Streptococcus pneumoniae hyaluronate lyase: An overview

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Hyaluronic acid is the most abundant glucoseaminoglycan of the extracellular matrix of connective tissues and is critical for cellular attachment and locomotion. This polysaccharide can be hydrolysed by various hyases that are widely distributed in nature. The degradation of HA and other glucoseaminoglycans by these enzymes facilitates the invasion of the host tissues, by bacterial pathogen, mostly Gram-positive *Streptococci*. The three-dimensional structures of *Streptococcus pneumoniae* hyaluronate lyase (SpnHL) have been determined recently by X-ray crystallography. The structure includes an N-terminal catalytic α-domain, C-terminal supportive β-domain connected by a short peptide linker. Based on the analysis of the crystal structures of native enzymes and their complexes with hexasaccharide substrate, a catalytic mechanism was proposed. The enzymes utilize a proton acceptance and donation mechanism to degrade their substrate. Crystal structure of hyaluronate lyase with vitamin C shows further possible structural requirement for the inhibitors to be designed specifically. Being a major surface protein of *S. pneumoniae* with potential antigenetically variable properties, SpnHL might represent another alternative for a pneumococcal vaccine.

HUMAN infections caused by Gram-positive bacterial pathogens are increasingly difficult to treat, predominantly due to emergence of resistant bacterial strains, not only against penicillin and penicillin-like antibiotics but even against novel antibiotics such as vancomycin[1]. One such Gram-positive bacterial organism is *Streptococcus pneumoniae*, a human pathogen that colonizes predominantly in the upper respiratory tract[2] (mostly nasopharynx) and is responsible for a number of diseases from the serious ones with significant mortality rates, such as pneumonia, bacteremia and meningitis to less serious but highly prevalent ones which cause significant morbidity, such as otitis media and sinusitis[3]. Disease rates are particularly high in young children, elderly and patients with immunosuppressive illness, particularly AIDS[4–6]. The asymptomatic carrier state, particularly in children, is thought to be the major reservoir of the pathogen. Around 5 million children in developing countries, younger than 5 years of age die each year from pneumonia[7]. Pneumococci account for several million cases of acute otitis media and an estimated 60,000 cases of invasive disease per year in USA[8]. Because of the worldwide increase in bacterial strains resistant to multiple antibiotics, treatment has become more difficult than in the past[9].

Prevention of pneumococcal disease relies today on vaccination of the susceptible population. However, vaccination encounters a number of problems such as the limited quantity of serotypes in the pediatric formulation, incomplete protection against colonization and selection of nonvaccine serotypes[10,11]. There is need for an alternative preventive strategy for situations where vaccination is insufficient, impossible or inefficient. Eradication or even reduction of nasopharyngeal carriage is likely to have a major impact on the transmission of *S. pneumoniae* and the incidence of infection. Antibiotic prophylaxis in controlled surrounding has shown limited success, but carries the risk of selective pressure resulting in an increase in resistant strains[12]. Until now, there has been no substance that can specifically reduce the number of pneumococci carried on human mucous membranes without affecting the normal endogenous mucosal flora.

Glycan

Oligosaccharide chains are ubiquitously present on cell, protein and lipid surfaces. They perform a variety of important biological functions and play an important role in host–microbe interactions. For higher organisms such as vertebrates, the fine regulation of synthesis and degradation of sugars (glycans) is essential not only for their primary function but also for many regulatory processes. Their primary function is often related to mechanical properties of polysaccharides and their complexes in environments such as the extracellular matrix (ECM) of tissues. Their regulatory processes involve lymphocyte homing, intercellular interaction, proliferation and locomotion. These physical regulatory processes are often facilitated by rapid turnover of sugar molecules and their complexes or assemblies[13–25].

Glucoseaminoglycans (GAGs), oligosaccharide chains of the ECM of tissues, are a specific type of glycan. They differ in the type of disaccharides they utilize as building blocks, and in the linkage between the building blocks[26]. Such diversification led to their division into three struc-

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tural groups: (i) cellbiose (e.g. hyaluronan), (ii) polylactosamine (e.g. dermatan, chondroitin and keratan sulphate) and (iii) polymaltose (heparin, heparan sulphate). Chondroitin, keratin sulphate and hyaluronan have a similar polymer backbone structure (Glcβ1 → 3Glcβ1 → 4).

Hyaluronan (hyaluronic acid)

Hyaluronic acid (HA) is a ubiquitous component of the ECM of vertebrates. It is widely distributed in various tissues such as vitreous humour of the eye, the aorta, blood, liver, synovial fluid, loose connective tissues and skin[27] (Table 1). This linear polysaccharide is composed of a repeating unit of chemical structure, [→ 3)GlcNAc (β1 → 4)GlcUA(β1 →]$_n$, where GlcNAc is *N*-acetyl-D-glucosamine and GlcUA is D-glucuronic acid (Figure 1). It has enormous size up to 25,000 disaccharide units of molecular weight ~10^7 Da. HA is highly hydrophilic and because of its ability to bind large quantities of water, it can form highly viscous solutions and thereby influence properties of the matrix[28,29]. One of the main mechanical/structural functions of HA is to lubricate joints and to absorb shock[30]. It has been implicated in many biological processes, including fertilization, embroynic development, cell migration, cell differentiation, wound healing, inflammation, and growth and metastasis of tumour cells. HA has also been assigned a variety of important physiological functions[31]. At physiological concentrations HA molecules form a random network of chains. Such a network may act as a sieve and regulate the distribution and transportation of plasma proteins[14,32].

