Unusual Structural Features of the Bacteriophage-associated Hyaluronate Lyase (hylp2)*

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Hyaluronate lyases are a class of endoglycosaminidase enzymes, which are of considerable complexity and heterogeneity. Their primary function is to degrade hyaluronan and certain other glycosaminoglycans and facilitate the spread of disease. Among hyaluronate lyases, the bacteriophage-associated enzymes are unique as they have the lowest molecular mass, very low amino acid sequence homology with bacterial hyaluronate lyases, and exhibit absolute specificity for one type of glycosaminoglycan, i.e. hyaluronan. Despite such unique characteristics significant details on structural features of these lyases are not available. The Streptococcus pyogenes bacteriophage 10403 contains a gene, hylp2, which encodes for hyaluronate lyase (HylP2) in this organism. HylP2 was cloned, overexpressed, and purified to homogeneity. The recombinant HylP2 exists as a homotrimer of molecular mass about 110 kDa, under physiological conditions. Limited proteolysis and guanidine hydrochloride denaturation studies demonstrated that the N-terminal region of the protein is flexible, whereas the C-terminal portion has a compact conformation. The enzyme shows sequential unfolding, with the N-terminal unfolding first followed by the simultaneous unfolding and dissociation of the stabilized trimeric C-terminal domain. We isolated a functionally active C-terminal fragment (Ser 128–Lys 337) of the protein that was stabilized in a trimeric configuration. Comparative functional studies with full-length protein, N:C complex, and isolated C-terminal domain demonstrated that the active site of HylP2 is present in the C-terminal portion of the enzyme, and the N-terminal portion modulates the substrate specificity and enzymatic activity of the C-terminal domain.

Many pathogenic bacteria produce extracellular products that have tissue-damaging effects. Some of these products serve as virulent factors in the pathogenesis of disease by facilitating the spread of bacteria or toxins through tissues. They are commonly known as "spreading factors." Hyaluronic acid has long been proposed as a virulence factor, in particular a spreading factor. Hyaluronate lyase (EC 4.2.2.1 or EC 4.2.99.1) are hyaluronidases produced by bacteria, which are capable of degrading glycans, mainly polymeric hyaluronan (HA) 3, (1, 2). There are two possible functions of the degradation of HA by hyaluronidase in the biology of the organism in which it is present. First being the enhancement of invasion and spread of organism during infection by destruction of the extracellular matrix. Apart from degradation of HA, hyaluronate lyases have been demonstrated to possess a nutritional role in group A streptococci, where they permit the organism to utilize host HA as an energy source (3–5). Both of these functions are important during pathogenesis of different diseases caused by the organism.

Hyaluronate lyases show different specificities for the polysaccharide substrate. The Streptococcus hyalurolyticus hyaluronate lyase specifically cleaves HA endolytically, producing un-saturated hexa- and tetrasaccharides. Whereas, the hyaluronate lyase isolated from group A streptococci or group B streptococci besides cleaving HA also show weak but significant activities toward chondroitin and/or chondroitin 4/6-sulfate.

Among the hyaluronate lyases, the most studied ones are those secreted by strains of group B streptococci and belong to the class of glycosaminoglycan-degrading enzymes (3). The group B streptococci hyaluronate lyases do not exhibit absolute specificity for one kind of glycosaminoglycan as both HA and certain chondroitin sulfates (CS) are cleaved by these enzymes. Structurally, the group B streptococci hyaluronate lyases are monomeric enzymes with their N-terminal domain being the catalytic domain. The group A streptococci produce a number of different hyaluronidases that include a chromosomally encoded hyaluronate lyase (6, 7), bacteriophage hyaluronidases (6, 8–11), and Spy1600 (9). Among the hyaluronidases of group A streptococci, most of the available information is on the phase-specific hyaluronidases (6–8, 12–14). Unlike most bacterial hyaluronidases, which act nonspecifically on both HA and CS, the enzyme specifically cleaves HA (13).

