THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 28, Issue of July 11, pp. 25509–25516, 2003
Printed in U.S.A.

Streptococcus pneumoniae Hyaluronate Lyase Contains Two Non-cooperative Independent Folding/Unfolding Structural Domains

CHARACTERIZATION OF FUNCTIONAL DOMAIN AND INHIBITORS OF ENZYME*

Received for publication, February 24, 2003, and in revised form, April 22, 2003
Published, JBC Papers in Press, April 27, 2003, DOI 10.1074/jbc.M301894200

Md. Sohail Akhtar and Vinod Bhakuni‡

From the Division of Molecular and Structural Biology, Central Drug Research Institute, Lucknow 226 001, India

Hyaluronate lyase contributes directly to bacterial invasion by degrading hyaluronan, the major component of host extracellular matrix of connective tissues. *Streptococcus pneumoniae* hyaluronate lyase (SpnHL) is built from two structural domains that interact through interface residues, in addition to being connected by a peptide linker. For the first time we demonstrate that the N- and C-terminal domains of SpnHL fold/unfold independent of each other suggesting the absence of any significant cooperative interactions between them. The C-terminal domain of SpnHL is less stable than the N-terminal domain against thermal and guanidine hydrochloride denaturation. The intact N-terminal domain was purified after limited proteolysis of SpnHL under conditions where only the C-terminal domain was unfolded. Isolated N-terminal domain of SpnHL had similar thermal stability as when present in the native enzyme and was found to be enzymatically active demonstrating that it is capable of carrying out enzymatic reaction on its own. Functional studies demonstrated that guanidine hydrochloride, guanidine isothiocyanate, L-arginine methyl ester, and L-arginine inhibit the enzymatic activity of SpnHL at very low concentrations. This provides a lead for new chemical entities that can be exploited for designing effective inhibitors of SpnHL.

Streptococcus pneumoniae is a major human pathogen that colonizes predominantly in the upper respiratory tract of humans. It is one of the key causes of life threatening disease such as pneumonia, bacteremia, and meningitis (1) and less dangerous but significantly more prevalent diseases like sinusitis or otitis media (2). The groups of human population especially affected by this organism are infants, the elderly, and immunocompromised individuals (3, 4).

Hyaluronidases constitute a special group of polysaccharide-degrading enzymes that have the ability to degrade primarily hyaluronan (HA),[1] yielding various lengths of oligosaccharides as the end product (5). The bacterial hyaluronidases are called hyaluronate lyase. Various Gram-positive microorganisms including species of *Streptococcus*, *Clostridium*, *Propionibacterium*, *Staphylococcus*, and *Peptostreptococcus* produce hyaluronate lyase (6). This enzyme is either cell-associated or released outside of cell. It primarily degrades hyaluronan and certain chondroitin sulfates, the major components of host extracellular matrix of tissue (5, 7, 8). The HA degradation directly contributes to bacterial invasion by allowing greater microbial access to, or migration between, host tissues for colonization (8, 9). Because of this reason hyaluronate lyase is also being called "spreading factor." The protein sequence of four bacterial hyaluronate lyase from *S. pneumoniae* (10), *Streptococcus agalactiae* (11), *Staphylococcus aureus* (12), and *Propionibacterium acnes* (13) have been reported so far. Their sequence homologies range from 28 to 68% suggesting their structural, functional, and evolutionary similarities.

The crystal structure of *S. pneumoniae* hyaluronate lyase (SpnHL) shows that the enzyme is built up of two domains, an N-terminal domain or catalytic domain, which is predominantly α-helical, and a C-terminal domain or the supportive domain, which is predominantly β-sheet (14). The two domains are connected by a short linker polypeptide. The interface between the N- and C- terminal domains is composed of several residues from the two domains forming several interactions. The cleft in between the α- and β-domains is the place where the HA binding and degradation occurs and is wide at the two ends and narrower in the middle. The crystal structure of *S. agalactiae* hyaluronate lyase is similar to *Spn*HL in whole structure architecture and active site geometry except that it has an additional β-sheet domain before the N-terminal α-domain present in the SpnHL (15).

Although crystal structure of hyaluronate lyase and *S. agalactiae* in complex with HA has been reported (14–16), detailed insight into important structural features like existence of co-operative interactions between C- and N-terminal domains and the specific role of C-terminal domain in functional activity of SpnHL have not been provided so far. Besides this, despite the importance of SpnHL in pneumococcal infection no sincere efforts to search suitable inhibitors for this enzyme have been made (5, 17).

We report detailed studies on the structural changes associated with the thermal denaturation and guanidine hydrochloride-, calcium chloride-, and magnesium chloride-induced unfolding of SpnHL using optical spectroscopic techniques, limited proteolysis, and enzymatic activity measurements. The N-terminal domain of SpnHL was isolated and purified after limited proteolysis. Comparative analysis of enzymatic activity and stability of the isolated N-terminal domain of SpnHL with that when present along with C-terminal in the native enzyme have also been reported. Furthermore, efficiency of guanidine hydrochloride (GdnHCl), L-arginine, L-arginine methyl ester

* This work was supported in part by the National Bioscience Award for Career Development Grant from Department of Biotechnology, New Delhi and Indian Council of Medical Research Grant SSP150 (to V. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 212411-18 (ext. 4282); Fax: 91-522-223405; E-mail: bhakuniv@rediffmail.com.

[1] The abbreviations used are: HA, hyaluronan; SpnHL, *S. pneumoniae* hyaluronate lyase; GdnHCl, guanidine hydrochloride; C_m, denaturant concentration where 50% denaturation of protein is observed; GdnHSCN, guanidine isothiocyanate; L-NAME, N_w-nitro-L-arginine methyl ester; T_m, mid-point of thermal denaturation.

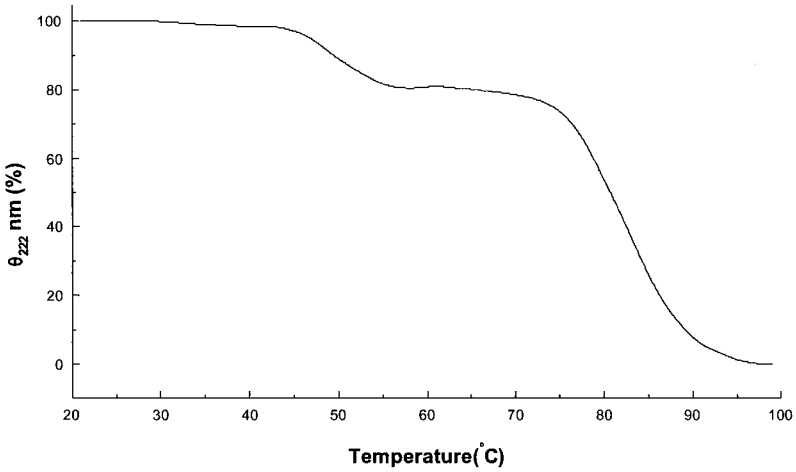

FIG. 1. **Thermal unfolding profile of native SpnHL measured by CD ellipticity at 222 nm.** The data are represented as percentages with the value observed for native enzyme taken as 100%. The experimental details are given under "Experimental Procedures."

