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INHIBITION OF PAPAIN BY ISOTHIOCYANATES *

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Summary

During the tapping of papaya latex for papain (EC 3.4.22.2), benzyl isothiocyanate is enzymatically produced from benzylglucosinolate, a major component of the latex fluid. Benzyl isothiocyanate inhibits papain hydrolysis of α-*N*-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt). Since the availability of the papain sulfhydryl group to 5,5′-dithiobis-2-nitrobenzoic acid (Nbs_2) is inversely related to the extensiveness of the isothiocyanate inhibition, and treatment by benzyl isothiocyanate reduces the affinity of activated papain to a mercurial-Sepharose column, it is proposed that the papain sulfhydryl reacts with the electrophilic functional group of this inhibitor.

Fifteen isothiocyanates were selected and both the chemical reactivity and their effectiveness as papain inhibitors determined. Three factors are involved in the isothiocyanate-papain inhibition: (1) chemical reactivity of isothiocyanates, (2) presence or absence of an aromatic substitution, and (3) the spatial relationship of the aromatic moiety to the -N=C=S group of isothiocyanates. These data further suggest the presence of an 'aromatic site' near the Cys-25 sulfhydryl group of activated papain. Based on the existing model of papain molecule, the imidazole group of His-159 coincides well with our proposed aromatic site. The prospects of using isothiocyanates as chemical probes for the study of chemical environment of active sites in other enzymes are briefly discussed.

Introduction

Benzyl isothiocyanate was originally identified in the crushed seeds of papaya (*Carica papaya* L.). Benzylglucosinolate, the precursor of benzyl isothio-

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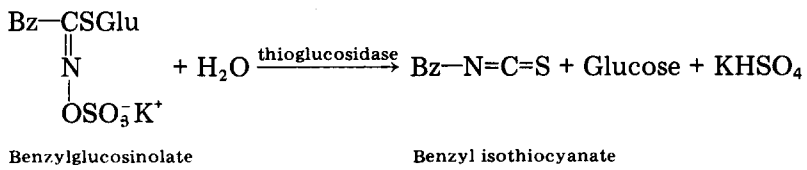
cyanate, was later found to be a major constituent of papaya latex; approximately 10% by weight of freshly dried latex may be attributed to this thioglucoside [1]. The dried latex is also known as crude papain (EC 3.4.22.2). The occurrence of benzylglucosinolate in papain products has been investigated in this laboratory [2].

During the tapping of papaya latex from green papaya fruit, benzyl isothiocyanate is invariably produced as a result of the incision of the tissue; a thioglucosidase is released into the latex fluid to cause the following enzymic hydrolysis:



Because of its electrophilic nature, benzyl isothiocyanate behaves as a naturally occurring papain inhibitor. We proposed that a thiocarbamic linkage was formed between the papain sulfhydryl and the -N=C=S functional group of benzyl isothiocyanate [2].

In the present study, we have used a series of naturally-occurring and synthetic isothiocyanates to evaluate the effect of their different substituents on the inhibition of papain. An attempt is made to rationalize our data based on the well studied chemical environment of the sulfhydryl active site of the papain molecule [3].

Materials and Methods

Activation of papain

Twice recrystallized papain (Sigma Chemical Co.) was activated in the presence of $3.5 \cdot 10^{-4}$ M cysteine, $6 \cdot 10^{-4}$ M EDTA and $5 \cdot 10^{-3}$ M phosphate buffer at pH 6 for 1 h at room temperature. The papain concentration in this solution was usually between 10^{-4} and $2 \cdot 10^{-4}$ M. Papain was also purified by affinity chromatography [4], the resulting Hg · papain was in a solution of standard buffer (10% dimethylsulfoxide, 0.5% butanol, 0.05 M sodium acetate, pH 5.0) and 0.5 mM $HgCl_2$. Activation was performed at room temperature for 1 h by adding cysteine and edta to a final concentration of 1 mM each. Protein concentration was determined by measuring the absorbance at 280 nm with $5.1 \cdot 10^{-4}$ $M^{-1} \cdot cm^{-1}$ as the molar extinction coefficient [5].

