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Inhibition of Papain with 2-Benzyl-3,4-epoxybutanoic Acid Esters. Mechanistic and Stereochemical Probe for Cysteine Protease Catalysis

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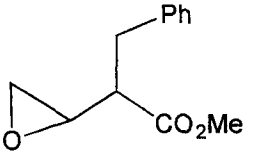
Abstract—Papain, a prototypic cysteine protease was inactivated by methyl and benzyl esters of (2*S*,3*S*)-2-benzyl-3,4-epoxybutanoic acid. On the other hand, methyl ester of (2*S*,3*R*)-2-benzyl-3,4-epoxybutanoic acid was shown to be a competitive inhibitor for the enzyme. It was inferred from the inactivation stereochemistry that in the papain catalytic reaction the nucleophilic attack of the side chain thioalkoxide of Cys-25 on the scissile peptide bond of substrates occurs in the '*re*' fashion. The papain inactivating potency of (2*S*,3*S*)-2-benzyl-3,4-epoxybutanoic acid methyl ester was enhanced over three-fold in a pH 8.0 solution compared with in the neutral solution. This together with our previous observation with α-chymotrypsin and the recent theoretical treatment of the enzymic reaction of papain, suggest that in the inactivation of papain by oxirane containing inhibitors, the oxirane does not need to be activated by prior protonation as thought previously. The oxirane ring is sufficiently labile that the unprotonated oxirane moiety can undergo an electrophilic reaction with the Cys-25 thiolate. © 1997 Elsevier Science Ltd.

Introduction

Cysteine proteases have been implicated in a number of pathological states such as progressive cartilage and bone degradation associated with arthritis,[1] and thus inhibitors of these enzymes are of medicinal interest. Of these cysteine proteases, papain[2] bears a special importance because as a much-studied and well-characterized prototypic cysteine protease, papain serves as a model target enzyme for the development of inhibitor design protocols. These design protocols can be useful for the design of inhibitors which are effective towards physiologically important cysteine proteases,[3] leading to the development of therapeutic agents.

Previously, we have reported on the design of 2-benzyl-3,4-epoxybutanoic acid (BEBA) methyl ester as an inactivator for α-chymotrypsin.[4] α-Chymotrypsin is a representative serine protease whose catalytic mechanism and active site structure are well established.[5] It was found from the study that while BEBA methyl ester having (2*S*,3*R*)-configuration inactivates the enzyme, its diastereomer having (2*S*,3*S*)-configuration is a competitive inhibitor.[4a] The other two diastereomers were shown to be substrates for the enzyme.[4a] Structures of the active sites of serine and cysteine proteases are similar, and both enzymes utilize a common catalytic pathway involving a nucleophilic attack at the scissile peptide bond of the bound substrates to form an acylenzyme intermediate, although the nature of the nucleophile is different: an alkoxide (Ser-195) for serine proteases and thioalkoxide (Cys-25) in the case of cysteine protesases. Accordingly, we were interested in evaluating the BEBA methyl esters that showed an inhibitory activity towards α-chymotrypsin as inhibitors of papain. We were also interested in knowing whether papain would exhibit the same stereochemistry in the inhibition as that shown by α-chymotrypsin. Although it has been known that oxirane derivatives such as E-64 and E-475 are potent inactivators for the enzyme,[6,7] stereochemistry associated with the inhibition has rarely been investigated. This report describes the stereochemistry exhibited by papain in the inactivation by BEBA methyl esters and its significance with respect to the stereochemistry of the nucleophilic attack of the cysteine thiolate on the amido carbonyl carbon atom of peptide substrate in its enzymic reaction. Furthermore, the present study showed that contrary to prevailing thought the oxirane ring of enzyme bound substrate analogues is sufficiently labile so that it can undergo electrophilic reaction with the thiolate of Cys-25 without being activated by protonation.



