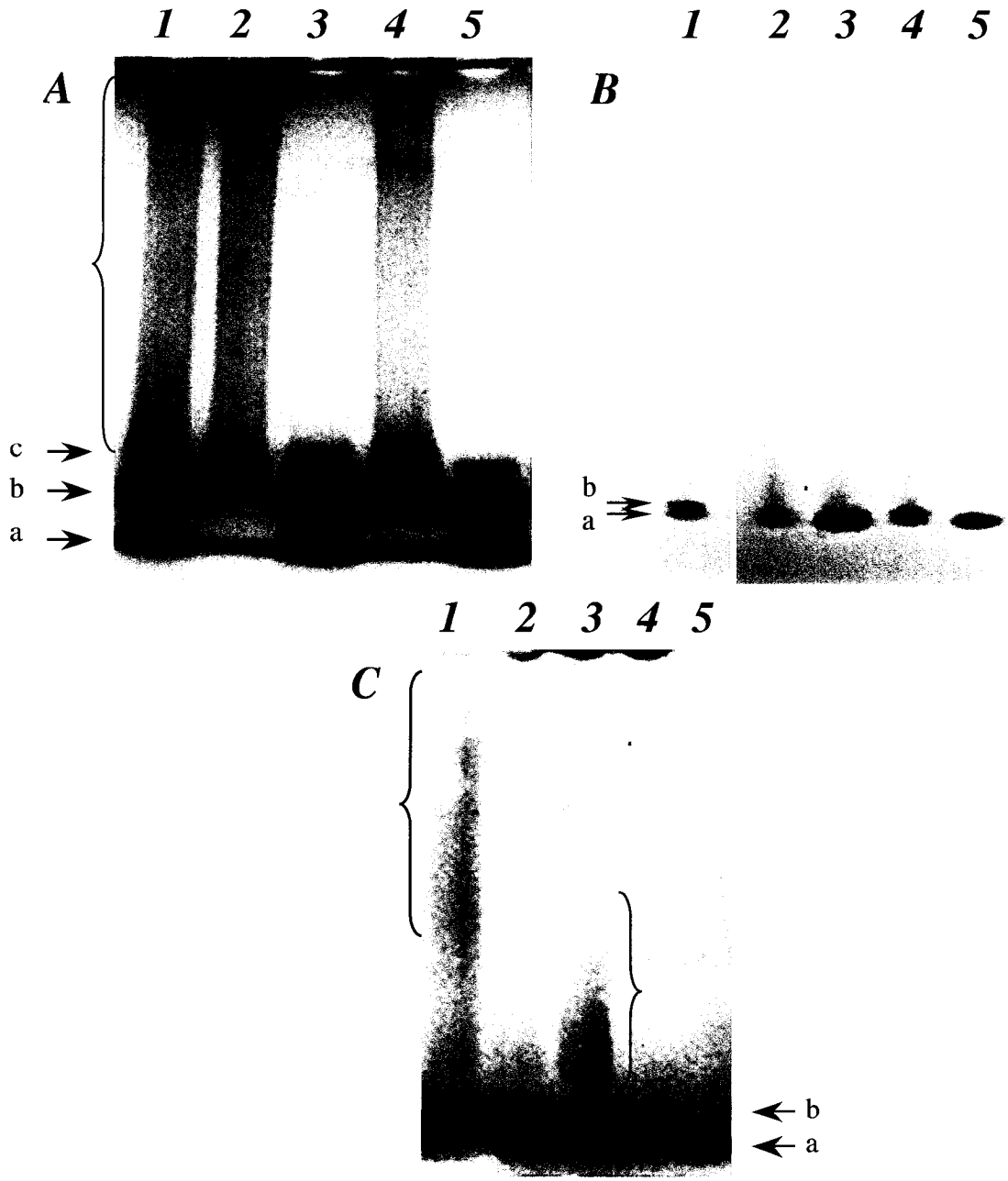


FIGURE 1.5: Polyacrylamide gel electrophoresis of LSA fractions stained with Alcian Blue. Gels are 4-20% with 5 μg LSA/lane. For gels **A** (SDS) and **B** (native) lanes are: **1**, whole LSA; **2**, >100K LSA; **3**, <100K LSA; **4**, >30K LSA; **5**, <30K LSA. In **A** and **B** LSA fractions were washed once against a centrifuge filter of the indicated cut off size. Thrice washed LSA fractions are shown in gel **C** (SDS) and indicate that LSA can be separated into distinct fractions via centrifuge filtration. Gel **C** lanes are: **1**, >100K LSA; **2**, <100K LSA; **3**, <100K >30K LSA; **4**, <30K LSA; **5**, <3K LSA.

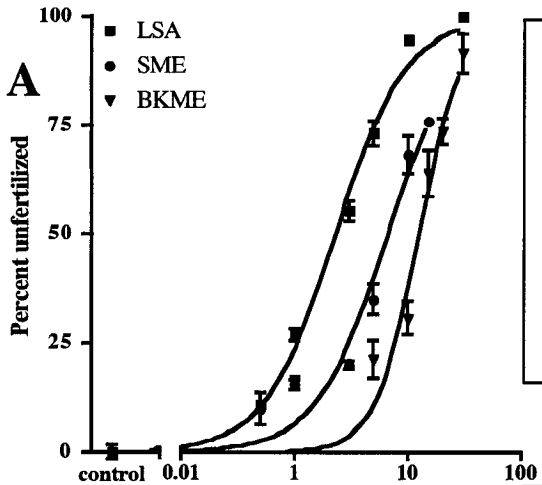
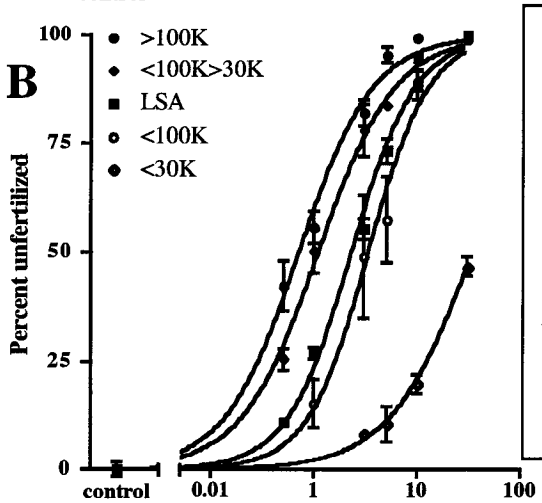


FIGURE 1.6. Inhibition of fertilization.

LDM	IC_{50}	95%C.I.	Slope	95%C.I.
LSA	2.3	2.1 to 2.5	1.33	1.23 to 1.58
SME	6.6**	5.6 to 7.8	1.40	1.04 to 1.75
BKME	12.4**	11.0 to 14.1	2.30*	1.57 to 3.01

** P<0.0001, *P=0.0063

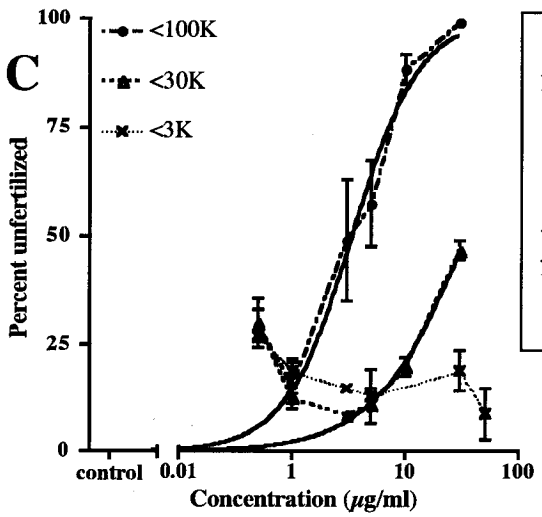
Toxicity of LSA and pulp mill effluent LDMs. Solid lines represent nonlinear sigmoidal curve fits.



Fraction	IC_{50}	95%C.I.	Slope	95%C.I.
>100K	0.7*	0.6 to 0.8	1.20	0.96 to 1.44
<100K>30K	1.1*	0.9 to 1.3	1.16	0.96 to 1.36
LSA, whole	2.3*	2.1 to 2.5	1.33	1.23 to 1.58
<100K	3.3*	2.6 to 4.3	1.49	0.87 to 2.10
<30K	≈34*	29 to 40	1.07	0.90 to 1.25

*P<0.0001

Toxicity of LSA fractions. The IC_{50} are different for each fraction, however the slopes are not significantly different (P=0.28). The highest concentration tested for <30K was 30μg/ml.



Concentration response data from LSA filtrate fractions. Each fraction shows a tendency to increase toxic response at low concentrations (<1 μg/ml) as indicated by the dashed lines. If concentrations <1 μg/ml are omitted from the <100K and <30K data sets, the remaining data fit sigmoidal response models (solid lines).

Chapter 2. The Inhibition of Acrosome Reaction Induction and Sperm to Egg Binding in the Sea Urchin by an Environmentally Present Polyanion, Lignosulfonic Acid

ABSTRACT

Breakdown products of the wood structural polymer lignin are major components in the effluent from commercial pulp and paper manufacturing mills. These lignin-derived macromolecules (LDMs) specifically inhibit a variety of cell-to-cell interactions. Our group has previously reported that a LDM from bleached Kraft mill effluent (BKME) inhibits sea urchin fertilization by binding to sperm cells and blocking the egg jelly induced acrosome reaction. Lignosulfonic acid (LSA), from sulfite mill effluent, is structurally distinct from BKME-LDM and is a more potent inhibitor of fertilization. Here the mode of action of LSA on sea urchin fertilization is characterized by examining its effects on two critical sperm-egg interactions: acrosome reaction induction and sperm-to-egg binding. The acrosome reaction in sea urchin is a receptor-ligand event induced by the fucose sulfate extracellular matrix of the egg known as the egg jelly. The egg jelly induced acrosome reaction is inhibited in a dose dependent manner when sperm are pre-exposed to LSA. Pre-exposure to LSA does not inhibit the artificial induction of the acrosome reaction with either ionomycin or NH_4Cl. The acrosome reaction is an exocytotic process that presents the protein bindin on the tip of the sperm to a sulfated glycoprotein receptor on the cell surface of the egg, resulting in sperm-to-egg binding. Sperm artificially induced to acrosome react (AR) were briefly exposed to LSA prior to egg introduction. Exposure to LSA dramatically reduced sperm-to-egg binding. In an

effort to identify cellular binding patterns for LSA, LSA was aminated with ethylenediamine then biotinylated with sulfo-NHS-LC-biotin. Biotinylated LSA (LSA-b) had a similar toxicological profile as "native" LSA for inhibition of fertilization; however, the aminated intermediate was only half as potent. LSA-b bound to the sperm head in a pattern similar to that observed for BKME-LDM. In AR sperm, the intensity of LSA-b label increased over the acrosomal region. The signal from LSA-b was quenched by the addition of a 100-fold excess of LSA. The observed binding patterns for LSA-b are consistent with its action as an inhibitor of the acrosome reaction and sperm-to-egg binding and indicate that LSA may bind to multiple cell surface proteins.

INTRODUCTION

Lignosulfonic acid (LSA, also commonly known as lignosulfonate) is a member of a family of related lignin-derived macromolecules (LDMs) that are formed as byproducts of the pulp and paper industry. LSA is derived from the sulfite pulping process whereby wood chips are extracted with acidic aqueous sulfur dioxide, resulting in the partial depolymerization, sulfonation and dissolution of lignin, to produce a cellulose fiber (Lin and Dence, 1992). The aqueous effluent of this process is believed to consist largely of the polar breakdown products of lignin such as LSA (Kringstad and Lindstrom, 1984). LSA, which ranges in molecular mass from 3 kd to several hundred kd, is a sulfonated polyanionic macromolecule that retains the substituted guaiacylpropane backbone of native lignin (Hassi, 1985; Lin and Dence, 1992; Pearl, 1967).

LSA has been shown to have interesting biological activities such as macrophage activation (Suzuki et al., 1989a) and inhibition the human immunodeficiency virus (HIV) *in vitro* (Sorimachi et al., 1990; Suzuki et al., 1989b). Similar to other polyanionic

macromolecules, notably sea urchin egg jelly fucose sulfate and fucoidan, LSA is a potent anticoagulant (Loomis and Beyer, 1953; Pereira et al., 1999). LSA is virtually nontoxic when administered orally to rats (LD_{50} 40 g/kg) and has been used for many years as an animal feed additive because of its antipepsin activity and the protection it provides against the development of gastric ulcers (Luscombe and Nicholls, 1973; Vocac and Alphin, 1968; Vocac and Alphin, 1969). As a component of pulp mill effluent the toxicologic effects of LDMs and LSA on freshwater and marine organisms have been investigated (Cherr et al., 1987; Higashi et al., 1992; Roald, 1977). Acute toxicologic effects to whole organisms were minor, however LDM derived from bleached Kraft mill effluent (BKME) has been shown to inhibit external fertilization and development in number of non-mammalian species (Cherr et al., 1993; Higashi et al., 1992; Pillai et al., 1997).

In the sea urchin, BKME-LDM inhibits fertilization without cytotoxicity. BKME-LDM binds to extracellular domains of the sperm cell and inhibits the acrosome reaction, yet pre-exposure to eggs does not inhibit fertilization (Pillai et al., 1997). The extracellular matrix of the sea urchin egg, a fucose sulfate polymer known as egg jelly, triggers the acrosome reaction. LSA inhibits fertilization in the sea urchin similar to BKME-LDM and its contraceptive activity is also aimed at the sperm cell (Chapter 1). The fertilization cascade in sea urchins can be inhibited at the level of the sperm cell in several ways including: sperm cell cytotoxicity, inhibition of acrosome reaction, inhibition of sperm-to-egg binding, and inhibition of sperm-to-egg fusion (Vacquier et al., 1995). Since the lack of cytotoxicity due to LSA was established in Chapter 1, this paper examines the ability of LSA to inhibit two initial receptor mediated and species

specific (Ohlendieck and Lennarz, 1996) events in sea urchin fertilization: acrosome reaction induction and sperm-to-egg binding. Both of these events involve the interaction of sulfated oligosaccharides with receptor proteins and have been shown to be inhibited by some sulfated polyanions (Dhume and Lennarz, 1995; Glabe et al., 1982; Pillai et al., 1997; Vacquier and Moy, 1997). Data presented herein shows that LSA inhibits both of these processes. This paper also describes the sub-cellular localization of LSA on sperm cells and lays the groundwork for subsequent receptor based investigations of LSA's mode of action.

MATERIALS AND METHODS

Chemicals

All chemicals used were obtained from Sigma or Aldrich unless otherwise specified. Lignosulfonic acid, sodium salt (LSA) was obtained from Aldrich and additionally purified as described previously.

Gamete collection.

Gametes from Pacific purple sea urchins (*Strongylocentrotus purpuratus*) were collected by injecting adults with 0.5 to 1.0 ml of 0.5-M KCl into the coelomic cavity via the oral peristomal membrane. Sperm were collected "dry" (undiluted) directly from the gonopores with a pipette and stored on ice in a capped tube. Eggs were spawned into 50-ml beakers filled with 0.7-μm filtered sea water (FSW), carefully decanted to remove large debris and kept at 4°C until used. Sperm motility and egg morphological quality were assessed using standard criteria (Chapman, 1995; Cherr et al., 1987).

Egg jelly isolation and quantification.

To isolate egg jelly, eggs were gently re-suspended and kept agitated with a slow stream of air bubbles. The pH of the suspension was lowered to 4.8-5.0 for 3-5 minutes using 1-M MES (2-morpholinoethanesulfonic acid) then raised to 7.8 with 1-M Tris (tris (hydroxymethyl) aminomethane). Eggs were sedimented by gentle hand centrifugation. The supernatant was removed and centrifuged at 30,000*g* (4°C) for 30 min (Lutz and Inoue, 1986; Weidman and Kay, 1986). The resulting egg jelly supernatant was concentrated using Centricon Plus-20 (Millipore, Billerica, MA) devices, according to manufacturers instructions, and stored in aliquots at –80°C until needed. Fucose content of egg jelly was determined by the phenol-sulfuric acid method (Dubois et al., 1956) as modified by Vacquier (Vacquier and Moy, 1997). Briefly, 0.1 ml of egg jelly was mixed with 0.1 ml of aqueous phenol (5%, v/v). The phenolic egg jelly solution was vortexed as 1 ml of concentrated H_2SO_4 was added. Absorbance (487 nm) was measured after 2 minutes at 23°C and compared to a fucose standard curve.

Acrosome reaction induction and evaluation.