In addition to ECM-based interactions of HA, there are numerous cell surface receptors for HA, including the receptor for hyaluronic acid-mediated motility (RHAMM) and CD44, which are present on the lymphocytes[33–36]. HA binds with high affinity (Kd = 1–2 × 10^{-9} M) to the surface of cells due to the interaction of a single molecule of HA with multiple receptor sites[37]. Binding of this type has been shown to occur in several types of cell lines. RHAMM has been associated with cell motility, which declines when the binding of HA to the cell is lost. CD44 has somewhat different affinities for HA depending on its origin and it is involved in lymphocyte homing, intercellular adhesion, endocytosis of extracellular HA and cell migration, etc.[38,39]. CD44 can mediate cell attachment to the ECM and metastatically spread tumour cell[14].

Table 1. Concentration of hyaluronan in human tissues and tissue fluids

Tissue/fluid	Hyaluronan concentration (mg/l)
Umbilical cord	14,100
Synovial fluid	1,420–3,600
Vitreous body	140–338
Dermis	200
Thoracic lymph	8.5–18
Urine	0.1–0.5
Serum	0.01–0.1

Pneumococcal invasion

Pneumococcal adherence to host cells is suggested to be a two-step process[40]. The first step involves targetting an atomic niche of the host, like the nasopharynx, to bind to the host-surface glycoconjugates on respiratory endothelial cells. Following this step, cytokine activation is induced, which results in expression of novel glycans on the surface of activated cells and increased pneumococcal adherence. An example of such cytokine activation is the expression of platelet activating-factor receptors on some host cell surface. These two steps lead to pneumococcal invasion of the host and to pneumococcal disease[41].

Hyases

Bacteria, in particular bacterial pathogen, often utilize glycan-degrading enzyme (hyases) to overcome the host defence mechanism to advance bacterial invasion of the host and to reach desired host sites that are essential for continuing bacterial invasion[42]. These enzymes can be grouped into two, hydrolases and lyases. Hydrolases degrade glycan via hydrolysis of glycosydic bond between sugars, and lyases degrade glycan using a β-elimination process.

Two independent lines of investigation led to the discovery of hyases. One area of research started with the observation about 70 years ago, that the extract from mammalian testis and other tissues contained a spreading factor substance that facilitated the diffusion of antiviral vaccine, dyes, toxins, etc. injected subcutaneously[43,44]. The other area was isolation and characterization of hyaluronan from vitreous humour[45]. Soon after the discovery of hyaluronan in 1934, it was demonstrated that extracts of type-2 pneumococcus (*S. pneumoniae*) depolymerize this macromolecule. Following these early observations, a number of investigators noted the presence of hyaluronan-degrading enzymes in microorganisms, including strains of *Micrococcus*, *Streptococcus*, *Peptococcus*, *Propionibacterium*, *Bacteroides* and *Streptomyces*. In subsequent years, it was demonstrated that at least three types of hyases exist and that they hydrolyse HA by different mechanisms. In addition, two additional prokaryotic hyases have been characterized. One is from *Clostridium perfringens*[46] and the other from a bacteriophage isolated from a strain of *Streptococcus pyogenes*[47].

Hyaluronidase

The term hyaluronidase was introduced to denote the enzyme which degrades HA[48]. Hyaluronidase activity has

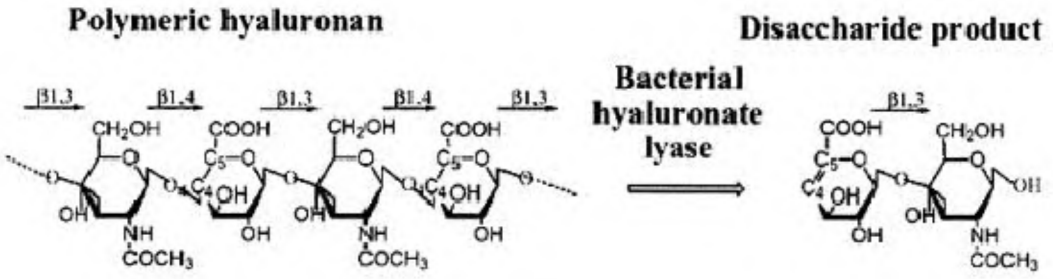

Figure 1. Structure and degradation of hyaluronan. Alternating units of glucuronic acid and *N*-acetylglucosamine are the building blocks of the hyaluronan polymer. The main digestive product of the action of bacterial hyaluronate lyase on hyaluronan is the disaccharide unit. The glycosidic linkage is also marked[42].