A wide variation is observed in the molecular mass of the hyaluronate lyases from different strains of bacteria. Whereas the molecular mass of bacterial hyaluronate lyase ranges between 90 and 120 kDa; the streptococcal bacteriophage hyaluronate lyases have the smallest molecular mass, ranging from 36 to 40 kDa (6). The crystal structure and functional details of several bacterial hyaluronate lyases are available (15–17), however, structure and functional information of only one bacteriophage, HylP1, has very recently been reported (18). The bacterial hyaluronidases are monomeric molecules containing two relatively large sized structural domains (about 40 kDa or more), whereas the bacteriophage hyaluronate lyases are oligomeric. It will be interesting to study the detailed structural and functional properties of the bacteriophage-associated enzyme, which corresponds to only half the molecular mass (i.e. about one structural domain) of the bacterial hyaluronate lyases.

Bacteriophages from two species of streptococci, Streptococcus pyogenes (6, 22) and Streptococcus equi (19), encode for hyaluronidase. The bacteriophage hyaluronidases have been shown to be hyaluronate lyase, like the chromosomally encoded enzyme (13). Absence of an N-terminal signal peptide in the bacteriophage hyaluronidase genes indicates
that the bacterial cell (6, 8) does not actively secrete these gene products. Hence, their likely function could be to degrade HA of the streptococcal capsule and allow the phage access to the cell surface, so that it can infect the encapsulated cells (20, 21). The hyaluronate lyase genes found in the bacteriophage genomes show a high degree of similarity to each other with one major difference being the deletion (or addition) of a 102-bp fragment that consists of a region that encodes a collagen-like motif, Gly-X-Y repeating units (6, 20). Based on this difference, they have been grouped into two types: the hylP-type, which contains a collagen-like repeat sequence and the hylP2-type, which lacks this repeat sequence. Based on the presence of collagen-like domain, it has been speculated that the HylP-type proteins might be stabilized as a triple helical structure (23). Recently reported three-dimensional structural hyaluronate lyase, hylP1, obtained from S. pyogenes revealed an unusual triple-stranded β-helical structure for this protein (18). For hylP2-type hyaluronidases, no other report except for the gene sequence is available.

For gaining insight into the structural properties of the bacteriophage-associated hyaluronidases, we carried out cloning and overexpression of the hylP2 gene from S. pyogenes bacteriophage 10403. The overexpressed protein was purified to homogeneity and its structural and functional properties were studied in detail. For understanding the role of the structural domains in modulating the functional activity and in stabilization of the oligomeric structure of the protein, studies on the isolated C-terminal domain of HylP2 were also carried out.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids—**Escherichia coli DH5α cells were used during the cloning of the gene. pET21d (+) vector (Novagen) and BL21(DE3) (Novagen) were used for expression of the HylP2 protein.

**Cloning of HylP2—**Plasmid pUC18 containing the hylP2 gene (1.030 kb) was used as template. An internal gene fragment of S. pyogenes bacteriophage 10403 encoding functional hylP2 (GenBank accession number U28144) was amplified by polymerase chain reaction. PCR was performed with primers (forward, 5′-CTAGCTAGCATGACT-GAAAATATACCATTAAGAGTCC-3′ and reverse, 5′-CCGCTC-GAGTTTTTATGAGGTGTTTTTACCTGAA-3′) with the C-terminal His, tag hylP2 and 5′-CCGCTGACATTTTTTAG-TATGACTTTTAC-3′ without histidine-tagged hylP2, with Nhel and Xhol sites (as underlined). PCR conditions used were: 1 time at 94 °C for 5 min; 30 times at 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 3 min; and 1 time at 72 °C for 10 min. The amplified fragments were cloned in pET21d (+) vector (Novagen) between Nhel and Xhol sites. DNA sequencing confirmed the homogeneity of the sequence. The resultant constructs were transformed into E. coli BL21(DE3) cells for checking the expression.

**Overexpression of HylP2—**A single colony from the BL21(DE3) plate was inoculated into 5 ml of LB broth (Hi-media) having ampicillin at a concentration of 100 μg/ml and allowed to grow overnight at 30 °C. It was then subcultured in 400 ml of LB broth containing a similar ampicillin concentration and allowed to grow at 30 °C until A600 of 0.6 was achieved. The culture was then induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside and incubated further at 30 °C for 3 h. The cells were harvested at 8000 × g for 10 min and the resultant pellet was then stored at −70 °C until further use.

