Inhibition of 1,4-β-D-Xylan Xylanohydrolase by the Specific Aspartic Protease Inhibitor Pepstatin

PROBING THE TWO-STEP INHIBITION MECHANISM*

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This is the first report that describes the inhibition mechanism of xylanase from Thermomonospora sp. by pepstatin A, a specific inhibitor toward aspartic proteases. The kinetic analysis revealed competitive inhibition of xylanase by pepstatin A with an IC50 value 3.6 ± 0.5 μM. The progress curves were time-dependent, consistent with a two-step slow tight binding inhibition. The inhibition followed a rapid equilibrium step to form a reversible enzyme-inhibitor complex (EI), which isomerizes to the second enzyme-inhibitor complex (EI*), which dissociates at a very slow rate. The rate constants determined for the isomerization of EI to EI* and the dissociation of EI* were 15 ± 1 × 10⁻⁵ and 3.0 ± 1 × 10⁻⁸ s⁻¹, respectively. The Kₐ value for the formation of EI complex was 1.5 ± 0.5 μM, whereas the overall inhibition constant Kₐ* was 28.0 ± 1 μM. The conformational changes induced in Xyl I by pepstatin A were monitored by fluorescence spectroscopy, and the rate constants derived were in agreement with the kinetic data. Thus, the conformational alterations were correlated to the isomerization of EI to EI*. Pepstatin A binds to the active site of the enzyme and disturbs the native interaction between the histidine and lysine, as demonstrated by the abolished isoidole fluorescence of o-phthalaldehyde-labeled xylanase. Our results revealed that the inactivation of xylanase is due to the interference in the electronic microenvironment and disruption of the hydrogen-bonding network between the essential histidine and other residues involved in catalysis, and a model depicting the probable interaction between pepstatin A with xylanase has been proposed.

In recent years considerable research efforts have been expended in the design and synthesis of glycosidase inhibitors not only to understand about the active site structures and mechanisms of these interesting enzymes but also in generating new therapeutic agents. Specific inhibitors of glycosidases have proved valuable in a number of applications ranging from mechanistic studies (1, 2) to possible therapeutic uses such as viral infectivity through interference with normal glycosylation of coat proteins (3), against cancer, bacterial infections, and as insecticides (4). Glycosidase inhibitors as therapeutics has been greatly facilitated by solving the crystal structure of family 10 xylanases (10, 11) have revealed the extended substrate binding cleft in which the surface residues are linked by an extensive hydrogen bond network. The cleft forms deep grooves, consistent with their endo-mode action, and comprise a series of subsites, each one capable of binding a xylose moiety (12). The active site of xylanase contains two essential catalytic groups, one playing the role of acid/base and the other functioning as a nucleophile (13). These two groups have been identified as carboxyl groups, and a covalent intermediate is formed that undergoes hydrolysis to afford hemiacetal with net retention of anomeric stereochemistry (14). The transition states leading to and from the covalent intermediate have substantial oxacarbonium ion character, as indicated by kinetic isotope effects and by the effects of electron-withdrawing substituents on the sugar ring upon reaction rate (15). Analysis of active site amino acids that play an important role in substrate binding and in catalysis has been greatly facilitated by solving the crystal structure of family 10 xylanases covalently linked to mechanism-based cello-

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bioaryl (2-deoxy-2-fluorocellobiosyl) and xylooligosaccharide (2-deoxy-2-
xylooligosaccharide) inhibitors (15). To gain further insight into
the details of the hydrolytic mechanism of glycosidases, specific
inhibitors are necessary that can act as mechanistic and struc-
tural probes. A diverse array of extremely potent, basic nitro-
gen-containing inhibitors has been developed over the years,
and they have been found to be of great utility in the study of
the glycosidase mechanism (7, 16). However, there have been
very few reports of naturally occurring inhibitors of xylanases.
Pepstatin is a naturally occurring low molecular weight potent
inhibitor specific for aspartic proteases (17). The unusual po-
tency of pepstatin toward aspartic proteases has been widely
exploited as a research tool for unraveling the mechanism of
this group of enzymes (18), its biological functions (19), and in
affinity chromatography (20, 21). It also has been tested as a
therapeutic agent for experimental control of gastric ulcer (17,
22), carrageenin edema (17), hypertension (23, 24), and infec-
tious diseases like human immunodeficiency virus (25). To our
knowledge there are no reports of pepstatin inhibition on xyl-
anolytic enzymes.

In this paper we present slow-tight binding inhibition of the
thermostable xylanase (Xyl I)1 from a Thermomonospora sp. by
a specific aspartic protease inhibitor, pepstatin A. The steady-
state kinetics revealed a two-step inhibition mechanism,
and the conformational modes observed during the binding of
inhibitor to the enzyme were conveniently monitored by fluo-
rescence analysis. The mechanism of inactivation of Xyl I by
pepstatin A was delineated by monitoring the isoindole flu-
orescence of the o-phthalaldehyde (OPTA)-labeled enzyme and
a model for the probable interactions have been proposed.