Key words: papain, inhibitory stereochemistry, active site directed inhibitor, competitive inhibitor, '*re*' face attack.
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Results

All four stereoisomers of BEBA esters were synthesized as described previously.[8] Two methyl esters having the *R*-configuaration at the 2-position were found to be substrates for papain, affording BEBA upon treatment with a catalytic amount of papain in the potassium phosphate buffer solution of pH 7.0. However, the other two diastereomers having the *S*-configuration at the 2-position resisted hydrolysis. Instead, they inhibited the catalytic activity of the enzyme. Detailed examination of the nature of the inhibition revealed that (2*S*,3*R*)-BEBA methyl ester is a competitive inhibitor having the K_i value of 710 μM as shown by the plots of Lineweaver–Burk[9] (not shown), and Dixon[10] (Fig. 1). However, in the case of (2*S*,3*S*)-BEBA methyl ester papain lost its enzymic activity in a time- and concentration-dependent manner (Fig. 2), suggesting that there occurs an irreversible inhibition of the catalytic activity of the enzyme. Kinetic parameters for irreversible inhibition were estimated from the double reciprocal plot (Fig. 3) of Kitz and Wilson[11] based on the kinetic equation (equation 1) derived for the scheme shown below (Scheme 1). The kinetic constants thus obtained are listed in Table 1.

$$E + I \overset{K_I}{\rightleftharpoons} E \cdot I \overset{k_{inact}}{\rightarrow} E - I \qquad \text{(Scheme 1)}$$

$$\frac{1}{k_{obs}} = \frac{1}{k_{inact}} + \frac{K_I}{k_{obs}} \cdot \frac{1}{[I]_0} \qquad (1)$$

The irreversibility of the papain inhibition by (2*S*,3*S*)-BEBA methyl ester was further supported by the dialysis experiment in which the inactivated enzyme failed to regain its catalytic activity. The rate of the irreversible inhibition was reduced by increasing the concentration of the substrate, indicating that the inhibition is active site directed. The benzyl ester of (2*S*,3*S*)-BEBA was shown to be a significantly more effective inactivator than the corresponding methyl ester for papain having the k_{inact}/K_I value of 49. The k_{inact}/K_I value of the (2*S*,3*S*)-BEBA methyl ester is 18 (Table 1).

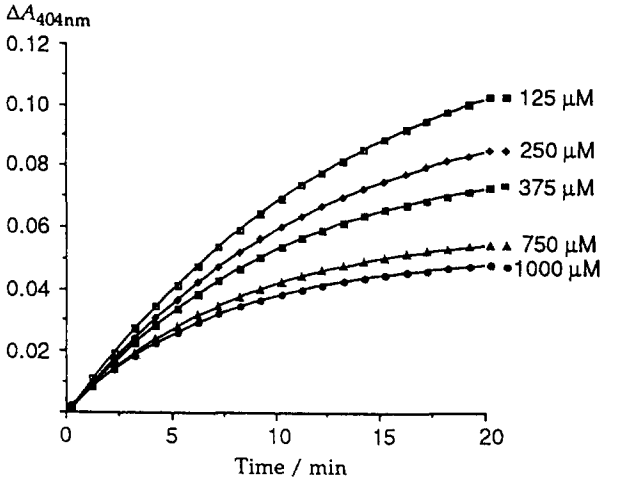


Figure 2. Inhibition of papain by (2S,3S)-BEBA methyl ester. Papain was added to a buffer solution (pH 7.0) of the inhibitor and substrate, and the hydrolysis of the substrate was followed by UV at 404 nm.

Discussion

Like α-chymotrypsin, the enzymic reaction of papain goes through an acylenzyme pathway involving a tetrahedral intermediate generated by the attack of the side chain alkylthiolate of Cys-25.[5] Seven subsites (S_4–S_3') have been identified at the active site of papain.[12] Unlike α-chymotrypsin in which the S_1 subsite plays a key role in substrate recognition, in the case of papain, however, the S_2 subsite is known to function as the primary substrate recognition site specific for an