Dry sperm was diluted 1:2000 (approx. 10^7 cells per ml) with 0.45-μm FSW buffered with 10-mM HEPES (pH 8.0) and pre-incubated with a dose range of LSA for 10 min at 12°C (Pillai et al., 1997; Vacquier, 1986). Acrosome reactions were induced by the addition of con-specific egg jelly or 10-μM ionomycin (Calbiochem, San Diego, CA). Ten minutes after induction, sperm were fixed with fresh 3% paraformaldehyde in artificial seawater, and 500 μl of the fixed sperm suspension was placed onto 12-mm poly-L-lysine coated glass coverslips in a 24-well ELISA plate. The plate was centrifuged at 700*g* for 10 minutes at 12°C. Coverslips were washed twice with HEPES

buffered saline (HBS, 10-mM HEPES, 150-mM NaCl, pH 8) and cells permeabilized with 0.1% Triton/ 0.2% BSA. After 2 more HBS washes, coverslips were blocked with 9% non-fat milk/HBS and a 1:40 dilution of phalloidin-Alexa 488 (Molecular Probes, Eugene OR) added. Coverslips were mounted onto glass slides with n-propyl gallate mounting media. Acrosome reacted (AR) sperm were scored using an Olympus BX50WI epifluorescence microscope under a UV-corrected 60x oil immersion objective (Figure 2.1) or via phase contrast microscopy with an Olympus BH-2 microscope and a 100x oil objective. Dim focal points of fluorescence at the sperm head, representing the F-actin-containing actomere (Tilney et al., 1978), were scored as background or non-reacted (AI, Figure 2.1 A). Bright and large or elongated fluorescence at the sperm head was scored as acrosome reacted (AR, Figure 2.1 B, C, D). Sperm from individual males was scored in replicate batches with at least 100 sperm counted per replicate. Dose response data was normalized to controls using Abbott's formula as described in Chapman (1995) and transformed to probit units to identify the 50% inhibitory concentration (IC_{50}) for LSA (Rand, 1995). For clarity, captured fluorescent pseudo-color images were overlaid on their transmitted light Nomarski differential interference contrast (DIC) counterparts using MetaMorph® image analysis software (Universal Imaging Corp., Downington, PA).

Sperm to egg binding assay.

Eggs dejellyed by gentle pH shock (as previously described) were rinsed with artificial sea water (Cavanaugh, 1978) buffered with 10-mM HEPES pH 8 (ASW-H, 490 mM NaCl, 27 mM $MgCl_2$, 28 mM $MgSO_4$, 10 mM $CaCl_2$, 10 mM KCl, 2.5 mM $NaHCO_3$) and adjusted to 10^4 eggs/ml. Fresh dry sperm was diluted 1:250 in ASW-H

containing 20-mM NH_4Cl in order to induce acrosome reaction (Garcia-Soto et al., 1985; Garcia-Soto et al., 1987). Thirty seconds were allowed for induction to commence then LSA was added to a final concentration of 5 mg/ml and the suspension gently mixed for 1 minute. Aliquots of this suspension were added to dejellyed eggs so that the final ratio of sperm to egg was 1000-3000. Sperm were allowed 30 seconds to bind eggs then the suspension was fixed with 1% glutaraldehyde. Fixed eggs were washed with ASW-H to remove unbound sperm, and then Hoechst 33342 dye was added for 5 minutes to assist in identifying sperm attached to the egg. Eggs were rinsed twice more to remove free Hoechst dye. Bound sperm were visualized on an Olympus BH-2 epifluorescence microscope with phase-contrast optics using a 40x objective and a UV filter. Sperm per egg were scored by counting the number of nuclei bound to the perimeter of the egg in the widest plane of focus in a stepwise manner (Figure 2.3 A). Only eggs where the vitelline layer had not yet elevated were scored and at least 100 eggs were scored per treatment.

LSA Conjugation with sulfo-NHS-LC-biotin.

Based on the presence of free aldehyde functional groups (Cherr et al., 1993; Hassi, 1985; Lin and Dence, 1992), LSA was biotinylated using a modified procedure for protein biotinylation (Hermanson, 1996) as we have previously described (Tollner et al., 2002). This procedure introduces an amine group at aldehyde sites in LSA then sulfo-NHS-LC-biotin binds to available amine groups (Figure 2.4 A). Purified LSA (as described above) was aminated by reaction with ethylenediamine in borate buffer. The reaction was stopped with 2-M glucose after 1 hour and the aminated LSA (LSA-NH) dialyzed extensively at 3500 molecular weight cutoff (3.5-K MWCO Slide-A-Lyzer,

Pierce Chemical Company, Rockford Il) against phosphate buffer, pH 7.2. Sulfo-NHS-LC-biotin (Pierce) was added to the dialyzed LSA-NH solution to a final concentration of 5 mM for 1 hour at room temperature. The reaction mixture was dialyzed (3.5-K MWCO) first against 5 mM HEPES, pH 8.0 for 24 hours and then into distilled water for an additional 24 hours. The resulting solution of biotinylated LSA (LSA-b) was divided into aliquots lyophilized and stored desiccated at –20°C.

Fertilization bioassays.

Fertilization bioassays were performed according to guidelines described by Chapman (1995) and Pillai et al. (1997). All experiments were conducted in ASW-H at 12°C. In order to insure that sensitivity to LSA was not diminished with excess sperm, a sperm to egg ratio resulting in 70% to ≤ 90% fertilization was determined for each batch of gametes immediately prior to experiments. Sperm concentration was determined using a hemocytometer. Sperm were pre-exposed to LSA for 10 minutes prior to the addition of eggs. Fertilization was allowed to proceed for 10 minutes then samples were fixed with 1% glutaraldehyde in ASW. In order to confirm that LSA did not affect eggs, eggs were pre-exposed to LSA for 10 minutes prior to the addition of sperm, given 10 minutes to fertilize then fixed as above. Two to four separate bioassays were performed for each LSA derivative. Each bioassay consisted of one pair of urchins run in 3-4 replicate batches with 100-300 eggs scored per replicate. Dose response data was normalized to controls using Abbott's formula as described in Chapman (1995) and transformed to probit units to identify IC_{50} values (Rand, 1995). Significant differences in IC_{50} values were evaluated using one-way ANOVA and specific differences between treatments

identified with a Bonferroni post-hoc test. Values with a $p<0.05$ were considered significantly different.

Localization of LSA on sperm.

Fresh motile sperm were fixed with 1% paraformaldehyde in ASW, diluted to 1:4000, and 500 μl aliquots added to a 24-well immunoassay plate (Nunc, Rochester NY) containing poly-L-lysine coated 12 mm round glass coverslips. Cell adhesion to coverslips was encouraged by centrifugation at 700*g* for 10 minutes. Coverslips were rinsed with HBS, nonspecific binding blocked with 9% non-fat milk in HBS, and incubated with LSA-b. After an additional HBS rinse, coverslips were immersed in ice cold 55% ethanol/HBS for 10 minutes to stabilize LSA binding (Pillai et al., 1997), then rinsed again with HBS and re-blocked with Pierce SuperBlock (Pierce Chemical Co., Rockford Il). Monoclonal mouse anti-biotin conjugated with Alexa 488 (Molecular Probes, Eugene OR) was added at a 1:100 dilution in SuperBlock for 1 hour, coverslips given a final rinse with HBS and mounted onto slides with n-propylene glycol mounting media and sealed with Revlon "Orange Flip" nail polish. Cells were evaluated on an Olympus BX50WI epifluorescence microscope using a UV-corrected 60x oil immersion objective. Captured fluorescent pseudo-color images were overlaid on their transmitted light Nomarski DIC counterparts using MetaMorph image analysis software. All images were captured with the same microscope, camera and software settings. No non-specific binding to sperm (+/- LSA) was observed in control slides where con-specific serum or ascites was substituted for antibody.

RESULTS

LSA inhibits the egg jelly induced acrosome reaction.

Acrosome reaction induction experiments were performed with the minimum concentration of egg jelly that produced at least 50% AR sperm. In order to determine this optimal egg jelly concentration, an egg jelly dose-response for acrosome reaction induction was evaluated at 0, 2, 6, 20, and 47 μg fucose equivalents/ml +/- 0.1 mg/ml LSA. The lowest optimal concentration was 20 μg fucose equivalents/ml for this isolated egg jelly. This egg jelly concentration induced acrosome reaction in an average of 58.5% of sperm at a cell density of 10^7 sperm/ml (Figures 2.1 B, 2.2 A). In the presence of 0.1 mg/ml LSA, the efficacy of this concentration of egg jelly to induce acrosome reaction was reduced to 20.5% (Figures 2.1 D, 2.2 A). As a positive control for each male, AR sperm were produced with ionomycin (10 μM) +/- 1.0 mg/ml LSA. Approximately 90% of sperm underwent acrosome reaction in the presence of ionophore irrespective of the presence of LSA (Figures 2.1 C, 2.2 A). A dose response relationship for LSA was evaluated using the egg jelly induced acrosome reaction (at 20 μg fucose/ml egg jelly) as an endpoint (Figure 2.2 B). The IC_{50} for inhibition of acrosome reaction was 0.063 mg/ml LSA at 10^7 sperm/ml.

LSA inhibits sperm to egg binding.

Sperm-to-egg binding was assessed microscopically by counting Hoechst 33342 stained sperm nuclei on the egg surface (Figure 2.3 A, B). Since the incubation period with Hoechst was brief, egg nuclei were not stained. Sperm binding to the egg was reduced by an order of magnitude in response to a brief pre-exposure to LSA (Figure 2.3

C). LSA inhibited sperm-to-egg binding whether or not sperm were induced to acrosome react.

Establishment of a stable LSA-biotin conjugate as a probe.

The two-step reaction scheme for LSA biotinylation is shown in Figure 2.4 A. Ethylenediamine was used to aminate free aldehyde groups on LSA then a biotin tag was conjugated to available amine groups with sulfo-NHS-LC-biotin (see Methods). Several attempts were made to attach labels to LSA using hydrazide methodology as previously described (Pillai et al., 1997). These attempts were successful, however the biotinylated product formed via hydrazide chemistry was less stable over time compared to the amination product (data not shown).

Fertilization bioassay with LSA conjugates.

LSA derivatives inhibit fertilization, however they differed in their potency (Figure 2.4 B). Slopes of probit regression were parallel and calculated IC_{50} values for LSA and LSA-b were not significantly different. By comparison, the intermediate product LSA-NH, was approximately 3-fold less potent (higher IC_{50}, p=0.002, 0.01 for LSA and LSA-b respectively) and exhibited a shallower probit slope. A change in IC_{50} and slope may indicate that the interaction of LSA-NH with sperm cells could be altered from that of LSA or LSA-b.

Localization of LSA-biotin on sea urchin sperm.

LSA-b bound to sperm cells in specific labeling patterns. Signal from the Alexa-488 label occurred over the head of the sperm in either AR or AI cells (Figure 2.5). Neither midpiece nor tail were labeled. The binding pattern in AI sperm was similar to

that of other LDMs (Pillai et al., 1997). In AR sperm, there was a marked localized increase in label intensity over the acrosomal region (Figure 2.5 B). Several different controls were used to evaluate binding specificity. No label was observed in control slides incubated with unconjugated LSA and secondary antibody. Very little to no label was observed in slides co-incubated with LSA-b and a 100-fold excess of unconjugated LSA (Figure 2.5 F). Control sperm, permeabilized with Triton prior to LSA-b addition showed a signal distributed throughout the cell (Figure 2.5 E), an indication that the signal seen in unpermeabilized sperm was extracellular. LSA binding was "fixed" via precipitation by the addition of 55% ice-cold ethanol according to the procedure of Pillai et al. (1997), in most labeling experiments. Controls that omitted this ethanol fixation step displayed similar, yet more specific binding patterns compared to ethanol fixed slides, particularly in AR sperm (Figure 2.5 C, D).

DISCUSSION

Lignin is one of the most abundant organic polymers on earth. It is a structural molecule found in all woody plants generally made up of randomly coupled substituted phenylpropane units (guaiacyl- and syringyl-), which form a three dimensional structurally heterologous scaffold (Pearl, 1967). The exact structure of lignin has eluded definition since the composition of the molecule varies across species and among individuals making it one of the most complex natural polymers known in terms of structure and heterogeneity (Lin and Dence, 1992). Lignin-derived macromolecules are generally polyanionic breakdown products of lignin that result from the commercial pulping of wood. Despite the fundamentally different chemical procedures used for lignin dissolution and the heterologous chemical nature of lignin itself, LDMs from

different pulp mill processes have surprisingly similar and specific biological activities (Chapter 1). Evidence presented here shows that LSA, an LDM from acid sulfite mills, binds to domains on the head of sea urchin sperm and inhibits two species-specific receptor mediated events in fertilization: acrosome reaction and sperm-to-egg binding.

In an effort to identify binding patterns for LSA, a novel procedure to create a biotinylated LSA probe for cellular labeling was developed. Several attempts were made to attach labels to LSA using hydrazide methodology as previously described (Pillai et al., 1997). While these methods were successful, biotinylated LSA thus labeled seemed to deteriorate over time (data not shown). The deterioration may be due to the breakdown of the hydrazone linkage created when using this chemistry. Unstable hydrazone linkages are typically reduced with sodium cyanoborohydride, in order to increase to reaction efficiency when carbohydrates are labeled (Hermanson, 1996). However, exposing LDMs to reducing agents has been shown to reduce their bioactivity (G. Cherr and R. Higashi, unpublished observations). The amine procedure (Figure 2.4 A) avoids the need to reduce the product in order to create a stable conjugation. Moreover, wide varieties of probes are commercially available, incorporating reactive sulfo-NHS esters as functional groups (Pierce Chemical Co., Rockford Il), which can be directly substituted in place of sulfo-NHS-LC-biotin according to this procedure.

Several attempts were made to quantify the extent of biotinylation on LSA using the HABA (2-(4'-hydroxyazobenzene) benzoic acid, Pierce Chemical Company, Rockford, Il) assay as described by Pillai (1997) for LDM from bleached Kraft mill effluent. This assay relies on a quantitative decrease of absorbance (at 500 nm) when biotin is added to a solution of HABA dye and avidin. While the assay worked with free

biotin in solution, it was not successful with LSA. Increasing concentrations of LSA and LSA-b resulted in increasing absorbance values. One explanation may be that the avidin protein bound to LSA, since changes in absorbance were concentration dependent regardless of its biotinylation status. Non-biotinylated LSA did not interact with NeutrAvidin™ (Pierce) nor streptavidin, however, neither of these molecules may be substituted in the HABA assay for avidin (Pierce Chemical Company, personal communication). NeutrAvidin™ is an avidin derivative that has been deglycosylated and had charged groups on its surface modified to give a relatively neutral isoelectric point. If LSA binds to avidin, presumably LDM from BKME does not. This observation illustrates how LDM's from different sources have different affinity for biological molecules. Such a difference suggests that a relationship exists between the structure of LDMs and their activity. One difference between the two LDM molecules that has been related to differences in biological activity is their molecular weight ranges (Chapter 1).

Somewhat surprisingly, the intermediate conjugation product, amine-LSA (LSA-NH), was less effective at fertilization inhibition than the biotinylated final product (LSA-b) and the original material (Figure 2.4 A, B). One explanation of this result is that the addition of the amine cation to LSA actually changed the way it interacts with binding sites on the sperm surface and/or altered the way anionic groups are presented on the surface of LSA-NH. Ethylenediamine will react with carboxyl groups in LSA as well as aldehydes (Hermanson, 1996; Lin and Dence, 1992). In neutral pH ranges, for every carboxyl that is replaced by an amine, there is a net change in charge of +2 (negative to neutral to positive) and for aldehydes the net change is +1 (neutral to positive). Adding such cationic functionalities to the structure of LSA would likely result in conformational

changes as salt bridges are formed between cationic (amine) and anionic (sulfonate) groups. Replacing the amine cation with neutral biotin results in a net change in charge of –1 at each point of substitution and would liberate LSA-b's anionic groups so that they can interact with the sperm surface more like "native" LSA. These results illustrate the importance of anionic charge and conformation to the overall bioactivity of LSA. Gestwicki (2002) points out that altering the valency of a multivalent biologically active ligand (like LSA) can alter its activity.