EXPERIMENTAL PROCEDURES

Materials—Oat spelled xylan, dinitrosalicylic acid, pepstatin A, and
o-phthalaldehyde (OPTA) were obtained from Sigma. Sephadex A-50
and Sephacryl S-200 were obtained from Amersham Biosciences . All
other chemicals used were of analytical grade.

Microorganisms Growth Condition and Purification of Xyl I—Ther-
monospora sp. producing Xyl I is an alkalophilic actinomycete
having optimum growth at pH 9 and 50 °C. It was isolated from
self-heating compost from the Barabanki district of Uttar Pradesh,
India (26). The Thermomonospora sp. was grown at 50 °C for 96 h for
the production of Xyl I. The enzyme was purified to homogeneity from
oat spelled xylan as the substrate under assay conditions. Protein concen-
tration was determined according to the method of Bradford (29) using
the extracellular culture filtrate by fractional ammonium sulfate pre-
paration and Sephacryl S-200 gel filtration chromatography (27).

Xylanase Assay and Inhibition Kinetics—Xylanase assay was carried
out in phosphate buffer, 0.05 M, pH 6.0, by mixing a specified concen-
tration of the enzyme with 0.5 ml of oat spelled xylan (10 mg/ml) in a
reaction mixture of 1 ml and incubating at 50 °C for 30 min. The reduc-
ing sugar released was determined by the dinitrosalicylic acid
method (28). One unit of xylanase activity was defined as the amount
of enzyme that produced 1 μmol of xylose equivalent per min using oat-
spelled xylan as the substrate under assay conditions. Protein concen-
tration was determined according to the method of Bradford (29) using
bovine serum albumin as the standard.

For initial kinetic analysis the kinetic parameters for the
substrate hydrolysis were determined by measuring the initial rate of enzymatic
activity. The inhibition constant (K) was determined by Dixon method
(30) and also by the Lineweaver-Burk analysis. The K was also
calculated from the double-reciprocal equation by fitting the data into
the computer software Microcal Origin. For the Lineweaver-Burk anal-
ysis Xyl I (2 μM) was incubated with pepstatin A at (1 μM) and (2 μM)
and assayed at increased concentrations of xylan (1–10 mg/ml) at 50 °C
for 30 min. The reciprocals of substrate hydrolysis (1/v) for each inhibi-
tor concentration were plotted against the reciprocals of the substrate
concentrations and the K was determined by fitting the resulting data.

In Dixon method (30), xylanolytic activity of Xyl I (2 μM) was mea-
sured in the presence of 5 and 10 mg/ml xylan at concentrations of pepstatin A ranging from 0 to 7 μM at 50 °C for 30 min. The reciprocals
of substrate hydrolysis (1/v) were plotted against the inhibitor concen-
tration and the K was determined by fitting the data using
Microcal Origin.

For the progress curve analysis assays were carried out in a reaction
mixture of 1 ml containing enzyme, xylan, and inhibitor at various
concentrations. The reaction mixture contained Xyl I (50 nM) in sodium
phosphate buffer, 0.05 M, pH 6.0, and varying concentrations of pepsta-
in A (0.3–3 μM) and xylan (10 mg/ml). The reaction was initiated by
the addition of Xyl I at 50 °C, and the release of products was monitored at
different time intervals by estimating the reducing sugar at 540 nm. In
each slow binding inhibition experiment, five to six assays were per-
formed with appropriate blanks. For the kinetic analysis and rate
constant determinations, the assays were carried out in triplicate,
and the average value was considered throughout. Further details of the
experiments are given in the respective figure legends.

Evaluation of Kinetic Parameters—Initial rate studies for reversible;
competitive inhibition was analyzed according to Equation 1,

\[ v = \frac{V_{max}S}{K_m + I + S} \]

(Eq. 1)

where K is the Michaelis constant, V is the maximal rate of sub-
saturating substrate concentration S, K + I is the dissociation
constant for the first reversible enzyme-inhibitor complex, and I is the
inhibitor concentration (31). The progress curves for the interactions
between pepstatin A and Xyl I were analyzed using Equation 2 (32, 33),

\[ [P] = \frac{v_0 - v}{k} \]

(Eq. 2)

where [P] is the product concentration at any time t, v is the
initial and final steady-state rates, respectively, and k is the apparent
first-order rate constant for the establishment of the final steady-state
equilibrium. As a prerequisite for tight binding inhibitors, corrections
have been made for the reduction in the inhibitor concentration that
occurs on formation of the enzyme inhibitor (EI) complex. This is be-
cause in the case of tight binding inhibition, the concentration of EI is
not negligible in comparison to the inhibitor concentration, and the free
inhibitor concentration is not equal to the added concentration of the
inhibitor. The corrections of the variation of the steady-state velocity
with the inhibitor concentrations were made according to Equation 3 and 4
as described by Morrison and Walsh (34),

\[ K = I_k + 2K \frac{S}{(S + K)} \]

(Eq. 3)

\[ K = K + (1 + S/K) \]

(Eq. 4)

\[ K = K \frac{1}{1 + (S/K)} \]

(Eq. 5)

The progress curves were analyzed by Equations 2 and 5 using non-
linear least-square parameter minimization to determine the best-fit
values with the corrections for the tight binding inhibition. The overall
inhibition constant is determined as given by Equation 6.