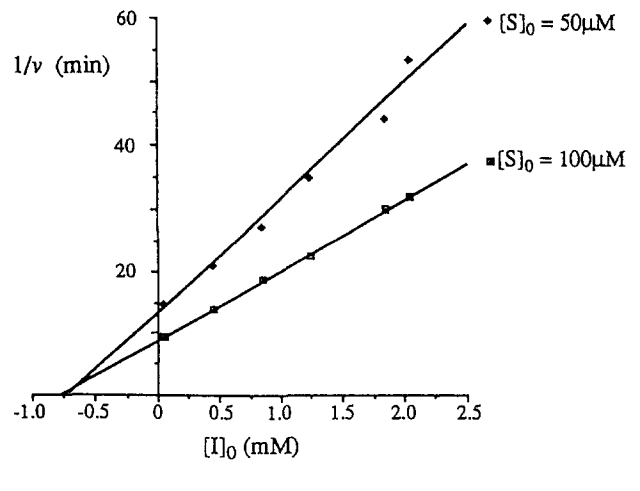


Figure 1. The Dixon plot for the inhibition of papain by (2*S*,3*R*)-BEBA methyl ester. $[S]_0$ and $[I]_0$ represent concentration of substrate (Cbz-Gly-*O*Np) and inhibitor [(2*S*,3*R*)-BEBA methyl ester], respectively.

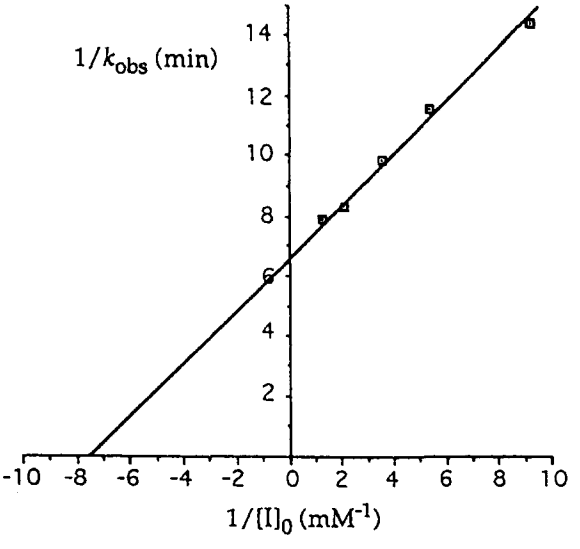


Figure 3. Double reciprocal plot of k_{obs} *vs* $[I]_0$ gives a straight line whose *y*-intercept corresponds to $1/k_{obs}$ and *x*-intercept shows $-1/K_I$.

Table 1. Kinetic parameters for inactivation of enzymes by BEBA esters

Inactivators	Enzyme	pH	K_I (μM)	k_{inact}(min^{-1})	k_{inact}/K_I (M^{-1} s^{-1})
(2S,3S)-BEBA methyl ester	papain	7.0	132	0.14	18
(2S,3S)-BEBA methyl ester	papain	8.0	96	0.32	55
(2S,3S)-BEBA benzyl ester	papain	7.0	116	0.34	49
(2S,3R)-BEBA methyl ester	α-chymotrypsin	7.0	3230*	5.24*	27

*Data from ref 4a.

amino acid residue having a hydrophobic side chain such as Phe and Leu.[13] The S_2 subsite is a large hydrophobic pocket consisting of Tyr-69, Tyr-67, Phe-207, Pro-68, Ala-160, Val-133, and Val-157.[13] The S_1 subsite of papain is much less well defined.

