# KINETIC STUDIES ON THE THIOL PROTEASE FROM *ACTINIDIA CHINENSIS*

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## 1. Introduction

The plant thiol proteases, papain, ficin and bromelain show a high degree of homology in the amino acid sequence near the active residues [1,2] and kinetic studies of papain and ficin indicate that the specificity and mechanism of action are similar [2,3]. A further thiol protease, actinidin [4], from the berries of *Actinidia Chinensis* (Chinese gooseberries) was first characterised by Arcus [4] and later studied by McDowall [5]. We have purified the enzyme from unripe berries to constant specific activity as a sulphenyl thiosulphate derivative and carried out kinetic studies to compare the behaviour of this enzyme with that of the other thiol proteases. The specificity of the enzyme towards small substrates and inhibitors, and its activity towards *N*-α-CBZ-lysine *p*-nitrophenyl ester are similar to those of ficin and papain.

## 2. Materials

All substrates and inhibitors were obtained from Cyclo, Sigma or BDH, and used without further purification. Dithioerythritol was a Sigma product and sodium tetrathionate was obtained from Fluka.

The enzyme was extracted from unripe fruit into 10 mM $Na_2S_4O_6$, 1 mM EDTA solution (1 l per kg) and centrifuged [6]. The enzyme was precipitated by 50% saturation with ammonium sulphate and the precipitate re-suspended in 1 mM $Na_2S_4O_6$, 1 mM EDTA solution (ca. 100 ml). Following dialysis against the same solution, aliquots of the enzyme solution (up to 50 ml) were loaded onto a DEAE-cellulose column 20 × 2.4 cm at pH 6.8. The column was developed at room temperature with 0.5 M phosphate buffer, pH 6.8, the enzyme eluting at 800–1000 ml. Enzyme from this preparation runs as a single fast moving band on disc gel electrophoresis at pH 8.9, gives a single band on ultracentrifugation and has a specific activity of 50 under assay conditions. Enzyme with higher specific activity was obtained by rechromatography on the same system, but using a column 1.5 m × 1.5 cm pumped at 30 p.s.i. Two proteins were resolved, the slower travelling one being active (fig. 1), with a specific activity of 67 under assay conditions.

## 3. Methods

Hydrolyses of *p*-nitrophenyl esters were followed at 348 nm on a Perkin Elmer PE 402 double beam spectrophotometer using a Smiths Servoscribe recorder. Spontaneous hydrolysis of the substrate was followed for about 20 sec and then enzyme in 0.01 M dithioerythritol added (dithioerythritol at the resulting concentrations, ca 0.1 mM, produced negligible catalysis of hydrolysis). Rates were calculated using $\Delta\epsilon = 5400$.

Assays were carried out using *N*-α-CBZ-lysine *p*-nitrophenyl ester (0.1 mM in 0.1 M phosphate buffer, pH 6.0). Since $[S]_0 >> K_m$, the initial rate of hydrolysis is equal to $V_{max}$ ($=k_{cat}[E]_0$).

The kinetic parameters $k_{cat}$ and $k_m$ for Z-lys-pNP* were determined from Eadie plots [7] of data from complete runs using the chords method [8]. The

*Abbreviation:*
Z-lys-pNP: *N*-α-CBZ-lysine *p*-nitrophenyl ester.



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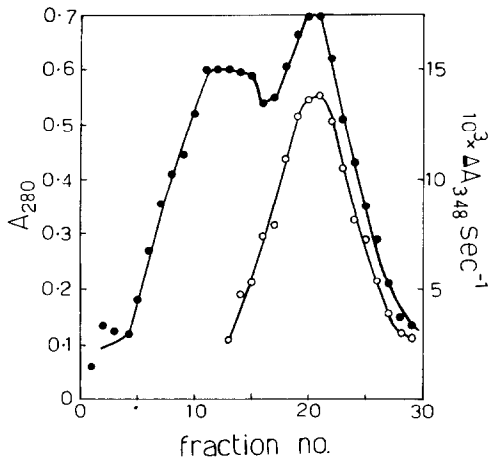


Fig. 1. Profile of elution of actinidin from 1.5 m × 1.5 cm DEAE-cellulose column. Absorbance at 280 nm is indicated by filled circles and enzyme activity is shown by open circles. Fractions were 20 ml and assays used 25 μl aliquots in a 3 ml reaction volume.

second order rate constants $k_{cat}/K_m$ for all substrates were obtained from first order plots with $[S]_0 < K_m$. Enzyme concentrations were calculated from the absorbance at 280 nm on the basis of $E_{280}^{0.1\%} = 2.12$ l g$^{-1}$ cm$^{-1}$ [5], and the molecular weight given in this communication.

The molecular weight was determined using the high speed equilibrium method [9] on solutions of three different concentrations of enzyme in 0.1 M NaCl, 0.01 M phosphate buffer, pH 6.8, in a Beckman model E ultra-centrifuge using interference optics. The partial specific volume was calculated [10] from the amino acid composition [11,12].