LSA binds to sea urchin sperm in a pattern similar to that reported for other LDMs (Pillai et al., 1997). Here the binding of LSA to acrosome reacted sea urchin sperm is reported for the first time (Figure 2.5). The specific pattern of LSA-b binding suggests that it may be interacting with specific cell surface receptors. The secondary antibody did not recognize unconjugated LSA and a 100x concentration of unconjugated LSA quenched the labeling specificity of LSA-b (Figure 2.5 F). The latter observation is an additional indication that LSA is binding to specific cell receptor sites. Due to the size and polarity of LSA it is thought to interact with extracellular domains of the sperm. Cells permeabilized with Triton X-100 prior to LSA-b treatment were labeled throughout the cell (Figure 2.5 E) in contrast to the localized labeling of unpermeabilized cells. These results confirm that LSA binds to localized surface sites on unpermeabilized cells. LSA binding is thought to be stabilized or "fixed" by the addition of 55% ethanol prior to slide processing (Pillai et al., 1997). Ethanol is known to be able to precipitate LSA out of aqueous solutions (Hassi, 1985; Lin and Dence, 1992) and may actually precipitate LSA onto the surface of the cell. In AI sperm, the general binding pattern was similar between ethanol treated and untreated cells (Figure 2.5 A, C). The label was localized to

the sperm head in both, but appeared more punctate in cells without ethanol. A more dramatic difference was seen in AR sperm, where the label in ethanol untreated sperm was focused on the acrosomal tip rather than over the entire head (Figure 2.5 B, D). Without further experimentation the reasons for this difference are difficult to explain. It is known that some sperm membrane proteins are shed during AR. What is clear is that in AR sperm LSA displays a strong affinity for the acrosomal tip, presumably the protein bindin. Other sulfated macromolecules have been reported to bind to sea urchin sperm bindin and to domains on mammalian sperm (DeAngelis and Glabe, 1987; DeAngelis and Glabe, 1990; Huang and Yanagimachi, 1984; Mahony et al., 1993). Using the same labeling techniques, we have recently reported the binding patterns of LSA on macaque sperm (Tollner et al., 2002).

The jelly coat of the egg stimulates the echinoid sperm acrosome reaction. The egg jelly component of the sea urchin egg consists of a large fucose-sulfate polymer mesh with associated proteins. While the majority, if not all, of the acrosome reaction inducing activity has been attributed to the purified fucose sulfate polymer (Alves et al., 1997; Vacquier and Moy, 1997) attaining greater than about 75% AR sperm requires whole egg jelly, including associated proteins (Vacquier and Moy, 1997). Female *S. purpuratus* can produce two structurally distinct types of egg jelly sulfated fucan, each with equal overall potency for acrosome reaction induction (Alves et al., 1998). Sperm from individual males, however, show considerable variability in response to the purified fucose sulfate isotypes (Vacquier and Moy, 1997). Since whole egg jelly was reportedly capable of full AR induction and the role that associated proteins play in the fertilization process

remains unclear, whole egg jelly was used for these investigations into the effects of LSA on fertilization rather than purified fucose-sulfate polymer.

The effects of LSA on the egg jelly induced acrosome reaction were evaluated. Since the actual concentration of jelly surrounding the egg is unknown, an optimal functional concentration was defined in order to approximate biological conditions. The optimal concentration of isolated egg jelly (20 μg fucose/ml) was defined as the concentration that induced greater than 50% acrosome reaction at a given sperm concentration. This concentration of egg jelly produced an average of 59% AR sperm cells from all males assayed (Figure 2.2 A). When sperm were exposed to 0.1 mg/ml LSA the incidence of acrosome reaction in response to egg jelly dropped to 21%. In the dose-response assay for acrosome reaction induction, the concentration of LSA that inhibits egg jelly induced acrosome reaction in 50% of sperm (IC_{50}, normalized to controls) is 0.063 mg/ml (Figure 2.2 B). There was a mean induction of 90% acrosome reaction using the Ca^{+2} ionophore ionomycin as a positive control (Figure 2.2 A). The presence of LSA had no effect on the ionophore induced acrosome reaction (Figure 2.1 C), similar to the results reported for BKME-LDM by Pillai et al. (1997). That sperm are still capable of ionophore induced acrosome reaction in the presence of LSA suggests that the Ca^{+2}-sensitive intracellular signaling processes, which mediate the acrosome reaction (Lievano et al., 1990), are still functional despite a high concentration of extracellular LSA. LSA's inhibition of the acrosome reaction must occur at a point prior to Ca^{+2} channel activation.

LSA likely exerts its bioactivity by binding to the sperm cell surface (Figure 2.5). It has been observed that when sperm are exposed to LDM containing seawater, then

removed by centrifugation, the toxicity of the supernatant seawater, in subsequent fertilization assays, is reduced (Cherr, unpublished data). The interpretation of this data is that sperm cells adsorb LDM and reduce its free concentration in solution. Therefore, any evaluation of LSA toxicity or bioactivity must be made in the context of the cell density used. For example, while the IC_{50} for acrosome reaction inhibition (Figure 2.2 B, 63 μg/ml) is much higher than that for fertilization inhibition (Figure 2.4 B, 2 μg/ml), the sperm concentration used in the acrosome reaction induction assay is also greater (by 2 orders of magnitude). Inhibition of the acrosome reaction is a more sensitive endpoint when one considers the concentration of sperm used.

The addition of 20 mM NH_4Cl to ASW elicits acrosome reaction in sea urchin, which is morphologically similar to that triggered by egg jelly, via an intracellular alkalinization of the sperm cell by NH_3 (Collins and Epel, 1977; Garcia-Soto et al., 1987). This method of induction was chosen over ionomycin for analysis of sperm-to-egg binding since ionomycin does result in some morphological distortion of the sperm cell (rounded heads as seen in Figure 2.5 B, D), which may have some effect on binding. Egg jelly could not be used since it would occupy potential binding sites on the sperm surface that LSA could occupy such as the bindin protein (DeAngelis and Glabe, 1987). To assess LSA effects on sperm-to-egg binding, sperm were exposed to a high concentration of LSA (5 mg/ml) for a very short duration (30 seconds) prior to egg introduction. Since the objective of this experiment was to show effect, not generate a dose response, enough LSA was used in order to insure an effect would be seen if there was one (Figure 2.3 A, B). Sperm binding was reduced by an order of magnitude with LSA pre-exposure. Sperm motility in 5 mg/ml LSA was equivalent to controls. When

compared to non-induced controls, induction of the acrosome reaction with NH_4Cl seems to have some effect on sperm-to-egg binding (Figure 2.3 C). This is to be expected since sperm rapidly lose the capacity to fertilize after AR induction (Vacquier, 1979). Furthermore, the timing of this experiment was crucial. The assessment of sperm-to-egg binding is kinetically dependent. Sperm bind to the egg during the first 25 seconds of exposure until one successfully penetrates the vitelline layer stimulating cortical granule breakdown and elevation of the visible vitelline envelope. From 25 to 50 seconds post-exposure sperm detach from the egg as cortical granule breakdown progresses (Vacquier and Payne, 1973).

Binding and subsequent fusion of sperm and eggs is thought to be the result from a multi-step interaction of the protein bindin, presented on the acrosomal process of the sperm, with a sulfated glycoprotein receptor on the egg surface distinct from the egg jelly (Stears and Lennarz, 1997; Vacquier et al., 1995). Bindin, a 24-kDa lectin protein containing many basic amino acids (DeAngelis and Glabe, 1988), interacts with three elements on its egg receptor: two peptide domains and sulfated O-linked oligosaccharide chains (non-species specific) (Stears and Lennarz, 1997). Work done by Dhume et al. (Dhume and Lennarz, 1995; Dhume et al., 1996) has shown that these same sulfated oligosaccharides are capable of inhibiting fertilization when sperm are pre-treated, and their potency increases with increasing valency. Multivalency alone does not determine biological activity, however, since dextran sulfate does not interfere with fertilization. Egg sulfated oligosaccharides are observed to only bind to AR sperm, whereas LSA binds to acrosome intact and reacted sperm. The egg sulfated oligosaccharides are thought to stabilize sperm-to-egg binding (Dhume et al., 1996). In AR sperm, LSA may

directly compete with sulfated oligosaccharides of the egg receptor for sperm bindin to prevent or destabilize binding. If LSA exposed sperm bind to an egg via a peptide recognition element, LSA may act to enhance the kinetics of sperm unbinding resulting in early detachment prior to sperm egg fusion. While it is likely that LSA inhibits bindin from interacting with the sulfated oligosaccharide chains, the possibility that LSA may also inhibit interaction with peptide domains of the egg receptor can not be ruled out.

Other multivalent sulfate- and sulfonate- containing polymers inhibit bindin activity *in vitro*. DeAngelis and Glabe (1990) identified several characteristics of the polyanions that contribute to their inhibitory activity: large size; the presence of multivalent sulfate- or sulfonate- groups; and proper spatial conformation of the anion. The polymeric backbone did not seem to be a critical factor in protein interaction except for proper anion presentation. These observations are consistent with the physico-chemical characteristics of LSA. The authors describe a model of sulfate- protein interaction, which involves the formation of a resonating, cyclic bonding system between the guanido group of arginine and the non-ester oxygen atoms of the sulfate moiety (DeAngelis and Glabe, 1988). Bindin's interaction with sulfated oligosaccharides of the egg receptor for sperm has been identified as a non-species specific component of sperm-to-egg binding (Stears and Lennarz, 1997). That the interaction is non-species specific may explain why many structurally diverse ligands have affinity for bindin. Bindin is a probable target for LSA's inhibition of sperm-to-egg binding. Chapter three takes a closer look at the interaction between LSA and bindin on acrosome reacted sea urchin sperm.

Interestingly, most of the polyanions that have affinity for the bindin protein *in vitro* do not inhibit fertilization when pre-incubated with sperm. The exceptions are

sulfated oligosaccharides from the egg receptor for sperm (Dhume and Lennarz, 1995) and the egg jelly fucose sulfate polymer (Glabe et al., 1982; Vacquier et al., 1979) and LSA. Fucose-sulfate polymer fragments block the acrosome reaction (Hirohashi and Vacquier, 2002). Most inhibitors of the acrosome reaction target ion movement through channels (Christen et al., 1983; Collins and Epel, 1977), but very few interfere with receptor-ligand interaction. The few receptor-ligand blockers that have been identified are species specific i.e. antibodies (Podell and Vacquier, 1984) and fucose sulfate polymer fragments. Perhaps this is a reflection of the evolved stringent species specificity of the acrosome reaction (Alves et al., 1998; Hirohashi et al., 2002b). The ability of a structurally heterologous molecule like LSA to inhibit these species-specific events is unique. LSA's binding pattern suggests that it is binding to specific proteins on the sperm surface. These proteins may be receptors for egg jelly ("suREJ") (Mengerink et al., 2000). One suREJ protein, suREJ3, is localized to the sperm head plasma membrane over the acrosome, overlapping the binding pattern of LSA. In chapter three, I will explore the interaction between LSA and suREJ3.

Lignin-derived macromolecules are unique inhibitors of sperm physiology. It is remarkable that such structurally heterologous molecules inhibit specific receptor-ligand events involving unrelated proteins. Data presented thus far strongly suggest that structural features of LSA contribute to its overall biological activity, notably poly-sulfonation, large molecular size, and the architectural conformation or presentation of sulfonate valences. It is possible that further analysis of the structure of LSA and the conformation of sulfonate groups could yield molecules with more targeted biological activities.

FIGURES

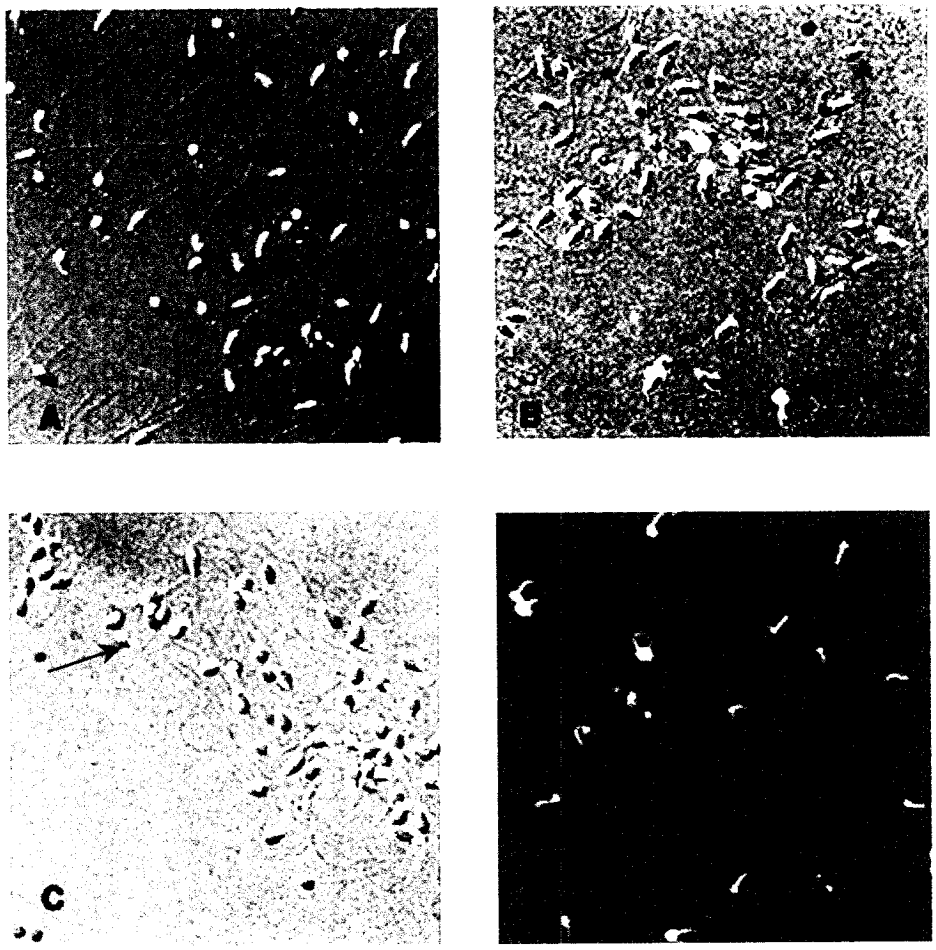


FIGURE 2.1: Detection of acrosome reaction with phalloidin-Alexa 488. Photomicrographs are fluorescent images overlaid on their DIC counterparts. Solid arrows indicate AR sperm; dashed arrows indicate AI sperm. **(A)** Background AI cells; **(B)** Egg jelly induced AR cells (20μg fuc equiv/ml EJ); **(C)** Ionomycin induced AR cells (1.0mg/ml LSA, 10μM ionomycin); **(D)** Egg jelly induced AR cells inhibited with LSA (0.1mg/ml LSA, 20μg fuc equiv/ml EJ).

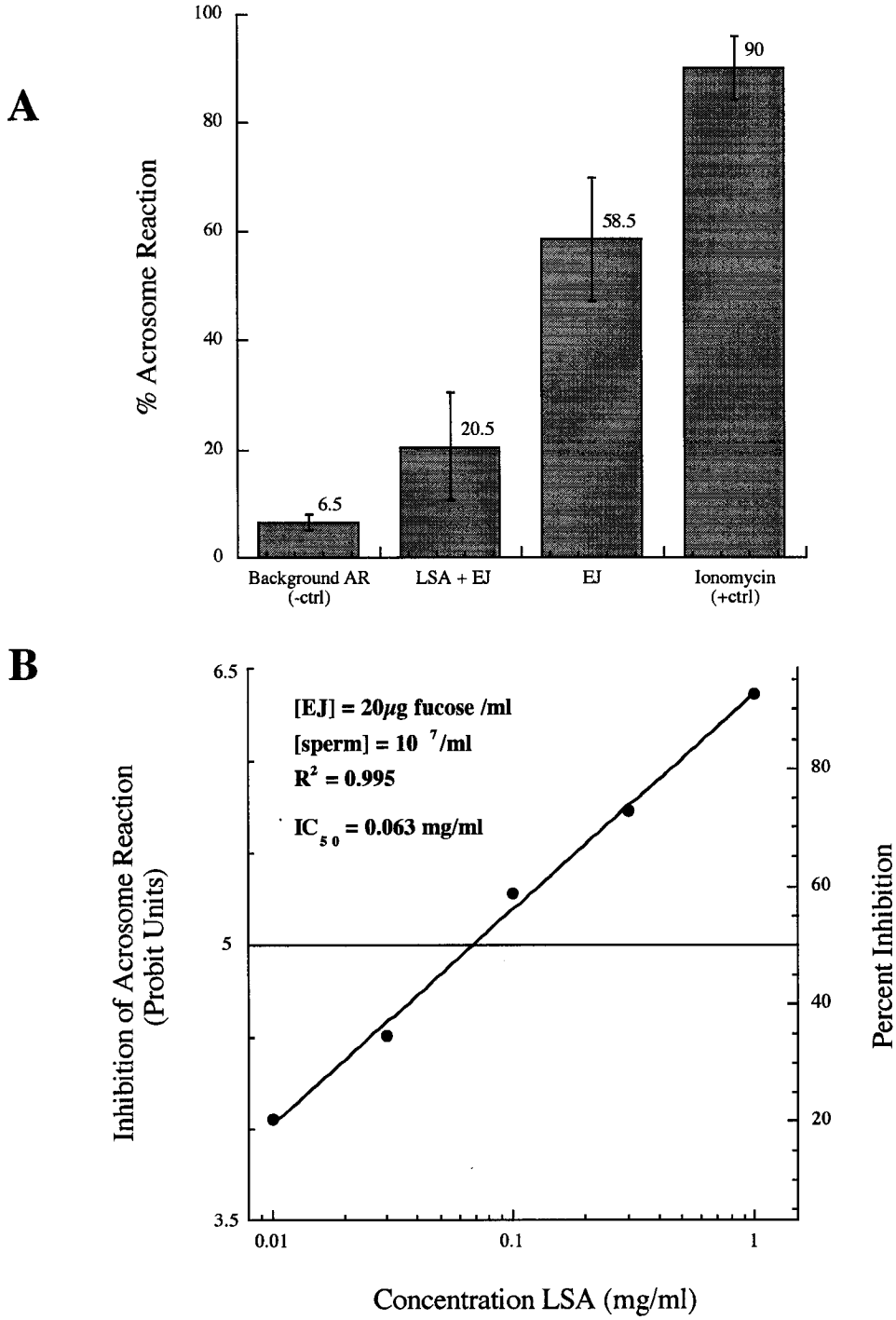


FIGURE 2.2: **(A)** LSA (0.1 mg/ml) inhibits egg jelly induced acrosome reaction at a biologically relevant concentration. **(B)** Dose-response graph, shown as probit, for inhibition of acrosome reaction by LSA. Right side ordinate indicates percent inhibition. Each data point represents 2 individual males with at least 300 sperm scored per individual. Data were normalized according to Abbots formula prior to probit transformation.