Previously, we proposed a three-dimensional schematic representation of the active site of α-chymotrypsin,[14] which was proven to be useful for designing various types of inhibitors for the enzyme.[4] A similar schematic representation is now proposed for the active site of papain. Figure 4 shows the three-dimensional model of the active site of papain, which is occupied by substrate. The substrate is depicted with the Newman's projection as it better reflects the stereochemical feature of the ligand and thus enables one to visualize the topology of the active site of the enzyme. In the representation the two subsites of S_1 and S_2 which play an impotant role in the recognition of substrate are shown as pockets. The thiolate nucleophile of Cys-25 is positioned in the catalytic site. We have observed that BEBA methyl esters bearing the *R*-configuration at the 2-position are substrates of the enzyme. These observations can be readily envisaged on the basis of the proposed active site model. In the Michaelis complex of the enzyme formed with these BEBA esters, the ester moiety rests at the catalytic site where it is subjected to a nucleophilic attack by the Cys-25 thiolate (Fig. 5). It is remarkable that while (2*S*,3*S*)-BEBA methyl ester inactivates the enzyme,[5] BEBA methyl ester having the (2*S*,3*R*)-configuration is a competitive inhibitor.[15] However, these observations may also be envisioned on the basis of the active site model. Methyl esters of (2*S*,3*S*)- and (2*S*,3*R*)-BEBA in which the stereochemistry at the 2-position belongs to the D-series are expected to bind the enzyme in a reverse fashion due to the configuration at the 2-position with the epoxide moiety being positioned at the catalytic site as shown in Figures 6 and 7, respectively. The chemically labile epoxide moiety of bound inhibitors, which is rested at the catalytic site can then undergo a chemical interaction with the Cys-25 thiolate, resulting in attachment of the inhibitor to the thiolate to cause an impairment of the enzymic activity permanently. The inactivation of papain only by (2*S*,3*S*)-BEBA methyl ester suggests that the S_N2 type nucleophilic attack of the Cys-25 thiolate occurs exclusively at the 3-position of the inhibitor. No stereospecificity would be expected if the nucleophilic attack takes place at the 4-position of the inhibitor. In the case of (2*S*,3*S*)-BEBA methyl ester the C_3–O bond of the epoxide moiety is apparently aligned in periplanar to the thiolate nucleophile when it binds the enzyme in view of the fact that as a S_N2 type ring opening reaction the attacking nucleophile is expected to approach the electrophilic carbon of the

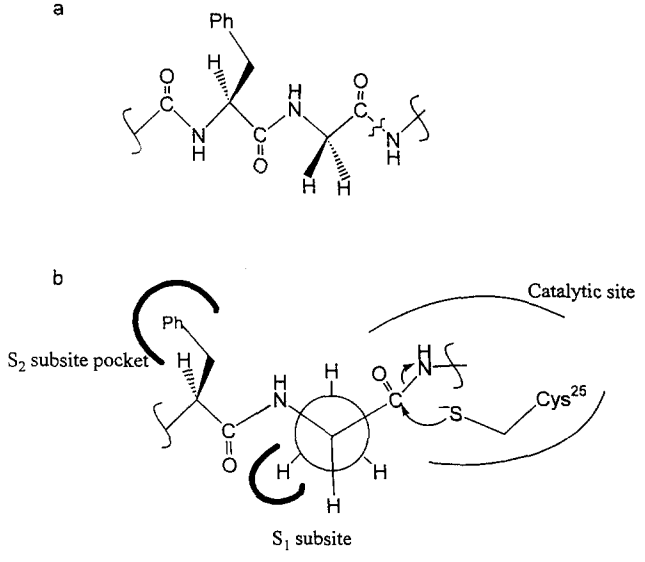


Figure 4. (a) Partial structure of substrate which shows cleavage site and sites (P_1 and P_2) of binding to the substrate recognition subsites of papain. (b) A three-dimensional representation of papain active site occupied by the substrate. The carbon atom at the front in the Newman projection is the α-carbon to the scissile peptide bond of the P_1 residue and the atom in the rear represents the P_1 methyl carbon atom of the substrate.

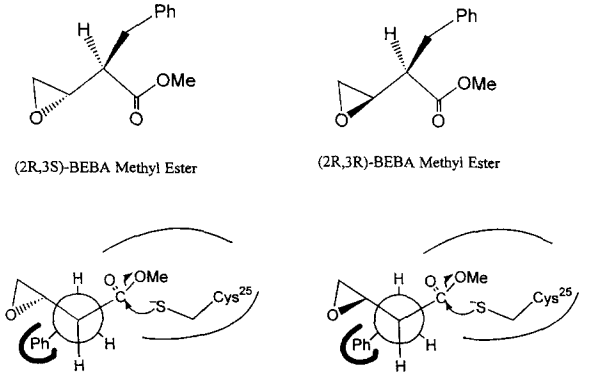
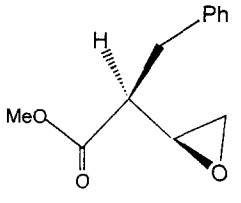