been detected in the venom of snake, fish, wasps, scorpions, spiders, etc.[49,50]. In the human body, hyaluronidase is found both in organs (testis, spleen, eye, liver, kidney, uterus and placenta) and body liquids (tear, blood and sperm). Testicular hyaluronidases have significant homology with the protein PH-20 present on the posterior head and the acrosomal membrane of mammalian sperm. PH-20 is originally synthesized as a polypeptide with an apparent molecular weight of 64 kDa and it is bound to the membrane via a glycosyl–phosphatidyl–inositol (GPI) anchor. In the course of sperm maturation, part of PH-20 is processed into two fragment linked by disulphide bridges: an amino terminal domain of 41–48 kDa and a carboxy terminal-domain of 27 kDa. It has been shown that PH-20 plays an essential role in fertilization[51].

In particular, bee-venom hyaluronidase has been studied in greater detail and its crystal structure is now known. It is a glycoprotein of molecular weight 66 kDa. Bee-venom hyaluronidase has 40% homology with the mammalian PH-20 (ref. 52) and greater than 50% sequence identity with other hymenoptera and, interestingly, with human lysosomal hyaluronidase. Recently, the human plasma hyaluronidase (Hya1-1) was purified, cloned and expressed. It shows over 40% sequence identity with PH-20, i.e. sperm-specific hyaluronidase[53]. Urine, in contrast to plasma, contains two hyaluronidase of 57 and 45 kDa; their isozymes recently purified and microsequenced. The lower Mr isozyme is believed to be an endo proteolytically processed form of the larger enzyme[54].

Hyaluronidases of animal origin hydrolyse HA by adding a water molecule between the disaccharide unit or by a process of transglycosylation[55]. Such a dramatic difference in the HA degradation mechanism between bacteria and animals may be the result of living competition in the course of evolution, in which the emergence of an alternative HA-degradation pathway would provide animals with certain means of resistance to bacterial invasion. This evolutionary force drives the branching of hyaluronidases into different catalytic mechanisms in bacteria and animals.

Classification

Hyaluronidase enzymes degrade hyaluronan, yielding various lengths of oligosaccharide units as end-products. There are three main classes of hyaluronidases: (i) hyaluronate 4-glycanohydrolase (hyaluronoglucosaminidase, EC 3.2.1.35), e.g. testicular hyaluronidases[56–58]; (ii) hyaluronate 3-glycanohydrolase (EC 3.2.1.36), e.g. leech hyaluronidases[59], and (iii) hyaluronate lyase (EC 4.2.99.1), e.g. bacterial hyaluronidases[60]. The first two groups of enzyme are hydrolases. Hyaluronate 4-glycanohydrolases cleave β-*N*-acetyl-hexosamine-(1 → 4) glycosidic bonds in HA, and chondroitin and chondroitin sulphates yield even-numbered oligosaccharide units of HA and *N*-acetylglucosamine at the reducing end. Hyaluronate 3-glycanohydrolases, such as leech hyaluronidase are specific for the glucuronic linkage in HA and are inert towards other polysaccharides. Finally, bacterial (*S. pneumoniae*, *S. agalactiae*, etc.) hyaluronate lyases cleave HA (β-GlcNAc-(1 → 4) glycosidic bonds[60–63]. In contrast to other hyaluronidases, they hydrolyse and yield 4,5-unsaturated oligosaccharides of various lengths, sometimes as small as disaccharides of HA. The most specific lyase is the commercially available enzyme prepared from *S. hyalurolyticus*, which is also an eliminase but gives mixtures of tetra- and hexa-saccharides as final degradation products of HA. It is inert towards other glucoseaminoglycans, e.g. chondroitin, chondroitin 4/6 sulphate, dermatan sulphate, keratin sulphate, heparan sulphate and heparin[64].

Some of the bacterial hyaluronate lyases also cleave certain chondroitin, chondroitin sulphates at every unsulphated disaccharide repeat, resulting in oligosaccharides with unsaturated hexuronic acid residues at their non-reducing ends. This ultraviolet chromophore provides a convenient method for the detection of the reaction product. The products can also be tagged through their free reducing groups with either of the fluorescent reporters, 2-aminopyridine or 2-aminoacredone, which makes it possible to separate and quantitate nanogram amounts of saturated mono and disaccharide (derived from the non-

reducing termini) and the unsaturated disaccharide (derived for their interior portion) produced by specific lyase digestion of hyaluronan, chondroitin and chondroitin sulphate chains in a mixture[65]. Currently, the activity of hyaluronate lyase is defined as its ability to breakdown HA to unsaturated disaccharide units, which is monitored by an increase in absorbance at 232 nm at 25°C (ref. 66). In addition, a variety of assays have been described to measure the activity of these different enzymes.