(L-NAME), and guanidine isothiocyanate (GdnHSCN) as inhibitors of SpnHL has also been studied.

EXPERIMENTAL PROCEDURES

Cloning of SpnHL—S. pneumoniae culture growth and the chromosomal DNA extraction were carried out as described previously (18). An internal gene fragment of SpnHL encoding functional hyaluronate lyase (19) was amplified by polymerase chain reaction. The different oligonucleotides (prepared based on the earlier reported gene sequence; GenBank™ accession number L20670) used were upstream, 5′-CTAGCTAGCGTTAAGGATACATACACAGACCGT and downstream, 5′-CCGCTCGAGTGCCTGCTCTAGTTTTTTAAAGAC for with C(6) histidine-tag and 5′-CCGCTCGAGTTATGCCTGCTCTAGTTTTTTAAAGAC for without histidine-tagged protein. Reactions were carried out in two phases for five and 30 cycles in a total volume of 50 μl with 2 units of Deep vent polymerase. The first five cycles for without overhang amplification was 94 °C, 30 s; 46.5 °C, 1 min; 72 °C, 3 min; and the second 30 cycles for with overhang amplification was 94 °C, 30 s; 56.5 °C, 1 min; 72 °C, 3 min with the final extension of 10 min. This amplified gene fragment was cloned into pET 21-d between *Nhe*I and *Xho*I sites. DNA sequencing confirmed the homogeneity of sequence. The resulting construct was transformed into BL21(DE3).

Overexpression and Purification—Overexpression and purification for histidine-tagged protein was carried out as described earlier (18). Purification of non histidine-tagged protein was carried out using various chromatographic techniques. Briefly, an overnight culture was harvested, and the cell pellet was resuspended in 20 mM Tris-Cl, pH 7.4, containing 2 mM EDTA. After sonication the cell lysate was loaded onto Mono-Q pre-equilibrated with the same buffer. The column was washed with the same buffer containing 100 mM NaCl, and the protein was eluted with the linear gradient of buffer containing 100–500 mM NaCl. The fractions containing SpnHL was pooled, and ammonium sulfate was added to a final concentration of 1.7 M. The protein solution was loaded onto phenyl-Sepharose (high substituted) pre-equilibrated with the same buffer containing 1.7 M ammonium sulfate. The protein was eluted using a linear gradient of 1.7 to 0 M ammonium sulfate. The active fractions were pooled, concentrated, and finally purified on a Superdex 200 HR 10/30 column on AKTA fast protein liquid chromatography equilibrated with the same buffer without ammonium sulfate. Eluted protein was finally concentrated, dialyzed against the 20 mM Tris-HCl, pH 7.4, and stored as a 10 mg/ml stock solution at 4 °C. The purity of purified enzyme was checked by electrospray ionization mass spectrometry and found to be 99% pure. The final purified protein sample was also subjected to the N-terminal protein sequencing.

Denaturation/Unfolding of SpnHL—SpnHL (1 μM) was incubated in the presence/absence of increasing concentration of GdnHCl or $CaCl_2$ or $MgCl_2$ and incubated for 1 h at 25 °C before taking the measurements. Initial studies were carried out with both the histidine-tagged and non-histidine-tagged enzyme. Similar results were obtained with both enzymes, so only histidine-tagged enzyme was used for further studies, as it was easy to purify.

Assay of Enzymatic Activity—The activity of SpnHL is defined as its ability to breakdown HA to unsaturated disaccharide units (20). In a 1-ml solution of 0.2 mg/ml HA in 50 mM sodium acetate buffer, 10 mM $CaCl_2$, pH 6.0 (or the different salt concentration was maintained in the HA solution), 10 μl of enzyme sample (4 ng/μl, diluted just before taking measurement or stated otherwise) was 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. Percent inhibition was determined as described previously (17).

Fluorescence Spectroscopy—Fluorescence spectra were recorded with a PerkinElmer Life Sciences LS 50B spectroluminescemeter in a 5-mm path length quartz cell at 25 °C. For monitoring tryptophan fluorescence, excitation wavelength of 290 was used, and the spectra were recorded between 300 and 400 nm.

Circular Dichroism Measurements—CD measurements were made with a Jasco J800 spectropolarimeter calibrated with ammonium (+)-10-camphorsulfonate. The results are expressed as the mean residual ellipticity [θ], which is defined as $[\theta] = 100 \times \theta_{obs}/(lc)$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in moles of residue per liter, and l is the length of the light path in centimeters. The values obtained were normalized by subtracting the base line recorded for the buffer having the same concentration of denaturant or salt under similar conditions.

Limited Proteolysis—SpnHL (0.2 mg/ml) incubated for 1 h under specified conditions was subjected to limited proteolysis with carboxypeptidase B under similar conditions like at 55 °C or in the presence of 0.75 M GdnHCl or 1.5 M $CaCl_2$ at 25 °C for 15 min. A protease to enzyme ratio of 1:100 (w/w) was used for the study. The protease reaction was stopped by adding 2 mM EDTA to reaction mixture, and the samples were analyzed on 10% SDS-PAGE.

Size-exclusion Chromatography—Gel filtration experiments were carried out on a Superdex 200 HR 10/30 column and Sephadex 75 HR 10/30 column on AKTA fast protein liquid chromatography (Amersham Biosciences) for the purification of native SpnHL and the N-terminal domain, respectively. Column was calibrated with various molecular weight standard markers (Amersham Biosciences) before use. Column was equilibrated and run with 20 mM Tris-HCl, pH 7.4, at 25 °C. 200 μl of the sample was loaded on the column and run at 25 °C, flow rate of 0.3 ml/min, with detection at 280 nm.

RESULTS AND DISCUSSION

The crystal structure of SpnHL shows that the enzyme is made of two distinct structural domains connected by a short polypeptide linker. Both the domains are spherical and of approximately the same size of about 41 kDa (14). The N-terminal α-helical domain (α-domain) contains the first 361 residues (Lys^{171}–Ser^{531}) of the enzyme and is composed of 13 α-helices connected by 12 loops. An 11-residue linker (Asp^{532}–Ser^{542}) is located between the two structural domains. The C-terminal β-sheet domain (β-domain) contains the following 347 residues (Tyr^{543}–Lys^{889}) and is composed of 24 β-strands, four short α-helices, and 25 connecting loops. The interface between the N- and C-terminal domains is 1776 $Å^2$ in area and is composed of 37 residues from the N-terminal domain and 34 residues from the C-terminal domain forming about 343 interactions. For analyzing the cooperative interactions existing between the two structural domains of SpnHL we first studied the

