Papain, a powerful proteolytic enzyme, belongs to the cysteine protease family and is a plant endoprotease. Its amino acid sequence, detailed X-ray structure, and extensive kinetic data, have generated new concepts and data on structure-function relationship of this enzyme. Although derived from a plant source, papain displays some structural and functional similarities to other cysteine protease of both plant and animal origin. Papain consists of a single polypeptide chain with 212 amino acid residues. The papain molecule is folded to form two interacting domains resulting in a cleft at the surface of the enzyme. The L-domain is alpha helical rich and it contains residues 10-111 and 208-212. It has got three alpha-helices in the secondary structural features. The R-domain is mainly made up of anti parallel β-sheet structure, but it also contains 2 helices, which are located at the opposite ends of the central β-structure. The active site residues Cys-25 and His 159 are located at the interface of this cleft on opposite domains of the molecule. Here Cys-25 is part of the L1 alpha helix at the surface of the left domain, while His-159 is in a β-sheet of the right domain. Apart from Cys and His, Asn 175 also plays a very important role in the catalytic mechanism and also in thermal stability of papain. The crystallographic structure of papain shows an extended network of interface interactions, making large domain movement unlikely. Small widening of the cleft seems to be required for substrate binding.

Metal ions serve a variety of functions in proteins, the most important being to enhance the activity including biological activity. But some of the metal ions are also known to inhibit certain enzymes. Since many proteins undergo structural transition in presence of divalent metal ions, studies on the unfolding of papain in the absence and presence of metal ions assumes significance. The effect of these metal ions on the conformation of proteins has been studied in some detail. Metal ions like zinc, cadmium, mercury, lead, copper and iron are inhibitors of papain. Bivalent ions like Fe^{2+}, Pb^{2+}, Cu^{2+} and Hg^{2+} have no preference for combining with papain as against cysteine. On the other hand Cd^{2+} has a 100 fold stronger affinity for papain than cysteine and for Zn^{2+} ratio is more than 1000 fold. Thus apparently the effects of divalent metal ions differ widely. Most of the active metal ions form octahedral complexes, whereas Zn^{2+} and Cd^{2+} can adopt tetrahedral stereochemistry. The mechanism of
inactivation by divalent metal ions such as zinc and cadmium is not clearly understood. The present study aims to understand the structure-function-activity relationship of papain in presence of metal ions.

Materials and Methods

Materials

Papain (two-times crystallized) lot # P 4762, was from Sigma Chemical Co., USA. This preparation was found to be homogeneous by electrophoresis in SDS polyacrylamide gel electrophoresis. Benzoyl-arg-p-nitroanilide, dimethyl sulphoxide and cysteine hydrochloride were also procured from Sigma Chemical Co., USA. Zinc chloride, cadmium chloride and sodium acetate were procured from Loba Chemical Co., USA. Arg-p-nitroanilide, dimethyl sulphoxide and cysteine hydrochloride were also procured from Sigma Chemical Co., USA. Zinc chloride, cadmium chloride and sodium acetate were procured from Loba Chemical Ltd, Bombay, India and all the other chemicals used were of analytical grade. Dialysis membrane of 23 mm flat width and 6000-8000 molecular weight cut-off was obtained from Spectrum Inc., Houston, Texas, USA.

Enzyme activity measurements

Activity of papain was quantified by measuring its ability to cleave an amide bond in a small molecular weight synthetic substrate, benzoyl-arg-P-nitroanilide. The assay is based on the method adopted by Arnon.\(^1\)

Thermal denaturation studies

The effect of specific metal ion binding on thermal denaturation profile of papain were studied using a Gilford Response-II UV-Vis spectrophotometer which had a six-position thermostated cuvette manifold. Here the thermal unfolding or precipitation of papain was monitored in acetate buffer pH 4.0, by recording absorbance at 287 nm as a function of temperature in the range of 30-95°C at 1°C increment. pH 4.0 was chosen because of the maximal difference between the denatured and the native protein spectra observed at this pH, and also to avoid the precipitation of metal ion-protein complex at higher temperature. Spectral data were stored and analyzed using special software supplied with the instrument. Using the apparent \(T_m\) values the thermodynamic parameters were calculated from the van't Hoff plot. From the thermal denaturation profile, fraction of papain unfolded was calculated using standard equation\(^1\).