Hyaluronate lyase of *S. pneumoniae*

Most strains of *S. pneumoniae* as well as other Gram-positive bacterial pathogens produce hyaluronate lyase[30,60,67,68]. Pneumococcal hyaluronate-lyase enzyme by breaking down hyaluronan, a ubiquitous and important constituent of connective tissues, is directly involved in the host invasion by *S. pneumoniae*. The exact mechanism of how the enzyme facilitates bacterial penetration against the physical defences of the host and the subsequent spread to its tissues, was poorly understood until recent structural studies were reported[5,69–72]. In *S. pneumoniae* cultures, the enzyme is found in both the culture and cell-associated fractions. This suggests that at least some amount of the enzyme may be released by the pathogen to the surrounding host tissues during infection to facilitate bacterial invasion.

Four bacterial hyaluronate lyase protein sequences from *S. pneumoniae*, *S. agalactiae*, *Staphylococcus aureus* and *Propionibacterium acnes* are known at present[73,74]. The sequence homologies range from 25 to 53%, suggesting their functional, structural and evolutionary similarities. *S. pneumoniae* hyaluronate lyase (SpnHL) has 21.3% homology to chondroitin AC lyase, another member of the GAG-degrading enzymes from the bacterium *Sphingobacterium heparinum*.

The full-length SpnHL has a molecular mass of 107 kDa when expressed in *Escherichia coli*[60]. Recently, the initial characterization of the gene for SpnHL was carried out. When expressed in *E. coli*, an active hyaluronate lyase of 107, 89, 91 and 94 kDa was observed, which shows the different proteolytic fragments of the enzyme. Interestingly, the major 89 kDa protein shows different translation initiation and lacks the first 162 *N*-terminal residues, and is suggested to be translated from a second initiation site at Met-163. The subsequent cloning and expression of the truncated but functional form of hyaluronate lyase (83 kDa) containing amino acids Ala 168 to Glu 891 lacking the C-terminal cell-wall anchoring domain was done by different research groups including us and the protein is now been extensively characterized[71,72,75]. The carboxy terminus of the mature enzyme contains a hydrophobic tail preceded by a signature sequence from Leu 919 to Gly 923 of LPQTG and a group of charged residues found in enzymes binding covalently to peptidoglycan structure to form a cell-wall anchor[76]. The general mechanism of such attachment comprise a group of such signature sequence properties. It requires a sorting signal located at the carboxy terminus of proteins, which consists of an LPXTGX (*X* represents any amino acid) motif followed by a carboxy-terminal hydrophobic domain and preceded by a tail of mostly positive-charged residues[76,77].

Activation of hyaluronate lyase

The analysis of the sequence and the three-dimensional structural information of two hyaluronate lyase enzymes from *S. pneumoniae* and *S. agalactiae*, suggests that the enzymes are synthesized by these two pathogens in their inactive, zymogen form. Then, they are secreted outside of the bacterial cell with activation by cleavage of the signal peptide, leaving an active form of the enzyme. Both these enzymes undergo significant degradation before becoming active. At least part of this process is caused by auto degradation. For example, the native, full-length *S. agalactia hyaluronate lyase* (SagHL) undergoes extensive proteolysis, resulting in a mixture of at least three molecular-weight products, 118, 111 and 92 kDa forms of the enzyme. The 118 kDa form is enzymatically inactive, while the 111 and 92 kDa forms are active[78]. The behaviour of SpnHL is similar even though not all forms of the enzyme were characterized, unlike the case of (SagHL).

Three-dimensional structure

The exact molecular mechanism of lyase action was largely unknown until recently when the first structural information on bacterial hyaluronate lyase was obtained by X-ray crystallography. Similar structural information for endogenous hyaluronidases is not available at present. Therefore details regarding this process are still unclear and are based on the comparison of other polysaccharide-degrading enzymes in the class of hydrolases.

Structure of SpnHL

The crystal structure of the SpnHL molecule has approximate dimensions of $59 \times 59 \times 88$ Å^3 and is divided into two distinct structural domains connected by one short polypeptide linker (Figure 2). Both domains are spherical and are of approximately the same size. The N-terminal alpha helical domain (α-domain) contains the first 361 residues (Lys171–Ser531) of the enzyme, and is composed of 13-α-helices connected by 12 loops (residues taking coil conformation, including various turns and 2–3 residues of short β-sheet and 3_{10}-helix). Only one 11-residue linker (Asp532–Ser 542) is located be-

tween the two structural domains. The C-terminal β-sheet domain (β-domain) contains 347 residues (Tyr 543–Lys889) in 24 β-strands (packed into five antiparallel β-sheets), four short α-helices and 25 connected loops. Interface between the α and β-domains is 1776 Å^2 in area and is composed of 37 residues from the α-domain and 34 residues from the β-domain forming 343 interactions[71,72].