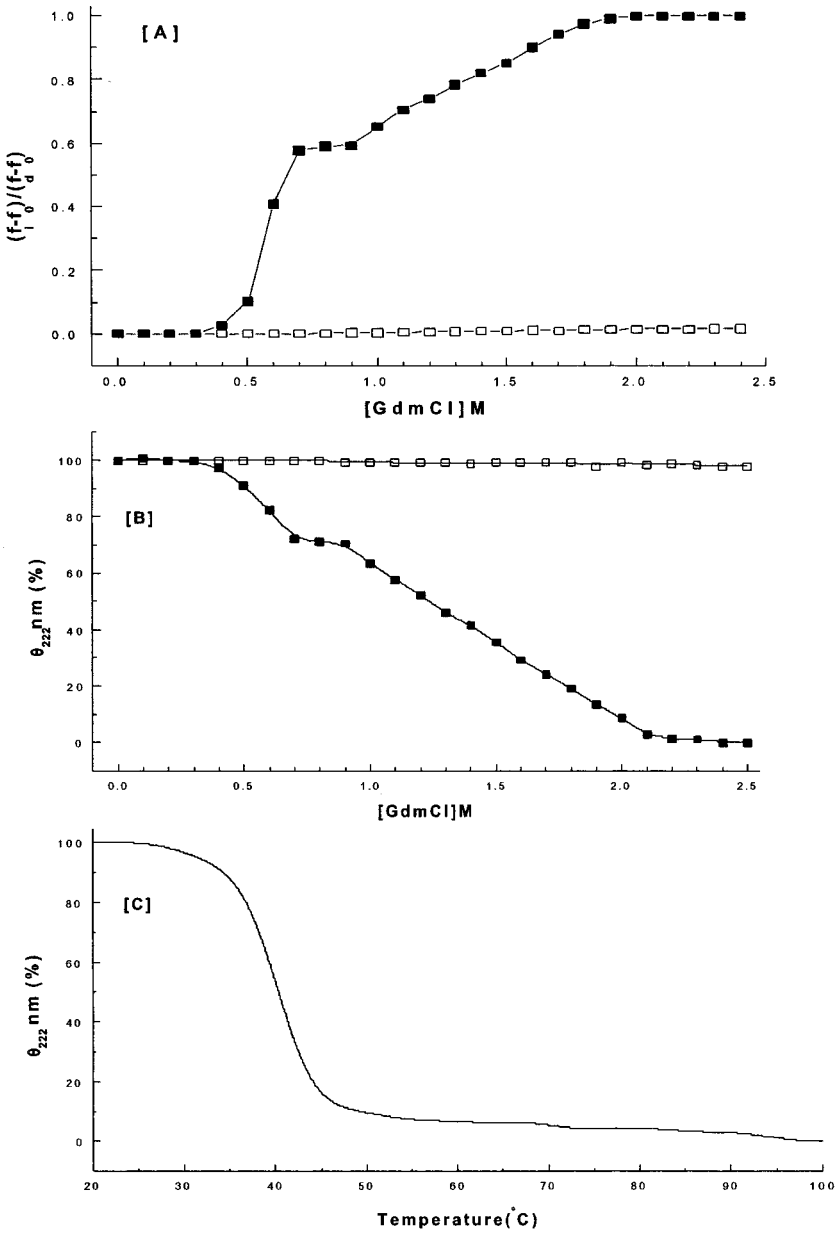

FIG. 2. **Guanidine hydrochloride-induced unfolding of SpnHL at 25 °C.** *A*, plot of fractional changes in wavelength of maximum fluorescence emission of tryptophan molecules in SpnHL, $(f_i - f_0)/(f_d - f_0)$, *versus* GdnHCl concentration. f_i is the wavelength for a particular sample, f_0 is the wavelength in absence of GdnHCl, and f_d is wavelength at GdnHCl concentration of or greater than 2.5 M. The *filled symbols* represent data for guanidine hydrochloride, and the *open symbols* represent that for NaCl. *B*, GdnHCl-induced changes in secondary structure of SpnHL as monitored by following changes in the CD ellipticity value at 222 nm. The data are represented as percentages with the value observed for native enzymes in absence of GdnHCl taken as 100%. The *symbols* are the same as in *A*. *C*, thermal unfolding of 0.75 M GdnHCl-treated SpnHL measured by CD ellipticity at 222 nm. The data are represented as percentages with the value observed for 0.75 M GdnHCl at 20 °C taken as 100%. The enzyme was incubated in 0.75 M GdnHCl for 1 h at 20 °C, after which the measurements were made.

relative stability of the N- and C-terminal domains by carrying out thermal- and GdnHCl-induced denaturation of enzyme.

The Two Structural Domains of SpnHL Have Significantly Different Stabilities

Thermal Denaturation—The thermal unfolding of SpnHL was characterized by monitoring the loss of secondary structure of enzyme with temperature. Fig. 1 summarizes the changes in ellipticity at 222 nm of SpnHL at increasing temperature, where two distinct transitions were observed between 20 and 100 °C. These transitions were in the temperature regions from 35 to 60 °C and 65 to 100 °C and centered at about 50 and 82 °C, respectively. Furthermore, the transitions were found to be reversible as they were also observed on cooling the SpnHL samples from 100 to 20 °C after the heating scan (data not shown). These observations suggest that the SpnHL molecule contains two distinct structural domains of significantly different thermal stabilities.

Guanidine Hydrochloride Unfolding—The guanidine hydrochloride-induced changes in the structural properties of SpnHL were studied using optical spectroscopic techniques. Time-dependent changes in structural parameters of SpnHL at increasing concentrations of GdnHCl showed maximum changes within 1 h of incubation and no further alteration up to the next 12 h (data not shown) suggesting that incubation time of 1 h is sufficient for achieving equilibrium under any GdnHCl concentration studied.

The GdnHCl-induced alterations in the tertiary structure of SpnHL were monitored by studying the changes in the tryptophan fluorescence of enzyme at increasing concentration of GdnHCl. If a significant difference exists between the fluorescence emission maxima of native and denatured protein, correlation between the GdnHCl concentration and the functional changes in wavelength at which fluorescence emission is maximal provides useful information on the stabilization of intermediates during protein unfolding (21). For SpnHL, the emission wavelength maximum in the absence of GdnHCl (f_o) and at GdnHCl concentration greater than 2.5 M (f_d) were 334 and 355 nm, respectively. The fractional change in wavelength at which the tryptophan fluorescence emission spectrum of SpnHL is maximal is plotted against the concentration of GdnHCl in Fig. 2*A*. Two well separated transitions were observed on increas-

