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Research article

Cyantraniliprole as papain inhibitor and its action in molecular docking studies

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Abstract

Importance of the work: Biosensors have emerged as an alternative method for the determination of cyantraniliprole; however, they still require expensive enzymes.

Objectives: A papain assay was applied as a model for the study of inhibition by cyantraniliprole.

Materials & Methods: Papain, an inexpensive enzyme found mainly in papaya resin, belongs to a proteolytic enzyme group capable of digesting large proteins into their smaller constituents. Papain activity was determined using a colorimetric reaction. *N*-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) was hydrolyzed to a yellow product, *p*-nitroaniline, which could be monitored spectrophotometrically at 430 nm.

Results: The enzyme was optimally active at pH 7. The Michaelis constant of papain toward this substrate was 2.40 mM and the kinetic constant V_{max} was 0.0169 µmol/min. This assay was investigated using the organic solvents dimethyl sulfoxide (DMSO) and acetonitrile. The enzyme activities were very similar when working in either phosphate buffer or phosphate buffer containing 3% (volume per volume, v/v) acetonitrile or 10% (v/v) DMSO. Then, the optimum conditions were applied to the quantitative analysis of cyantraniliprole. The activity of papain was strongly inhibited by cyantraniliprole. The preliminary results showed a wide linear range from 4.5 to 47.0 parts per million. The molecular docking studies confirmed that cyantraniliprole interacted with papain similarly to BAPNA, with the limit of detection being in the low parts per million.

Main finding: The work represents the first investigation of papain inhibition by cyantraniliprole. With its simplicity and low cost, this assay has high potential for use in the field.

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Introduction

Cyantraniliprole is an anthranilic diamide insecticide and ryanodine receptor modulator; targeting of the ryanodine receptors results in their unregulated activity, causing lethargy, paralysis, and eventually death (Cordova et al., 2007; Jeanguenat, 2013). For this reason, cyantraniliprole plays an important role in maintaining and increasing agricultural productivity. However, excess insecticide usage can harm humans as well as plants, so the maximum residue limit for cyantraniliprole in *Cucurbitaceae* is 0.3 mg/kg (Kwannimit et al., 2021). The determination methods for this class of insecticides are different process using high-performance liquid chromatography (Hu et al., 2013; Kwannimit et al., 2021) and immunoassay (Zhang et al., 2015; Xu et al., 2021). Even though these techniques provide high sensitivity, they have certain limitations, such as the cost of instruments, complex sample preparation and the requirement of expert operators to perform the assays (Zhang et al., 2015; Xu et al., 2021). Such limitations have led to a rise in the popularity of biosensors, which are determinations based on enzyme activity. Many enzymes have been reported, including acetylcholinesterase, organophosphorus hydrolase and organophosphorus acid hydrolase (Verma and Bhardwaj, 2015). These inhibition-based sensors offer high sensitivity, but their utility is hampered by poor specificity and a high cost (Verma and Bhardwaj, 2015).

Papain is an enzyme produced from papaya belonging to the protease enzyme family (EC 3.4.22.2) (Amri and Mamboya, 2012; Novinec and Lenarcic, 2013). It has been utilized in many fields, including the food and beverage industry, due to its well-understood mechanism and the low cost of its production (Harrison et al., 1997). The structure of this enzyme is composed of two domains: the L-domain and the R-domain (Paul et al., 2013), with the amino acids cysteine (Cys25) and histidine (His158) playing important roles at the active sites. Most of the inhibitors have been reported for papain based on the nucleophilic attack of the active site cysteine resulting in disulfide bond formation (Katunuma et al., 1999, Reddy et al., 2002, Beavers et al., 2008). The involved functional groups include aldehydes, (Katunuma et al., 1999) amides, nitriles (Reddy et al., 2002) and esters (Beavers et al., 2008). For example, N-acetyl-phenylalanyl-2-amino-propionitrile utilizes nitrile carbon to form a thioimidate intermediate covalently attached to the Cys of papain (Reddy et al., 2002). Papain's hydrophobic pocket can also bind to the hydrophobic or non-polar atoms of inhibitors. For example, Chowdhury *et al. (2002)* removed the aromatic ring of noncovalent inhibitors resulting in a significant loss of inhibition. Some inhibitors cause conformational changes in the protein structure that inactivates the enzyme function (Juettner et al., 2020). Therefore, the current research developed a biosensor for the quantification of cyantraniliprole belonging to anthranilic diamide derivatives bearing a cyano group. The assay used the colorimetric BAPNA method to measure papain activity. The interaction was also between cyantraniliprole and papain compared to the natural substrate, in molecular docking studies. This approach could be applied to cyantraniliprole detection in remote locations where an advanced laboratory is not available.