Circular dichroic spectrum

Far-ultraviolet circular dichroic studies were performed from 200 to 260 nm using a Jasco J-715 spectropolarimeter calibrated with d-10-camphorsulfonic acid. To measure the CD spectrum of papain, samples were scanned from 200-260 nm, with 0.2 nm increment, 50 nm/min and averaged for atleast two samples. The blank spectra without enzyme was subtracted from the sample spectra. Protein concentration of 7x10^4 M was dialyzed exhaustively for 24 hr versus different concentration of metal ions at 4°C, centrifuged at 6000xg for 30 min and the supernatant was used for circular dichroic spectral measurements. The mean residue ellipticities were calculated using a mean residue weight of 110.80 for papain using the amino acid sequence data\(^1\).

Differential scanning calorimetric measurements

All the Differential scanning calorimetric (DSC) experiments were performed on a MicroCal MC-2 Ultrasensitive Differential scanning calorimeter. Protein solutions were exhaustively dialysed against the indicated metal ions for 24 hr at 4°C. The final dialysate (dialysed for atleast 12 hr) was used for the reference cell. For all the experiments protein concentration of 2x10^4 M was used. Concentration of protein samples was determined spectrophotometrically by using a value of 25±0.1 as the extinction coefficient at 278 nm for papain. All the protein solution and buffer were degassed with gentle stirring under vacuum before being loaded into the calorimeter. Experiments were performed over a range of 30-100°C at a scan rate of 1.5°C per min. Normalized heat capacity \(C_p\) data were corrected for buffer baseline. Raw data from the DSC run were analyzed using Origin\(^{TM}\) (version 2.9) scientific plotting software. The DSC heat capacity data were curve fit by using non-two state fit with fixed \(T_m\) for papain.

Results

In order to investigate the effect of metal ions on the enzyme activity, the activity of papain in the presence of various concentrations of Zn\(^{2+}\) and Cd\(^{2+}\) determined by measuring its ability to cleave an amide bond with a small molecular weight synthetic substrate. Both zinc and cadmium were potent inhibitors of the enzyme activity. In the case of ZnCl\(_2\), the enzyme loses nearly 70% of its activity at 1x10^-4 M concentration, and the enzyme is completely inactivated above 1x10^-3 M concentration of ZnCl\(_2\). In the case of cadmium 60% of the enzyme activity is
lost at 1×10⁻⁴ M concentration and is completely inactivated above 1×10⁻³ M concentration of CdCl₂ (Fig. 1). In order to see the effect of chloride ions, the effect of MgCl₂ and KCl on the enzyme activity was also checked, wherein there was no inhibition of enzyme activity. Thus the inhibitory effect was mainly due to the cation, namely zinc and cadmium.

The reversibility of the metal ion inhibition was checked by treating the enzyme with EDTA with proper controls. At lower concentration of metal ions (less than 1×10⁻³ M) the inhibition is completely reversible while at higher concentration, the inhibition is not completely reversible. Kinetics of the inhibition of papain at lower concentration of metal ions (Fig. 2) indicates competitive inhibition. The kᵢ for zinc and cadmium was 5×10⁻⁵ M and 8×10⁻⁵ M respectively.

The destabilization of papain molecule as a result of metal ion interaction was further probed through measurements of the apparent thermal transition temperature of the enzyme. From the denaturation profile a graph of fraction unfolded vs temperature was obtained using the standard equation.

\[ F_u = Y - Y_f - Y_u \]  \hspace{1cm} \ldots (1)

where \( F_u \) is the fraction unfolded, \( Y_f \), \( Y_u \) and \( Y \) are absorbance of completely folded (native), completely

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**Fig. 1**—Enzyme activity of papain in presence of varying concentrations of metal ions \((\text{A): ZnCl}_2\) where \((\bullet)\) is control in \(0.02 \text{ M acetate buffer pH 5.6; (O), 1×10^{-3} M; (▲), 5×10^{-3} M; (X), 1×10^{-4} M and (■), 2×10^{-4} M.} \)