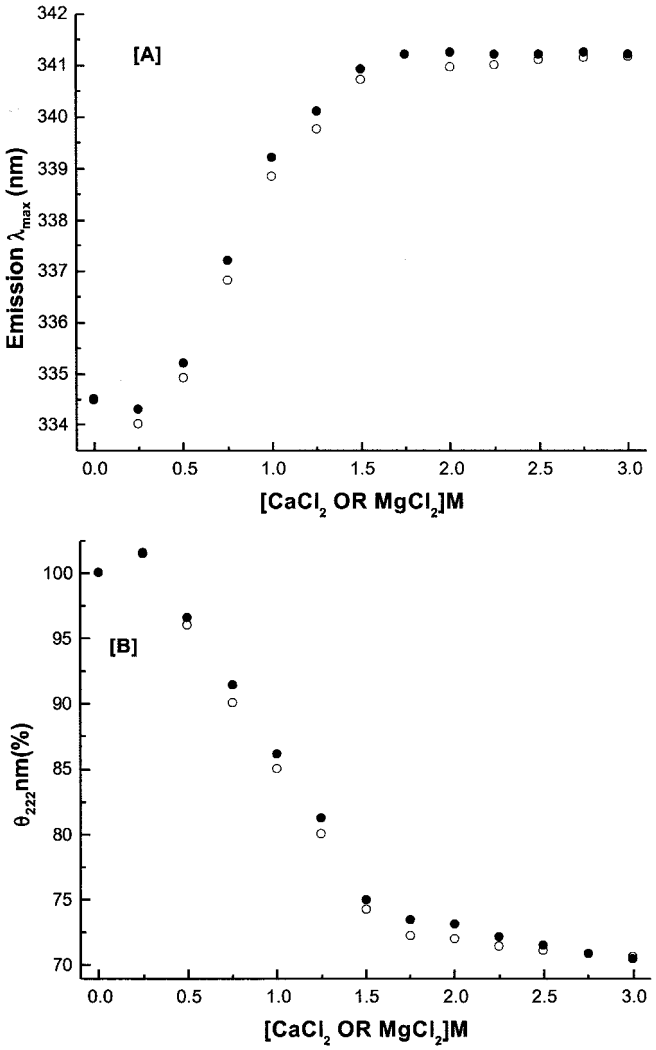

FIG. 3. **Calcium chloride- and magnesium chloride-induced changes in tertiary and secondary structure of SpnHL at 25 °C.** *A*, plot of changes in wavelength of maximum fluorescence emission of tryptophan molecules in SpnHL at increasing concentration of $CaCl_2$ or $MgCl_2$. The *filled symbols* represent data for $CaCl_2$, and the *open symbols* represent that for $MgCl_2$. The enzyme was incubated at increasing concentrations of $CaCl_2$ or $MgCl_2$ for 1 h at 25 °C, after which the measurements were made. *B*, $CaCl_2$- or $MgCl_2$-induced changes in secondary structure of SpnHL as monitored by following changes in the CD ellipticity value at 222 nm. The data are represented as percentages with the value observed for native enzyme in absence of $CaCl_2$ or $MgCl_2$ taken as 100%. The *symbols* are the same as in *A*.

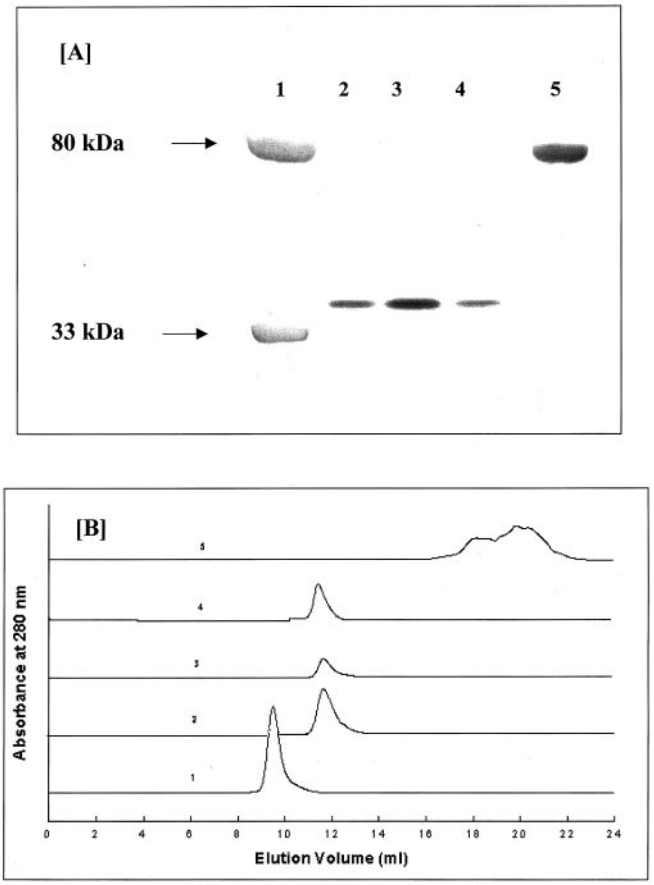

FIG. 4. **Characterization and purification of N-terminal domain of SpnHL.** *A*, SDS-PAGE profile of native SpnHL and SpnHL treated under various conditions on proteolysis with carboxypeptidase B. *Lanes 2-5* represent proteolytic fragments of SpnHL heated at 55 °C, 0.75 M GdnHCl-stabilized SpnHL, 1.5 M $CaCl_2$-stabilized SpnHL, and native SpnHL, respectively. *Lane 1* represents protein standards of molecular masses of 80 and 33 kDa. *B*, size exclusion profile on Superdex 75 HR column of the various fragments obtained on limited proteolysis of SpnHL by carboxypeptidase B as shown in *A*. The various *curves* represent proteolytic fragments of native SpnHL (*1*), native SpnHL heated at 55 °C (*2*), 0.75 M GdnHCl-stabilized SpnHL (*3*), 1.5 M $CaCl_2$-stabilized SpnHL (*4*), and 2.5 M GdnHCl-stabilized SpnHL (*5*).

ing the GdnHCl concentration between 0 and 2.5 M. The first transition was very sharp and occurred between 0.4 and 0.7 M GdnHCl. A plateau region existed between 0.7 and 0.9 M and was followed by a broad second transition between 0.9 and 2 M GdnHCl. Furthermore, the changes in emission wavelength associated with the first transition was only from 334 to 341 nm, suggesting that only partial unfolding of the SpnHL molecule is associated with the first transition. At 2 M GdnHCl, an emission wavelength of 355 nm was observed indicating that all the tryptophan moieties present in the SpnHL are exposed to solvent under these conditions (22). Such a situation can arise only when the protein is completely unfolded.

The effect of GdnHCl on the secondary structure of SpnHL was studied by monitoring changes in the far-UV CD profiles of the enzyme. In the far-UV region, the CD spectrum of native SpnHL shows the presence of substantial α-helical and β-sheet conformation. Fig. 2*B* summarizes the effect of increasing GdnHCl concentration on loss of ellipticity at 222 nm for SpnHL. Two well separated transitions, a sharp transition between 0.4 and 0.75 M GdnHCl, and a broad transition between 0.9 and 2.2 M GdnHCl were observed. Furthermore, only about 30% loss of secondary structure was found to be associated with the first transition indicating only partial unfolding of SpnHL molecules under these conditions. However, at GdnHCl concentrations greater than 2 M, a complete loss of far-UV CD signal was observed suggesting complete unfolding of SpnHL molecules at higher GdnHCl concentrations.

As only partial unfolding of SpnHL molecules was found to be associated with the first transition observed at low GdnHCl concentrations, it seems that probably only one of the structural domains of enzyme unfolds under these conditions whereas the other domain remains intact. The thermal denaturation profile of 0.75 M GdnHCl-treated SpnHL further strengthened this possibility as only a single transition with mid-point of thermal transition (T_m) of about 40 °C was observed for this sample (Fig. 2*C*).