Assay of papain activity

The esterase activity of papain on α-*N*-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt, Sigma Chemical Co.) was followed by the rate of addition of approximately 0.05 M NaOH to keep a constant pH of 6.0 with a pH-stat (Radiometer, London). The NaOH solution was standardized daily with 0.1 M potassium phthalate. The assay mixture consisted of 10 mM Bz-Arg-OEt, 0.2 M NaCl, 3% CH_3CN (with or without inhibitor) and 10^{-6}—$2 \cdot 10^{-6}$ M activated papain in a final volume of 6 ml. The pH of the mixture was adjusted to 6.0 prior to the addition of the enzyme.

Detection of papain-SH as the reaction site of benzyl isothiocyanate

Two methods were used for this purpose:

1. Reaction with 5,5′-dithiobis-2-nitrobenzoic acid (Nbs_2, Sigma Chemical Co.). Activated papain was dialyzed 3 times against deonized water with a fast dialysis apparatus to remove cysteine. 1.5 ml of this papain solution was reacted with various amounts of benzyl isothiocyanate in 0.1 ml CH_3CN. After 15 min at room temperature, a 0.1-ml aliquot was withdrawn for the assay of Bz-Arg-OEt hydrolysis. The remaining solution was mixed with 0.1 ml of saturated Nbs_2 solution in 0.01 M phosphate buffer, pH 7.5 and the absorbance at 412 nm [6] measured 15 min later. Benzyl isothiocyanate itself did not interfere with the above color reaction.

2. Affinity chromatography [4]. Hg · papain purified by affinity chromatography was first activated and the cysteine removed by rapid dialysis. One half (3.5 ml) of this solution with a concentration of $2.6 \cdot 10^{-5}$ M was allowed to react with 0.5 ml of 4 mM benzyl isothiocyanate in CH_3CN for 1 h before being applied to a mercurial-Sepharose column (2 × 1.5 cm) which had been equilibrated with standard buffer containing 1 mM EDTA. This column was first eluted with the same buffer until the absorbance at 280 nm was below 0.1. A small volume of standard buffer was then added to wash off EDTA. Finally, standard buffer containing 0.5 mM $HgCl_2$ was applied to elute any mercurial-bound protein molecules. As a control, the other half of the papain solution was mixed with 0.5 ml of CH_3CN and subjected to the same chromatographic procedure.

Sources of isothiocyanates

Cyclohexylmethyl, phenylpropyl, phenylbutyl, *p*-chlorobenzyl, *tert*-2-phenylcyclopropyl and 1-naphthylmethyl isothiocyanates were synthesized from their corresponding amines according to the method of Joshims and Seelinger [7]:

$$R{-}NH_2 + CS_2 + \text{dicyclohexylcarbodiimide} \xrightarrow[-10^\circ C]{\text{ether}} R{-}N{=}C{=}S + \text{dicyclohexylthiourea}$$

Structures of these synthesized isothiocyanates were verified by gas chromatography/mass spectrometry. A Varian Aerograph 1400 Gas Chromatograph interfaced with a Finnigan Model 3000 Peak Identifier was used. For gas chromatography, a 2 m × 2.5 mm stainless steel column packed with 5% Silar-5CP (Supelco Co., Inc.) on 80—100 mesh Chromosorb W, AW-DMCS was used. The helium carrier gas flow rate was 25 ml/min. The injector and oven temperature were usually 210°C. Conditions used for the mass spectrometer were: sensitivity, 10^{-6} A/V; electron multiplier high voltage, —2.00 kV; and electron energy, —69.5 V.

All synthesized isothiocyanates showed a parent peak (P) at the calculated molecular weight and a base peak at P minus -N=C=S, except for cyclohexylmethyl isothiocyanate, which has a base peak at P minus $-CH_2-N{=}C{=}S$. Infrared spectra of these compounds showed a strong band in 2100 cm^{-1} region, which is characteristic of the -N=C=S functional group.