**Purification of HylP2—**The cells were resuspended in lysis buffer containing 50 mM HEPES, 10 mM EDTA (pH 7.0), disrupted using a probe-type ultrasonicator, and centrifuged at 12,000 × g for 1 h at 4 °C. The supernatant was loaded onto a CM-Sepharose column equilibrated with lysis buffer. The column was initially washed with lysis buffer and subsequently with the same buffer containing 50 and 100 mM imidazole, respectively. The protein was eluted using 300 mM imidazole in the lysis buffer. The active fractions were pooled, concentrated, and finally purified on a Superdex 200 HR 10/300 column on AKTA fast performance liquid chromatography equilibrated with 20 mM Tris, 150 mM sodium sulfate, and 10% glycerol (pH 7.0). The eluted protein was tested for purity by SDS-PAGE and ESI-MS and was found to be about 95% pure.

A similar enzymatic activity and proteolytic pattern with α-chymotrypsin was observed for both the histidine-tagged and nonhistidine-tagged protein. Hence, because of ease in purification and advantage of characterizing the C-terminal domain of the protein, histidine-tagged protein was used for studies dealing with the isolated C-terminal domain.

**Size-exclusion Chromatography (SEC)—**Gel filtration experiments were carried out on a Superdex 200 HR 10/300 column (manufacturer’s exclusion limit 600 kDa) with AKTA fast performance liquid chromatography (Amersham Biosciences). The column was calibrated with various molecular weight standard markers (Amersham Biosciences). The column was equilibrated and run with 20 mM Tris-HCl, 150 mM sodium sulfate (pH 7.0). For GdnHCl-treated protein samples the column was equilibrated and run with the above mentioned buffer containing the desired concentration of GdnHCl. 200 μl of the sample was loaded on the column and run at 25 °C at a flow rate of 0.3 ml/min, with detection at 280 nm.

**Tryptophan Fluorescence—**Fluorescence spectra were recorded with a PerkinElmer Life Sciences LS 50B spectrophotometer in a 5-mm path length quartz cell at 25 °C. Excitation wavelength of 290 nm was used and the spectra were recorded between 300 and 400 nm. Protein concentration of 1 μM was used for the studies.

**Circular Dichroism Measurements—**CD measurements were made with a Jasco J810 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The results are expressed as mean residual ellipticity [θ], which is defined as [θ] = 100 × θ_obs/(lc), where θ_obs is the observed ellipticity in degree, c is the concentration in moles of residue/liter, and l is the length of the light path in centimeters. The values obtained were normalized by subtracting the baseline recorded for the buffer having the same concentration of the denaturant under similar conditions. 3 μM protein was used for the studies.

**Limited Proteolysis—**0.2 mg/ml protein was subjected to limited proteolysis with α-chymotrypsin, at a protease to protein ratio of 1:500 and 1:1 (w/w), for 1 and 5 h, respectively, at 30 °C. The protease reaction was stopped by adding phenylmethylsulfonyl fluoride to a final concentration of 1 mM in the reaction mixture, and the samples were analyzed on 12% SDS-PAGE.

**Cross-linking Using Glutaraldehyde—**The native protein or the C-terminal domain, at a concentration of 0.1 mg/ml, was used for cross-linking studies. The cross-linking of protein samples was carried out with 1% glutaraldehyde as described earlier (24). The molecular mass of the cross-linked products were determined by 10% SDS-PAGE.
Assay of Enzymatic Activity—The activity of the enzyme was determined by measuring its ability to breakdown HA or CS to unsaturated disaccharide units (25). In 1 ml of solution of 0.3 mg/ml HA (or mentioned otherwise), 50 mM sodium acetate buffer, 15 mM CaCl₂ (pH 6.0), and 1.5 or 3 μg of enzyme sample (diluted just before taking measurement) were added. The reaction mixture was incubated for 5 min during which the measurements were carried out by monitoring the increase in absorbance at 232 nm at 25 °C.