**(B): CdCl}_2\) where \((\bullet)\) is control in \(0.02 \text{ M acetate buffer pH 5.6; (O), 1×10^{-3} M; (▲), 5×10^{-3} M; (X), 1×10^{-4} M and (■), 2×10^{-4} M.} \)

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**Fig. 2**—Double reciprocal plot of papain in presence of varying concentrations of metal ions \((\text{A): ZnCl}_2\) where \((\bullet)\) is control in \(0.02 \text{ M acetate buffer pH 5.6; (O), 1×10^{-3} M; (▲), 5×10^{-3} M; (X), 1×10^{-4} M and (■), 2×10^{-4} M.} \)

**(B): CdCl}_2\) where \((\bullet)\) is control in \(0.02 \text{ M acetate buffer pH 5.6; (O), 1×10^{-3} M; (▲), 5×10^{-3} M; (X), 1×10^{-4} M and (■), 2×10^{-4} M.} \)
denatured and experimental values respectively. The apparent thermal transition temperature values of native papain and papain in presence of metal ions was obtained by van't Hoff plot. The apparent thermal transition temperature of native papain was $83 \pm 1^\circ C$, which decreased as a function of $ZnCl_2$ concentration (Fig. 3). The apparent thermal transition temperature of papain decreased from a control value of $83 \pm 1^\circ C$ to $78 \pm 1^\circ C$ in presence of $1 \times 10^{-2} M$ $ZnCl_2$ and was 82, 80 and $78 \pm 1^\circ C$ in presence of $1 \times 10^{-3}$, $5 \times 10^{-3}$ and $1 \times 10^{-2} M$ $ZnCl_2$ respectively. Similar effect was also observed in presence of cadmium. Fig. 4 shows the fraction unfolded vs temperature of papain in presence of different concentrations of cadmium. The apparent thermal transition temperature of papain decreases to a value of $78 \pm 1^\circ C$ in presence of $1 \times 10^{-2} M$ $CdCl_2$. In presence of both the metal ions the $\Delta H_{th}$ of papain decreases as a function of metal ion concentration. The plot of $\Delta H_{th}$ as a function of metal ion concentration is shown in Fig. 5, which clearly shows the reduced thermal stability of papain in presence of metal ions at higher concentration, while there is no significant change at lower concentration of metal ions.

It is well known that calorimetric measurements provide independent determination of transition enthalpies. Thermodynamic analysis of the calorimetric data of thermal denaturation of papain carried out by Tiktopulo and Privalov13, has shown that denaturation of papain is not a two state transition, and the two domains of papain molecule are unfolded independently. In our data, when the thermogram was fitted with non-two state transition with fixed $T_{m}$ two transitions were obtained. The

![Graph showing temperature vs fraction unfolded for papain in presence of different metal ions concentrations.](image)

Fig. 3—Thermal denaturation of papain in (O), $0.02 M$ acetate buffer at pH 4.0; (△), $5 \times 10^{-3} M$ $ZnCl_2$ and (●), $1 \times 10^{-2} M$ $ZnCl_2$ by recording the absorbance at 287 nm as a function of temperature in the range of 30-95°C. [Inset: Apparent thermal transition temperature of papain as a function of $ZnCl_2$ concentrations.]
thermal unfolding of papain under these conditions is irreversible in that cooling and rescanning of the sample in the calorimetric cell reveals a complete loss of the transition. Although the thermal denaturation appears to be irreversible, protein aggregate or precipitation does not appear. Corrected thermograms of papain in buffer alone is shown in Fig. 6. In the absence of metal ions, the two transitions are tightly linked and overlap extensively, while upon binding of metal ions, these two transitions are well separated. The calorimetric scan of papain in buffer alone is characterized by two transitions by a peak A at 83±0.2°C and another peak B at 90±0.2°C, both of which, shift to lower temperature and is less pronounced with increased ZnCl₂ concentrations. Thermodynamic parameters characterizing the thermal transition of papain as a function of ZnCl₂ concentration is summarized in Table 1. Comparison of the differential scanning calorimetric scans of papain in buffer alone and in presence of different concentrations of ZnCl₂ (Fig. 7) shows significant change in the shape of transition. It can be seen from Table 1, that the thermal transition temperature of both the lower transition and higher transition decreased as ZnCl₂ concentration is increased. The thermal transition temperature of papain in presence of 1×10⁻² M ZnCl₂ was 80±0.2 and 86±0.2°C for transition A and transition B respectively. The differential scanning calorimetric studies of papain in presence of cadmium chloride shows that maximum destabilization effect was in presence of 1×10⁻² M CdCl₂ wherein the transition temperature for both the transitions decrease from a control value of 83±0.2°C and 90±0.2°C to a value of 80±0.2 and 88±0.2°C respectively (Fig. 8). All the thermodynamic parameters characterizing the thermal transitions of papain as a function of CdCl₂ concentration is summarized in Table 2.