To examine their inhibitory effect on papain, synthesized isothiocyanates were first purified on a Varian Aerograph 1800 Gas Chromatograph equipped

with a Carle Micro Thermistor Detector (Carle Instrument, Inc.). The conditions used were similar to those used in the gas chromatography/mass spectrometry. The isothiocyanate fraction was collected in a glass capillary tube from the gas chromatograph and the amount measured by the difference in weight of the capillary before and after the collection of the effluent. Isothiocyanate/CH_3CN solutions of desired concentrations were prepared by washing the contents of the glass capillary into redistilled CH_3CN.

The following isothiocyanates were available commercially: Methyl, allyl and 2-naphthyl isothiocyanate, Aldrich Chemical Co.; *n*-butyl, 1-naphthyl and *n*-octadecyl isothiocyanate, Eastman Organic Chemicals; benzyl and phenylethyl isothiocyanate, K & K Chemical Co.; and phenyl isothiocyanate, Matheson, Coleman and Bell. The purity of commercial isothiocyanates was examined gas chromatographically and they were used as papain inhibitors without further purification.

Chemical reactivity of isothiocyanates

The reactivities of isothiocyanates as electrophiles were estimated by measuring their half-lives (L_{50}) in NH_4OH/CH_3OH at 25 ± 1°C. The L_{50} value is hence inversely related to the electrophilicity.

$$R{-}N{=}C{=}S + NH_4OH \xrightarrow[25\pm1^\circ C]{CH_3OH} R{-}NH{-}\underset{\underset{S}{\|}}{C}{-}NH_2 + H_2O$$

Approximately 1 mg of isothiocyanate was dissolved in 5 ml of redistilled CH_3OH. The reaction was initiated by the addition of 0.5 ml conc. NH_4OH. Quantities of the remaining isothiocyanate during the course of reaction were determined with a Varian Aerograph 1800 Gas Chromatograph equipped with flame ionization detectors. The conditions used were similar to those used in the gas chromatograph of gas chromatograph/mass spectrometry, in addition, hydrogen and air flow rates of 25 ml/min and 300 ml/min, respectively were used. In each case, a suitable n-alkane ranging from $C_{18}H_{38}$ to $C_{24}H_{50}$ was selected as internal standard for the normalization of the peak heights of the isothiocyanate. L_{50} was measured as the time in minutes required for the reduction of isothiocyanate peak height to half of that prior to the addition of NH_4OH. Since NH_4OH does not respond to the flame ionization detector and the product thiourea is non-volatile, the remaining isothiocyanate appears in each case as the only peak on the gas chromatogram, and the kinetics of the system may therefore be determined without any interference. The L_{50} of butyl isothiocyanate in H_2O was estimated in a similar manner except a water-acetone mixture was used instead of NH_4OH/CH_3OH.

Results

Effect of benzyl isothiocyanate on papain

When Bz-Arg-OEt was used as the substrate, inhibition of crystalline or affinity-chromatographically purified papain by benzyl isothiocyanate showed a non-competitive type of double reciprocal plot (Fig. 1) similar to that obtained

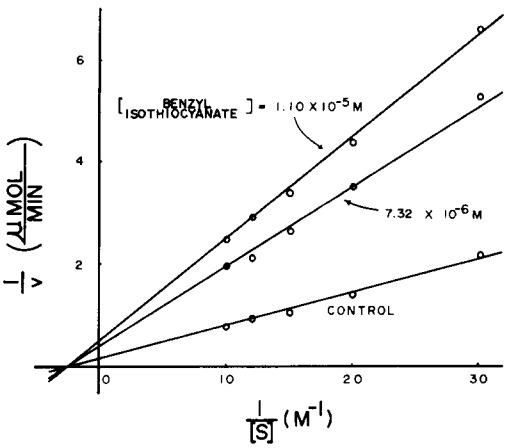


Fig. 1. Double reciprocal plot of the hydrolysis of Bz-Arg-OEt by affinity-chromatographically purified papain with or without the presence of benzyl isothiocyanate. Papain concentration was $3 \cdot 10^{-7}$ M.

using *N*-carbobenzoxyglycine-*p*-nitrophenyl ester (N-CBz-Gly-*p*-NP) [2]. The average K_i was calculated as $3 \cdot 10^{-6}$ M.