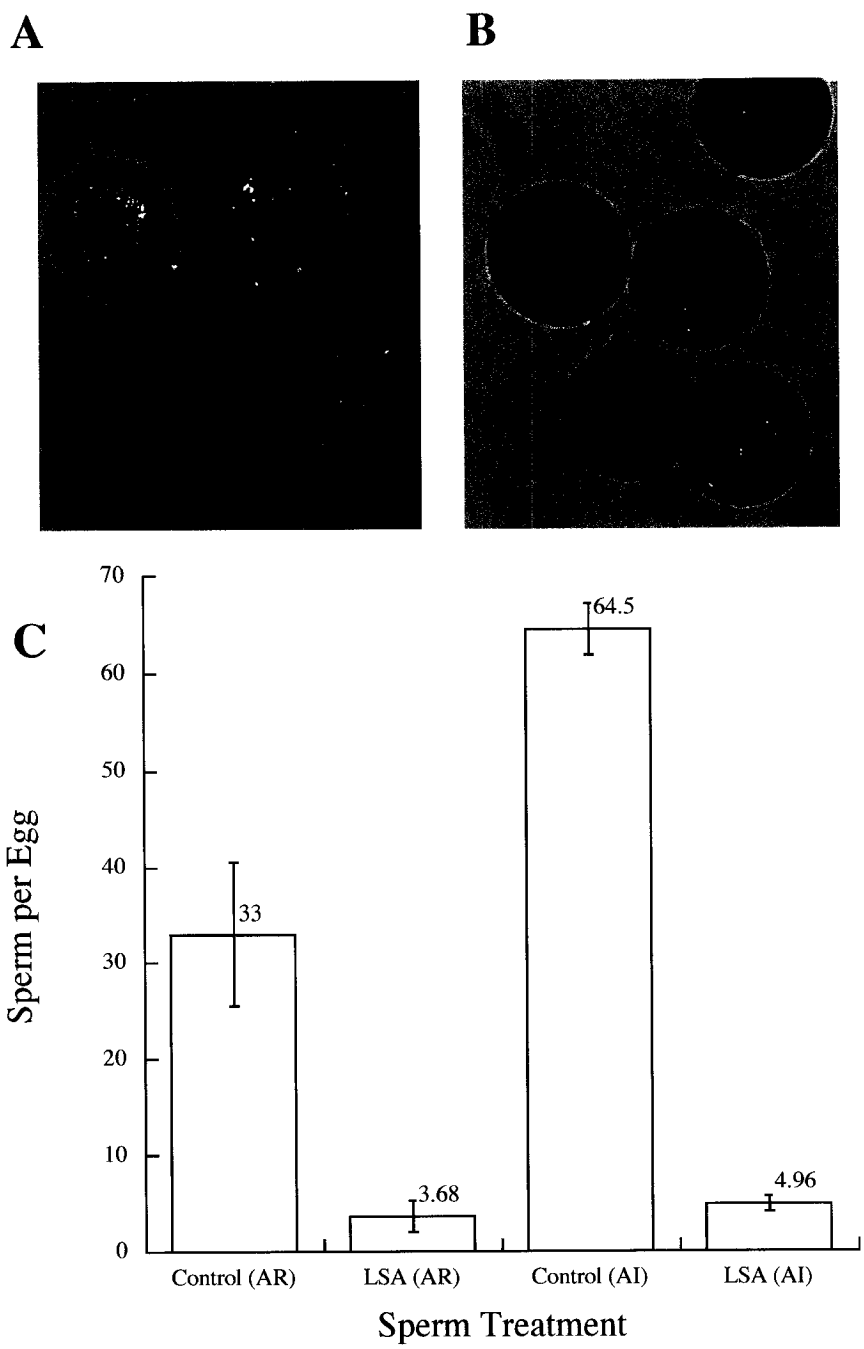


FIGURE 2.3: LSA inhibits sperm to egg binding. In the photomicrographs, Hoechst stained sperm nuclei show up as bright spots on the surface of dejellied sea urchin eggs when exposed to UV light **(A)** control; **(B)** LSA pre-exposure. **(C)** Sperm binding to de-jellied eggs decreases by 10-fold with LSA pre-exposure.

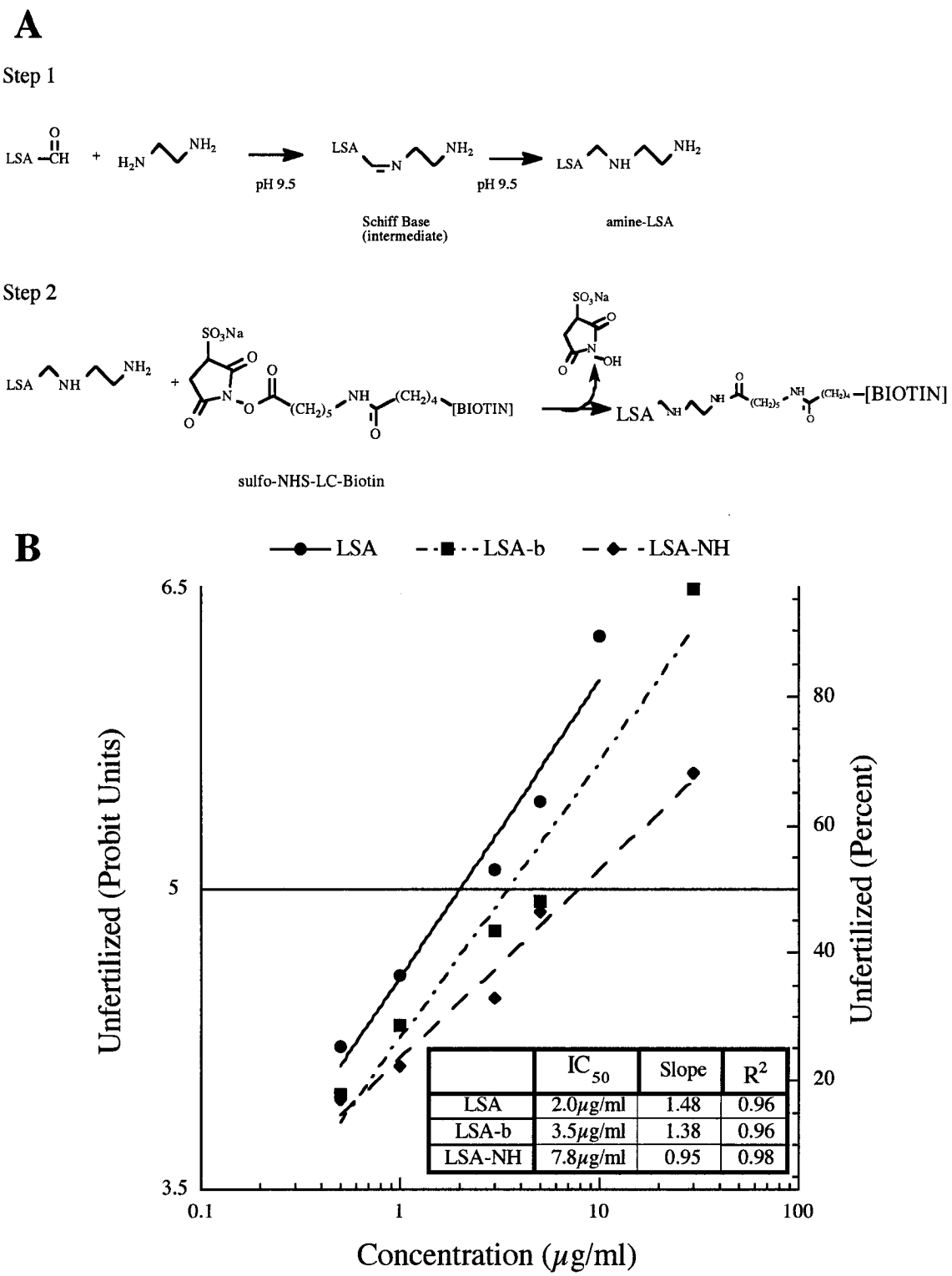


FIGURE 2.4: **(A)** LSA conjugation reaction scheme. Step 1 LSA amination. Step 2 LSA biotinylation. **(B)** Inhibition of fertilization by LSA derivatives expressed as probit.

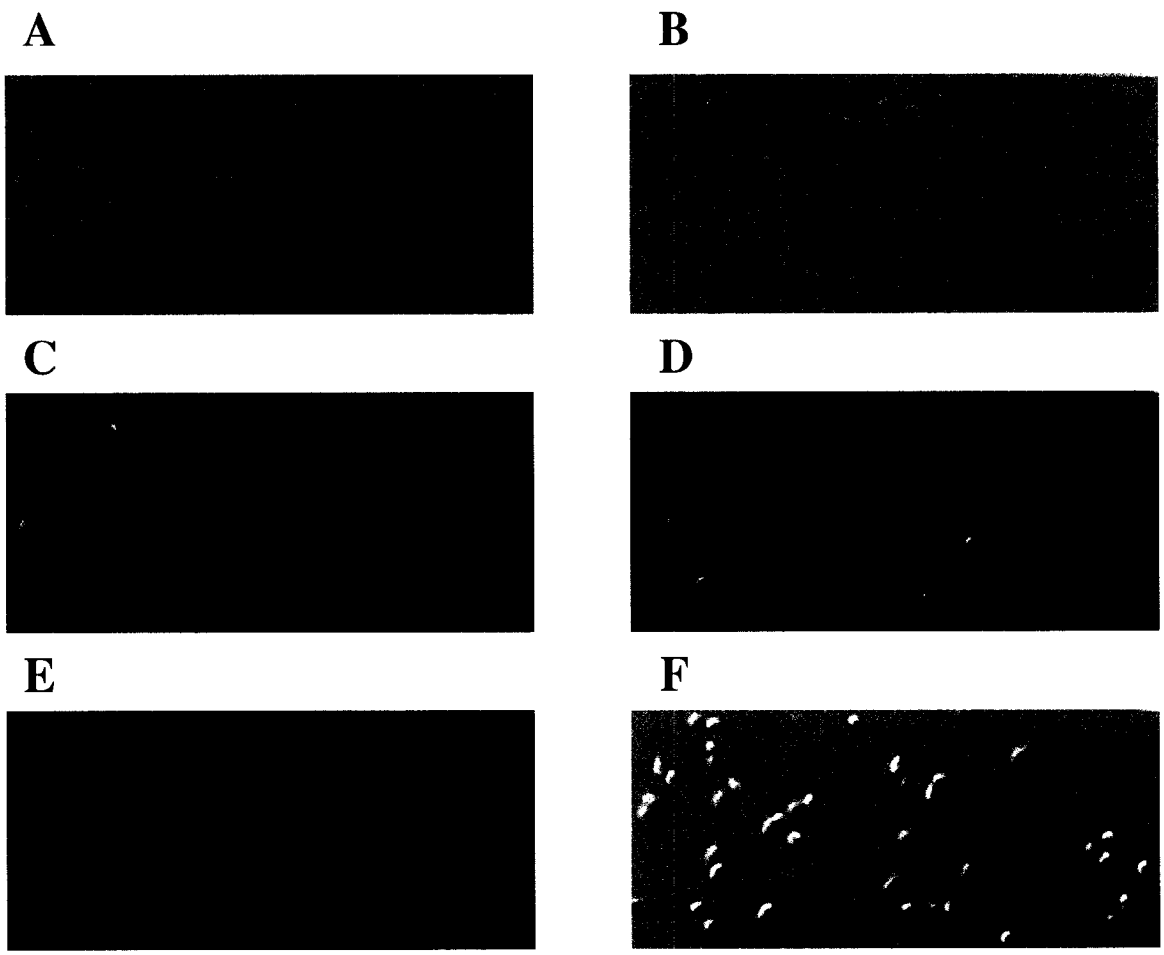


FIGURE 2.5: LSA-b bound to AI and AR sperm detected with mouse anti-biotin Alexa 488. Photomicrographs are fluorescent pseudo-color images overlaid on their DIC counterparts. **(A)** AI sperm; **(B)** AR sperm, ionomycin; **(C)** AI sperm, no ethanol step; **(D)** AR (ionomycin) sperm, no ethanol step; **(E)** AI triton permeabilized sperm; **(F)** AR, 100x unlabeled LSA control.

Chapter 3. Lignosulfonic Acid Specifically Binds to Sea Urchin Sperm Cell Surface Proteins, suREJ3 and Bindin.

ABSTRACT

Lignosulfonic acid (LSA) is a polydisperse macromolecular breakdown product of lignin produced as a byproduct of the sulfite pulping of wood. This polyanion and related lignin derived macromolecules are potent inhibitors of fertilization in diverse species, which act by interfering with specific aspects of sperm physiology. In the sea urchin, LSA binds to domains on the sperm head and inhibits two receptor-ligand mediated events required for fertilization, acrosome reaction induction and sperm to egg binding. A quantitative solid phase whole-cell binding assay was developed to characterize the binding behavior of biotinylated LSA (LSA-b) to cell surface sites on sea urchin sperm. The binding of LSA-b to sperm was specific and saturable indicating a finite number of binding sites. Acrosome reacted (AR) sperm had a greater capacity to bind LSA-b than acrosome intact (AI) cells. The binding of LSA-b to sperm had a kinetic component that is also concentration dependent. Structurally diverse polyanionic competitors including an endogenous ligand, sea urchin egg jelly, inhibited LSA-b binding to various degrees. The efficacy of competitive polyanions to inhibit LSA-b binding to sperm cells parallels their ability to inhibit fertilization. Charge density alone, however, does not govern the ability of LSA to bind sperm surface domains suggesting that other structural features of the macromolecule are involved. LSA significantly inhibited the binding of antibodies against sperm surface proteins, suREJ3 and bindin, which are key regulatory molecules respectively involved in acrosome reaction and

sperm-to-egg binding. These data support the hypothesis that the mechanism by which LSA inhibits fertilization involves binding to the receptor proteins suREJ3 and bindin.

INTRODUCTION

Sulfated polysaccharides have been identified in diverse phyla as ligands for receptor proteins that mediate key fertilization events (Dell et al., 1999; Mengerink and Vacquier, 2001; Vacquier, 1998). In the sea urchin (*Strongylocentrotus purpuratus*), the fucose sulfate polymer fraction of the egg extracellular matrix (egg jelly) prepares the sperm cell for fertilization by inducing the acrosome reaction. The fucose sulfate polymer of the egg jelly is thought to interact with a family of signaling proteins on the sperm cell surface known as suREJ, sea urchin receptor for egg jelly (Mengerink et al., 2000). The binding of egg jelly fucose sulfate polymer to suREJ initiates a cascade of intracellular signaling events culminating in the exocytotic extension of the acrosomal filament (Shapiro et al., 1990). The acrosomal filament is coated with the protein bindin. Bindin attaches to polypeptides and sulfated oligosaccharides on the egg surface resulting in sperm to egg binding and eventual cell fusion (Dhume et al., 1996). Both acrosome reaction induction and sperm-to-egg binding are mediated by species specific ligands (Glabe and Lennarz, 1979; Hirohashi et al., 2002a).