$CaCl_2$ Interaction with SpnHL Results in Unfolding of Only One Domain

Calcium ions are essential for the normal enzymatic activity of SpnHL (18, 23). It has been speculated that the calcium ions most likely bind to the C-terminal domain of SpnHL and induce struc-

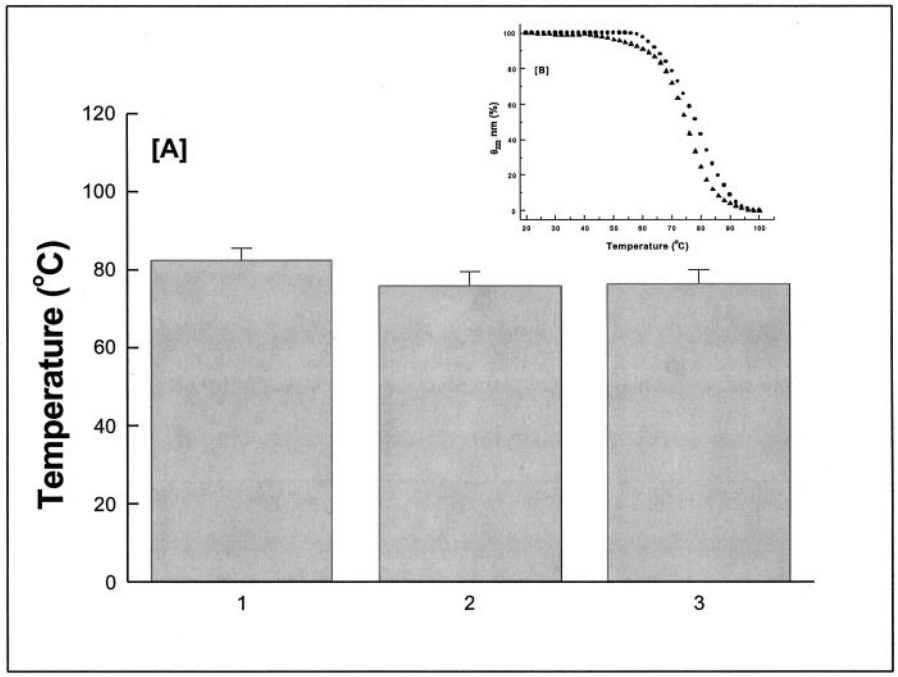

FIG. 5. **Thermal denaturation of N-terminal fragment of SpnHL.** The figure shows comparative mid-point of thermal transition of isolated N-terminal fragment purified by size exclusion chromatography as shown in Fig. 4*B*. The T_m were obtained from the thermal unfolding profiles of these samples measured by CD ellipticity at 222 nm and is represented as percentages with the value of these samples at 25 °C taken as 100% (*inset*). *Numbers* represent N-terminal domain in native SpnHL (Fig. 1) (*1*), N-terminal domain obtained after proteolysis at 55 °C (*circles* in *inset*) (*2*), and N-terminal domain obtained after proteolysis in the presence of 0.75 M GdnHCl (*triangles* in *inset*) (*3*).

tural changes in the loop of the catalytic cleft (24). We studied the effect of $CaCl_2$ and $MgCl_2$ on the domain structure of SpnHL.

The $CaCl_2$- and $MgCl_2$-induced alterations in the tertiary structure of SpnHL were studied by monitoring the changes in the tryptophan fluorescence of enzyme. Fig. 3*A* summarizes the changes in the emission wavelength of tryptophan fluorescence of SpnHL at increasing concentrations of $CaCl_2$ and $MgCl_2$. A sigmoidal dependence of enhancement of emission wavelength (from 334 to 341 nm) with increasing concentration of $CaCl_2$ or $MgCl_2$ was observed. However, even at 1.5 M $CaCl_2$ or $MgCl_2$ an emission wavelength of about 341 nm was observed suggesting only partial unfolding of enzyme under these conditions (as discussed under "GdnHCl Denaturation"). The far-UV CD studies on SpnHL with increasing concentrations of $CaCl_2$ or $MgCl_2$ also support the suggestion of partial unfolding of enzyme by these salts. Fig. 3*B* shows the loss of CD ellipticity at 222 nm of SpnHL with increasing concentration of $CaCl_2$ or $MgCl_2$. A maximum of only about 30% loss of secondary structure was observed for $CaCl_2$- or $MgCl_2$-induced transition. As $CaCl_2$ or $MgCl_2$ induces only partial unfolding of SpnHL molecules it seems that these salts probably induce unfolding of only one of the structural domains of enzyme whereas the other domain remains intact.

As GdnHCl (at low concentration), $CaCl_2$, or $MgCl_2$, which are all electrolytes, induced partial unfolding of SpnHL, the possibility of a nonspecific ion-induced effect on the enzyme was also explored. For this, the effect of NaCl on structural parameters of SpnHL was studied and is summarized in Fig. 2, *A* and *B*. NaCl even up to 2 M concentration showed no significant change in the tryptophan fluorescence or to the secondary structure of SpnHL indicating that NaCl does not induce any change in enzyme structure probably because of lack of its interaction with the enzyme. These observations collectively suggest that GdnHCl, $CaCl_2$, and $MgCl_2$ have specific interaction/binding sites with/in SpnHL.

Identification of Structural Domain Susceptible to Low Temperature or Low GdnHCl Concentration or $CaCl_2$

The factors determining the vulnerability for proteolysis of a protein by protease depend 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 (25).

To characterize the structural domains corresponding to the transitions observed during thermal-, GdnHCl-, and $CaCl_2$-induced unfolding of SpnHL (see Figs. 1–3) limited proteolysis studies were carried out under conditions where only one transition was complete. Fig. 4*A* summarizes the SDS-PAGE profile of the protein fragments obtained on limited proteolysis with carboxypeptidase B of the SpnHL molecule heated at 55 °C or treated with 0.75 M GdnHCl or 1.5 M $CaCl_2$. For all three samples, a single protein band corresponding to a molecular mass of about 40 kDa was observed on proteolysis. There are about 96 cleavage sites of Carboxypeptidase B spread throughout the SpnHL molecule, including one in the linker region. However, limited proteolysis of SpnHL by the protease in all the conditions studied resulted in only a single fragment of about 40 kDa. This suggests that the obtained fragment is a folded one in which the proteolytic sites are buried in the protein interior and not accessible to protease for cleavage. The N-terminal amino acid sequencing of the 40-kDa fragments were carried out following Western blotting of protein bands on polyvinylidene difluoride membranes. In all the three cases the nine amino acids obtained corresponded to the first nine residues (ASVKDTYTD) of the N-terminal domain of SpnHL. These observations suggest that heating of the SpnHL molecule to 55 °C or treating it with 0.75 M GdnHCl or 1.5 M $CaCl_2$ results in unfolding of only the C-terminal domain. The N-terminal domain of SpnHL obtained on limited proteolysis was purified by size exclusion chromatography (Fig. 4*B*). For characterization of the carboxypeptidase B cleavage site in SpnHL that generates the N-terminal fragment on proteolysis, electrospray ionization mass spectrometry analysis of the N-terminal fragments obtained were carried out. Only two peaks corresponding to molecular massed of 40501 and 42414.5 Da with relative intensity ratios of 3:1, respectively, were observed (data not shown). As the major peak corresponded to a molecular mass of 40501 Da, it suggests that the carboxypeptidase B cleavage site in the SpnHL is between Tyr^{520} and Lys^{521}. This site is located at the end of N-terminal domain just before the polypeptide linkers analyzed from the crystal structure.