Site of benzyl isothiocyanate reaction

The curves in Fig. 2 show that as the degree of inhibition of papain by benzyl isothiocyanate increases, concentration of titratable thiol groups decreases.

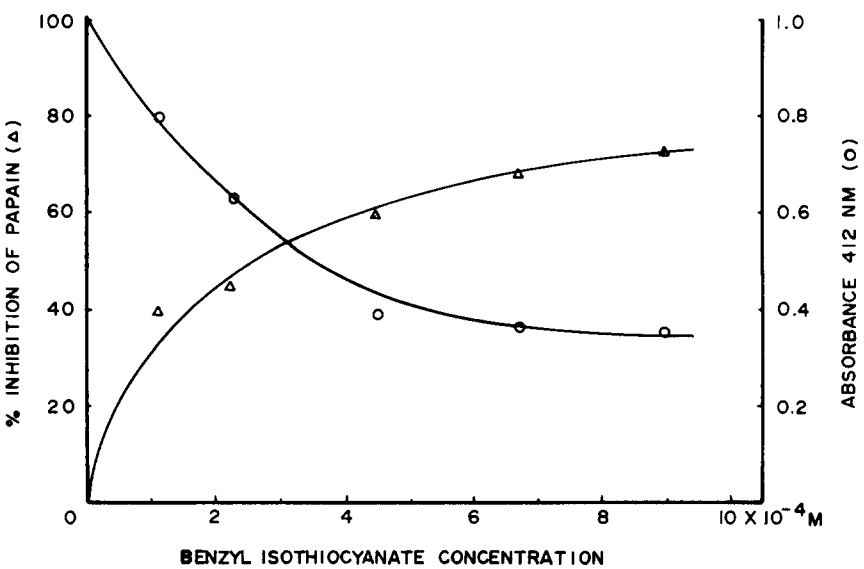


Fig. 2. Effects of benzyl isothiocyanate concentration on the availability of -SH to Nbs_2 (- ○ -) and percent inhibition of papain (- △ -). Activated papain ($1 \cdot 10^{-4}$ M) was dialyzed to remove cysteine. A 1.5-ml aliquot was used to incubate with various amounts of benzyl isothiocyanate in 0.1 ml CH_3CN for 15 min. 0.1 ml of this solution was withdrawn to assay for papain activity and the rest was mixed with Nbs_2 solution. Absorbance at 412 nm was measured 15 min later.

These results suggest that benzyl isothiocyanate inhibits papain catalytic activity by modifying the Cys-SH of papain. However, complete inhibition is not obtained under these conditions because of the limited solubility of benzyl isothiocyanate in the 6% aqueous CH_3CN reaction solution.

A similar conclusion can be drawn from the chromatograms shown in Fig. 3. When benzylisothiocyanate-inhibited papain is chromatographed on a mercurial-Sepharose column, most of the proteins are eluted by standard buffer, EDTA. Only a relatively small quantity remains on the column, requiring Hg-containing buffer for complete elution. The reverse results are obtained with activated papain not treated with benzyl isothiocyanate. The inhibitor itself does not bind to the column.

Isothiocyanates as papain inhibitors

Three types of isothiocyanates (Table I) were investigated for their effectiveness as papain inhibitors:

Group I: Aliphatic isothiocyanates without aromatic substitution on the side chain. These compounds are active inhibitors only if the alkyl substitutions are relatively small; no inhibitory effect was detected for cyclohexylmethyl and *n*-octadecyl isothiocyanate.