Lignosulfonic acid (LSA), a sulfonated macromolecular polyanion derived from pulp mill effluent, is a potent contraceptive (Tollner et al., 2002). In the sea urchin, LSA specifically inhibits acrosome reaction induction and sperm to egg binding, fertilization events mediated by distinct sulfated polysaccharides. LSA binds to specific cell surface domains on the sperm cell and is thought to mediate its biological action by interfering with protein receptors for endogenous sulfated polysaccharides. Other investigators have

reported that LSA inhibits protease activity (Naess et al., 1973), microbiologically active substances (Naess and Sandvik, 1973), viral infectivity (Sorimachi et al., 1990; Ward and Tankersley, 1980) as well as cell adhesion and growth (Sorimachi, 1992). These specific biological actions are counterintuitive when one considers that LSA is a polydisperse heterologous breakdown product of lignin with an undefined molecular architecture. LSA's backbone structure consists largely of lignacious aromatic alcohol polymers that have been heavily sulfonated during the pulping process (Hassi, 1985). The presence of many sulfonate moieties governs LSA's polarity despite a hydrophobic backbone and a molecular weight range from 3k to <300K.

Multivalent sulfated or sulfonated polyanions are capable of acting as effectors or inhibitors of extracellular biological interactions. Investigations into the structural basis for biological activity of these and other multivalent ligands have consistently revealed that multivalency is required for activity and not the backbone structure *per se* (DeAngelis and Glabe, 1990; Gestwicki et al., 2002; Haroun-Bouhedja et al., 2000; Moreno et al., 2001). The backbone structure of multivalent ligands does contribute to their overall potency by presenting recognition elements in a proper spatial orientation (Gestwicki et al., 2002). Large molecular weight is also another key contributor to biological activity. Data presented in Chapter 1 demonstrated that larger fractions of LSA are better inhibitors of fertilization than smaller molecular size fractions. Similarly, DeAngelis showed that larger molecular weight fractions of fucoidan (a.k.a. fucoidin), a sulfated fucan isolated from the marine alga *Fucus spp.* (Bilan et al., 2002), have a better affinity for sea urchin bindin (DeAngelis and Glabe, 1987). Due to the size and polarity of sulfated polyanions their principal effects occur at the level of the cell surface

(Kiessling et al., 2000; Lynch et al., 1994) and it has been shown that some interact with specific protein receptors. Sulfated and sulfonated polyanions that inhibit HIV infectivity interact with specific cell surface proteins (McClure et al., 1992) and inhibit the binding of monoclonal antibodies (Parish et al., 1990). Heparin-mimicking polyanions, such as polyanetholesulfonic acid, bind to and antagonize fibroblast growth factor (Liekens et al., 1999). In addition to binding sea urchin bindin, fucoidan also binds to mammalian sperm proacrosin/acrosin and prevents antibody binding to polysulfate binding epitopes of the protein (Moreno et al., 2002).

The present study characterizes the interaction of LSA with binding sites on the sperm surface using a quantitative solid phase whole-cell binding assay and a biotinylated LSA (LSA-b) probe. LSA-b behaved in a manner consistent with receptor-ligand binding models. LSA inhibited the binding of antibodies to proteins that entirely co-localize within its binding domain, suREJ3 and bindin. These results support the hypothesis that LSA exerts its biological action by binding to key molecules involved in gamete recognition.

MATERIALS AND METHODS

Reagents

All chemicals used were obtained from Sigma or Aldrich unless otherwise specified. Lignosulfonic acid, sodium salt (LSA) was obtained from Aldrich and additionally purified and biotinylated by the amine method as previously described (Chapter 2) using sulfo-NHS-LC-biotin (Pierce Chemical Company, Rockford, Il). Dr. Victor Vacquier graciously donated affinity-purified antibodies to *S. purpuratus* sperm surface proteins, suREJ3 and 350kD. Two antibodies to suREJ3 were used, anti-S/C (chicken) and anti-IH

(rabbit) (Mengerink et al., 2002) as well as a mouse monoclonal antibody (anti-J18/5) to a 350kD sperm surface protein. An affinity-purified rabbit antibody to *S. purpuratus* bindin protein was a generous gift from Dr. Charles Glabe. Alexa-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR).

Gamete collection.

Gametes from Pacific purple sea urchins (*Strongylocentrotus purpuratus*) were collected by injecting adults with 0.5 to 1.0 ml of 0.5-M KCl into the coelomic cavity via the oral peristomal membrane. Sperm were collected "dry" (undiluted) directly from the gonopores with a pipette and stored on ice in a capped tube. Eggs were spawned into 50-ml beakers filled with 0.7-μm filtered sea water (FSW), carefully decanted to remove large debris and kept at 4°C until used. Sperm motility and egg morphological quality were assessed microscopically for each batch of gametes (Chapman, 1995; Cherr et al., 1987).

Egg jelly isolation and quantitation.

To isolate egg jelly, eggs were gently re-suspended and kept agitated with a slow stream of air bubbles. The pH of the suspension was lowered to 4.8-5.0 for 3-5 minutes using 1-M MES (2-morpholinoethanesulfonic acid) then raised to 7.8 with 1-M Tris (tris (hydroxymethyl) aminomethane). Eggs were sedimented by gentle hand centrifugation. The supernatant was removed and centrifuged at 30,000*g* (4°C) for 30 min (Lutz and Inoue, 1986; Weidman and Kay, 1986). The resulting egg jelly (EJ) supernatant was concentrated using Centricon Plus-20 (Millipore, Billerica, MA) devices (according to manufacturers instructions) and stored in aliquots at –80°C until needed. Fucose content of EJ was determined by the phenol-sulfuric acid method (Dubois et al., 1956) as

modified by Vacquier (Vacquier and Moy, 1997). Briefly, 0.1 ml of EJ was mixed with 0.1 ml of aqueous phenol (5%, v/v). The phenolic EJ solution was vortexed as 1 ml of concentrated H_2SO_4 was added. Absorbance (487 nm) was measured after 2 minutes at 23°C and compared to a fucose standard curve.

Estimation of relative charge to mass ratio

Charge to mass ratio relative to LSA was estimated for certain compounds. Charge was estimated using the alcian blue dye binding assay (Gold, 1979) as described by Cherr (1993). Samples were read in a spectrophotometer at 480 nm. Purified lyophilized LSA was used to construct a standard curve. Three lyophilized replicate samples were carefully weighed, reconstituted to a known concentration, and measured in the assay against the standard curve.

Fertilization bioassays.

Fertilization bioassays were performed according to guidelines set forth by Chapman (1995) and as described in Pillai et al., 1997. All experiments were conducted at 12°C in pH 8 HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffered artificial seawater (ASW-H, 490-mM NaCl, 27-mM $MgCl_2$, 28-mM $MgSO_4$, 10-mM $CaCl_2$, 10-mM KCl, 2.5-mM $NaHCO_3$, 10-mM HEPES). In order to insure that assay sensitivity was not diminished with excess sperm, a sperm to egg ratio resulting in 70% to $\leq$ 90% fertilization was determined for each batch of gametes immediately prior to experiments. Sperm concentration was determined using a hemocytometer. Sperm were pre-exposed to a fertilization inhibitor for 10 minutes prior to the addition of eggs. Fertilization was allowed to proceed for 10 minutes, then samples were fixed with 1% glutaraldehyde in ASW. Each bioassay consisted of one pair of urchins run in 3-4 replicate batches with

100-300 eggs scored per replicate. Concentration response data was normalized to controls using Abbott's formula as described by Chapman (1995) and transformed to probit units to identify the 50% inhibitory concentration (IC_{50}) (Rand, 1995).

Quantitative solid phase whole-cell binding assays.

A solid phase binding assay was devised to assess the qualitative and quantitative binding characteristics of LSA to whole sperm cells and to specific cell surface proteins. Similar whole sperm cell binding assays with biotinylated ligands or sperm specific antibodies have been used by other investigators (Huang and Yanagimachi, 1984; Kurpisz et al., 1993; Mahony et al., 1993). Data from each experiment represents sperm from 2-3 individual males assessed separately and in triplicate. Specific experiments required specific variations on the assay. Generally, dry sperm were diluted 1:50 in ASW-H and washed twice to remove pigmented cells (200*g* for 5 min at 4°C). Acrosome reaction was induced in subsets of sperm by the addition of 10-μM ionomycin (Calbiochem, La Jolla, CA) from a 10 mM stock in dimethylsulfoxide. Sperm were fixed using fresh 3% paraformaldehyde/ 0.1% glutaraldehyde in ASW-H (Vacquier, 1986) and the concentration adjusted to 10^7 cells/ml. One hundred microliters of fixed sperm were added to wells of a flat bottom 96-well plate (Nunc MaxiSorp) in triplicate. Cell to plate adhesion was encouraged by centrifugation at 700*g* for 10 min. Microscopic examination revealed that sperm generally adhered to plates in a monolayer and were evenly distributed. Wells were washed with HEPES buffered saline (HBS, 150-mM NaCl, 10-mM HEPES, pH 8) at least thrice in between each step of the assay. Whenever a protein label was used, wells were first blocked with 9% non-fat milk HBS for 30 minutes. Non-specific binding of biotinylated LSA (LSA-b) to the plate was not detectable. Non-

specific binding to cells was defined as the signal detected in the presence of a 100-fold excess of unlabeled LSA after background correction. The first label, usually LSA-b ± a competitor, was allowed 1 hour for incubation. In all competition assays the concentration of LSA-b was 0.1 mg/ml, based on range finding studies (data not shown). LSA binding was fixed by the addition of ice cold 55% ethanol in HBS (Pillai et al., 1997). Wells were then blocked for protein and incubated with the second label; NeutrAvidin™ conjugated horseradish peroxidase (Pierce), for 1 hour. Unlike avidin, LSA did not bind non-specifically to NeutrAvidin™ (Chapter 2). Plates were developed using o-phenylenediamine (OPD) substrate, the reaction terminated with 2.5-M H_2SO_4, and read on a BioRad 550 plate reader at 490 nm.

LSA binding time course: This assay was conducted on a 96-well plate as described above with the exception that live sperm (10^7 cells/ml) were used and the assay conducted at 12°C. In order to minimize spontaneous acrosome reaction, Ca^{+2}-free artificial seawater (500 mM-NaCl, 27-mM $MgCl_2$, 28-mM $MgSO_4$, 10 mM-KCl, 2.5-mM $NaHCO_3$, 1-mM EGTA, 10-mM HEPES, pH 8) was used as the main buffer in this experiment. LSA-b was allowed to interact with the sperm cells for various time intervals and the interaction terminated by a 4x wash with HBS. All wells were treated with ice-cold 55% ethanol/HBS to fix the cells and LSA and the plates developed as described above.

Antibody binding inhibition: Plates were prepared as described above with fixed sperm cells. Sperm were exposed to 1-mg/ml LSA in HBS for 1 hour (positive controls with HBS only). All wells were then blocked for protein (as above) and incubated with the primary antibody (diluted in 2% NFM/HBS) for an hour, washed and blocked again

in 2% NFM/HBS to remove residual azide. An appropriate HRP conjugated secondary antibody (diluted in 2% NFM/HBS) was added for an hour and the plates developed with OPD substrate as described above. Reductions in specific antibody binding compared to controls were evaluated using a Student's T-test.

Microscopic labeling and evaluation.

Fresh motile sperm were fixed with 1% paraformaldehyde in ASW, diluted to 1:4000, and 500 μl aliquots added to a 24-well immunoassay plate (Nunc) containing poly-L-lysine coated 12-mm round glass coverslips. Cell adhesion to coverslips was encouraged by centrifugation at 700*g* for 10 minutes. Coverslips were rinsed with HBS, non-specific binding blocked with Pierce SuperBlock (Pierce) for 30 minutes. Primary antibody (diluted in SuperBlock) was allowed to incubate for 1 hour then coverslips were rinsed with HBS and an appropriate Alexa-conjugated secondary antibody (diluted in SuperBlock) was added for an additional hour in darkness. Secondary antibodies used were: goat anti-chicken Alexa 568; goat anti-rabbit Alexa 488; goat anti-mouse Alexa 350 (Molecular Probes). Rinsed coverslips were mounted onto slides with n-propylene glycol mounting media and sealed with Revlon "Orange Flip" nail polish. Cells were evaluated on an Olympus BX50WI epifluorescence microscope using a UV-corrected 60x oil immersion objective. Captured fluorescent pseudo-color images were overlaid on their transmitted light Nomarski differential interference contrast (DIC) counterparts using MetaMorph image analysis software (Universal Imaging Corp., Downington, PA). All images specific to a fluorophore were captured with the same microscope, camera and software parameters. Non-specific binding to sperm (+/- LSA) was not observed in control slides where con-specific serum or ascites was substituted for primary antibody.

RESULTS

Saturation curves for LSA-b binding to sperm.

The binding of LSA-b to the surface of sperm cells is saturable and dose dependent as shown in Figure 3.1. Specific binding of LSA-b to sperm was calculated by subtracting nonspecific bound (NSB) from total bound. Figure 3.1 further illustrates a difference in apparent affinity of LSA-b to sperm surface sites between acrosome reacted (AR) and acrosome intact (AI) sperm. LSA-b seemed to have a greater affinity for AR sperm as indicated by the steeper slope of total LSA-b bound at low concentrations and higher overall absorbance. The specific bound data indicated that the total sites available for binding LSA-b were greater in AR sperm than AI sperm and/or that AR sperm receptors have a greater affinity for LSA-b. The total bound curve for AR sperm had a distinct biphasic inflection point, which could be indicative of at least two receptor populations with distinctly different affinities for LSA-b. In AI sperm, the total bound curve did not appear to be biphasic, however the possibility exists that LSA may be binding to multiple receptors with similar affinity. The two total bound curves seemed to converge at the highest concentration, while corrected specific bound LSA-b indicated a clear distinction between AR and AI sperm. This is an indication that at the highest LSA-b concentration, specific binding sites have been largely occupied in both cell states and non-specific binding makes up a greater fraction of the total LSA-b bound to sperm.

Kinetics of LSA-b binding to sperm.

The kinetics of LSA-b binding to live sperm were observed over 10 minutes (Figure 3.2). Live sperm were used in order to give an estimation of how LSA behaves under bioassay conditions. The binding pattern was similar at both concentrations with the main

difference being the magnitude of the response signal corresponding to the higher concentration. Data suggests that some pre-incubation with sperm is required to achieve maximal binding. Some binding saturation seemed to occur at about 120 sec, in both, and then there is an increase of about the same slope. The kinetic data appear to behave in a biphasic manner indicating at least two binding sites with differing rates of association for LSA. The gradual increase in binding at later time points could be due to changes in binding site availability caused by sperm death and/or non-specific interactions. Data are corrected for NSB with a 100x control at the longest time point. A more complete interpretation of these data would be possible with the inclusion of 100x controls at each time point.

LSA-b competes with sulfated and sulfonated macromolecules for binding sites on the sperm surface.

The specificity of LSA interaction with sperm was further characterized by comparing the ability of sulfonated and sulfated macromolecules to compete with LSA-b for sperm surface binding sites. The data are presented as the Hill (logit) transformation of specifically bound LSA-b as a function of the concentration of unlabeled competitor (Figure 3.3A). Values of constants obtained from log regression of the data are summarized in Figure 3.3B. The binding of LSA-b to sperm was inhibited in a dose dependent manner by increasing concentrations of unlabeled LSA, an aromatic sulfonated polymer polyanetholesulfonic acid (PASA) ((E)-1-methoxy-4-(1-propenyl) benzene homopolymer, sulfonated, sodium salt), and to a far lesser extent fucoidan (FDN), a sulfated heteropolymer of L-fucose (Bilan et al., 2002). Based on IC_{50} values, PASA was the most potent inhibitor of LSA-b binding in both AI and AR sperm.