\[ K = \frac{[E][I]}{[E][I]} = K \frac{K}{K + K} \]

(Eq. 6)

For the time-dependent inhibition, there exists a time range in the
progress curves wherein formation of EI is small. Within this time
range, it is possible to directly measure the effect of the inhibitor on v.
i.e. to measure K directly. Values for K were obtained from Dixon
analysis at a constant substrate concentration as described in Equation
7.

\[ \frac{1}{v} = \frac{1}{V_{max}} + \frac{1}{V_{max}} \frac{S}{(1 + /K)} \]

(Eq. 7)

The rate constant K for the dissociation of the second enzyme-
inhibitor complex was measured directly from the time-dependent
inhibition. Concentrated Xyl I and pepstatin A were incubated in a
reaction mixture to reach equilibrium followed by large dilutions in

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1 The abbreviations used are: Xyl I, xylanase from Thermomonospora
sp.; IC50, 50% inhibitory concentration; OPTA, o-phthalaldehyde.
assay mixtures containing near-saturating substrate. Xyl I (2 mM) was preincubated with equimolar concentrations of pepstatin A for 120 min in sodium phosphate buffer, 0.05 M, pH 6.0. 5 µl of the preincubated sample was removed and diluted 5000-fold in the same buffer and assayed at 50 °C using xylan at (150 mg/ml) at different time intervals.

Fluorescence Analysis—Fluorescence measurements were performed on a PerkinElmer Life Sciences LS50 luminescence spectrometer connected to a Julabo F20 water bath. Protein fluorescence was excited at 295 nm, and the emission was recorded from 300–400 nm at 25 °C. The slit widths on both the excitation and emission were set at 5 nm, and the spectra were obtained at 100 nm/min.

For inhibitor binding studies, Xyl I (2 µM) was dissolved in sodium phosphate buffer, 0.05 M, pH 6.0. Titration of the enzyme with pepstatin A was performed by the addition of different concentrations of the inhibitor to a fixed concentration of enzyme solution. For each inhibitor concentration on the titration curve a new enzyme solution was used. All the data on the titration curve were corrected for dilutions, and the graphs were smoothed. The magnitude of the rapid fluorescence decrease (F₀ – F) occurring at each Pepstatin A concentration was computer-fitted to Equation 8 to determine the calculated value of Kᵢ and ΔFₓₘₐₓ (35).

\[
(F₀ - F) = \Delta F_{\text{max}} \left[\frac{1}{1 + (Kᵢ/I)}\right]
\]

(Eq. 8)

The first order rate constants for the slow loss of fluorescence kₓₐₓ at each inhibitor concentration [I] were computer fitted to the Equation 9 (31) for the determination of kₓ under the assumption that for a tight binding inhibitor, kₓ can be considered negligible at the onset of the slow loss of fluorescence.

\[
kₓₓₓ = k[I]/(Kᵢ + [I])
\]

(Eq. 9)

Time course of the protein fluorescence after the addition of inhibitor was measured for 300 s with excitation and emission wavelengths fixed at 295 and 340 nm, respectively, with data acquisition at 0.1-s intervals. Corrections for the inner filter effect were performed as described by Equation 10 (36),

\[
Fₓ = F \text{ antilog}[\left(A₊₊ + A₋₋\right)/2]
\]

(Eq. 10)

where Fₓ and F stand for the corrected and measured fluorescence intensities, respectively, and A₊₊ and A₋₋ are the absorbances of the solution at the excitation and emission wavelengths, respectively. Background buffer spectra were subtracted to remove the contribution from Raman scattering.

Effect of Pepstatin A on the Isoindole Fluorescence of OPTA-labeled Xyl I—Fresh OPTA solution was prepared in methanol for each experiment. The modification was carried out by incubating Xyl I (2 µM) in 1 ml of 0.05 M sodium phosphate buffer, pH 6, with 50 µM OPTA at 25 °C. Methanol had no effect on the activity of the enzyme and was always less than 2% (v/v). The formation of Xyl I-isoindole derivative was followed spectrophotometrically by monitoring the increase in fluorescecence with the excitation wavelength fixed at 330 nm. To monitor the effect of pepstatin A on the isoindole fluorescence of Xyl I, the enzyme was preincubated with pepstatin A (2 µM) for 20 min, and then OPTA was added, and the formation of isoindole derivative was monitored as described above.