The change in the secondary structure of papain as a function of metal ion concentration can be followed by measuring the far-UV circular dichroic spectra of
Fig. 5—$\Delta H_m$ as a function of metal ion concentration in 0.02 $M$ acetate buffer, $pH$ 4.0 (▲), ZnCl$_2$ and (●), CdCl$_2$.

[Inset: van't Hoff plot from where $\Delta H_m$ is calculated and plotted; (○), in buffer alone (▲), 1 $\times$ 10$^{-2}$ $M$ ZnCl$_2$ and (●), 1 $\times$ 10$^{-5}$ $M$ CdCl$_2$]

Fig. 6—Differential scanning calorimetric profile of papain in 0.02 $M$ acetate buffer, $pH$ 5.6.

[The curve fitting is done to indicate the bimodal contribution of the pattern.]
Table 1—Thermodynamic parameters characterizing the thermal transition of papain as a function of ZnCl₂ concentration. [Here the T_{ml} relates to the lower temperature peak in the bimodal differential pattern and T_{m2} relates to the higher temperature peak in the same pattern]

<table>
<thead>
<tr>
<th>ZnCl₂ (M)</th>
<th>T_{ml} Temp. (°C)</th>
<th>ΔH_{ml} (KJ.mol⁻¹)</th>
<th>ΔH_{m2} (KJ.mol⁻¹)</th>
<th>T_{m2} Temp. (°C)</th>
<th>ΔH_{m2} (KJ.mol⁻¹)</th>
<th>ΔH_{m2} (KJ.mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>83±0.2</td>
<td>550±20</td>
<td>190±8</td>
<td>90±0.2</td>
<td>502±17</td>
<td>400±16</td>
</tr>
<tr>
<td>1x10⁻³</td>
<td>83±0.2</td>
<td>280±12</td>
<td>210±8</td>
<td>90±0.2</td>
<td>506±17</td>
<td>370±16</td>
</tr>
<tr>
<td>5x10⁻³</td>
<td>82±0.2</td>
<td>440±16</td>
<td>170±8</td>
<td>88±0.2</td>
<td>470±16</td>
<td>360±16</td>
</tr>
<tr>
<td>7.5x10⁻³</td>
<td>81±0.2</td>
<td>380±16</td>
<td>235±8</td>
<td>87±0.2</td>
<td>420±16</td>
<td>380±16</td>
</tr>
<tr>
<td>1x10⁻²</td>
<td>80±0.2</td>
<td>350±16</td>
<td>230±8</td>
<td>85±0.2</td>
<td>420±16</td>
<td>370±16</td>
</tr>
</tbody>
</table>

Fig. 7—Differential scanning calorimetric profile of papain in presence of different concentrations of ZnCl₂ in 0.02 M acetate buffer pH 5.6 [(a), Pattern in buffer alone is shown for comparison; (b), 7.5x10⁻³ M and (c), 1x10⁻² M|

The control papain and papain-metal ion complexes.

The circular dichroic spectra of papain in the range of 200-260 nm is presented in Fig. 9, along with those in presence of metal ions. The spectrum of native papain had a trough, at 208 nm. From Fig. 9 we can see that there is also a small change in the CD spectrum of papain upon binding of these metal ions. The α-helix and β-structure contents of native papain was 20 and 13% respectively.