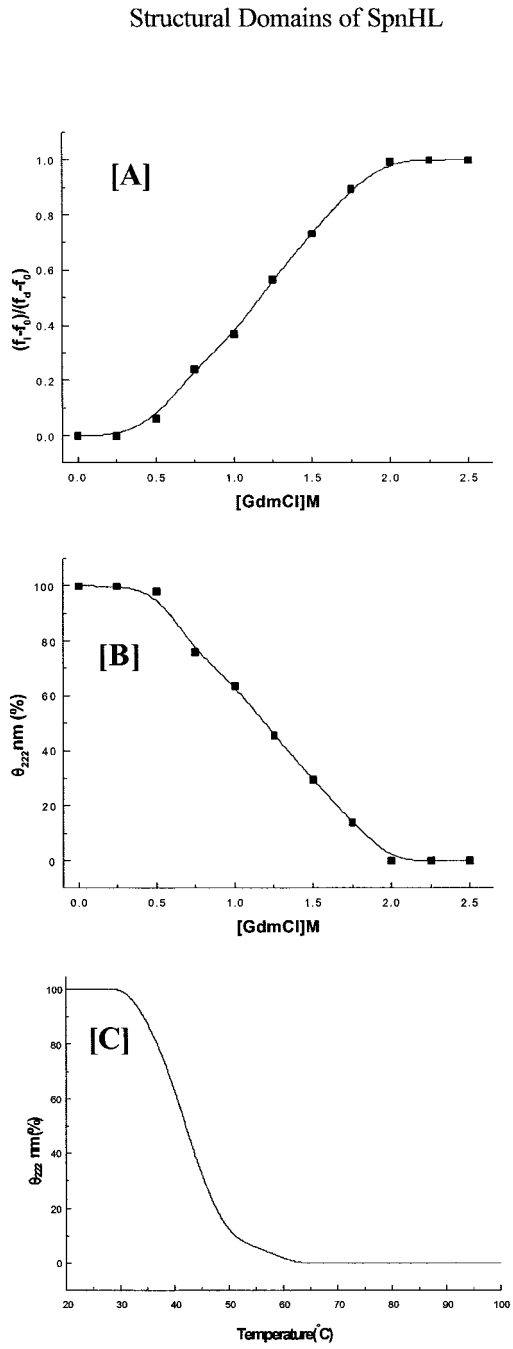

FIG. 6. **Guanidine hydrochloride-induced unfolding of N-terminal fragment of SpnHL obtained on limited proteolysis of SpnHL at 55 °C.** *A*, plot of fractional changes in wavelength of maximum fluorescence emission of tryptophan molecules in N-terminal fragment of SpnHL, $(f_i - f_o)/(f_d - f_o)$, *versus* GdnHCl concentration. The studies were carried out at 25 °C. *B*, GdnHCl-induced changes in secondary structure of N-terminal fragment of SpnHL as monitored by following changes in the CD ellipticity value at 222 nm. The data are represented as percentages with the value observed for enzymes in the absence of GdnHCl taken as 100%. The studies were carried out at 25 °C. *C*, thermal unfolding of 0.75 M GdnHCl-treated N-terminal fragment of SpnHL measured by CD ellipticity at 222 nm. The data are represented as percentages with the value observed for 0.75 M GdnHCl at 20 °C taken as 100%. The enzyme was incubated in 0.75 M GdnHCl for 1 h at 20 °C, after which the measurements were made.

Characterization of N-terminal Domain

The size exclusion chromatographically purified N-terminal domains of SpnHL (Fig. 4*B*) were analyzed for thermal stability, GdnHCl denaturation, and enzymatic activity.

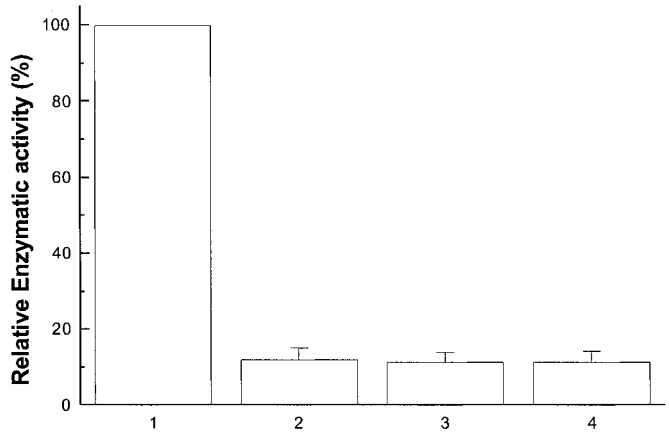

FIG. 7. **Enzymatic activity of the N-terminal fragment of SpnHL.** The figure shows comparative enzymatic activity of native SpnHL and isolated N-terminal fragment purified by size exclusion chromatography as shown in Fig. 4*B*. The *numbers* correspond to native enzyme (*1*), N-terminal domain obtained after proteolysis at 55 °C (*2*), N-terminal domain obtained after proteolysis in the presence of 0.75 M GdnHCl (*3*), and N-terminal domain obtained after proteolysis in the presence of 1.5 M $CaCl_2$ (*4*). The enzymatic activity is represented in percentages with that corresponding to native SpnHL taken as 100%.

Thermal Stability—The thermal denaturation profiles of the isolated N-terminal fragments were obtained by monitoring loss of CD ellipticity at 222 nm with increasing temperature. Fig. 5 summarize the T_m observed for the isolated N-terminal domain obtained on limited proteolysis of SpnHL at 55 °C and on treatment with 0.75 M GdnHCl. A single transition with the T_m of about 76 and 75 °C was observed for the two samples, which is very close to the T_m of 82 °C (Fig. 1) observed for N-terminal domain in the native enzyme. This observation demonstrates that the fragments obtained on proteolysis of SpnHL (Fig. 4*B*) are intact N-terminal domains. Furthermore, it also demonstrates that the stability of N-terminal domain was not affected by removal of C-terminal domain suggesting the absence of cooperative interactions between the two domains of SpnHL.

GdnHCl Denaturation—The GdnHCl denaturation profile of the purified N-terminal domain obtained on limited proteolysis of SpnHL at 55 °C and on treatment with 0.75 M GdnHCl was studied using tryptophan fluorescence and far-UV CD spectroscopies and is summarized in Fig. 6. The alterations in the tertiary structure of N-terminal fragment of SpnHL were monitored by studying the changes in the tryptophan fluorescence at increasing concentration of GdnHCl. When fractional change in emission wavelength at which the fluorescence intensity of N-terminal fragment of SpnHL is maximal is plotted against the increasing concentration of GdnHCl, a sigmoidal transition in the GdnHCl concentration range between 0.5 and 2 M was observed (Fig. 6*A*). A C_m (denaturant concentration where 50% denaturation of protein is observed) of about 1.25 M GdnHCl was found to be associated with this transition.