All competitors have shallow Hill slopes ($|n_H|<1$). Typically this is an indication that negative cooperativity describes the behavior of the unlabeled ligand interacting with binding sites in common with the labeled ligand; that is, the binding of the unlabeled ligand to one site causes subsequent unlabeled ligands to bind with less affinity. Since negative cooperativity is indicated, this data cannot be used for a mathematical calculation of K_i (dissociation constant for the competitor) according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

The competition data (Figure 3.3) show that LSA has the greatest affinity for AR sperm and less for AI sperm, corroborating data presented in Figure 3.1. By contrast, PASA has lower affinity (higher Kd') for AR sperm than AI sperm. In both AR and AI sperm, high concentrations of unlabeled LSA (>0.6 mg/ml) inhibit LSA-b more effectively than high concentrations of PASA. The potency of competitive polyanions to inhibit LSA-b binding to sperm cells parallels their ability to inhibit fertilization (Figure 3.4). At equivalent concentrations, PASA is a better contraceptive than LSA. A 100x higher concentration of fucoidan only results in meager inhibition. By comparison, at 3 μg/ml the >100K LSA fraction inhibits fertilization by 83% (Chapter 1). Higher molecular weight fractions of LSA-b may be more recalcitrant to competition from PASA. The data for fucoidan show that this polyanion is a weak competitor with LSA-b on AI sperm (Figure 3.3). While fucoidan is known to interact strongly with bindin (Glabe et al., 1982), it does not appreciably inhibit fertilization (Figure 3.4). Unfortunately reproducible competition data with fucoidan and LSA-b on AR sperm was not obtained, but it is expected that this polyanion would be a much better competitor on these cells due to the presence of bindin.

Con-specific egg jelly inhibits LSA-b binding in AI sperm at a biologically relevant (AR inducing) concentration (Figure 3.5). The amount of egg jelly used is the lowest concentration that gives greater than 50% acrosome reaction in *S. purpuratus* sperm at 10^7 cells/ml. The same concentration of contra-specific egg jelly does not affect LSA-b binding nor does a much higher concentration of fucoidan. If the interaction of LSA with sperm were solely a function of its charge density, compounds with a higher charge to mass ratio may be expected to be better competitors with LSA, however, the results do not support this hypothesis (Figure 3.6). These results suggest that LSA-b binds to species-specific egg jelly receptors.

LSA selectively inhibits antibody binding.

LSA-b binds specifically to domains over the sperm head (Figure 2.6). The ability of LSA to inhibit the binding of antibodies to specific proteins that are present within the binding region of LSA-b is shown in Figure 3.7. LSA significantly inhibited the binding of two antibodies raised against proteins mediating acrosome reaction and sperm-to-egg binding respectively, yet did not inhibit the binding of an antibody (anti-J18/5) to a 350kD protein that localized over the entire sperm surface (Figure 3.7A). LSA dramatically inhibited the binding of a suREJ3 antibody (anti-S/C) that targets epitopes corresponding to the sea urchin egg lectin domain and carbohydrate recognition domain of the protein (Mengerink et al., 2002). An antibody specific to the intracellular C-terminal region of suREJ3 (anti-IH) did not bind to non-permeabilized cells irrespective of the presence of LSA (data not shown). Within the context of this experiment, the ability of LSA to inhibit antibody binding is limited to antigens that entirely co-localize with its binding domain.

DISCUSSION

Compared to internal fertilization, external fertilization takes place in a much less controlled environment where gametes can be exposed to a variety of environmental stressors (turbulence, oxidative stress, ultraviolet radiation, competing gametes and contaminants) that can affect fertilization. As external spawners, sea urchins have evolved a variety of mechanisms to deal with these stressors (Mead and Epel, 1995) and ensure the species specificity of their gamete interactions. Indeed the species specificity of fertilization is guarded by multiple mechanisms (Ohlendieck and Lennarz, 1996; Vacquier, 1998). One such mechanism is the use of specific conformations of sulfate groups on extracellular matrix polysaccharides as ligands for key proteins in the fertilization process (Hirohashi et al., 2002a). This strategy works to ensure echinoid species specificity since most species produce unique sulfated polysaccharides as part of their "egg jelly" extracellular matrix (Alves et al., 1997; 1998).

Earlier data, reported herein, shows that LSA, a lignacious component of pulp mill effluent, inhibits fertilization in the sea urchin at the sperm cell by preventing both acrosome reaction and sperm-to-egg binding. Furthermore, a biotinylated LSA conjugate (LSA-b) specifically labels particular domains of the sea urchin sperm head (Chapter 2). The binding pattern of LSA-b suggests that it may bind to specific receptor molecules that regulate the inhibited fertilization events.

A quantitative solid phase whole-cell binding assay was devised in order to further explore the nature of LSA-b binding to sperm. Traditionally, quantitative binding data is obtained with a radiolabeled probe. Using a biotinylated probe to assess ligand-receptor interactions as an alternative to a radioactive one presents its own challenges and

limitations, but is not without precedent (Vieira, 1998). Since the detection method for biotinylated ligands involves more intermediary molecules than radioactive or fluorescent ligands, a greater degree of inter-assay variability exists. The activity of each intermediary molecule affects the detectable end result e.g. moles biotin/mg LSA, concentration of NeutrAvidin™, activity of HRP, concentration of substrate, length of color development. Appropriate controls for each of these variables allow meaningful interpretation of the data. Despite these caveats, other investigators have successfully employed biotinylated ligands to obtain quantitative binding data with whole sperm cells (Huang and Yanagimachi, 1984; Mahony et al., 1993). An additional constraint unique to this solid phase assay was the inability to directly detect free LSA-b in solution or calculate the free concentration based on the fraction bound since the specific activity (moles biotin/mg LSA) of the ligand was unknown.

As described in Chapter 2, the ratio of moles of biotin per mg of LSA was unable to be determined due to interference problems with the HABA assay used to quantify biotin. LSA bound non-specifically to avidin protein in this assay. While no such non-specific binding to Pierce NeutrAvidin™ or streptavidin was detected, neither can be substituted in the HABA assay (Pierce Chemical Company, personal communication). Without a quantitative correlation between signal strength and the mass of ligand bound, absorbance measurement cannot be used to calculate the amount of LSA bound even though a proportional relationship exists. Due to the inability to accurately estimate the concentration of free LSA-b (required for Scatchard analysis), competition data was analyzed via the Hill transformation based on percent of maximum LSA-b bound. Future

studies could employ a fluorescent LSA ligand, facilitating determination of specific activity.

Presented herein is strong evidence that LSA inhibits at least two species specific ligand-receptor mediated interactions required for fertilization in *S. purpuratus* at the level of the protein receptor. These data show that LSA-b bound to intact sperm cells in a manner consistent with ligand-receptor binding models. The binding of LSA-b to sperm was saturable and specific (Figure 3.1) as well as kinetically dependent (Figure 3.2). Acrosome reacted (AR) sperm appear to have a greater capacity to bind LSA-b than acrosome intact (AI) cells (Figure 3.1) suggesting the presence of additional binding sites. The binding curve for AR sperm also had an inflection point indicative of at least two receptor populations with distinct affinities for LSA-b. While part of the inflection may be due to non-specific binding not measured at lower concentrations, the apparent existence of different affinity sites in AR sperm can be explained by the availability of the bindin protein as a potential target for specific LSA-b binding. Competition data confirm that LSA had a greater affinity for AR sperm than AI sperm (Figure 3.3A, B). The binding of LSA-b to AI sperm was inhibited by a biologically relevant concentration of con-specific egg jelly, but not by contra-specific egg jelly (Figure 3.5). LSA significantly inhibited the binding of antibodies specific to a protein involved in acrosome reaction, suREJ3, in AI sperm and to the sperm adhesion protein bindin in AR sperm (Figure 3.7). This pattern of data supports the hypothesis that the ability of LSA to inhibit the acrosome reaction and sperm-to-egg binding is due to its affinity for these key receptor proteins.

The Hill transformation of competition data revealed that all LSA-b competitors behave with negative cooperativity (Figure 3.3) as indicated by shallow slopes. This effect may be seen if the binding sites are clustered or if several sites are present per receptor molecule. The presence of heterologous binding sites (expected on whole sperm cells) or receptors that do not all recognize the unlabeled ligand with the same affinity may also produce a shallow Hill slope. Glabe (1982) noted that the binding of fucoidan to bindin particles behaves with negative cooperativity and that it may be an inherent property of large multivalent ligand binding since molecules that bind first may interact at multiple sites and limit the availability of sites for further binding. Each of the multivalent competitors used is a polydisperse mixture of molecular sizes. Larger molecules, with greater valency, will bind better than smaller ones leading to an inherently variable affinity for each of the inhibitors used. The apparent affinity constant, Kd', is an amalgamation of the affinity constants for each size of ligand in the mixture towards each binding site on the sperm. The values of Kd' in this experiment give us an indication of the overall affinity of each competitor for common binding sites relative to the affinity of LSA-b, with a smaller number corresponding to higher affinity. As such, these Kd' values should be treated as empirical descriptions of the data and not true binding constants. The increased IC_{50} and decreased Kd' of LSA towards AR sperm (Figure 3.3) indicate that higher affinity receptors (e.g. bindin) that recognize LSA are present on AR sperm, in agreement with binding data (Figure 3.1). As a competitor with LSA-b, PASA has lower affinity (higher Kd') for AR sperm than AI sperm suggesting that PASA may not interact as strongly with bindin relative to LSA. The higher Kd' value for PASA (Figure 3.3B) may reflect the greater affinity of LSA-b for AR sperm

and/or the loss of receptor sites on the acrosomal cap membrane with high affinity for PASA.

Three signaling proteins have been identified in sea urchin sperm that belong to the sea urchin receptor for egg jelly (suREJ) family, each with a suspected role in acrosome reaction induction (Mengerink et al., 2000). The fucose sulfate polymer portion of egg jelly has been shown to bind to suREJ1 (Moy et al., 1996; Vacquier and Moy, 1997) and induce acrosome reaction. Even though suREJ3 shares a similar REJ domain and carbohydrate recognition domain with suREJ1 and possesses a sea urchin egg lectin domain, it has not been conclusively demonstrated that fucose sulfate polymer or other ligands in egg jelly bind to suREJ3 (Mengerink et al., 2002). While the ligands for these domains have yet to be identified, their extracellular presence strongly suggest that suREJ3 interacts with some aspect of the extracellular matrix of the egg. Of the 3 members of the REJ protein family identified thus far, only suREJ3 localizes solely to regions on the sperm head (Mengerink et al., 2000), entirely within the labeling region of LSA-b. Sperm that are pre-incubated with LSA have a dramatically reduced antigenicity for an antibody (anti-S/C) to the sea urchin egg lectin domain and carbohydrate recognition domain of suREJ3. The ability of LSA to markedly inhibit the binding of an antibody to suREJ3 is consistent with the hypothesis that this protein is a receptor for sulfated ligands on the egg surface.

Some antibodies that localize to regions in the tail as well as the head are capable of inducing the acrosome reaction, including anti-J18/5 (K. Mengerink, personal communication) as well as antibodies to suREJ1 (Moy et al., 1996). Antibodies to suREJ3 including anti-S/C do not induce acrosome reaction (Mengerink et al., 2002).

Acrosome reaction induction likely requires participation of proteins in the tail or midpiece of sperm in addition to the head. Since anti-J18/5 induces the acrosome reaction, but LSA does not inhibit its binding despite considerable co-localization in the head region (Figure 3.7) the acrosome reaction is likely the result of an activation cascade involving multiple proteins. The ability of anti-J18/5 to induce acrosome reaction in the presence of LSA was not evaluated. Inhibition of the acrosome reaction by LSA is reversible with a sufficient concentration of the endogenous inducer, egg jelly. In Chapter 2, data indicate that 20% of sperm are still capable of acrosome reaction in the presence of 0.1-mg/ml LSA by an egg jelly concentration of 20 μg fucose/ml. These data show that the same concentration of egg jelly is able to reduce the fraction of LSA-b bound to sperm by 32%. The conclusion can be drawn that while LSA occupies receptor sites for egg jelly it may also occupy other binding sites. LSA inhibits at least one egg jelly receptor, which is sufficient to block acrosome reaction induction. Based LSA's pattern of inhibition and binding, there are other specific sites with which it interacts that have yet to be identified. These binding sites may be proteins involved in key physiologic processes.

The attachment of bindin to its ligand on the egg surface is high affinity and highly species specific (Dhume and Lennarz, 1995; Lopez et al., 1993). The ability of LSA to inhibit sperm-to-egg binding and to do so by interacting (at least in part) with bindin, is consistent with the observations of DeAngelis and Glabe (1990). They found that bindin displays a remarkable affinity for sulfate- or sulfonate- containing polymers and that the selectivity for these over polymers containing other anionic groups suggests the interaction is more complex than the formation of simple salt bridges. DeAngelis and

Glabe (1990) postulate that all three non-ester oxygen atoms of the sulfur moiety interact with a "docking site" on bindin and that the orientation of these oxygen atoms may govern binding specificity and affinity. The ester linkage in the sulfate moiety does not appear to be critical given the high affinity of sulfonate polymers for bindin. The stereochemical conformation of sulfate groups on oligosaccharides associated with the egg receptor for sperm enable them to attach to bindin, however they do not attach to AI sperm (Dhume and Lennarz, 1995) despite the presence of sulfate responsive receptors, the suREJ proteins (Mengerink et al., 2000). LSA has a high degree of sulfonation, which increases the probability that a $-SO_3$ will be in the correct conformation for binding (DeAngelis and Glabe, 1987). LSA is intrinsically heterologous in backbone structure and in its pattern of sulfonation (Hassi, 1985). Similarly, fucoidan also has a variable and heterologous structure (Bilan et al., 2002), yet binds to bindin with high avidity. High affinity multivalent binding requires multiple interactions to occur in a precise orientation (Bertozzi and Kiessling, 2001). LSA may be able to mimic the interaction of sulfated fucans with bindin as described by DeAngelis and Glabe (1988). Initially salt bridges could form between sulfonate groups on LSA and a cationic receptor site. This is a long-range interaction. Once multiple ionic bonds are formed, some sulfonate groups may be induced to form more stable ring bonds with guanidino moieties of arginine (DeAngelis and Glabe, 1988). The basic amino acids arginine and lysine have been implicated in the binding of polysulfonated macromolecules to proacrosin (Urch and Patel, 1991) as well as fibroblast growth factor (Liekens et al., 1999). The structure of LSA may allow enough conformational flexibility to permit sulfonate groups to be induced to fit into a binding site as has been suggested for some polysaccharides

(Letourneur et al., 1993). Since multivalent ligands, like LSA, possess multiple copies of a binding-recognition element many potential mechanisms have been postulated for receptor interaction (Gestwicki et al., 2002; Kiessling et al., 2000).

Given LSA's heterologous and ill defined structure (Hassi, 1985; Lin and Dence, 1992) the question can be raised that charge density on LSA governs its affinity to sperm rather than more specific structural or conformational factors. If this were the case, it would be expected that compounds with a higher charge to mass ratio would make better LSA competitors. This is not the case (Figure 3.6). Other structural or conformational features of LSA also contribute to its affinity. While multivalency does play a key role in binding, the architecture of the ligand determines its activity (Gestwicki et al., 2002). With some multivalent ligands, structural hydrophobic groups assist in stabilizing ionic bonds via van der Waals interactions. Other investigators have noticed that aromatic sulfonated polyanions have enhanced biological activity over their aliphatic counterparts (Tan et al., 1993). Watanabe and co-workers (1993) have demonstrated that LSA in aqueous solution binds to hydrophobic as well as hydrophilic surfaces, suggesting that LSA is able to undergo conformational rearrangement to avail its aromatic functionalities for binding.

The specificity of bindin and suREJ proteins for their endogenous ligands is governed, at least in part, by ligand stereochemistry (DeAngelis and Glabe, 1990; Hirohashi et al., 2002a). It has been noted that sea urchin bindins are not related to any other proteins (Vacquier, 1998), yet the analogous sperm binding mechanism in mammals, zona pelucida – proacrosin binding, is inhibited by similar multivalent sulfated and sulfonated compounds (Howes et al., 2001). We have observed that LSA is capable

of inhibiting fertilization processes in unrelated echinoderm species and a marine worm *Urechis caupo* (unpublished results), as well as in mammals (Tollner et al., 2002). We have also observed developmental effects of LSA on echinoderm embryos as a result of specific binding to primary mesenchyme cells (unpublished results). Other investigators have reported that LSA inhibits specific extracellular processes such as coagulation (Loomis and Beyer, 1953), viral binding (Sorimachi et al., 1990), and cell growth and adhesion (Sorimachi, 1992). LSA has the plasticity to specifically bind to and inhibit receptor molecules with little or no sequence homology and that do not display effector cross reactivity. The biological processes that LSA has been observed to inhibit are consistently mediated by diverse sulfated polysaccharides. The ability of LSA to bind to receptors with divergent specificity for ligand architecture can be conceptualized in two ways. Either LSA's structure is so heterologous that it expresses multiple copies of many different binding-recognition elements or the structure of LSA allows enough conformational flexibility that sulfonate valences (and/or other binding-recognition elements) can assume the proper spatial orientation to fit in a variety of receptor types. These hypotheses have been proposed to explain the activity of other polyanions (Gestwicki et al., 2002; Kiessling et al., 2000; Letourneur et al., 1993).