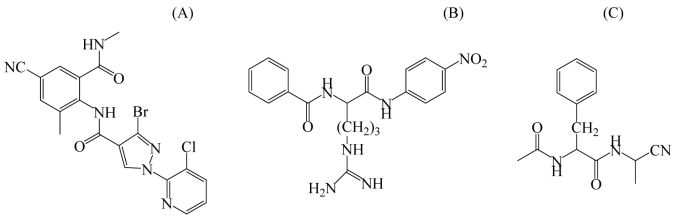

Fig. 1 Structure of: (A) cyantraniliprole; (B) N-α-benzoyl-arginine-p-nitroanilide; (C) N-acetyl-phenylalanyl-2-amino-propionitrile

Materials and Methods

Materials

Food-grade papain was purchased from MySkinRecipes, Thailand. The stock was dissolved in phosphate buffer pH 6. Potassium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Kemaus, Australia, and Merck, Germany, respectively. N-α-benzoyl-arginine-p-nitroanilide (BAPNA), purchased from Sigma-Aldrich, China, was prepared by dissolution in dimethyl sulfoxide (DMSO) obtained from RCI Labscan, Thailand. Cyantraniliprole standard (purity, 92.59%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). The stock solution was prepared in acetonitrile.

Papain assay

Enzyme activity was determined by mixing 0.04 g/mL of papain with 2 mM BAPNA, adjusting the total volume to 1,000 µL with 50 mM of phosphate buffer pH 7 and shaking well. Then, the absorbance was measured at a wavelength of 430 nm for a total of 2 min. The enzyme activity was calculated based on Equation 1.

$$\text{Activity (µmol/min, U)} = \frac{\frac{\Delta\text{Absorbance}}{\Delta\text{minutes}}}{\frac{1.08\times10^{4}}{\text{M cm}}} \times \frac{10^{6}\,\mu\text{M}}{1\ \text{M}} \times 0.00100\ \text{L} \quad (1)$$

where the molar extinction coefficient (ε) of p-nitroaniline is 1.08×10^4 /M/cm at 430 nm.

Optimization of enzymatic activity measurement

First, enzyme kinetic assays were performed under different pH conditions ranging from 3 to 9 at fixed enzyme and substrate concentrations. The buffer was a 50 mM phosphate (H_3PO_4/KH_2PO_4 for pH 3–4 and K_2HPO_4/KH_2PO_4 for pH 5–9). Second, the effect of the enzyme concentration was investigated in the presence of a constant concentration of substrate (2 mM). Various enzyme concentrations (0.005–0.080 g/mL) were investigated. The enzyme activity under each condition was calculated according to Equation 1. Lastly, the enzyme kinetics were studied using BAPNA at various concentrations (0.1200–8.0000 mM). Then, Lineweaver-Burk plots were created to determine the kinetic constants (K_m and V_{max}). K_m and V_{max} were obtained according to Equation 2 where [S] defined as the substrate concentration:

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}}\right)\left(\frac{1}{[S]}\right) + \frac{1}{V_{max}} \quad (2)$$

Effect of organic solvents

The effects were studied of the organic solvents (acetonitrile and DMSO, which are used for insecticide extraction and substrate preparation, respectively). An aliquot of 700 uL of phosphate buffer solution (pH 7) containing a known amount of papain and various percentages of either acetonitrile (from 0.6% to 10.0% (v/v)) or DMSO (from 0% to 10.0% (v/v)) in the absence of cyantraniliprole were used. The enzyme activity was measured using Equation 1.

Application of developed system for quantitative analysis of cyantraniliprole insecticides

Cyantraniliprole was quantified under the optimum conditions to express the feasibility of this developed method. The final BAPNA and papain concentrations were 1 mM and 0.03 g/mL, respectively. The cyantraniliprole concentration varied in the range 12–70 parts per million (ppm). To increase the sensitivity, the enzyme was incubated with cyantraniliprole for 1 hr before initiating the reaction by the addition of BAPNA. The activity was calculated according to Equation 1. The relative activity was calculated according to Equation 3:

$$\text{Relative activity} = \frac{A_I}{A_0} \quad (3)$$

where A_0 is the enzyme activity in the absence of cyantraniliprole and A_I is the enzyme activity in the presence of cyantraniliprole at various concentrations.