For kinetic measurements, varying concentrations of HA/CS between 0.012 and 0.5 mg/ml were used. Protein concentrations of 1.5 and 3 μg for full-length protein and protease-treated protein samples, respectively, were taken. The kinetic parameters were calculated using extinction coefficient of 5.5 × 10⁻³ M⁻¹ cm⁻¹ for the disaccharide products.

ESI-MS—The mass spectra were recorded on a MICRO-MASS QUIATTRO II mass spectrometer (Micromass, Altricem, United Kingdom) equipped with an electrospray ionization (ESI) ion source as described earlier (26).

RESULTS AND DISCUSSION

Expression and Purification—The expression of recombinant HyIP2 was good and the expressed protein was present predominantly (>90%) in the soluble fraction. The protein present in the soluble fraction was purified by the method described under “Experimental Procedures.” The yield of the purified recombinant HyIP2 was in the range of 8–10 mg/liter. The purified protein was homogeneous as indicated by a single protein band on SDS-PAGE (Fig. 1A) and a single peak in SDS-PAGE analysis of the glutaraldehyde cross-linked HyIP2 at pH 7.0 (Fig. 1, B and C).

The calculated molecular mass (from primary amino acid sequence) of 37.2 kDa for the HyIP2 corroborated well with the molecular mass of about 36.0 and 36.9 kDa observed from SDS-PAGE (Fig. 1A) and ESI-MS experiments (data not shown), respectively. The results of the subunit mass (as determined by SDS-PAGE and ESI-MS) along with SEC and glutaraldehyde cross-linking studies demonstrate that HyIP2 of the S. pyogenes bacteriophage exists as a homotrimer under physiological conditions.

Structural Features of HyIP2—Studies on the model polypeptides and proteins revealed that the α-helical and β-sheet proteins show characteristic far-UV CD spectra. The α-helical proteins have two minima at 222 and 208 nm and the β-sheet proteins have a single minimum at 216 nm (28). For HyIP2 protein, the far-UV CD spectrum characteristic of a protein having both α-helix and β-sheet secondary structures was observed (Fig. 2A). Hence, HyIP2 is a αβ-protein.

The fluorescence spectrum of HyIP2 is shown in Fig. 2B. The emission wavelength maximum of the tryptophan fluorescence for the recombinant protein was observed at about 326 nm. The buried tryptophan residues in the folded protein show fluorescence emission maxima at 330–335 nm (29). Hence, in the HyIP2 protein the tryptophan molecule is buried in the hydrophobic environment. According to the primary amino acid sequence, HyIP2 has a single tryptophan residue at position 19 (23). So the presence of the buried tryptophan residue in native protein suggests that the N-terminal region of HyIP2 be in folded conformation.

The HyIP2 Undergoes Sequential Unfolding—The unfolding characteristic of HyIP2 was studied by monitoring the GdnHCl-induced changes in the structural properties of HyIP2. Time-dependent changes in the structural parameters of HyIP2 at increasing GdnHCl concentrations (0.5, 1, and 4 M) were monitored to standardize the incubation time required to achieve equilibrium under these conditions. Under all the conditions studied, the changes occurred within a maximum of 3 h with no further alterations in the values obtained up to 12 h (data not...
shown). These observations suggested that a minimum time of about 3 h is sufficient for achieving equilibrium under any of the denaturing conditions studied.

To study the GdnHCl-induced changes in the secondary structure of the HylP2 protein, far-UV CD studies were carried out. Fig. 3A summarizes the effect of increasing concentrations of GdnHCl on the CD ellipticity at 216 nm of the HylP2 protein. A biphasic loss of CD signal was observed between 0.25 and 4 M GdnHCl. An initial sharp decrease of about 55% in the CD signal was observed between 0.25 and 1.0 M GdnHCl. This was followed by a further gradual sigmoidal decrease in CD signal from about 55 to 0% between 1.25 and 4 M GdnHCl. A similar unfolding profile was observed when the changes in CD ellipticity at 222 nm were monitored. The above presented results suggested that GdnHCl-induced unfolding of the HylP2 protein is non-cooperative and occurs in two steps.