RESULTS

Kinetic Analysis of the Inhibition of Xyl I—The aspartic protease inhibitor (pepstatin A) was well documented for its inhibitory activity toward pepsin, renin, cathepsin D, and human immunodeficiency virus 1 protease (37, 38, 39, 25). The bifunctional nature of pepstatin A was established by its potency toward Xyl I, the xylanase purified from the Thermomonospora sp. Xyl I, a member of family 10 xylanase, is highly thermostable with half-lives of 86, 30, and 15 min at 80, 90, and 100 °C, respectively, and is stable in an expansive pH range of 5–10 with more than 75% residual activity. Initial kinetic studies revealed that pepstatin A is a competitive inhibitor of Xyl I with an IC₅₀ value of 3.6 ± 0.5 µM (Fig. 1). In the absence of pepstatin A, the steady-state rate of xylanolytic activity of Xyl I was reached rapidly, whereas in its presence a time-dependent decrease in the rate as a function of the inhibitor concentration was observed. Examination of the progress curves revealed a time range where the initial rate of reaction did not deviate from linearity (Fig. 2), and the conversion of EI to EI⁺ was minimal. For a low concentration of pepstatin A this time range was 10 min, within which classical competitive inhibition experiments was used to determine the Kᵢ values (Equation 5). The value of the inhibition rate constant Kᵢ associated with the formation of the reversible enzyme-inhibitor complex (EI) determined from the fits of data to the reciprocal equation, was 1.5 ± 0.5 µM (Fig. 3A), which was corroborated by the Dixon method (Fig. 3B). The apparent rate constant kᵢ derived from the progress curves when plotted versus the inhibitor concentration, followed a hyperbolic function (Fig. 4), revealing that a fast equilibrium precedes the formation of the final slow dissociating enzyme-inhibitor complex (EI⁺), indicating a two-step, slow-tight inhibition mechanism (Scheme I). Indeed, the data could be fitted to Equation 5 by non-linear regression analysis, which yielded the best estimate of the overall inhibition constant Kᵢ of 28.0 ± 1 µM.

In another method, the rate constant kᵢ for the conversion of EI⁺ to EI, was determined by preincubating high concentrations of enzyme and inhibitor for sufficient time to allow the system to reach equilibrium. Dilution of the enzyme-inhibitor complex into a relatively large volume of assay mixture containing saturating substrate concentration causes dissociation of the enzyme-inhibitor complex and, thus, regeneration of enzymatic activity. Under these conditions, v₀ and the effective inhibitor concentration can be considered approximately equal to 0, and the rate of activity regeneration will provide the kᵢ value. After preincubating Xyl I with pepstatin A, the enzyme inhibitor mixture was diluted 5000-fold into the assay mixture containing the substrate at 50 Kᵢ. By least-squares minimization of Equation 2 to the data for recovery of enzymatic activity, the determined kᵢ was 3.0 ± 1 × 10⁻⁵ s⁻¹ (Fig. 5), which clearly indicated a very slow dissociation of EI⁺. The final steady-state rate vₛ was determined from the control that was preincubated without the inhibitor. The value of the rate constant Kᵢ, associated with the isomerization of EI to EI⁺, was 15 ± 1 × 10⁻⁵ s⁻¹ as obtained from fits of Equation 3 to the onset of inhibition data using the experimentally determined values of Kᵢ and kᵢ (Table 1). The overall inhibition constant Kᵢ⁺ is a function of kᵢ/kᵢ⁺ + kᵢ and is equal to the product of Kᵢ and this function. The kᵢ⁺ value indicated a slower rate of dissociation of EI⁺ complex and the half-life tᵢ₀ for the reactivation of EI⁺ as determined from kᵢ⁺ values was 64 ± 2 × 10² h, suggesting higher binding affinity.
of pepstatin A toward Xyl I. Where \( E \) stands for free enzyme, \( I \) is free inhibitor, \( EI \) is a rapidly forming pre-equilibrium complex, and \( EI^* \) is the final enzyme-inhibitor complex. \( E \) may undergo inter-conversion into another form, \( E^* \), which binds to the inhibitor by a fast step, where \( k_d \) and \( k_{-d} \) stand for the rate constants for forward and backward reaction, respectively, for the conversion of the enzyme.

Scheme I describes two alternative models for the time-dependent inhibition. The mechanism in Scheme Ia, where the binding of the inhibitor to the enzyme is slow and tight but occurs in a single step, is eliminated based on the data of Table I. Scheme Ic represents the inhibition model where the inhibitor binds only to the free enzyme because the inhibitor has a slow dissociation constant. This model may be used to eliminate the initial rapid decrease in fluorescence due to the binding of pepstatin A to the active site and changes in the native conformation. Our ongoing results for the inactivation of Xyl I were, therefore, consistent with a slow-tight binding mechanism as described in Scheme Ib.