The effect of GdnHCl on the secondary structure of N-terminal fragment of SpnHL was studied by monitoring changes in the far-UV CD profiles of the enzyme. The CD spectrum of N-terminal fragment of SpnHL in the far-UV region shows the presence of substantial α-helical conformation. Fig. 6*B* summarizes the effect of increasing GdnHCl concentration on the loss of ellipticity at 222 nm for N-terminal fragment of SpnHL. A single transition in the GdnHCl concentration range between 0.5 and 2 M was observed. A C_m of about 1.25 M GdnHCl was found to be associated with this transition. As for both tryptophan fluorescence and far-UV CD, a single transition between 0.5 and 2 M GdnHCl was observed, which suggests that the

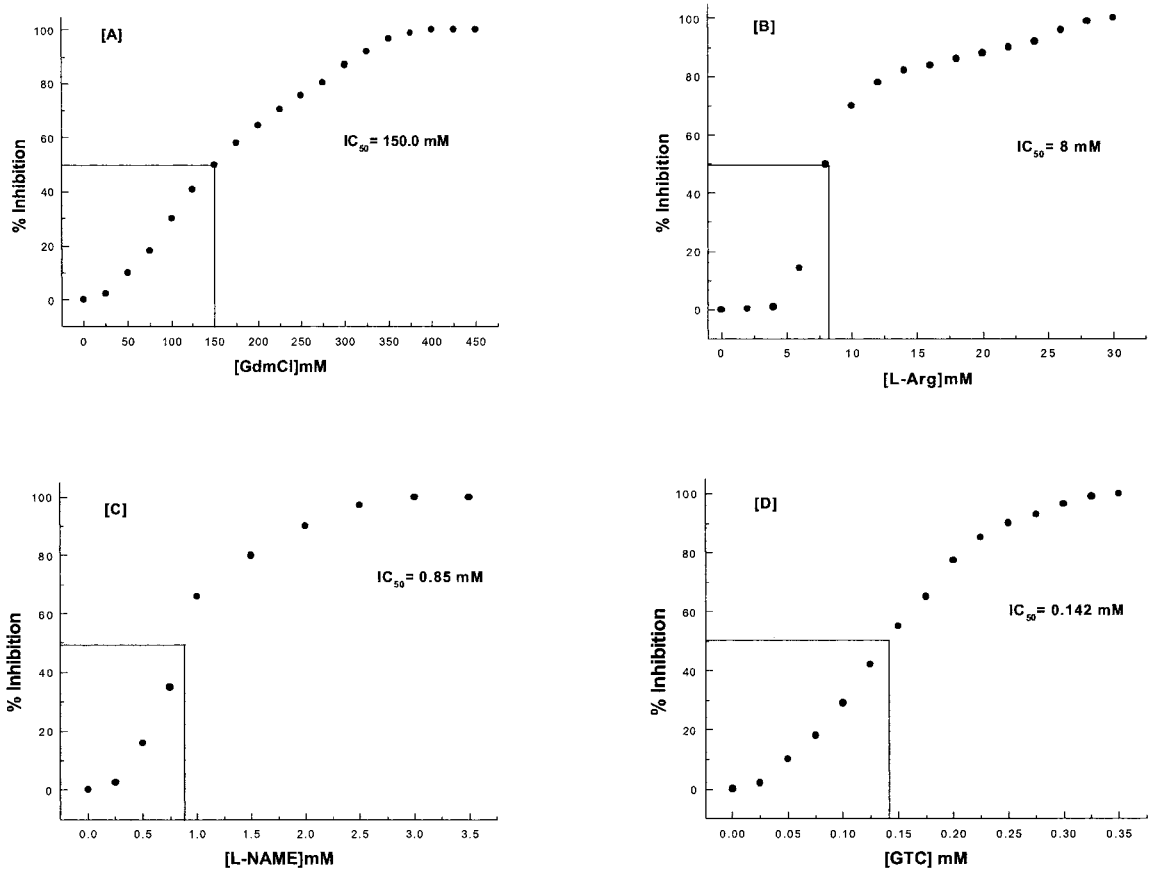

FIG. 8. **Enzyme activity of SpnHL in the presence of GdnHCl (*A*), L-arginine (*B*), L-NAME (*C*), and GdnHSCN (*D*).** Each *point* represents averaged value for four parallel measurements. The experimental details are given under "Experimental Procedures."

N-terminal fragment of SpnHL undergoes a cooperative unfolding with simultaneous loss of both the secondary and the tertiary structure.

The thermal denaturation profile of N-terminal fragment of SpnHL obtained as described above, treated with 0.75 M GdnHCl, was studied. A single transition with a T_m of about 40 °C (Fig. 6*C*) was observed, which is similar to that observed for the 0.75 M GdnHCl-treated SpnHL (Fig. 2*C*).

In native SpnHL, for the second transition observed at higher concentrations of GdnHCl, a C_m of about 1.25 M was observed by both tryptophan fluorescence and far-UV CD (Fig. 2, *A* and *B*), which is similar to that reported above for the purified N-terminal fragment of SpnHL. These observations lend further support to the contention that the N-terminal fragments obtained on limited proteolysis of SpnHL treated under specified conditions is in folded conformation.

N-terminal Domain Is the Functional Domain Capable of Carrying Out Enzymatic Reaction on Its Own

The reported structure of SpnHL in complex with hyaluronic acid (16) showed that the three residues, Tyr^{408}, His^{399}, and Asn^{349}, from N-terminal domain and HA are perfectly placed to support the mechanism of HA degradation (14, 16). This indicates that probably the C-terminal domain does not play any significant role in the enzymatic activity of SpnHL. This possibility is further supported by the evidence from some structurally related enzyme like glucoamylase (26) and alginate lyase (27) in which only the α-domain is present.

As we were able to obtain an intact N-terminal domain of SpnHL we carried out enzymatic activity measurements of this domain. Fig. 7 summarizes the comparison of enzymatic activity of isolated N-terminal domain with that of the native enzyme. The N-terminal domain showed about 12% enzymatic activities compared with the native enzyme. This demonstrates that the N-terminal domain is the functional domain of SpnHL and is itself sufficient for carrying out the enzymatic reaction. The C-terminal domain seems to modulate the enzymatic activity probably by holding the substrate, hyaluronic acid, in a suitable conformation for enzymatic reaction as speculated earlier (14).

Inhibitors of SpnHL

The degradation of hyaluronan in the connective tissues by SpnHL is an important step in the pneumococcal invasion. The bacterial strains producing more hyaluronate lyase are more virulent than those strains producing less enzyme (28). Furthermore, *S. pneumoniae* strains with SpnHL and cell toxin pneumolysin double mutations showed significant additive attenuation in virulence (29). Therefore, the inhibition of SpnHL is expected to reduce the spreading of *S. pneumoniae* pathogen in the early stages of the pneumococcal invasion. Furthermore, hyaluronate lyase from bacteria and *Streptomyces* differ from hyaluronidases from other sources, including mammals, by their mode of action. They catalyze an elimination reaction, rather than hydrolysis of the β1,4-glycosidic linkage between *N*-acetyl-β-D-glucosamine and D-glucuronic acid residues in hyaluronan. Because of these reasons SpnHL becomes a potential target for developing antimicrobial agents.