Fig. 3B summarizes the changes in tryptophan emission wavelength maxima of the HylP2 protein on incubation with increasing concentrations of GdnHCl. On increasing the GdnHCl concentration between 0.20 and 1.0 M GdnHCl, a sigmoidal shift in emission wavelength maxima from 326 to 353 nm was observed (Fig. 3B), indicating the GdnHCl-induced unfolding of the protein at about 1 M GdnHCl.

Interestingly, the GdnHCl-induced unfolding observed by monitoring of the tryptophan fluorescence (Fig. 3B) coincides with the first transition (between 0.20 and 1.0 M GdnHCl) as observed from far-UV CD studies (Fig. 3A). This demonstrates that it corresponds to only the partial unfolding; probably the unfolding of the N-terminal portion of the protein molecule. The second transition observed in far-UV CD studies at higher GdnHCl concentrations corresponds primarily to the unfolding of the C-terminal domain (discussed in detail later).

To study the effect of unfolding of the N-terminal region on the quaternary structure of the protein, SEC and glutaraldehyde cross-linking studies were carried out on the 1.0 M GdnHCl-stabilized partially folded intermediate of HylP2 (Fig. 3C). A retention volume of 12.4 ml was observed for 1.0 M GdnHCl-stabilized HylP2, which is slightly less than the retention volume of 13.0 ml as observed for the native protein. This indicated that perhaps the GdnHCl-stabilized intermediate of HylP2 is in a trimeric configuration but with slightly larger hydrodynamic radii than the native protein. This can be attributed to the fact that the N-terminal portion of enzyme is unfolded under these conditions. The trimeric configuration of the 1.0 M GdnHCl-stabilized partially folded intermediate of HylP2 was confirmed by glutaraldehyde cross-linking studies. Fig. 3C, inset, shows the comparative SEC profiles of the glutaraldehyde cross-linked native protein and the 1.0 M GdnHCl-stabilized partially folded intermediate of HylP2.
presence of the C-terminal His, moiety in Fragment I suggests that α-chymotrypsin cleaves HylP2 between amino acids Tyr^{126} and Ser^{127} during limited proteolysis. This results in release of a C-terminal domain corresponding to the amino acid sequence Ser^{127}–Lys^{337} (calculated molecular mass of about 23.15 kDa) of native HylP2.

Fragment II showed N-terminal sequence similar to that observed for the native protein and no affinity for the nickel-nitrotriacetic acid-agarose matrix and anti-His antibody on Western blot analysis demonstrating that this fragment contains the same N-terminal as the native protein and does not contain C-terminal His, moieties. Hence, Fragment II corresponds to the N-terminal domain of HylP2.

There are about 20 cleavage sites for α-chymotrypsin that are spread throughout the length of HylP2. However, on limited proteolysis, only a single large fragment corresponding to the C-terminal of the proteins was obtained. This suggests that the C-terminal domain of the protein is in a compact folded conformation because several of the proteolytic sites present in this domain are not accessible to protease for cleavage.

As an intact C-terminal domain HylP2 was obtained on limited proteolysis of protein with α-chymotrypsin we tried to purify this fragment and study its quaternary structure. Fig. 5, A and B, shows the column profile of the proteolysed samples on the S-200 column. For the protein sample proteolysed at a protein:protease ratio of 500:1, no significant difference in the retention volume (about 13.2 ml) as compared with the native protein (13.03 ml) was observed. However, the SDS-PAGE analysis of the sample under the peak showed the presence of both Fragments I and II in the proteolysed samples (data not shown). These results demonstrate that the two fragments obtained on proteolysis under these conditions have a tendency to associate and as a result they elute as a single protein species in SEC but get separated in the SDS-PAGE. Hence, a single protein fragment of HylP2 could not be isolated from the protein sample proteolysed at a protein:protease ratio of 500:1.