The enzymes that are closely related to SpnHL in terms of fold and structure include SagHL, *Flavobacterium heparinum* chondroitin AC lyase (FheCACL) and to a lesser extent, the Sphingomonas species alginate lyase AI-III (SspAL)[62,79–82]. SpnHL, SagHL and FheCACL are composed of two domains, a catalytic domain that is a highly α-helical α6/α6-barrel domain or α-domain, and a neighbouring domain, rich in β-sheet, β-domain connected by only one flexible linker peptide composed of several residues. Therefore, it is feasible that both domains have a significant degree of movement with respect to one another, which could account for at least two properties for these enzymes: (i) widening and narrowing of the catalytic cleft to accommodate substrate binding prior to catalysis, and (ii) attenuation of activity by regulating access to the active site cleft via blocking its entrance.

In addition, *S. agalactiae* has a third domain at its N-terminal, from residue Ala171 to Val244, which is primarily built from β-sheet structure and is termed as β-2 domain. The SpnHL structure reflects a closed conformation of the enzyme, whereas SagHL structure exists in an open conformation (Figure 3).

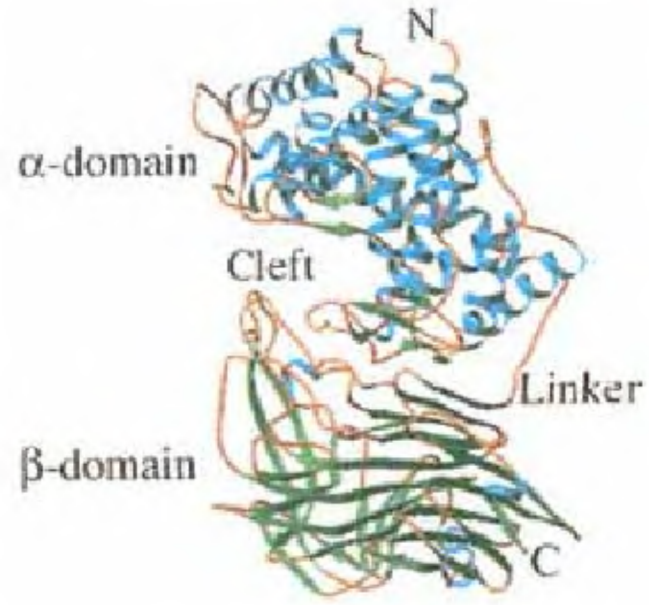

Figure 2. Three-dimensional structure of *SpnHL*. Both domains of the enzyme are shown, the α-helical domain (top) and the β-sheet domain (bottom). Structure of the enzyme is colour-coded by secondary structure elements: α-helices, Blue; β-sheet, Green; Others, brown. Figure has been generated using RASMOL[113] from the protein crystal structure 1egu in the PDB[72].

A comparison of SpnHL and SagHL suggests that the dimensions of the catalytic cleft can be altered. The cleft of these two enzymes differs primarily in the width, with the SagHL cleft being wider. Although both the domains of these enzymes are similar in structures, the structures of the whole enzyme have less similarity due to the different positioning of the two domains relative to one another. However, both the enzymes degrade the same substrate, hyaluronon, and they utilize the β-elimination process that produces disaccharides of HA as the final degradation product[80,83].

The other structures which have been elucidated include *Streptococcus glucomylase*, endoglucanase CelA, CelD and endo/exocellulase[84–87]. They all share one common structure motif, a barrel-like fold with α6/α6, α6/α5 or α5/α5 topology but differ mostly in the number of helices and details regarding the barrel-like fold. All of them contain a cleft transversing the molecule where the substrate, a polysaccharide chain, can bind and is degraded through hydrolysis or β-elimination involving selected residue from the substrate binding and the catalytic cleft.

Mechanism of HA degradation

The mechanism of all lyase enzymes that degrade polysaccharides is based on the β-elimination process. For at