Figure 5. Methyl esters of (2*R*,3*S*)- and (2*R*,3*R*)-BEBA anchor in the active site of papain with the ester group being positioned at the catalytic site where the nucleophile attack of the Cys-25 thiolate on the ester carbonyl occurs.

epoxide ring from the rear in the direction periplanar to the cleavable C–O bond. The competitive inhibition of papain by (2*S*,3*R*)-BEBA methyl ester may be rationalized on the ground that the C_3–O bond is not favorably aligned with respect to the Cys-25 thiolate for the nucleophilic attack. Because of the nonperiplanar alignment of the C_3-O bond of (2*S*,3*R*)-BEBA methyl ester with respect to the thiolate, the nucleophile fails to cause the cleavage of the oxirane ring, and thus the inhibitor just binds the enzyme in competition with substrate to function as a competitive inhibitor (Fig. 7).

In papain, the S_2 subsite pocket is known to accommodate a bulky hydrophobic side chain of the P_2 amino acid residue of substrate.[13] Therefore, it was thought to be of interest to evaluate benzyl ester of (2*S*,3*S*)-BEBA as an inactivator for papain because upon binding to the enzyme the hydrophobic aromatic ring of the benzyl group of the inhibitor is expected to rest in the S_2 subsite, enhancing the binding to lead to an improvement of the inactivation (Fig. 8). Indeed, the k_{inact}/K_I value of (2*S*,3*S*)-BEBA benzyl esters was shown to be improved by about three-fold to that of the corresponding methyl ester (Table 1). It is worthy of noting that the improvement of the inactivating potency is largely due to the increase of the k_{inact} value (Table 1), which is in line with what was observed with substrates; the S_2 specificity for large hydrophobic residues results in an increase of k_{cat} value.[16] The 3-position of the inhibitor, which is the nucleophilic center for the Cys-25 thiolate attack in the interaction with papain, corresponds to the scissile peptide carbonyl carbon of the substrate. Accordingly, it can be inferred from the inactivation stereochemistry observed with (2*S*,3*S*)-BEBA methyl ester that in the papain catalyzed proteolytic reaction, the nucleophilic attack of the Cys-25 thiolate on the scissile carbonyl carbon occurs on the '*re*' face of the carbonyl bond. It is interesting to note that this stereochemistry of the thiolate attack is opposite of that exhibited by α-chymotrypsin, i.e. the '*si*' face attack.[4a]

It has been postulated on the basis of theoretical calculations that in the case of papain-catalysed proteolysis there occurs protonation on the scissile amide at either the oxygen or the nitrogen prior to or concurrent with the thiolate nucleophilic attack.[17] Thus, the selective inactivation of papain by epoxysuccinyl derivatives such as E-64 was explained on the basis of the protonation of the oxirane moiety of the enzyme bound inhibitor most likely by water, rendering the epoxide to be a strong electrophile.[6g,7,18] In this connection, it is worth remembering that α-chymotrypsin is inactivated by BEBA methyl ester.[4] The inactivation of α-chymotrypsin by BEBA methyl ester suggests that the oxirane ring of the inhibitor is sufficiently liable to undergo an electrophilic reaction with the alkoxide of Ser-195. As can be seen from Table 1, BEBA esters bind papain better but is slower in



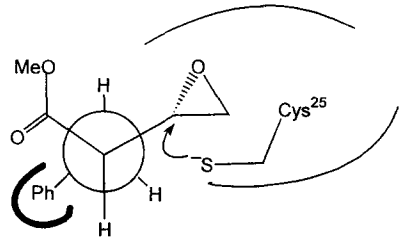
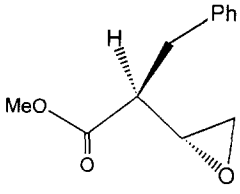


Figure 6. (2*S*,3*S*)-BEBA methyl ester anchors in the active site of papain with the epoxide ring being positioned at the catalytic site. The C_3–O bond is aligned periplanar to the thiolate nucleophile so that the S_N2 type ring cleavage reaction occurs.