For the protein sample proteolysed at a protein:protease ratio of 1:1, only a single protein fragment, the C-terminal domain (as discussed above), was obtained. Interestingly, this isolated C-terminal domain showed a retention volume similar to the native protein trimer on SEC, suggesting that the two proteins have similar hydrodynamic radii. Such a high hydrodynamic radius for the C-terminal domain of molecular mass of about 24 kDa is possible only when it exists as a trimer. This was confirmed by the glutaraldehyde cross-linking studies where a molecular mass of about 70 kDa corresponding to the trimer of this truncated part of the full-length protein (Fig. 5C) was observed. These results demonstrate that the isolated C-terminal domain (Ser^{128}–Lys^{337}) of HylP2 is stabilized as a homotrimer under physiological conditions.

GdnHCl-induced unfolding of the isolated C-terminal domain was performed to study whether it exists in a folded conformation or not. Fig. 3A shows the effect of increasing concentrations of GdnHCl on the CD ellipticity at 216 nm for the C-terminal domain of HylP2. A sigmoidal loss of CD signal was observed on increasing the GdnHCl concentration from 1 to 4 M. Comparison of the GdnHCl-induced unfolding of the C-terminal domain with that of the full-length protein shows that it corresponds mainly to the second transition observed at higher GdnHCl concentrations during unfolding of the full-length protein. These observations demonstrate that the isolated C-terminal domain exists in a folded conformation and is more stable than the N-terminal domain.

The Active Site Is Present in the C-terminal Portion of HylP2—Hyaluronate lyases degrade HA and CS by cleaving the β1,4-glycosidic linkage between the glycan units of these substrates. The mechanism of this degradation process is based on the β-elimination and involves selective residues of a well defined catalytic site of these enzymes (31).

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GdnHCl-treated HylP2. For both samples, molecular mass of about 110 kDa was observed, thus confirming that both the native protein and the 1.0 M GdnHCl-treated HylP2 are stabilized as trimers.

Identification, Purification, and Characterization of a Compact C-terminal Domain of HylP2—The factors determining the vulnerability of a protein for proteolysis by protease depends on the conformational parameters such as accessibility, segmental motion, and protrusions. For this reason limited proteolysis has been effectively used to monitor structural domains in proteins, ligand-induced conformational changes, and protein folding/unfolding (30).

Fig. 4 summarizes the SDS-PAGE profile of the protein fragments obtained on limited proteolysis of the recombinant HylP2 with α-chymotrypsin. Two different patterns of proteolysis, depending on the protein to protease ratio used, were observed (Fig. 4, A and B). At a protein to protease ratio of 500:1, two protein fragments, namely Fragment I and II, corresponding to molecular mass of about 24 and 14 kDa, respectively (Fig. 4A), were obtained. However, when the proteolysis was carried out at a protein to protease ratio of 1:1, only a single protein band corresponding to the molecular mass of about 24 kDa was obtained (Fig. 4B). The Fragment I, obtained at low and high protease concentrations (Fig. 4, A and B), showed affinity for the nickel-nitrotriacetic acid-agarose matrix and anti-His antibody on Western blot analysis (Fig. 4, A and B, inset) demonstrating that this fragment contains the C-terminal His, moieties. Because, in the recombinant HylP2 protein, the histidine tag was present at the C terminus so these fragments correspond to the C-terminal portion of the protein. Furthermore, both fragments showed the N-terminal sequence SSSTG and molecular mass of 23.2 kDa (by ESI-M5), suggesting that they correspond to the same protein sequence of HylP2. The N-terminal sequence of SSSTG along with the
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ysis of the x-ray structure of the co-crystals of HA with Streptococcus pneumoniae hyaluronate lyase (SpnHL) and Streptococcus agalactiae hyaluronate lyase (SagHL) shows that the catalytic residues Asn, His, and Tyr are placed in a highly specific manner in the amino acid sequence of these proteins. There are 50 amino acid residues between Asn and His, whereas the His and Tyr residues are nine amino acid residues apart (Asn349 to Tyr408 and Asn429 to Tyr488 of SpnHL and SagHL, respectively, in the primary amino acid sequence). In the amino acid sequence of HylP2, a similar definite placement of these residues having 39 amino acid residues between Asn206 and His316 and 8 amino acid residues between His316 and Tyr322 is observed. The CLUSTALW alignment of several proteins show high homology with HylP2 on BLAST and also show that hyaluronidase activity was carried out. The interesting observation from the multiple sequence alignment was that placement of the above mentioned probable catalytic residues are conserved in the amino acid sequence of all these proteins (supplementary Fig. 1). This further supports the contention that these may be the catalytic residues in these proteins. The presence of the probable catalytic residues in the C-terminal portion of HylP2 authenticates the view that probably the C-terminal domain is the catalytic domain of the protein. Furthermore, as the isolated C-terminal domain of HylP2 is stabilized as trimer like the full-length protein we thought it worthwhile to see whether this domain does possess functional activity or not.