Discussion

The effect of divalent metal ions namely zinc and cadmium on the structure activity-stability relationship of papain has been investigated by activity measurements, thermal denaturation studies, differential scanning calorimetric and circular dichroic studies. The results from the activity measurements in presence of these metal ions indicate that both zinc and cadmium are extremely potent inhibitors of papain. The enzyme loses its activity by nearly 70% in case of ZnCl₂ and 60% in case of CdCl₂ at 1x10⁻⁴ M concentration. Since there is no ligand field stabilization effect in Zn²⁺ and Cd²⁺ ions because of their completed d-shells, their stereochemistry is determined solely by considerations of size; electrostatic forces and covalent bonding forces. The protein apparently selects metal ions on the basis of both charge and size, the higher the charge, the
better the binding. Zn$^{2+}$ and Cd$^{2+}$ are considerably more electropositive than their neighbours in the transition group in the periodic table. Chemical nature [i.e., hardness or softness] according to Glusker$^4$ is important for the recognition of a metal ion by the enzyme. Cd$^{2+}$ is a soft metal ion with the electrons bound loosely, having ionic radii of 0.91Å and thus more polarizable. Whereas Zn$^{2+}$ has a smaller ionic radii of 0.71Å and can bind comparatively tightly. Zinc is a metal of such hardness that it can easily accommodate nitrogen, sulphur and oxygen atoms in its coordination polyhedra.

The inhibition of papain activity at lower concentration is a reversible one and is also competitive. Here the metal ions compete with the substrate and the affinity of both zinc and cadmium (K$_a$ value of 5×10$^5$ M and 8×10$^5$ M respectively) is far greater than the affinity of substrate (K$_a$ value of 4×10$^5$ M) towards enzyme. The effect of metal ions in the enzyme activity is accompanied by only a small change in the secondary structure of protein only at higher concentrations of metal ions whereas no change in the conformation of the papain molecule is observed at lower concentrations of the metal ions. The alpha helical content of the native papain molecule is 20%. Of the 2 domains, the left domain of papain molecule has large alpha helical content, while the right domain is mainly of $\beta$-structure$^2$. In presence of metal ions there is no significant change in the alpha helical content, while there was a small change.

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Table 2—Thermodynamic parameters characterizing the thermal transition of papain as a function of CdCl$_2$ concentration. [Here the $T_{m1}$ relates to the lower temperature peak in the bimodal differential pattern and $T_{m2}$ relates to the higher temperature peak in the same pattern]

<table>
<thead>
<tr>
<th>CdCl$_2$ (M)</th>
<th>Temp. ($^\circ$C)</th>
<th>$\Delta H_{m1}$ (KJ mol$^{-1}$)</th>
<th>$\Delta H_{m2}$ (KJ mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>83±0.2</td>
<td>550±20</td>
<td>190±8</td>
</tr>
<tr>
<td>1×10$^{-3}$</td>
<td>82±0.2</td>
<td>190±28</td>
<td>220±28</td>
</tr>
<tr>
<td>5×10$^{-3}$</td>
<td>82±0.2</td>
<td>295±28</td>
<td>215±28</td>
</tr>
<tr>
<td>7.5×10$^{-3}$</td>
<td>81±0.2</td>
<td>280±28</td>
<td>205±28</td>
</tr>
<tr>
<td>1×10$^{-2}$</td>
<td>80±0.2</td>
<td>140±28</td>
<td>276±28</td>
</tr>
</tbody>
</table>

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Fig. 8—Differential scanning calorimetric profile of papain in presence of different concentrations of CdCl$_2$ in 0.02 M acetate buffer pH 5.6 [(a), Pattern in buffer alone is shown for comparison; (b), 7.5×10$^{-3}$ M and (c), 1×10$^{-2}$ M].
explained that papain denaturation is a quasi-independent transition. Further Arana and Garcia\textsuperscript{15} have detected and characterized this stable intermediate state formed during the thermal unfolding of papain by circular dichroic spectra. The X-ray structure of papain, shows that it exists as two domains. Although the native papain shows only a single DSC transition at pH 5.6, this transition uncouples into two overlapping transition as the metal ion concentration is increased. The two individual overlapping transitions were obtained by deconvolution of the experimental curve, as followed by Brands et al.\textsuperscript{16} for phosphoglycerate kinase. Destabilization effect of the protein molecule upon binding of metal ions can be seen by decreased thermal transition temperature, wherein native papain, when fitted with non-two state model, has two transition, first transition at 83±0.2°C and a second transition at 90±0.2°C. The thermal transition temperature of both the transition decreases as a function of ZnCl\textsubscript{2} concentration, maximum being at 1×10\textsuperscript{-2} M ZnCl\textsubscript{2}, wherein the apparent T\textsubscript{m} was 80±0.2 and 85±0.2°C for transition A and B respectively. While in presence of 1×10\textsuperscript{-2} M CdCl\textsubscript{2} the transition temperature of both the systems decreased to 80±0.2 and 88±0.2°C respectively. It is important to note that efficient enzymatic activity in papain requires not only correct active site geometry but is also influenced by domain packing properties in some region remote from the active site\textsuperscript{3}.