Group II: Aliphatic isothiocyanates with aromatic substitution. They are strong inhibitors; concentrations required for the 50% inhibition (I_{50}) of papain activity under the present experimental conditions ranged from $6.0 \cdot 10^{-7}$ to $6.4 \cdot 10^{-6}$ M.

Group III: Aromatic isothiocyanates. The three representative compounds examined are relatively inactive even though they are good electrophiles.

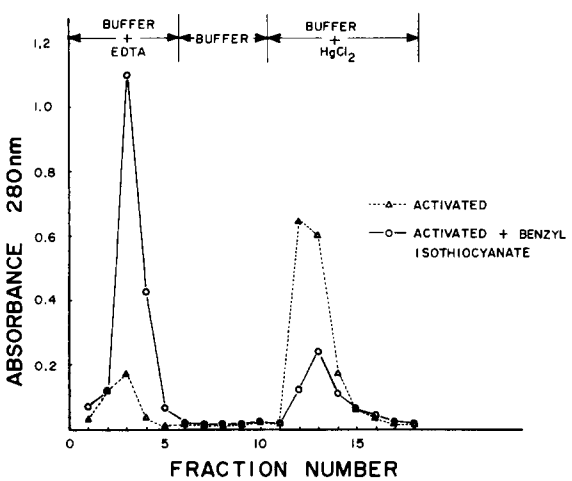
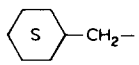
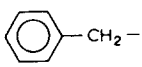
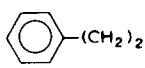
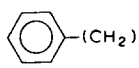
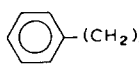
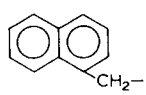
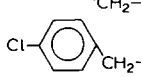
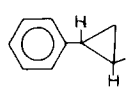
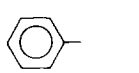
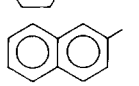
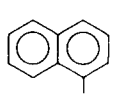


Fig. 3. Affinity-chromatograms of papain with or without benzyl isothiocyanate treatment. Affinity-chromatographically purified papain was activated in 1 mM EDTA and cysteine and rapidly dialyzed against 1 mM EDTA solution for 3 times. The final papain concentration was $2.6 \cdot 10^{-5}$ M. 3.5 ml of this solution with 0.5 ml Ch_3CN was applied to a mercurial-Sepharose column and washed successively with standard buffer + EDTA, standard buffer and standard buffer + $HgCl_2$. Fractions of 3 ml were collected and their absorbance measured at 280 nm. Another 3.5 ml was incubated with 0.5 ml of benzyl isothiocyanate (4 mM in CH_3CN) for 1 h before being applied to the same column and subjected to the same treatment.

TABLE I

CONCENTRATION OF ISOTHIOCYANATE (R—N=C=S) WHICH INHIBITS 50% OF THE PAPAIN ACTIVITY (I_{50}) AND THE HALF-LIFE (L_{50}) OF ISOTHIOCYNATES IN METHANOLIC AMMONIA

Group	R	I_{50}(M)	L_{50}(min)
I	CH_3-	1.7×10^{-5}	8.7
	$CH_2=CHCH_2-$	1.9×10^{-5}	6
	$n-C_4H_9-$	3×10^{-5}	37.5
	$n-C_{18}H_{37}-$	N.E. *	—
		N.E.	60
II		3.7×10^{-6}	8.7
		6.4×10^{-6}	26
		5.6×10^{-6}	37
		6.0×10^{-6}	60
		4.3×10^{-6}	10
		1.0×10^{-6}	8
		6×10^{-7}	~3
III		$\sim 10^{-4}$	~3
		$\sim 10^{-4}$	13.7
		N.E.	8

* N.E., no inhibitory effect at 1×10^{-4} M.

Chemical reactivity of isothiocyanates

The electrophilicities of isothiocyanates measured as their half-life (L_{50}) in NH_4OH-CH_3OH are listed in Table I. Typical curves (remaining isothiocyanate concentration vs. time) from which L_{50} were obtained showed pseudo-first-order reaction kinetics due to the excess NH_4OH used in the reactant mixture.