The potency of competitive polyanions to inhibit LSA-b binding to sperm cells parallels their ability to inhibit fertilization (Figures 3.3 and 3.4). Other competitive binding studies with sulfated polyanions have found similar parallels between competitive potency and *in vivo* activity (Belford et al., 1993; Brimacombe et al., 1999). PASA is a better inhibitor of sea urchin fertilization and more potent at quenching LSA-b binding to sperm than is LSA. The reasons for differences in activity are not clear.

PASA may simply be more heavily sulfonated than LSA resulting in a higher valency. More likely, the reason lies in the way the sulfonate groups are distributed in PASA. Sulfonate groups on PASA may be distributed or positioned in such a way as to result in a better "fit" in receptor sites relative to LSA. They may better mimic the conformation of sulfate groups in *S. purpuratus* egg jelly for example. PASA shares many of the same activities as LSA including anticoagulant, anti-viral (Tan et al., 1993), and contraceptive properties. As with LSA, other structural features of PASA may also contribute to its overall potency including structural flexibility, shell of hydration, availability of hydrophobic character, and over all three-dimensional architecture (Kiessling et al., 2000; Letourneur et al., 1993; Liekens et al., 1999). Many investigators have observed that the molecular size of a multivalent polymer is related to its biological activity (DeAngelis and Glabe, 1987; Parrish et al., 1989; Powis et al., 1992; Tan et al., 1993; Vocac and Alphin, 1968). In both AR and AI sperm, high concentrations of unlabeled LSA (>0.6 mg/ml) inhibit LSA-b more effectively than high concentrations of PASA (Figure 3.3). PASA may compete with LSA more effectively at some receptors and less so at others as indicated by the shallower slope of the PASA competition curves compared to those of LSA. Alternatively, the PASA size fractions used may compete effectively against a certain fraction of LSA-b and less so against a remaining fraction. The reported molecular weight range for PASA is 9K to 11K (Tan et al., 1993) whereas LSA ranges from 3K to greater than 300K. Larger LSA size fractions are more effective inhibitors of fertilization (Chapter 1). If affinity for the sperm surface correlates with increased inhibition then one would expect that the most inhibitory LSA fractions (>100K) would

have the highest affinity for sperm. The high MW LSA-b fractions would therefore be more recalcitrant to competition from PASA than smaller size fractions.

While PASA and LSA share aromatic character, fucoidin lacks such hydrophobic functionalities suggesting that hydrophobic character (in addition to sulfonation) of polyanions plays a role in biological activity in the sea urchin as it does in HIV inhibition (Tan et al., 1993). The high concentrations of fucoidan required for any appreciable competition with LSA in AI sperm indicate that the observed effect may be due to mass-action rather than specific competition at receptors (Figure 3.3A). Alternatively, it may be that only a minor component of whole fucoidan is able to compete effectively with LSA at specific receptors. Fucoidan is a structurally variable sulfated fucan (Bilan et al., 2002) and it is possible that a minor fraction shares a similar structural conformation as *S. purpuratus* fucose-sulfate polymer.

Interestingly, PASA does not appear to have an enhanced affinity for AR sperm, unlike LSA (Figure 3.3). Given that PASA is not cytotoxic to sperm, very effective at inhibiting fertilization, and a potent competitor with LSA-b on sperm it is reasonable to hypothesize that PASA inhibition occurs prior to the acrosome reaction and likely involves inhibition of a REJ protein. Since fucoidan is known to be an effective inhibitor of bindin function but a poor contraceptive (Glabe et al., 1982), one can conclude that inhibition of fertilization in sea urchins is more effective when it is targeted prior to induction of the acrosome reaction. Since bindin is proximal to its ligand on the egg at the time of acrosome reaction and needs to hold a moving sperm, its rate of association would have to be very high. It is reasonable then that a high concentration of fucoidan must be used to see any reduction in fertilization since a sufficient concentration must be

available at the site of sperm-to-egg binding. LSA binds to sperm inhibiting both induction of the acrosome reaction and sperm-to-egg binding; however, a sufficient concentration of egg jelly can overcome LSA's inhibition of the acrosome reaction in at least a fraction of sperm (Figure 3.5). Conceptually, LSA can be carried on the sperm surface to the site of sperm-to-egg binding resulting in a locally high concentration of LSA. Assuming that the concentration of egg jelly is highest at the surface of the egg, and that egg jelly removes the same fraction of LSA from all sperm cells, any LSA that is remaining is available for interaction with bindin in the event that acrosome reaction occurs. Since LSA has an increased affinity for AR sperm it is plausible that inhibition of sperm-to-egg binding may contribute considerably to its overall potency as an inhibitor of fertilization.

Sulfated and sulfonated polyanions represent a class of compounds with vastly different structures yet similar and specific biological activities. Generally these compounds can inhibit biological processes mediated by the interaction of sulfated polysaccharides with their receptors. While their general activity has been attributed to multivalency, aspects of their molecular architecture moderate their potency and efficacy in specific interactions. The analysis presented here has identified and characterized LSA's interaction with two specific receptors on the surface of sea urchin sperm. LSA's interactions with other receptors within its sperm-binding domain have yet to be characterized and further study may reveal insights to sperm cell biology and fertilization. LSA may prove to be very useful as a biological tool to investigate extracellular biological interactions or as a therapeutic agent in a variety of applications.

Given LSA's plasticity for receptor recognition, further investigation on its effects in different systems may unveil novel interactions.

FIGURES

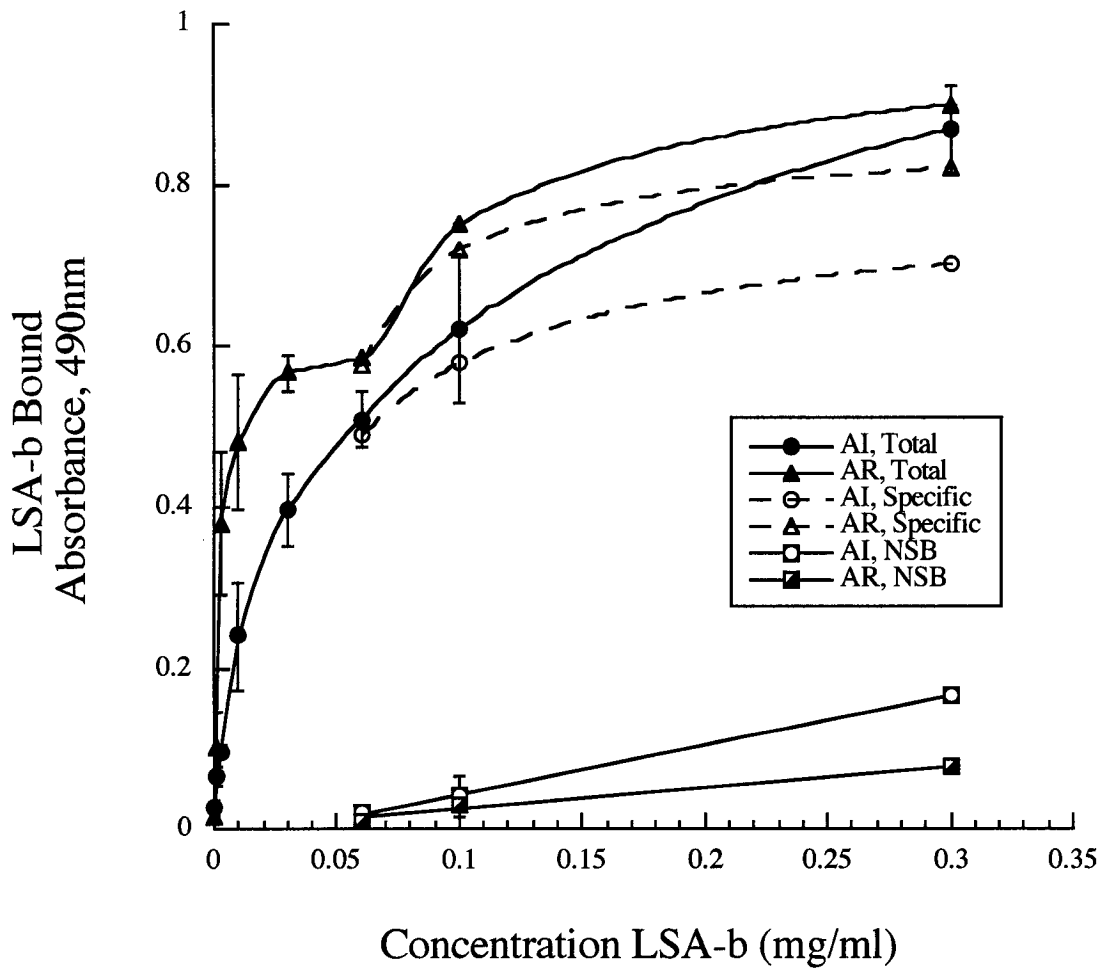


FIGURE 3.1: LSA-b binding to *S. purpuratus* sperm cells (10^7 cells/ml), acrosome reacted (AR, ionomycin) and intact (AI). Data is represented as total, specific and non-specific bound (NSB) LSA-b. Specific binding was calculated by subtracting NSB from total bound. The curve fits are interpolated between data points.

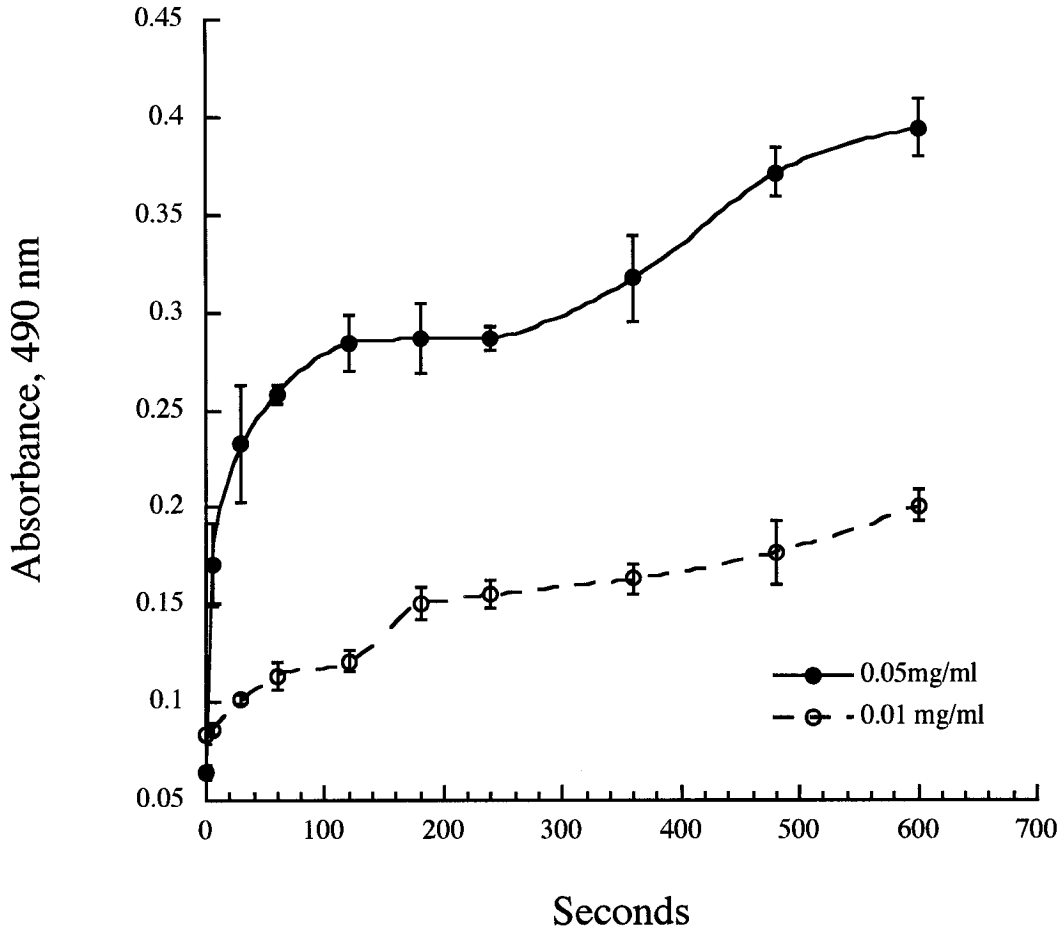
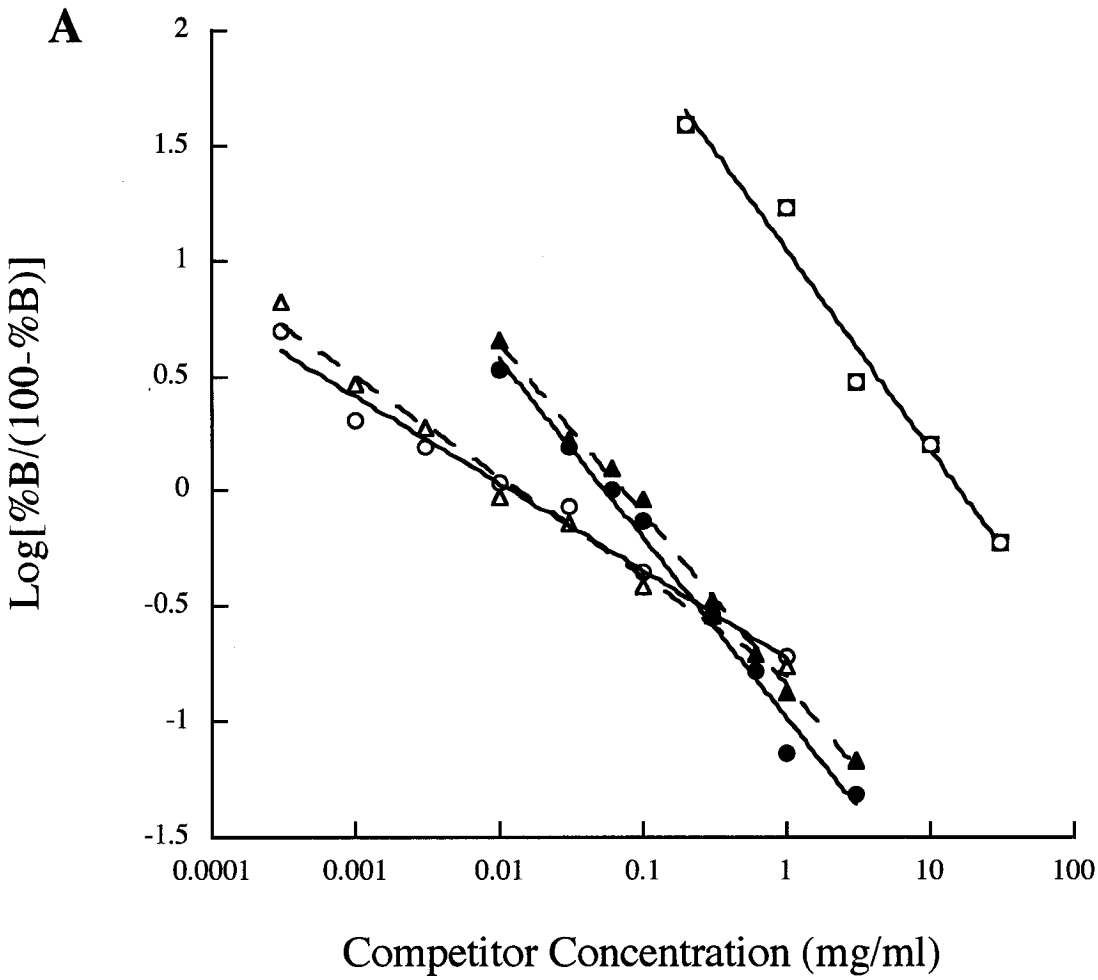


FIGURE 3.2: Time course showing the binding of LSA-b to live *S. purpuratus* sperm cells (10^7 cells/ml) over 10 minutes.