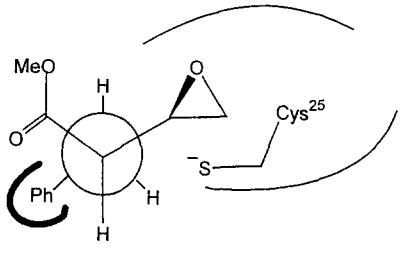


Figure 7. (2*S*,3*R*)-BEBA methyl ester anchors in the active site of papain with the epoxide ring being positioned at the catalytic site, but the C_3–O bond is not aligned favorably with the Cys-25 thiolate for the S_N2 type ring cleavage reaction.

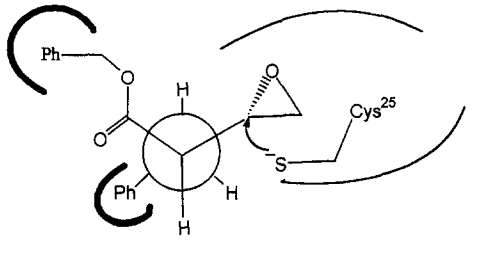


Figure 8. The aromatic ring of the benzyl ester moiety in (2*S*,3*S*)-BEBA benzyl ester anchors in the S_2 subsite pocket, leading to an improvement of the inactivating potency.

inactivating the enzyme compared with α-chymotrypsin. The slower inactivation (k_{inact} = 0.14 min^{-1}) of papain by the alkylthiolate compared with the inactivation (k_{inact} = 5.24 min^{-1}) of α-chymotrypsin by the alkoxide may be understood on the grounds that under the non-solvating environments of the enzyme active site, alkoxides are better nucleophiles than thiolate.[19] The very recent report of Harrison et al.[20] is noteworthy. These investigators have arrived at the conclusion, from theoretical calculations of the enzymic hydrolysis reaction using a hybrid QM/MM potential, that papain catalyzed proteolysis is a concerted process with no involvement of a tetrahedral intermediate, apparently not neccecitating the involvement of the prior protonation of the peptide bond. Thus, it appears that there is no need to invoke the proposition that epoxysuccinyl and related inhibitors require a prior protonation of the epoxide moiety for inactivation of papain. As a further support, the papain inactivating potency of (2*S*,3*S*)-BEBA methyl ester was improved by over threefold when tested in the solution of pH 8.0 (Table 1). The improvement of the inactivation potency (k_{inact}/K_I) in the alkaline medium compared with that in neutral pH (Table 1) bears considerable significance with respect to the hypothesis of the prior protonation of the epoxide moiety especially in view of the fact that the source of the proton for the epoxide protonation was thought to be the aqueous reaction medium.[17] These experimental results and the theoretical treatment of the enzyme reaction[20] support the view that in the inactivation of papain by oxirane containing inhibitors the unprotonated oxirane is sufficiently electrophilic to undergo reaction with the Cys-25 thiolate.

Conclusion

We have shown that methyl and benzyl esters of (2*S*,3*S*)-BEBA are active site directed inactivators and methyl ester of (2*S*,3*R*)-BEBA is a competitive inhibitor for papain. A three-dimensional representation of the active site of papain is proposed, with which the observed inhibitory stereospecificity may be envisaged. It can be inferred from the inactivation stereochemistry that in catalytic hydrolysis of papain the nucleophilic attack of the side chain thiolate of Cys-25 on the scissile peptide carbonyl carbon occurs on the '*re*' face of the amide carbonyl bond. On the basis of the present study together with our previous one performed with α-chymotrypsin, it can be concluded that there is no need to invoke the proposition that the prior protonation of the epoxide moiety is required for the inactivation in the case of the epoxide containing irreversible inhibitors for papain.