## 4. Results and discussion

### 4.1. *Molecular weight*

Linear plots were obtained from interference photographs. A molecular weight of 26,000 ± 500 was obtained using a calculated partial specific volume of 0.720. This value of the molecular weight does not agree with that obtained by Sephadex gel filtration [5] but the enzyme has subsequently been found to show anomalous behaviour on gel filtration [11].

Since the two proteins resolved on rechromatography have the same molecular weight and charge, it is thought that the inactive protein may be enzyme in which the active thiol has been oxidised to a sulphonate.

Table 1

Kinetic constants for the hydrolysis of Z-lys-pNP catalyzed by actinidin, papain and ficin.

| | Actinidin[a] | Papain[b] | Ficin[c] |
|---|---|---|---|
| $k_{cat}$, sec$^{-1}$ | 29 ± 2 | 44.5 ± 1.8 | 32.4 ± 0.5 |
| $K_m$, μM | 22 ± 2 | 1.71 ± 0.25 | 2.7 ± 0.2 |

a) In pH 6.0 phosphate buffer, using rechromatographed enzyme.
b) At pH 6.2 [13].
c) At pH 6.6 [14].

### 4.2. *Activity towards N-α-CBZ-lysine p-nitrophenyl ester*

The values of the kinetic constants, $k_{cat}$ and $K_m$, for this substrate at pH 6.0 are given in table 1, together with those for papain [13] and ficin [14]. The latter were obtained at pH values different from those used here but for both enzymes a broad plateau covers this range of pH [13,14]. A broad pH optimum from pH 5.0 to pH 6.5 is also observed for the $k_{cat}$, $K_m$ and $k_{cat}/K_m$ values for actinidin. The $k_{cat}$ value for actinidin is similar to those for papain and ficin but the $K_m$ value is considerably higher.

### 4.3. *Specificity*

The rates of hydrolysis of *p*-nitrophenyl esters of various CBZ-amino acids were determined at pH 6.0 in 20% $CH_3CN$–water and values of $k_{cat}/K_m$ are given in table 2. The results indicate that a basic side chain on the substrate is required for maximum catalytic activity, as found for papain [2].

Inhibition of the hydrolysis of Z-lys-pNP at pH 6.0 by a series of arginine derivatives was studied. $K_i$ was obtained from values of the first order rate constant in the absence ($k_0$) and presence ($k_i$) of inhibitor at the same [E] by plotting $k_0/k_i$ against [I], and the values are given in table 3.

A comparison of the values of *N*-acetyl-L-arginine and *N*-benzoyl-L-arginine indicates that the aromatic acylamido group contributes significantly to binding.

Table 2

Second order rate constants for the actinidin-catalyzed hydrolysis of *N*-CBZ-amino acid *p*-nitrophenyl esters*.

| Amino acid | $10^{-4}$ Rate constant ($M^{-1}$ $sec^{-1}$) |
|---|---|
| Lysine | 11.9† |
| Tryptophan | 1.4 |
| Alanine | 1.3 |
| Tyrosine | 0.6 |
| Leucine | 0.6 |
| Glycine | 0.2 |

* In pH 6.0 phosphate buffer, 20% $CH_3CN$–water, at 17°.

† The decrease in the second order rate constant for Z-lys-pNP, relative to the value implicit in table 1, represents mainly an effect of the organic solvent on $K_m$.

Studies of the binding of synthetic peptide substrates and inhibitors to papain have shown the presence of an acylamido binding site with high affinity for aromatic groups [15]. A measure of the specificity of this site can be obtained from the observation that the $K_m$ for the papain-catalysed hydrolysis of the nitrophenyl ester of acetyl-glycine is 100 times that for the corresponding benzoyl-glycine ester [16]. Actinidin therefore, appears to show a smaller preference for an aromatic acylamido function than papain.

The greater affinity of the enzyme for benzoyl-arginine relative to its ethyl ester may be due to the presence of a cationic group at the active site. The involvement of such a residue at the active site of papain has been suggested to explain the observation that halo acids are more reactive to the essential thiol group than their amides at neutral pH [17,18].

## 5. Conclusion

The specificity of actinidin is similar to that of papain. The actinidin-catalysed hydrolysis of Z-lys-pNP has a comparable $k_{cat}$ to that of papain and ficin, although the $K_m$ is much higher. We are now carrying out rapid kinetic studies of this hydrolysis in order to determine the detailed mechanism of the reaction.

Table 3

$K_i$ values for competitive inhibitors of the hydrolysis of Z-lys-pNP (50 μM) at pH 6.0.

| Inhibitor | $K_i$ (mM) |
|---|---|
| *N*-Acetyl-L-arginine | 110 |
| *N*-Benzoyl-L-arginine | 24 |
| *N*-Benzoyl-L-arginine ethyl ester | 40 |

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