Limit of detection and limit of quantification values

Ten sample blanks were tested in the developed system. The limit of detection (LOD) and limit of quantification (LOQ) were estimated using blank and calibration standards. The concentration of cyantraniliprole that produced an S-to-N ratio of 3 was considered to be the LOD, while the LOQ was defined by an S-to-N ratio of 10.

Molecular docking

The binding of natural substrate (BAPNA) and cyantraniliprole in papain was determined using a molecular docking approach with the iGEMDOCK v.2.1 software (Hsu et al., 2011). The initial structures of BAPNA and cyantraniliprole were obtained from PubChem as CID 24891801 and CID 11578610, respectively. The three-dimensional structure of papain complexed with inhibitor benzyloxycarbonyl-phenylalanylalanyl-chloromethyl ketone (ZPACK) retrieved from the protein DATA bank (PDB: 6PAD) was used as the receptor. For docking each ligand against a protein, the accurate docking (very slow) with population size n = 800, 80 generations and 10 solutions was applied**.** Then, the docking pose with the lowest binding energy value for each ligand-protein complex was analyzed and imaged using the BIOVIA Discovery Studio Visualizer package (Dassault Systemes; San Diego; USA).

Results and Discussion

Effect of pH and enzyme concentration

According to other studies, the optimal pH for papain activity ranges from 3.0 to 9.0 and varies between substrates (Edwin and Jagannadham, 2000). Herein, the effect of pH in the range 3.0–9.0 was investigated. The results in Fig. 2A show that the activity gradually increased with increasing pH values until the maximum value were reached at pH 7.0, above which the activity then started decreasing because the active site of papain contains the Cys25-His159 ion pair at neutral pH (Reddy et al., 2002). Therefore, a phosphate buffer solution at pH 7 was chosen for subsequent experiments**.**

When the substrate concentration was fixed, increasing the enzyme concentration increased the enzyme activity. The enzyme activity increased with the increase in papain concentration. Up to an enzyme concentration of 0.05 g/mL, the enzyme activity was constant, possibly as a result of substrate depletion (Fig. 2B).

Effect of substrate concentration

Detection methods in biosensor applications are highly dependent on the substrate concentration and colorimetric measurements should obey Michaelis-Menten kinetics and show hyperbolic saturation (Verma and Bhardwaj, 2015). The kinetic experiment of the hydrolysis reaction of BAPNA by papain enzyme was studied to determine the efficiency of the enzyme's action on the substrate. From the experimental results in Fig. 2C, the activity values increased with increasing concentration of BAPNA. Furthermore, the K_m value of the hydrolysis of BAPNA by papain was 2.40 mM, and the V_{max} value was 0.0169 µmol/min. The calculated K_m value was consistent with reported values (3.00 ± 0.35 mM), according to Mole and Horton (1973).

Study of effect of organic solvents

Most enzymes are inactivated by organic solvents, including papain (Pietsch et al., 2009). Since cyantraniliprole is normally extracted using acetonitrile, the acetonitrile effect on the activity was studied to suggest the proper sample volume to use in this method. A substantial decrease in activity was obtained when the acetonitrile percentage in the phosphate buffer was 5.0 % (v/v), 10.0% (v/v) and 50.0% (v/v), as shown in Fig. 3A. It was concluded that acetonitrile should be set lower than 2.5% (v/v), which could still provide about 80% of maximal enzyme activity. In addition, the enzyme-substrate complex is soluble in DMSO. A similar experiment was conducted in the presence of DMSO at different percentages. As shown in Fig. 3B, the same activity was obtained when the DMSO percentage in the phosphate buffer was 2–10% (v/v). Therefore, DMSO had no major direct effect on activity.