**Effect of Inhibitor Binding on the Fluorescence of Xyl I**—The kinetic analysis revealed a two-step inhibition mechanism, where the \( EI \) complex isomerizes to a tightly bound, slow dissociating \( EI^* \) complex. This isomerization is a consequence of the conformational changes induced in Xyl I due to the binding of pepstatin A. The tryptophanyl fluorescence of Xyl I exhibited an emission maxima \( \lambda_{max} \) at 340 nm as a result of the radioactive decay of the \( \pi \)-\( \pi \) transition from the Trp residues (Fig. 6). The binding of pepstatin A resulted in a concentration-dependent quenching of the fluorescence with saturation reaching at above 6 \( \mu \)M pepstatin A (inset of Fig. 6). The absence of blue or red shift in \( \lambda_{max} \) negated any drastic gross conformational changes in the three-dimensional structure of the enzyme due to inhibitor binding. The subtle conformational changes induced during the isomerization of \( EI \) to \( EI^* \) were monitored by analyzing the tryptophanyl fluorescence of the complex as a function of time. Binding of pepstatin A resulted in an exponential decay of the fluorescence intensity as indicated by a sharp decrease in the quantum yield of fluorescence followed by a slower decline to a stable value (Fig. 7). Furthermore, titration of pepstatin A against Xyl I revealed that the magnitude of the initial rapid fluorescence loss \( (F_0 - F) \) increased in a saturation-type manner (Fig. 8), which corroborated the two-step slow tight binding inhibition of Xyl I by pepstatin A. From the data in Fig. 8, the magnitude of the rapid fluorescence decrease at a specific pepstatin A concentration was found to be close to the total fluorescence quenching observed in Fig. 6, indicating that the \( EI \) and \( EI^* \) complexes have the same intrinsic fluorescence. The value of \( K \) determined by fitting the data for the magnitude of the rapid fluorescence decrease \( (F_0 - F) = 1.2 \pm 0.5 \mu \)M, and the \( k \) value determined from the data derived from the slow decrease in fluorescence was \( 14 \pm 1 \times 10^{-5} \) s\(^{-1} \). These rate constants are in good agreement with that obtained from the kinetic analysis; therefore, the initial rapid fluorescence decrease can be correlated to the formation of the reversible complex \( EI \), whereas the slow, time-dependent decrease reflected the accumulation of the tight bound slow dissociating complex \( EI^* \).

**Effect of Pepstatin A on the Isoindole Fluorescence of Xyl I** by OPTA—In our earlier report we have investigated the role of essential histidine and lysine residues in the active site of the Xyl I and shown that binding of the chemoaffinity label OPTA to these residues of the active site resulted in the formation of an isoindole derivative (40, 41). The active site of Xyl I is composed of the catalytic carboxyl groups and the histidine residue, which play a crucial role in catalysis. To investigate the binding of pepstatin A to the active site and changes in the native intermolecular interactions, we have monitored the changes in the interaction of the lysine and histidine due to pepstatin A binding and their influence on the isoindole fluorescence of Xyl I (Fig. 9). The unbound enzyme did not show fluorescence when excited at 338 nm; however, incubation of OPTA with Xyl I resulted in an increase in the fluorescence with a \( \lambda_{max} \) at 417 nm due to the formation of the isoindole derivative. The pepstatin A preincubated Xyl I failed to react with OPTA as revealed by the total loss of isoindole fluorescence, which not only confirmed the binding of pepstatin A to the active site of Xyl I but also further revealed that the binding of pepstatin A resulted in the formation of a new set of hydrogen bonding and other nonionic interactions. These altered weak interactions cause disruption of the native hydrogen-bonding network of the histidine and lysine residues, which are essential for the formation of isoindole derivative.

**DISCUSSION**

In recent years a number of naturally occurring reversible glycosidase inhibitors such as nojirimycin, castanospermine, and swainsone have been reported (1). Nojirimycin and castanospermine are naturally occurring polyhydroxylated alkaloids known as azasugars. Nojirimycin is produced by Bacilli and Streptomyces sp. and shows inhibitory activity against plant and fungal glucosidases (42). Castanospermine was isolated from the seeds of Castanospermum australe (43) and was found to be a powerful inhibitor of \( \alpha \) and \( \beta \) glucosidases (44). Swainsone is an indolizidine alkaloid was isolated from the plant Swainsona canescens. Swainsone is a specific and potent inhibitor of \( \alpha \)-mannosidase (45). Another class of inhibitors is the covalent, irreversible type, typically affinity labels. These are generally synthetic analogues of sugars containing reactive groups such as epoxides, isothiocyanates, and \( \alpha \)-halocarboxyls (1, 46). There are reports of mechanism-based inhibitors such as conduritol epoxides (47), the quinone methide-generating glycosides (48), and the glycosylmethyl triazenes (49). These are more selective inhibitors whose efficacy depends upon binding and subsequent enzymatic action to generate a reactive species. Although a plethora of synthetic inhibitors has been reported, there is a lacuna of naturally occurring low molecular weight inhibitors of glycosidases from microorganisms. It is noteworthy to point out that several extremophiles are known to produce highly thermostable xylanases and cellulases; how-
ever, these organisms have not been studied extensively for their potential exploitation toward isolation of inhibitors of important enzymes. Pepsin and Xyl 1 catalyze hydrolytic cleavage of two different substrates made of peptide bond and glycosidic bond, respectively. Pepsin, a model enzyme for aspartic protease, did show any activity on xylan, a sugar polymer made of glycosidic bond. The Xyl 1 did not show any activity against protein substrates, such as hemoglobin and casein, made of peptide bond (data are not shown), indicating these enzymes are absolutely specific toward their natural substrates. Here we present the first report of an aspartic protease specific inhibitor, pepstatin A, exhibiting slow-tight binding inhibition against xylanase from *Thermomonospora* sp. There are several reports on the inhibition of pepstatin against aspartic proteases (17, 25, 37, 38, 39), but this is the first study that explores the inhibition mechanism of this specific aspartic protease inhibitor against a xylanase. The inhibitor showed exceptionally high potency against Xyl 1, the thermostable xylanase from a *Thermomonospora* sp., and its 1:1 molar ratio of interaction with the enzyme indicated its “tight binding” nature. The two-step inhibition mechanism was corroborated by the equilibrium binding studies of the enzyme and inhibitor and the correlation of the kinetic data with the conformational changes induced in the enzyme-inhibitor complexes.