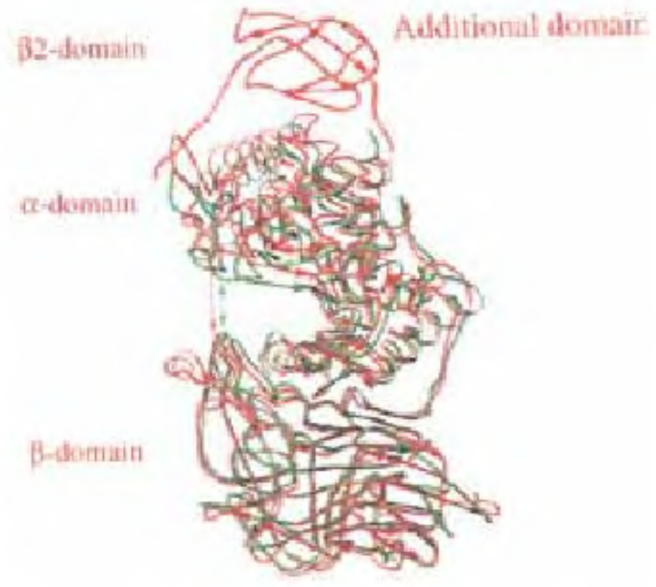

Figure 3. Structure alignment of three-dimensional structures of hyaluronate lyase from streptococcus species. The alignment is based on the structure of *S. pneumoniae* (green) with that of *S. agalactiae* hyaluronate lyase (red). *S. agalactiae* enzyme structure shows the presence of an additional β-sheet domain, β_2-domain, at its N-terminal. Cleft area in *S. agalactiae* enzyme is significantly wider (described as open conformation) than in the pneumococcal enzyme (described as closed conformation). Approximate width of the cleft is marked by arrows, red for SagHL enzyme structure and green for SpnHL structure. Figure has been generated using RASMOL[113] from the protein crystal structure 1egu and 1f1s in the PDB[80].

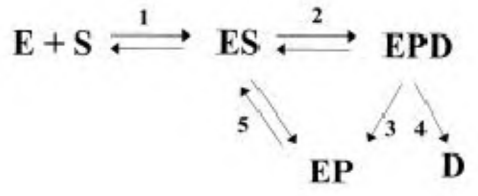

Figure 4. Schematic diagram of hyaluronan degradation. E, Enzyme; S, Hyaluronan substrate; EPD, Enzyme with bound truncated hyaluronan substrate and a disaccharide product; EP, Enzyme with bound truncated hyaluronan substrate and disaccharide product released form the active site of the enzyme[63].

least some polysaccharide lyase (SpnHL and SagHL) this process is based on the proton acceptance and donation (PAD) mechanism (Figure 4). Catalysis through this mechanism involves five general steps: (i) the polysaccharide substrate binds to the catalytic cleft, (ii) the carboxyl group of the glucuronic moiety of HA on the C5 carbon atom is neutralized by Asn349, (iii) a proton from the C5 carbon is extracted by His399 and an unsaturated bond is formed between C4 and C5 of the leaving polysaccharide group, (iv) the glycosidic bond is broken after a proton is donated from the protein by Tyr 408 (SpnHL numbering scheme) and finally (v) the cleaved product leaves the active side, while the catalytic residues His399 and Tyr408 balance the proton by their exchange with water. During the process the C5 carbon atom changes its hybridization from sp^3 to sp^2 with respective changes in the product conformation of the sugar ring (puckering of the sugar ring)[71,72].

On the other hand, hydrolysis of the polysaccharide seems to follow a different mechanism, either a direct or a double displacement (DD) mechanism involving two glutamic acid or aspartic acid residues separated by 6 Å. The steps involved in this mechanism are as follow: (i) binding of polysaccharide substrate, (ii) cleaving the glycosidic bond in the substrate and forming a covalently linked glycosyl enzyme intermediate with the inversion of the anomeric C1 atom configuration (glycosylation), and (iii) cleaving the enzyme glycosyl bond involving a water molecule with the assistance of the deprotonated carboxylate residue, leading to the second inversion of the configuration of C1 (deglycosylation)[88,89].

The structure of SpnHL and its complexes with hexasaccharide substrate and disaccharide product was also determined and the properties including the mechanism of catalysis were proposed. The different catalytic residues Asn349, His399, Tyr408 and the residues responsible for substrate binding and translocation were clearly identified.

The active-centre residues in SagHL are also conserved and are similar to SpnHL. The catalytic residues of SagHL include Asn429, His479 and Tyr488, the hydrophobic patch is composed of Glu468, Asp478 and Thr480 (ref. 80). These residues structurally align closely with homologous residues of the SpnHL. In addition, the catalytic residues are strictly conserved in the sequence of all known bacterial hyaluronate lyase, chondrotin lyase and FheCACL.

The cleft between the α- and β-domains of the SpnHL molecule surface is about 30 Å in length and 10 Å in width. The cleft is the place where HA binds and is degraded. The widely examined key catalysing residue, His399, is located inside the cleft at the edge of this surface[66], and is in contact with Glu577 from loop β L3 in the β-domain. Most of the residues along the cleft are either charged or aromatic. The surface electrostatic potential distribution shows that the centre of the cleft is highly positively charged. Four negatively-charged residues, Glu388, Asp398, Thr400 and Glu577, accumulate at one end of the cleft and no positively charged residues are present in this region. This makes one side of the end of the cleft slightly negative, forming a negative patch. Three aromatic residues Trp291, Trp292 and Phe343 are aligned at one side of the cleft, opposite to the above negative patch and His399. These three residues form a small aromatic patch. The existence of this aromatic patch in the cleft provides the SpnHL molecule with a way to select the cleavage sites on the substrate[71]. The SpnHL sequence comparison with known hyaluronate lyase sequences from other species, *S. agalactiae*, *S. aureus* and *P. acnes* showed 25 conserved residues along the cleft. Most of these residues are arranged along the middle narrow segment of the cleft and are also conserved in the chondroitin lyase sequence from *S. heparinum* (*Flavobacterium heparinum*).