Salicylate, vitamin C, heparin, dicumarene, and flavonoids have been reported to have certain inhibitory effects on the enzyme activity of hyaluronidase (5). However, recent studies by Li *et al.* (17) have reported that of the above mentioned molecules only vitamin C inhibits SpnHL activity. They also reported the crystal structure of SpnHL·vitamin C complex

(17), which shows that only one vitamin C molecule binds to SpnHL at the active site and forms about 25 interactions with seven residues (mainly from N-terminal domain and only one from C-terminal domain) of SpnHL.

The structural studies on SpnHL presented in this paper demonstrated that GdnHCl interacts with enzyme and induces significant structural changes in it. As the active site of an enzyme is very susceptible to subtle adjustments or conformational variations in the enzyme we thought it worthwhile to study the effect of GdnHCl and some related molecules (having guanidino moiety) on the enzyme activity of SpnHL so that their efficiency as inhibitors can be explored. Fig. 8 summarizes the effect of increasing concentrations of GdnHCl, L-arginine, L-NAME, and GdnHSCN on the inhibition of enzymatic activity of SpnHL. All four molecules inhibited the SpnHL activity in a dose-dependent manner. The IC_{50} of enzyme activity by GdnHCl, L-arginine, L-NAME, and GdnHSCN was found to be 150, 8.0, 0.85, and 0.14 mM, respectively. The interesting observation is that at the concentration of GdnHCl at which there is complete inhibition of SpnHL activity (about 350 mM), no significant effect on structural properties of enzyme was observed (Fig. 2). Similar observations were obtained for GdnHSCN, L-NAME, and L-arginine (data not shown). These observations suggest that GdnHCl or L-arginine or L-NAME and GdnHSCN affect the active site of enzyme at a concentration much lower than that required for bringing out any significant structural change in enzyme. This is possible only if these molecules bind directly to the active site first, followed by binding to other sites in the enzyme, which results in structural changes. For vitamin C, which has been reported as an inhibitor of SpnHL, an IC_{50} of ~5.8 mM was observed (17). In the present study an IC_{50} of ~0.85 and 0.14 mM were observed for L-NAME and GdnHSCN, respectively, which are significantly lower (about seven and 40 times) than vitamin C. These studies present a new chemical entity that can be exploited for better designing of inhibitors for SpnHL.

Acknowledgments—Dr. A. Ayyagari, Department of Microbiology, Sanjay Gandhi Institute for Post Graduate Studies, Lucknow, India is thanked for providing the *S. pneumoniae* culture. Dr. C. M. Gupta is thanked for constant support provided during the studies. Council of Scientific and Industrial Research, New Delhi, is thanked for financial assistance (to M. S. A.).

REFERENCES

1. Mufson, M. A. (1990) *Principles and Practice of Infectious Diseases* (Mandell, G. L., Douglas, R. G., Jr., and Bennett, J. E., eds) pp. 1539–1550, Publisher, Location of Publisher
2. Jonston, R. B., Jr. (1991) *Rev. Infect. Dis.* **13,** S509–S517
3. Musher, D. M. (1991) *Clin. Infect. Dis.* **14,** 801–807
4. Gray, B. M., Converse, G. M., III, and Dillion, H. C., Jr. (1980) *J. Infect. Dis.* **142,** 923–933
5. Menzel, E. J., and Farr, C. (1998) *Cancer Lett.* **131,** 3–11
6. Park, Y., Cho, S., and Linhardt, R. J. (1997) *Biochim. Biophys. Acta* **1337,** 217–226
7. Boulnois, G. J. (1992) *J. Gen. Microbiol.* **138,** 249–259
8. Jedrzejas, M. K. (2000) *Microbiol. Mol. Biol. Rev.* **65,** 187–207
9. Feldman, C., Munro, N. C., Jeffery, P. K., Mitchell, T. J., Andrew, P. W., Boulnois, G. J., Guerreiro, D., Rhode, J. A., Todd, H. C., and Cole, P. J. (1992) *Am. J. Respir. Cell Mol. Biol.* **5,** 416–423
10. Berry, A. M., Lock, R. A., Thomas, S. M., Rajan, D. P., Hansman, D., and Paton, J. C. (1994) *Infect. Immun.* **62,** 1101–1108
11. Lin, B., Hollingshead, S. K., Coligan, J. E., Egan, M. L., Baker, J. R., and Pritchard, D. (1994) *J. Biol. Chem.* **269,** 30113–30116
12. Farrell, A. M., Taylor, D., and Holland, K. J. (1995) *FEMS Microbiol. Lett.* **130,** 81–85
13. Steiner, B. M., Romero-Steiner, S., Cruce, D., and George, R. (1997) *Can. J. Microbiol.* **43,** 315–321
14. Li, S., Kelly, S. J., Lamani, E., Ferraroni, M., and Jedrzejas, M. K. (2000) *EMBO J.* **19,** 1228–1240
15. Li, S., and Jedrzejas, M. J. (2001) *J. Biol. Chem.* **276,** 41407–41416
16. Ponnuraj, K., and Jedrzejas, M. J. (2000) *J. Mol. Biol.* **299,** 885–895
17. Li, S., Taylor, K. B., Kelly, S. J., and Jedrzejas, M. J. (2001) *J. Biol. Chem.* **276,** 15125–15130
18. Paton, J. C., Berry, A. M., Lock, R., Hansman, D., and Manning, P. A. (1986) *Infect. Immun.* **54,** 50–55
19. Jedrzejas, M. J., Mewbourne, R. B., Chantalat, L., and McPherson, D. T. (1998) *Protein Expr. Purif.* **13,** 83–89
20. Pritchard, D. J., Lin, B., Willingham, T. R., and Baker, J. R. (1994) *Arch. Biochem. Biophys.* **315,** 431–437
21. Praksh, K., Prajapati, S., Ahmad, A., Jain, S. K., and Bhakuni, V. (2002) *Prot. Sci.* **11,** 46–57
22. Akhtar, M. S., Ahmad, A., and Bhakuni, V. (2002) *Biochemistry,* **41,** 7142–7149
23. Pritchard, D. J., Trent, J. O., Zhang, P., Egan, M. L., and Baker, J. R. (2000) *Proteins Struct. Funct. Genet.* **40,** 126–134
24. Fethiere, J., Eggimann, B., and Cygler, M. (1999) *J. Mol. Biol.* **288,** 635–641
25. Hubbard, S. J. (1998) *Biochim. Biophys. Acta* **1382,** 191–206
26. Sevcik, J., Hostinova, E., Gasperik, J., Solovicova, A., Wilson, K. S., and Dauter, Z. (1998) *Acta Crystallogr. Sect. D Biol. Crystallogr.* **54,** 854–866
27. Yoon, H. J., Mikami, B., Hashimoto, W., and Murata, K. (1999) *J. Mol. Biol.* **290,** 505–514
28. Rollend, K., Marois, C., Siqyier, V., Cattier, B., and Quentin, R. (1999) *J. Clin. Microbiol.* **37,** 1892–1898
29. Berry, A. M., and Paton, J. C. (2000) *Infect. Immun.* **68,** 133–140