**FIG. 3. Initial rate of enzymatic reaction of Xyl 1 in the presence of pepstatin A.** A, enzymatic activity of Xyl 1 was estimated using oat-spelled xylan in sodium-phosphate buffer, 0.05 M, pH 6.0, and the xylose equivalent was determined at 540 nm. Xyl 1 (2 μM) was incubated without (☐) or with the inhibitor at 1 μM (●) and 5 μM (▲) and assayed at an increased concentration of xylan (1–10 mg/ml) at 50 °C for 30 min. The reciprocal of substrate hydrolysis (1/v) for each inhibitor concentration was plotted against the reciprocal of the substrate concentration. B, xylanase (2 μM) was assayed using xylan at 5 mg/ml (●) and 10 mg/ml (☐) with increasing concentrations of pepstatin A at 50 °C for 30 min. The reciprocal of substrate hydrolysis (1/v) was plotted against inhibitor concentration. The straight lines indicated the best fits for the data obtained by non-linear regression analysis and analyzed by Lineweaver-Burk reciprocal equation (A) and the Dixon method (B), respectively.
In the presence of competitive inhibitors, a number of enzymatic reactions do not respond immediately but display a slow-onset of inhibition, which is referred as slow binding inhibition (50–54). The establishment of the equilibria between enzyme, inhibitor, and enzyme-inhibitor complexes in slow binding inhibition occurs slowly on the steady-state time scale (54–58). Enzyme-catalyzed reactions, where the concentrations of the enzyme and inhibitor are comparable and the equilibria are set up rapidly are referred as tight binding inhibition. Kinetically the slow binding inhibition can be illustrated by three mechanisms (Scheme 1). When an inhibitor has a low $K_i$ value and the concentration of I varies in the region of $K_i$, both $k_6$ and $k_4$ values would be low. Thus, a simple second-order interaction between enzyme and inhibitor and low rates of association and dissociation would lead to slow binding inhibition. Alternatively, a two-step model depicts the rapid formation of an initial collisional complex EI, which slowly isomerizes to form a tightly bound slow dissociating complex EI*. Slow binding inhibition can also arise due to an initial slow interconversion of the enzyme E into another form, $E^*$, which binds to the inhibitor by a fast step. Understanding the basis of the isomerization of EI to $E^*$ could lead to the design of inhibitors that allow titration of the lifetime of the $E^*$. In case of slow-tight binding inhibition, the inhibitor will inhibit the enzyme competitively at the onset of the reaction; however, at increasing concentrations of inhibitor the rate of substrate hydrolysis will decrease hyperbolically as a function of time. In tight binding inhibition, corrections have to be made for the reduction in the inhibitor concentration that occurs on formation of the EI complex, since the concentration of $E^*$ is not negligible in comparison to the inhibitor concentration and the free inhibitor concentration is not equal to the added concentration of the inhibitor. The kinetic analysis of the xylanase inhibition in this paper provides a unique opportunity for the quantitative determination of these rates and affinities, which can be extended to other slow-tight binding inhibition reactions. The formation of EI complex between Xyl I and pepstatin A was too rapid to be measured at steady-state kinetics and was likely to be near diffusion control. However, the isomerization of EI to the second tightly bound enzyme-inhibitor complex, $E^*$, was too slow and relatively independent of the stability of the EI or the ability of the inhibitor to stabilize the $E^*$. The $k_a$ values revealed very slow dissociation of the inhibitor from the $E^*$, indicating a highly stable, non-dissociative nature of the second complex. Therefore, for slow-tight binding inhibition the major variable is $k_a$, the first-order rate constant associated with the conversion of $E^*$ to EI, and the apparent inhibitor constant $K_i*$ depends on the ability of the inhibitor to stabilize the $E^*$. The half-life as derived from the $k_a$ value indicated a longer half-life of the $E^*$, which is an essential parameter for an inhibitor to have biomedical applications.

The characteristic feature of slow binding inhibition is the induction of conformational changes in the enzyme-inhibitor...
complex, resulting in the clamping down of the enzyme to the inhibitor, thus, the formation of a stable enzyme-inhibitor complex. The two-step inhibition mechanism of Xyl I by pepstatin A was reflected in the quenching pattern of the fluorescence of the enzyme-inhibitor complexes. The rate constants derived from the fluorescence analysis of the complexes corroborated the values derived from the kinetic analysis. Therefore, we propose that the initial rapid fluorescence loss reflected the formation of the reversible complex $Ei$, whereas the subse-
Slow-Tight Binding Inhibition of Xylanase by Pepstatin