Reactivity of the isothiocyanate functional group is enhanced by effects of conjugation (e.g. R=phenyl), induction (e.g. R=*p*-chlorophenyl) and by the small size of the substitution (e.g. R=methyl). Large aliphatic substitution renders it inert to nucleophilic attack as in the case of *n*-octadecyl isothio-

cyanate. The half-life of isothiocyanates in water is much longer, phenyl and butyl isothiocyanates in an H_2O/acetone solution have estimated L_{50} values of 50 min and 10 h, respectively.

Discussion

Our present data suggest that the sulfhydryl functional group of Cys-25 on activated papain is no longer available after benzyl isothiocyanate inhibition. Fig. 2 in which increasing inhibitor concentration inversely relates to the availability of papain sulfhydryl to Nbs_2, and Fig. 3 in which the treated papain fails to bind with Hg^{2+} on the Sepharose-mercurial column support the assumption that this inhibitor reacts with the papian active site. This view is further supported by works on isocyanates, the oxygen analog of isothiocyanates: Twu and Wold [8] reported that butyl isocyanate modified the functional sulfhydryl group of yeast alcohol dehydrogenase to form an *S*-butylcarbamoyl derivative. Papain was inactivated by reaction with a stoichiometric amount of butyl isocyanate at low pH and in this case, it was demonstrated that the active site SH group was derivatized. Using [^{14}C]butyl isocyanate, Brown and Wold[9] further confirmed that the interaction took place at the active site serine of chymotrypsin and elastase. Isothiocyanates and isocyanates closely resemble one another in their reaction mechanisms except that the sulfur analogs are usually much more stable. Butyl isothiocyanate decomposes slowly in an acetone/water solution, having and L_{50} of longer than 10 h, while butyl isocyanate shows a half-life of approx. 1 min in an aqueous buffer solution [10]. With a weaker electrophilic functional group, the side chain of an isothiocyanate would assume a greater influence in the process of enzyme-inhibitor complex formation. The importance of the side chain will be discussed later.

The highly reactive nature of the papain sulfhydryl group in comparison with that of cysteine [11] also leads us to believe that the nucleophilic addition takes place at the papain active site. However, direct evidence such as isolating the thiocarbamic ester of benzyl isothiocyanate and cysteine from the hydrolysate of the inhibited papain would be difficult, if not impossible, due to the unstable nature of the thiocarbamic bond [12]. Attempted spectroscopic study of thiocarbamic bond formation was hindered by the fact that the product did not cause any significant change in absorbance from that of the reactant mixture.

We reported in a previous paper [2] that when CBz-Gly-*p*-NP was used as the substrate for papain assay, the double reciprocal plots showed "non-competitive" inhibition. Similar results were obtained in the present study using Bz-Arg-OEt as the substrate (Fig. 1). However, because of the aforementioned observations, we believe the non-competitive plots obtained in both cases suggest that once the papain-inhibitor complex (papain-SH · Bz-N=C=S) is formed, it has a very small dissociation constant in comparison with that of the papain-substrate complex, rather than that the inhibitor reacted with the enzyme at a different site as a non-competitive type of plot would usually imply [13].

$$\text{Papain—SH} + \text{Bz—N=C=S} \rightleftharpoons \text{Papain—SH} \cdot \text{Bz—N=C=S} \rightarrow \text{Papain—S—}\underset{\underset{\text{S}}{\|}}{\text{C}}\text{—NH—Bz}$$