Different hyaluronate lyases have different specificity for their polysaccharide substrates. The phage H4489A and S. hyalurolyticus hyaluronate lyases have strict specificity for HA, not cleaving chondroitins of any kind (29, 30), whereas SpnHL and SagHL not only process HA but certain CS also (33). To study the kinetic properties of enzymatic reaction as well as substrate specificity, a comparative analysis of the kinetic properties of the full-length protein, the N:C complex, and the isolated C-terminal domain using HA and CS as substrates was carried out and summarized in Fig. 6. Native HylP2 and the N:C complex obeyed Michaelis-Menten kinetics (Fig. 6, A and B) with $K_m$ values...
as for degradation of the HA substrate, calculated from the curve, being 0.31 \pm 0.02 and 0.23 \pm 0.02 mg/ml for the native HylP2 protein and the N:C complex, respectively. However, both proteins showed no activity with CS (Fig. 6, A and B). Studies on the kinetic properties of the isolated C-terminal domain with HA showed interesting results (Fig. 6C). The C-terminal domain at the low substrate concentration, up to 0.012 mg/ml, followed a profile similar to that of the native protein and N:C complex, however, on increasing the substrate concentration further, a drop in velocity was observed, indicating that the protein shows the property of “high substrate inhibition.” Furthermore, unlike the full-length protein and the N:C complex that exhibited absolute specificity for only one type of glucosaminoglycan, i.e. HA, the C-terminal domain showed a similar kinetic profile with both HA and CS. A possible explanation for the loss of substrate specificity observed for the isolated C-terminal domain is that the removal of the N-terminal domain probably results in stabilization of a conformation of protein having an enlarged substrate binding pocket. This large binding pocket besides accommodating the smaller substrate HA can also take in a bulkier functional properties of bacteriophage hyaluronate lyase.

Calcium ions have been reported to activate hyaluronate lyase (34). The effect of calcium ions on enzyme activity can be because of a direct effect either on the enzyme, on the HA substrate, or both. Fig. 7, A and B, summarizes the results of the effect of increasing calcium chloride concentrations on HA lyase activity of HylP2 and the isolated C-terminal domain. A maximum activation of about 2- and 1.7-fold was observed at about 15 and 20 mM CaCl_2 concentration for native HylP2 and the isolated C-terminal domain, respectively. A similar activation of both the full-length protein and the isolated C-terminal domain of HylP2 was observed with magnesium ions also (Fig. 7, A and B).

The dependence of pH on the enzymatic activity of the HylP2 protein and the isolated C-terminal domain was also studied between the pH range 2 and 10 and is summarized in Fig. 7C. For both samples, a bell-shaped curve centered at about pH 6 was observed suggesting that the native enzyme as well as the C-terminal domain works with maximum efficiency at about pH 6.0. At pH below 4 and above 10, a complete loss of activity was observed. The pH-dependent curves for both the native protein and isolated C-terminal domains were not absolutely symmetrical, which suggests that probably the substrate HA also undergoes pH-dependent structural change.

The results presented in this paper demonstrate that the C-terminal domain of HylP2 is the catalytic domain and can carry out the catalytic function on its own. However, specific interactions between N- and C-terminal domains seem to be required for achieving maximum activity and generating substrate specificity for the enzyme. The present work for the first time provides detailed insight into the structural and functional properties of bacteriophage hyaluronate lyase.

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