Conformational integrity of the active site of an enzyme is essential for its catalysis, and investigations on the molecular orientation of the functional groups of the active site as well as their microenvironment are areas of growing scientific interest. Chemo-affinity labeling is a powerful technique to assign the conformational changes of ligand-macromolecule complexes, which combines some of the advantages of both the photoactivated and electrophilic affinity labeling (61). OPTA is a bifunctional, fluorescent chemoaffinity label, which until recently was known to have absolute specificity for amino and thiol groups (62) for the formation of an isoindole derivative. However, application of OPTA as a probe to ascertain the conformational flexibility and polarity of the active site of Xyl I by the formation of a fluorescent isoindole derivative with the lysine and histidine residue has been reported in our laboratory (40). OPTA contains two aldehyde groups, one of which reacts with the primary amine of lysine while the second group reacts with the secondary amine of the imidazole ring of histidine, resulting in the formation of the isoindole derivative. Our foregoing results revealed that, when Xyl I was preincubated with pepstatin A, OPTA failed to form the isoindole derivative, as reflected by the loss of fluorescence. The inability of OPTA to form the isoindole derivative with the pepstatin A-bound Xyl I could be attributed to the interaction of the inhibitor with either lysine or histidine or both the residues, thereby changing the native molecular interactions of these residues. The chemical structure of pepstatin is isovaleryl-l-valyl-l-valyl-statyl-l-alanyl-statyl, which contains two residues of unusual amino acid statin ((3S,4S)-4-amino-3-hydroxy-6-methyl-heptanoic acid). The statin is the major structural component responsible for the pepstatin inhibition against the aspartic protease. Aspartic proteases consist of two carboxyl groups at the active site, one of which has to be protonated and the other deprotonated for the enzyme to be active. Aspartic protease undergoes a general acid-base catalysis that may be called a "push-pull" mechanism. The nucleophilic attack is achieved by two simultaneous proton transfers, one from a water molecule to the diad of the two carboxyl groups and a second one from the diad to the carbonyl oxygen of the substrate with the concurrent CO-NH bond cleavage. Pepstatin is an analog of the transition state of peptic catalysis that tightly binds to active site of the enzyme (37). In the case of xylanase the active site contains two essential catalytic groups, one playing the role of acid/base and the other functioning as a nucleophile. We propose that pepstatin makes several interactions with the active site residues of Xyl I both through hydrogen bonds and non-bonded interactions (Fig. 10A). The hydroxyl group of statin residue of the inhibitor form hydrogen bonding with the amines of Lys and Gin. The model also depicts the hydrogen bonding between the carbonyl oxygen of Ala of the inhibitor with the secondary amine of the His of Xyl I. The catalytic site of xylanases consists of two carboxyl groups and an essential lytic water molecule and follows a general acid-base catalytic mechanism (63). Fig. 10B demonstrates the probable interaction of the essential His and Lys residues of Xyl I with the hydroxyl and carbonyl functional groups of the inhibitor. Based on the existing experimental evidences, we further propose that the other residues of the inhibitor could form many intermolecular hydrogen bonds and other weak interactions with the residues in or near the active site of Xyl I. We also visualize that the tight binding nature of pepstatin A in conjunction with the multiple nonbonded interactions may be sufficient to interfere in the native weak interactions between the carboxyl groups, the lytic water molecule, and the essential histidine residue of the active site, leading toward the inactivation of Xyl I.

Based on our observations, we conclude that the inhibition of Xyl I by pepstatin A followed slow-tight binding inhibition mechanism and the induced conformational changes are conveniently monitored by fluorescence spectroscopy. Chemo-affinity labeling of the enzyme active site has demonstrated that the inactivation of xylanase is due to the interference in the electronic microenvironment and disruption of the hydrogen-bonding network between the essential histidine and other residues involved in catalysis. However, the crystal structure of the enzyme-inhibitor complex will aid to understand the mechanism of inactivation of Xyl I in depth and will further shed light on the molecular interactions between the enzyme and inhibitor.

![Graph](https://www.jbc.org/Downloadedfrom)
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REFERENCES