Experimental

All four stereoisomers of BEBA methyl esters and (2*S*,3*S*)-BBEBA benzyl ester were prepared as described in the literature.[8] Benzyloxycarbonyl-glycine *o*-nitrophenyl ester (Z-Gly-*O*Np), a chromophoric substrate for papain, was synthesized by the literature procedure.[22] Papain obtained from Sigma was activated before assay in 100 mM potassium phosphate buffer, containing 0.5 mM dithiothreitol and 2 mM EDTA at 25 °C for 1 h. The activated papain solution was stored at 4 °C and used as the enzyme stock solution. Enzyme concentration was determined from the absorbance at 280 nm (E = 58.5 mM^{-1} cm^{-1}).[6f] Buffers used for assay were the same as those used in the enzyme activation. The solutions of Z-Gly-*O*Np and BEBA esters were prepared in acetonitrile (HPLC grade). The final concentration of acetonitrile in assays was kept constant at 5% (v/v).

Enzymic hydrolysis of (2*R*, 3*S*) and (2*R*,3*R*)-BEBA methyl esters with papain

To a suspension of BEBA methyl ester (190 mg, 1 mmol) in 0.01 M phosphate buffer (pH 7.0, 10 mL) was added papain (~20 mg) and the resulting mixture was stirred at room temperature. The pH of 7.0 was maintained by titrating the reaction mixture with 0.01 N NaOH using a pH-stat. After 10 mL (1 mmol) was consumed, the solution was acidified to pH 2 and extracted with ether (3 × 20 mL). The combined ether layer was dried over $MgSO_4$, filtered and evaporated to give an oil (~160 mg, 90%), the NMR and MS spectra of which were identical with those of the authentic BEBA.[8]

Inhibition of papain with (2*S*,3*R*)-BEBA methyl ester

Into the 1.0 mL cuvette containing the buffer solution of Z-Gly-*O*Np and (2*S*,3*R*)-BEBA methyl ester, the enzyme stock solution (pH 7.0) was added to start the hydrolysis. The final concentrations of the inhibitor, substrate, and papain in the assay solution were 0.4–3.0 mM, 50–100 μM and 2–3 μM, respectively. The rate of hydrolysis of Z-Gly-*O*Np at 25 °C was monitored continuously for 60 s at 404 nm in a computer-assisted UV spectrophotometer equipped with a thermocontroller set.[7] The K_i values were calculated from the plots of $1/v$ vs $[I]_0$ according to the method of Dixon (Fig. 1).[10]

Inactivation of papain with (2*S*,3*S*)-BEBA methyl ester and (2*S*,3*S*)-BEBA benzyl ester

Into the 1.0 mL cuvette containing the buffer solution of Z-Gly-N*O*p and (2*S*,3*S*)-BEBA methyl ester, the papain stock solution was added to start the hydrolysis. The final concentrations in the assay mixture were 0.4–3.0 mM, 50–200 μM and 0.2–0.3 μM for inhibitor, substrate and papain, respectively. The change in absorbance at 404 nm was recorded over such a time interval that the control curve in the absence of the inhibitor was linear. The pseudo-first-order rate constants, k_{obs}, were obtained from the computer-assisted UV spectrophotometer (Fig. 2). A replot of $1/k_{obs}$ vs $1/[I]_0$ yielded the inactivation parameters k_{inact} and K_I

(Fig. 3).[11] Similarly were determined the inactivation parameters of (2*S*,3*S*)-BEBA benzyl ester.

Dialysis

The buffer solution of papain (475 μL) was incubated with (2*S*,3*S*)-BEBA methyl ester stock solution (25 μL) for 4 h at room temperature to ensure complete inactivation of the enzyme. The final concentrations of papain and the inhibitor in the assay mixture were 20 M and 3 mM, respectively. After dialysis using a dialysis kit against 500 mL of the same buffer at 4 °C for 24 h, aliquots (100 μl) were diluted into the substrate buffer solution (900 μL) and the remaining enzyme activity was determined immediately to find no enzymic activity being regained. The control experiment carried out in the absence of the inactivator showed that 90% of the enzymic activity remain.

Acknowledgements

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