B

Competitor	Sperm	IC_{50}	Kd'	$\lvert n_H \rvert$	R^2
LSA	AI	0.055	9.6	0.78	0.98
LSA	AR	0.073	6.9	0.74	0.99
PASA	AI	0.012	5.3	0.38	0.98
PASA	AR	0.014	6.3	0.43	0.99
FDN	AI	16.14	11.1	0.87	0.97

FIGURE 3.3: **(A)** Increasing concentrations of competitor reduces the binding of LSA-b (0.1 mg/ml) to sperm cells. Regression lines were fit to the data according to the Hill equation: $\log[B/Bmax-B] = n_H \log[C] - \log Kd'$. **(B)** Legend and summary of regression values. Key: LSA, lignosulfonic acid; PASA, polyanetholesulfonic acid; FDN, fucoidan; AI, acrosome intact; AR, acrosome reacted; IC_{50}, concentration of competitor which inhibits binding of LSA-b by 50%; Kd', amalgamated affinity constant; $\lvert n_H \rvert$, absolute value of slope (Hill coefficient); R^2, regression coefficient.

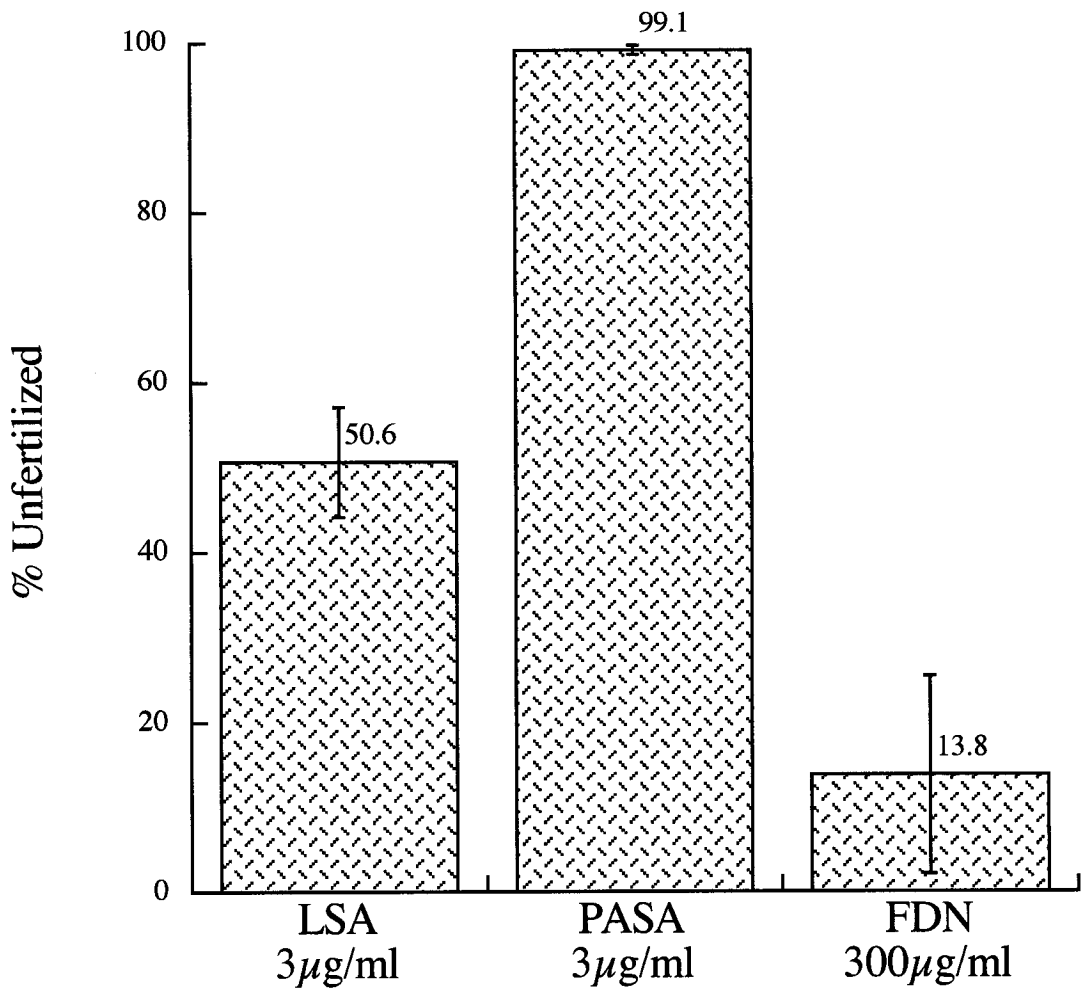


FIGURE 3.4: Inhibition of fertilization by LSA binding competitors. At equivalent concentrations, PASA is a better inhibitor than LSA. A 100x higher concentration of fucoidan only produces modest inhibition.

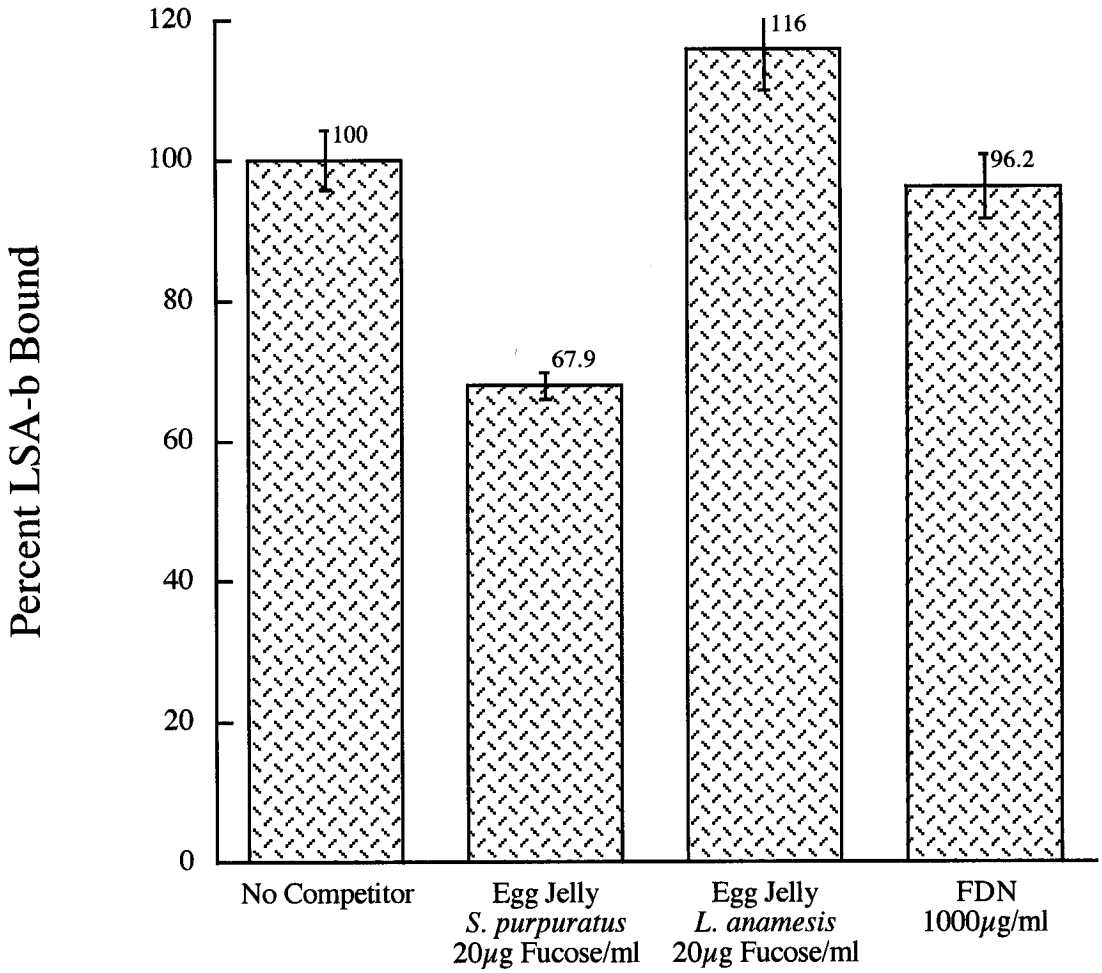


FIGURE 3.5: Con-specific (*S. purpuratus*) egg jelly inhibits LSA-b (0.1 mg/ml) binding to acrosome intact sperm cells at a biologically relevant concentration. Contra-specific egg jelly (*L. anamesis*) has no inhibitory effect on LSA-b binding nor does a high concentration of the fucose-sulfate polymer fucoidan.

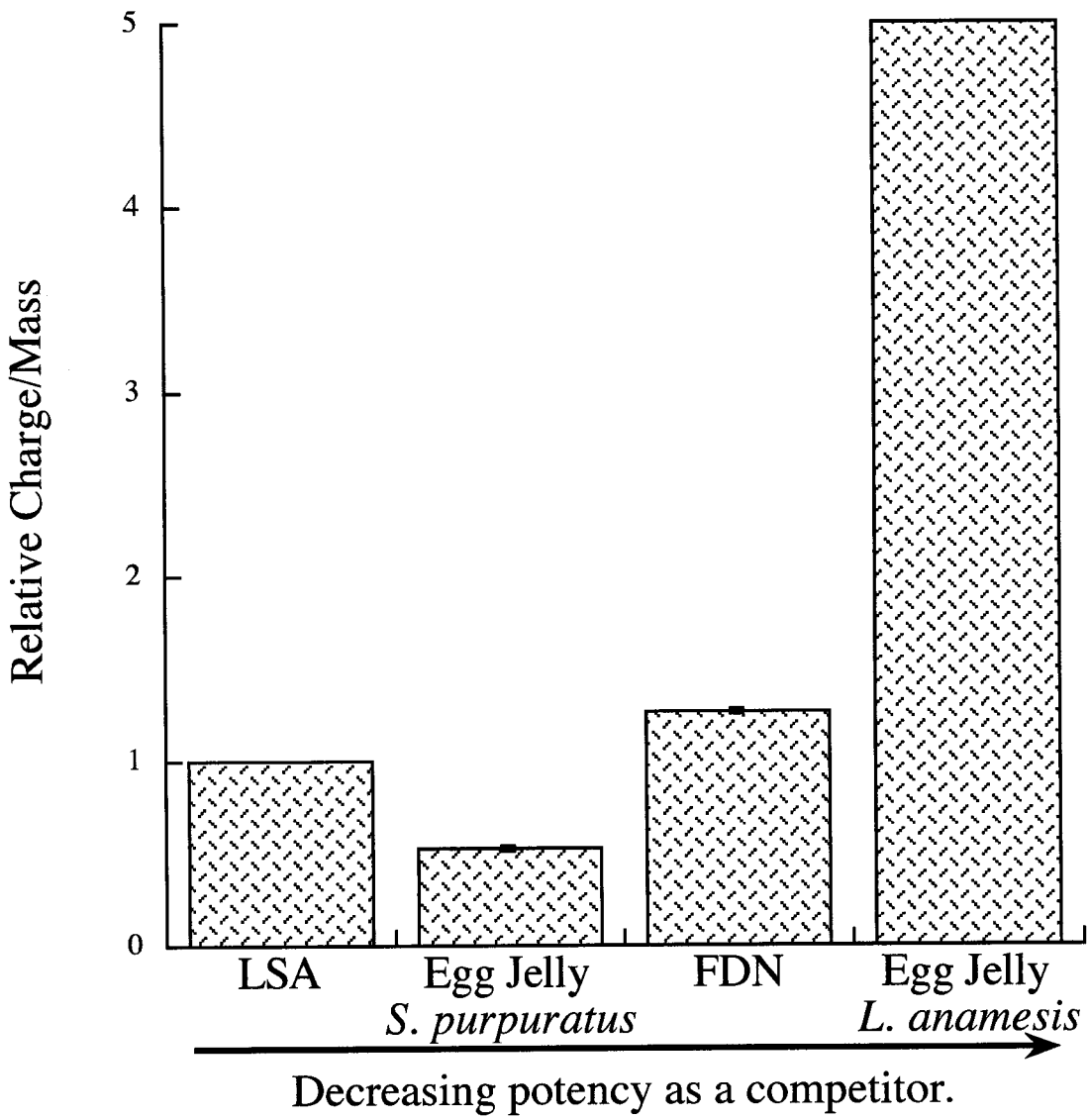


FIGURE 3.6: Relative charge to mass ratios of LSA-b competitors. Competitors are ranked in order of decreasing potency from left to right. Charge density is not the key factor determining LSA/sperm binding. Charge to mass ratio for PASA was not calculated.

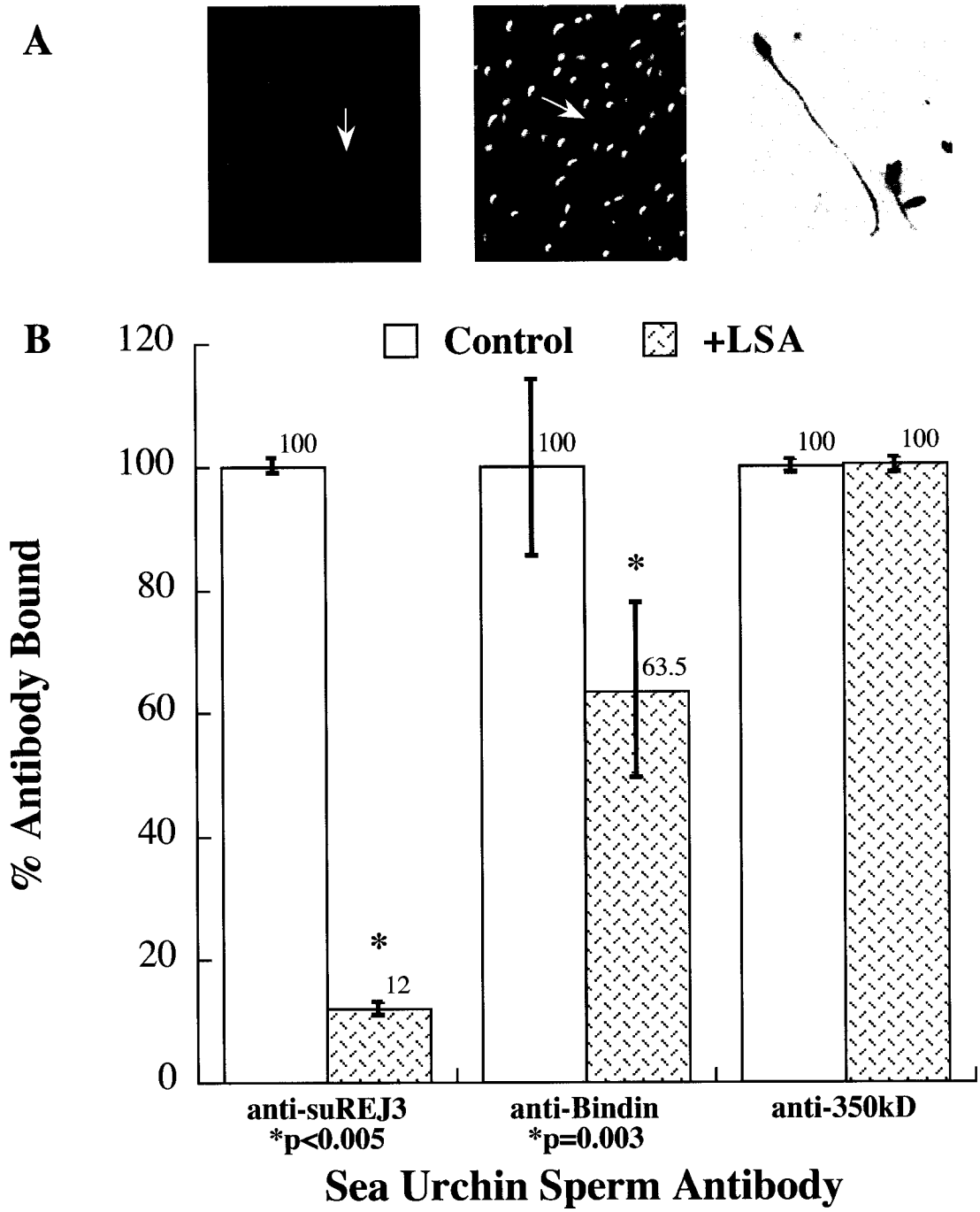


FIGURE 3.7: LSA selectively inhibits antibody binding. **(A)** Photomicrographs (fluorescent pseudo-color images overlaid on their DIC counterparts) indicating localization of antibodies on sperm, from left to right: anti-suREJ3 (red), anti-bindin (green, AR sperm), anti-350kD (blue). White arrows indicate specific binding to regions that co-localize with the binding of LSA-b. **(B)** LSA inhibits the binding of anti-suREJ3 and anti-bindin antibodies in AI and AR sperm (respectively). The binding of an antibody to a 350kD protein that localizes over the entire surface of the sperm cell is not inhibited.

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