1. Legler, G. (1990) Adv. Carbohydr. Chem. Biochem. 48, 319–358
2. Sinnott, M. L. (1990) Chem. Rev. 90, 1171–1202
3. Elbein, A. D., Legler, G., Trusty, A., McDowell, W., and Schwarz, R. (1984) Arch. Biochem. Biophys. 235, 579–588
4. Leroy, E. and Reymond, J.-L. (1999) Org. Lett. 1, 775–777
5. Truscheit, E., Frenmer, W., Junge, B., Muller, L., Schmidt, D. D., and Wingender, W. (1981) Angew. Chem. Int. Ed. Engl. 20, 744–761
6. Fenton, R. J., Murley, P. J., Owens, J. J., Gower, D., Parry, S., Crossman, L., and Wong, T. (1999) Antimicrob. Agents Chemother. 43, 2642–2647
7. Biely, P. (1985) Trends Biotechnol. 3, 286–290
8. Kulkarni, N., Sheddy, A., and Rao, M. (1999) FEBS Microbiol. Rev. 23, 411–456
9. Tarvainen, K., and Keskinen, H. (1991) Clin. Exp. Allergy 21, 609–615
10. Harris, G. W., Jenkins, N. A., Connerton, I., Cummings, N., Lo Leggio, L., Scott, M., Hazlewood, G. P., Laurie, J. I., Gilbert, H. J., and Pickersgill, R. W. (1994) Structure 2, 1107–1116
11. White, A., Withers, S. G., Gilkes, N. R., and Rose, N. R. (1994) Biochemistry 33, 12546–12552
12. Davies, G. J., Wilson, K., and Henrissat, B. (1997) Biochem. J. 321, 557–559
13. Royers, J. C., and Nakas, J. P. (1989) Enzyme Microb. Technol. 11, 405–410
14. Ly, H. D., and Withers, S. G. (1999) Ann. Rev. Biochem. 68, 695–698
15. White, A., Tull, D., Johns, K., Withers, S. G., and Rose, D. R. (1996) Nat. Struct. Biol. 3, 149–154
16. Stutz, A. E. (1999) Immunosugars as Glycosidase Inhibitors: Anjirmycin and Beyond, pp. 95–120, Wiley-VCH, Weinheim, Germany
17. Umezawa, H., Anyagi, T., Morishima, H., Hamed, M., and Takeuchi, T. (1970) J. Antibiot. (Tokyo) 23, 259–262
18. Sachdev, G. P., Brownstein, A. D., and Fruton, J. S. (1973) J. Biol. Chem. 248, 6292–6299
19. Barrett, A. J., and Dingle, J. T. (1972) Biochem. J. 127, 439–441
20. Murakami, K., Inagami, T., Michelakis, A. M., and Cohen, S. (1973) Biochem. Biophys. Res. Commun. 54, 482–487
21. Carvol, P., Devaux, C., and Menard, J. (1973) FEBS Lett. 34, 189–192
22. Umezawa, H. (1972) Enzyme Inhibitors of Microbial Origin, p. 31, University Park Press, Baltimore, MD
23. Miller, R. P., Poper, C. J., Wilson, C. W., and De Vito, E. (1972) Biochem. Pharmacol. 21, 2941–2944
24. Lazar, J., Orth, H., Mochring, J., and Gross, F. (1972) Nauyn Schmiededbergs Arch. Pharmacol. 275, 114–118
25. Von der Helm, K., Gurtler, L., Eberle, J., and Deinhardt, F. (1989) FEBS Lett. 247, 349–352
26. George, S. P., Ahmad, A., and Rao, M. (2001) Bioresour. Technol. 77, 171–175
27. George, S. P., Ahmad, A., and Rao, M. (2001) Biochem. Biophys. Res. Commun. 282, 48–54
28. Miller, G. L. (1959) Anal. Chem. 31, 426–428
29. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
30. Dixon, M. (1955) Biochem. J. 55, 170–171
31. Cleland, W. W. (1979) Methods Enzymol. 63, 103–138
32. Beith, J. G. (1995) Methods Enzymol. 248, 59–84
33. Morrison, J. F., and Stone, S. R. (1985) Comments Mol. Cell. Biophys. 2, 347–368
34. Morrison, J. F., and Walsh, C. T. (1988) Adv. Enzymol. 62, 201–302
35. Houtzager, V., Ouleit, M., FalgueyreJ.-P., Passmore, L. A., Bayly, C., and Percival, M. D. (1996) Biochemistry 35, 10974–10984
36. Lakowicz, J. R. (1983) Principles of Fluorescence Spectroscopy, pp. 180–195, Plenum Press, New York
37. Marrininsyn, J., Jr., Hartseck, J. A., and Tang, J. (1976) J. Biol. Chem. 251, 7688–7694
38. McKown, M. M., Wrokman, R., and Gregerman, R. I. (1974) J. Biol. Chem. 249, 7770–7774
39. Knight, C. G., and Barrett, A. J. (1976) Biochem. J. 155, 117–125

FIG. 10. Schematic representation of the model depicting the probable mechanism of pepstatin A binding to the active site of the Xyl I. The active site of the Xyl I has been modeled based on the x-ray crystallographic structure of a similar thermostable family 10 xylanase from Thermosaus aurantiacus (54) (Protein Data Bank code 1TUX), and the pepstatin structure (shown in red) was obtained from the Protein Data Bank code 1PSO (65). A, the interactions were modeled by using the software MOLMOL. The active site of Xyl I includes the essential Glu, His, Gln, and Lys residues. The dotted lines indicate the hydrogen bonding between the inhibitor and the catalytic acidic residues of Xyl I. Based on our results we propose that the hydroxyl group of statin residues of the inhibitor form hydrogen bonding with the free amine groups of the Lys and Gln. The model also depicts the hydrogen bonding between the carbonyl oxygen of Ala of the inhibitor with the secondary amine of the His of Xyl I. The other residues of the inhibitor can also form many non-bonded interactions with the active site residues of Xyl I. B, probable interactions of the amine groups of essential Lys (A) and His (B) residues of Xyl I with the hydroxyl (C) and carboxyl (D) groups present in the inhibitor.
Inhibition of 1,4-β-d-Xylan Xylanohydrolase by the Specific Aspartic Protease Inhibitor Pepstatin: PROBING THE TWO-STEP INHIBITION MECHANISM
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