HA is a linear polysaccharide built of repeating units of β-D-glucuronic acid and 2-acetamino-2-deoxy-β-D-glucose. Since the pKa of the glucuronic acid carboxylate group is approximately ~3.2, HA is negatively charged under physiological conditions[29]. The interaction between SpnHL and HA is dependent in part upon ionic interactions and involves basic amino acids[35]. Therefore, salt bridges and hydrogen bonds play a key role in this interaction. The positive charges in the cleft facilitate the attachment of the SpnHL molecule to the negatively charged HA, and these charges hold the substrate chain in position during catalysis. The negative patch in the cleft provides an electrostatic force for the release of the unsaturated and negatively charged disaccharide product. The aromatic patches in the cleft accurately bind the cleavage sites on the substrate chain, anchoring them in their correct position[71].

Role of β-domain

In the structurally related enzymes glucoamylase and alginate lyase, only the α-domain is present. In the SpnHL structure only one residue from the β-domain, i.e. Asn580 in β-L3 participates in catalysis. These observations suggest that

the β-domain of SpnHL most likely plays a supportive role, probably by holding the HA chain during the glycosidic bond cleavage, rather than contributing any of its residues directly for the catalytic process. The other possibility is that β-domain regulates access of the substrate to the active cleft of the enzyme. Such regulation might be dependent on Ca^{2+} binding to this domain which induces structural changes to the loops close to the catalytic cleft. Such Ca^{2+} binding might stabilize the active conformation of SpnHL. Similar behaviour has been observed for *Clostridium thermocellum* endoglucanase CelD a cellulose-degrading enzyme[90].

It is well known that the SpnHL enzyme activity depends upon the presence of Ca^{2+}, which is most likely to bind to the β-domain. Functional significance of the existence of β-domain might be to modulate SpnHL activity through controlling the substrate entrance, accomplished by the binding of small molecules such as Ca^{2+}. Recent folding studies where the intact N-terminal domain was proteolytically cleaved and purified showed enzymatic activity, which suggests that the N-terminal domain is the functional domain and the C-terminal domain seems to modulate the enzymatic activity probably by holding the substrate[71,72].

Effect of mutations on catalysis of SpnHL

Role of different residues in the mechanism of catalysis shows that the mutation in five residues R243V, N349A, H399A, Y408F and N580G changes the enzyme activity up to 67, 6, 12, 0, and 115% respectively. All these five residues are strictly conserved among all known bacterial hyaluronate lyases. In addition, recent studies in the double or triple mutation of the amino acid residues of the hydrophobic patches show complete loss in enzyme activity[91]. As expected, mutations of the catalytic residues in SagHL also completely inactivated the enzyme[71,72,80].

Treatment of pneumococcal infection

The currently licensed 23 valent polysaccharide pneumococcal vaccine is only moderately effective, and is not prescribed for children younger than two years due to poor antibody response to the polysaccharide. The vaccine contains 23 purified capsular polysaccharide antigens of *S. pneumoniae* that represent at least 85–90% of the serotype that causes invasive infection[92–95]. The serotypes 6B, 9V, 19A, 19F and 23F cause most drug-resistant infections. Although antimicrobial drugs such as penicillin have diminished the risk for pneumococcal disease, the proportion of strains that are resistant to antibiotics is steadily increasing. Recently, however, vancomycin tolerance also emerged in pneumococci[1]. This reinforces the need for an effective improved vaccine and/or effective new drugs against pneumococcal infection.

Individual contributions of known virulence factors of *S. pneumoniae* to the pathogenesis of this organism and the development of a pneumococcal vaccine have recently been investigated. The antigens that reduced the virulence of the organism and are probably the best candidates for vaccine development are pneumolysin (ply), two neuraminidase (NanA, NanB), major autolysis (LytA), choline-binding protein A (CbpA), pneumococcal surface antigen (PsaA) and pneumococcal surface protein (PspA)[96–101]. These proteins are involved in interactions with the host complement system (PspA), degradation of hyaluronon of the ECM (hyaluronate lyase), lysis of cholestrol containing membrane (Ply), degradation of peptidoglycan layer of pneumococci most likely to release cytoplasmic Ply and inflammatory degradation products of cell wall (LytA), and binding of metals (divalent cations) such as Mn^{2+} or Zn^{2+} (PsaA) followed by their transport inside the cytoplasm of pneumococci (ABC-type transporters)[42].

The functions of all the above proteins facilitate significant aspects of pneumococcal colonization and/or invasion; compromising these functions leads to compromised pathogenicity of *S. pneumoniae*. Therefore these proteins can serve as targets for the development of novel therapy to treat pneumococcal disease. On one hand, the antibodies against the majority of these surface antigens are protective against the disease, and therefore these antigens can be used as protein-based vaccine candidates. The poor immunogenicity of the polysaccharide vaccine is due to a poor antibody response elicited by these vaccines and to the fact that the T-cell independence of the response fails to induce memory. The development of conjugated vaccine by coupling the polysaccharide with protein carrier should increase the potency of the vaccine. Vaccines composed of a mixture of polysaccharide and protein antigens are likely to provide better protection against *S. pneumoniae* than vaccines based only on one or a limited mixture of the possible single-type (polysaccharide or protein) protective component[42].