Data in Table I show that the effectiveness of isothiocyanates as a papain inhibitor is affected by the following factors: (1) the chemical reactivity or, the electrophilicity of the -N=C=S. It is generally true that within the same group in Table I that an isothiocyanate that is more reactive with ammonia is a more potent inhibitor of papain; (2) aromatic substitution on the side chain enhances enzyme inhibitory potency; benzyl isothiocyanate is roughly four times as effective as methyl isothiocyanate even though both of them have the same electrophilicity as measured by L_{50} values. In another case, butyl isothiocyanate is a better electrophile than phenylbutyl isothiocyanate, yet the latter is five times more effective than the former as an inhibitor; (3) however, aromatic isothiocyanates in group III are poor papain inhibitors in spite of their high chemical reactivity (low L_{50} values). While the first of the above characteristics conforms well with one's expectations, the second and the third indicate that there is an enhancement of the inhibitory activity by an aromatic substitution and this effect requires at least one carbon atom (e.g. a methylene group) spaced in between the aromatic moiety and the -N=C=S functional group.

To explain this, we propose the existence of an 'aromatic site' in the vicinity of Cys-25 of the papain molecule. The inhibitory effect of group I isothiocyanates without any aromatic substitutions is not facilitated by this 'aromatic site'. Rather, it relies mainly on the electrophilicity of the -N=C=S. Group II isothiocyanates with at least one carbon atom in betweem the aromatic moiety and -N=C=S are capable of interacting with both the 'aromatic site' and the sulfhydryl group of Cys-25. The first four isothiocyanates in this group have similar I_{50} values in spite of the increasing number of carbon atoms between phenyl and -N=C=S and increasing L_{50} values. These results suggest that the phenyl ring of these isothiocyanates is of primary importance to the inhibitory reaction; once the phenyl group is attached to the aromatic site of papain, the freely rotating sp^3 C-C bonds of these isothiocyanates may align the -N=C=S for nucleophilic attack by papain-SH as illustrated by phenylbutyl isothiocyanate and *t*-2-phenylcyclopropyl isothiocyanate in Fig. 4. The latter was found to be the most potent inhibitor among all isothiocyanates tested. It could be argued that this molecule not only satisfies the steric requirement, but also is chemically most reactive ($L_{50} \approx 3$) due to the partially unsaturated nature of the cyclopropane ring. For compounds in Group III, once the aromatic ring is bound to the 'aromatic site', the center carbon of -N=C=S may not be able to reach the sulfhydryl group of Cys-25, either because of lacking of the 'arm' in the case of phenyl isothiocyanate (Fig. 4), or of the rigid structure in the cases of naphthyl isothiocyanates.

Based on the current understanding of the structure of papain active site [3], the imidazole ring of His-159 appears to be the most reasonable choice as the proposed 'aromatic site'. Chemical evidence obtained by Husain and Lowe [14] indicates that the distance between the Cys-25 sulfhydryl group and the imidazole ring is approximately of 5 Å. X-ray studies by Drenth et al. [3] reveal that the sulfur atom and the N-1 atom of imidazole are 3.4 Å apart, or the distance between the sulfur and the center of the imidazole ring is approximately 4.5 Å. We have estimated, based on the reported values of bond length [15], the distances from the center of the phenyl ring to the center carbon of -N=C=S to be 4.87 Å for benzyl isothiocyanate and 4.08 Å for phenyl isothio-