It is concluded that the inhibitory effect of divalent metal ions namely Zinc and Cadmium at lower concentrations is completely reversible and the inhibition is competitive in nature. At higher concentrations the loss of activity is not completely reversible and there is no significant change in secondary structure. The calorimetric results shows that the two domains in the native papain interact strongly and the two transitions are tightly linked and overlap extensively. Upon binding of metal ions this transition uncouples into two transitions indicating that the interaction between the domains is decreased. Thus the interaction of the two metal ions at 1×10\textsuperscript{-2} M completely inhibits the enzyme activity. As a result of binding of the metal ions, the thermal stability of the enzyme is decreased as reflected by apparent T\textsubscript{m} measurements and differential scanning calorimetric data. The differential scanning calorimetric results indicate the fine difference between cadmium and zinc and their effect on the heat capacity of protein as

in the β-structure content upon binding of these metal ions.

These small changes in the secondary structure of papain may also effect the thermal stability of papain molecule. The thermal denaturation results show that the binding of metal ions destabilizes the papain molecule. This was indicated by a shift in the apparent thermal transition temperature of papain from a control value of 83±1°C to 78±1°C. The calculated values of ΔH\textsubscript{m} and apparent T\textsubscript{m} for the thermally induced conformational transition of papain in buffer alone and in different concentration of metal ions clearly demonstrate the destabilization effect of these metal ions on papain.

Effect of these metal ions on the conformation of protein was further studied by differential scanning calorimetric studies, where the thermal transition temperature decreases as a function of ZnCl\textsubscript{2} concentration. Papain denaturation is not a two state process, but in contrast the two domains unfold independently. Tiktopulo and Privatov\textsuperscript{13} have

Fig. 9—Far ultraviolet circular dichroic spectra of papain in the presence of selected concentrations of metal ions in 0.02 M acetate buffer, pH 5.6 [O], in buffer alone ; [●, 1×10\textsuperscript{-2} M ZnCl\textsubscript{2} and (▲), 1×10\textsuperscript{-2} M CdCl\textsubscript{2}]

in the β-structure content upon binding of these metal ions.
judged in the temperature range of 30 to 100°C. If one closely looks at the effect of cadmium as shown in Fig. 8 it is a broad peak with trailing edges spreading between 65 to 89°C with peak at 89°C and the leading edge sharply comes down but if one sees the curve fitting of data (Fig. 6) one can only fit this data into two resolved systems one with a transition of 83°C and the other one at 89°C. On the other hand, if one looks at the effect of ZnCl₂ on the excess heat capacity of protein one sees a clear bimodality with the percentage of higher apparent Tₘ versus low apparent Tₘ peaks namely leading peak and trailing peak increasing concomitantly in per cent fraction of trailing peak as ZnCl₂ concentration is increased from $7.5 \times 10^{-1}$ to $1 \times 10^{-6}$ M This signifies the role of Zn²⁺ in probably reaching the two domains of protein where stabilization for temperature is grossly altered as a result of binding of Zn²⁺. Compared to CdCl₂ pattern one sees here, the clear bimodality as compared to the skewness in cadmium which indicates the effect of Zn²⁺ being much more high in the destabilization of native structure of protein at equivalent concentrations. As a matter of fact, at higher concentrations of divalent metal ions the enzyme is almost inactivated, indicating structural role of areas of domains with reference to binding site of metal ion related to activity.

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References