Recently, it has been shown that the purified pneumococcal lytic enzyme (pal) is able to kill about 15 common serotypes of pneumonia, including highly penicillin-resistant strains and prevents its colonization in the nasopharynx[102].

The search for an inhibitor for hyaluronate lyase started long back when it was found that certain chemicals inhibit hyaluronidase activity. The activity of hyaluronidase is modulated by various activators (adrenaline, histamine and acid phosphatase formed in the prostrate, spleen, kidney, erythrocytes and platelets) and inhibitors (vitamin C, salicylate, heparin, dicumarene, antihistaminica and flavonoids)[103]. The recently known crystal structure of vitamin C in complex with SpnHL showed the real mechanism of inhibition. The IC_{50} of inhibition for vitamin C was about 5.8 mM. The structural basis of this inhibition is due to the structural similarities of vitamin C and glucuronate residues in hyaluronan, the substrate of

hyaluronate lyase[104]. The inhibitory effect shows that vitamin C is probably directly involved in bacterial invasion, in addition to its antioxidant and free radical scavenger properties[105,106].

Significance

In addition to the above-mentioned properties, hyaluronidase and HA also have other biologically significant roles.

Therapeutic uses of hyaluronidase and HA

Hyaluronidase and HA are widely used therapeutically in many fields like ophthalmology, surgery, gynaecology, etc.[107]. During surgical intervention in the eye, HA is often injected intraoperationally to keep the anterior eye chamber intact or to protect the corneal endothelium during lens transplantation. The concomitant increase in intraocular pressure can be efficiently counteracted by injection of hyaluronidase[108,109]. Hyaluronidase can be used as an alternative or adjunct to conventional mechanical vitrectomy[110].

Physiological role of hyaluronidase

HA fragmentation by hyaluronidase stimulates angiogenesis. Fibrotic healing of adult and late gestational wounds correlates with increased hyaluronidase activity and removal of HA[111]. In general, hyaluronidase is thought to play a role in HA homeostasis and metabolism, e.g. in the turnover of anterior chamber HA, and in the degradation of highly concentrated HA used as viscoelastic.

Biological role

As mentioned above, the major end product obtained with hyaluronidase is unsaturated hexuronido-*N*-acetylglucosamine, except that unsaturated tetra- and hexasaccharides are obtained with the hyaluronidase from *S. hyalurolyticus*. Most, if not all, the unsaturated disaccharide-producing microorganisms also have a beta-glucuronidase that produces an alpha-keto acid and *N*-acetylglucosamine from the disaccharide. Therefore, HA in the host mucosal layers and in the host diet is probably the significant source of carbon and energy, particularly for bacteria present in the human colonic micro flora[112].

Commercial importance

Generally, hyaluronidase is extracted from bovine and sheep testis or from bacteria. For therapeutic purposes, only highly purified hyaluronidases should be used. Highly purified preparations include testicular hyaluronidase from bovine testes and have an activity of 40,000–50,000 IU/mg. The most frequent contaminating activities in less pure preparations are proteolytic and glucuronidase activities[113].

Concluding remarks

Certain proteins or enzymes displayed on the surface of Gram-positive organisms significantly contribute to pathogenesis and might be involved in the disease process caused by these pathogens. Often, these proteins are involved in direct interaction with host tissues or in concealing the bacterial surface from the host defence mechanism. *S. pneumoniae* is not an exception in this regard. In the past, the polysaccharide capsule was considered the primary virulence factor of *S. pneumoniae* because the noncapsulated bacteria are almost completely harmless compared to the encapsulated strain. Recent studies however have suggested that certain pneumococcal proteins, could be used as potential vaccine candidates. If antibodies to these proteins could offer better protection to humans, they could provide the source of a pneumococcal vaccine. Some of these proteins such as PspA or Ply, have already shown significant promise for use in an alternative vaccine approach. For example, PspA can elicit antibodies in mice that protect against inocula more than 100 times the 50% lethal dose.

Hyaluronate lyase is another major surface protein of *S. pneumoniae* with potential antigenetically variable properties that might be essential for full pneumococcal virulence. Thus it might represent an alternative for a pneumococcal vaccine or drug target, especially when combined with other pneumococcal virulence factors such as PspA or pneumolysin.

The availability of three-dimensional structural information about pneumococcal proteins with tetra and hexasaccharide substrate and inhibitors will most probably facilitate the elucidation of the function and detailed mechanism involved in such functions. Such knowledge will aid the development of treatment strategies or to design specific inhibitors for pneumococcal disease, which may have therapeutic utility as well as help in the progress of science in general.

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