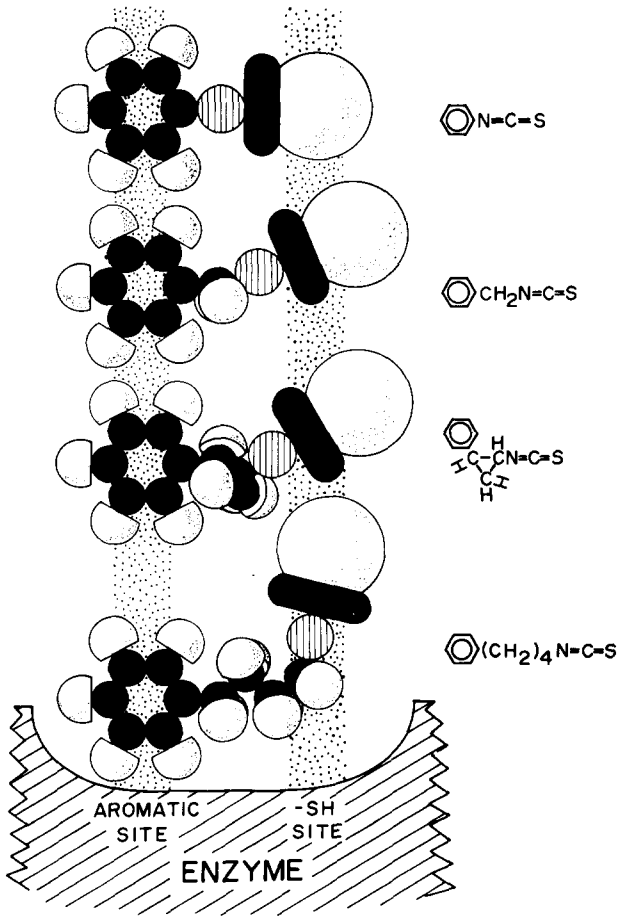


Fig. 4. Proposed alignment of phenyl, benzyl, *t*-2-cyclohexylmethyl and phenylbutyl isothiocyanates with the aromatic site and Cys-25 sulfhydryl on activated papain based on Godfrey molecular models. The aromatic site is believed to be the imidazole ring of His-159. Once the aromatic interaction has occurred between the phenyl ring and the aromatic site, the central carbon of -N=C=S of phenyl isothiocyanate is the only one incapable of reaching the Cys-25 SH site.

cyanate. These measurements agree with our interpretation that Group II isothiocyanates are highly inhibitory because the papain sulfhydryl group is within the reach of the -N=C=S after phenyl-imidazone interaction. Yet, for phenyl isothiocyanate, this aromatic interaction seriously hinders the thiocarbamic bond formation and an unexpected high I_{50} value (low inhibitory effect) consequently results.

The pK of His-159 imidazole has been assigned either a low value near 4 [16] or, a high value above 8 [17,18]. In our case, a high pK is preferred because it facilitates π to π charge transfer complex formation with the aromatic moiety of an isothiocyanate. Using spectrophotometric method Polgar [17] suggested that the mercaptide-imidazolium ion-pair was the major species of active papain. Recently, Drenth et al. [18] proposed that in addition to the ion-pair formation at the active site, oscillational rotation of His-159 imidazole ring

was essential to the catalytic action. The mechanism proposed by Drenth et al. may be used to explain the inhibitory behavior of isothiocyanates in Table I: Group II compounds are the most potent inhibitors because only these compounds are capable of blocking the papain active site by both charge transfer complex formation and nucleophilic addition of Cys-25 mercaptide to -N=C=S. The charge transfer complex would also restrict the freedom of rotation of the imidazole ring.

Our preliminary studies showed that benzyl isothiocyanate is much less effective toward chymotrypsin which is a serine protease or, toward non-proteolytic sulfhydryl enzymes such as rabbit muscle lactate dehydrogenase or yeast alcohol dehydrogenase. However, it does inhibit other homologous plant sulfhydryl proteases such as chymopapain and ficin effectively. Bromelain was inhibited to a lesser extent. All these plant proteases are quite similar to papain in their structure around the active site (histidine in the vicinity of cysteine). The amino acid sequence of chymopapain near the cysteine is different from that of papain although it was found from immunological studies that they have similar determinants around the catalytic site [19]. The similarity between papain and ficin is probably closer than that between papain and bromelain, because papain and ficin showed about the same k_{cat} toward Bz-Arg-OEt and α-*N*-benzoyl-arginine amide hydrolysis while bromelain exhibits a 140 fold difference [20]. Our finding that benzyl isothiocyanate is more effective toward papain, chymopapain and ficin than bromelain suggests that is is a very specific active site inhibitor.

Isothiocyanates with various side chains may be synthesized [7]. Purification of volatile isothiocyanates and determination of their chemical reactivities can be readily achieved by procedures described in this study. With these advantages, it is hoped that these compounds may be utilized as chemical probes of the active site of plant sulfhydryl proteases.

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