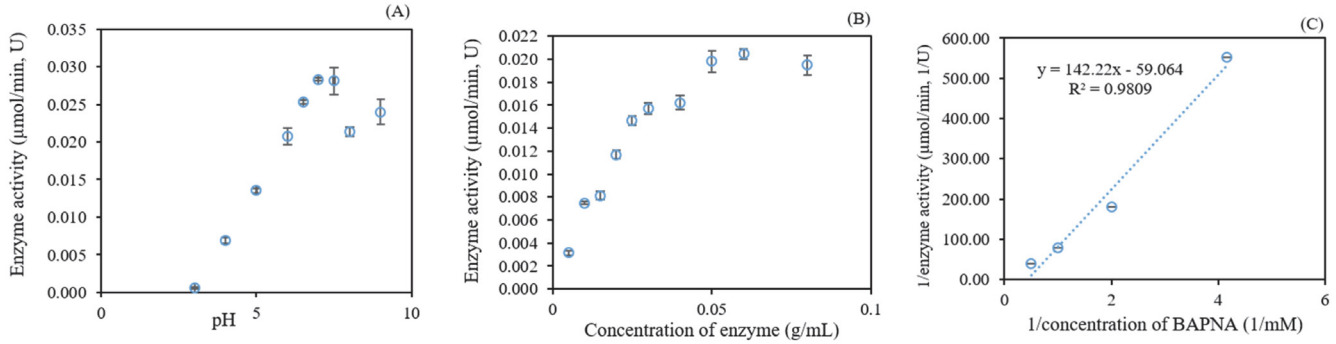

Fig. 2 Optimization of hydrolysis of *N*-benzoyl-L-arginine-*p*-nitroanilide (BAPNA) by papain: (A) effect of pH on papain activity; (B) effect of enzyme concentration on enzyme activity; (C) effect of substrate concentration on papain activity, where R^2 = coefficient of determination, errors bars indicate ± SD and U stands for enzyme unit expressed as µmol substrate converted per minute.

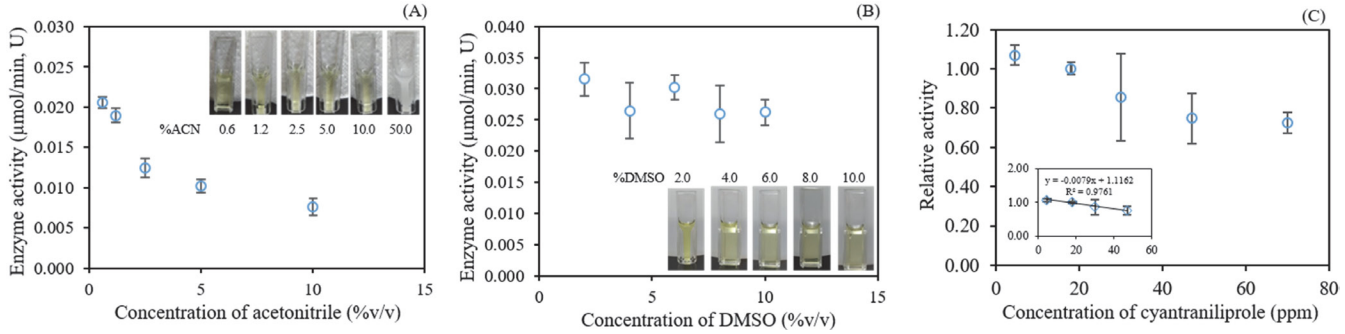

Fig. 3 (A) Effect of acetonitrile (ACN) on papain activity; (B) Effect of dimethyl sulfoxide (DMSO) on papain activity; (C) Inhibitory effect of cyantraniliprole on papain, where errors bars indicate ± SD, ppm = parts per million and U stands for enzyme unit expressed as µmol substrate converted per minute

Application of developed system for quantitative analysis of cyantraniliprole insecticides

The inhibitory effect of cyantraniliprole on acetylcholinesterase (AchE) activity has been reported (Atta et al., 2022) who showed that there were different inhibition degrees in the presence of 18 ppm and 60 ppm cyantraniliprole. In the current work, the detection of cyantraniliprole was based on the inhibition of papain activity. A yellow product, *p*-nitroaniline, results from papain hydrolysis and can be detected spectrophotometrically at 430 nm. Insecticide detection was carried out by incubating the enzyme-cyantraniliprole mixture for 1 h, using an insecticide concentration in the range 4.5–70 ppm. Surprisingly the presence of cyantraniliprole enhanced the papain activity at low concentrations, while the high concentrations substantially decreased the formation of the yellow product, according to Fig. 3C. This effect might have resulted from the changes in enzyme conformation by the cyantraniliprole-enzyme interaction, similar to the one observed with tannin-acid phosphatase (Adamczyk et al., 2017). The investigation of the kinetics and inhibitory mechanism was carried out. The calibration range was linear from 4.5 ppm to 47 ppm, with a mean coefficient of determination (R^2) of 0.9761. At the same concentration of cyantraniliprole, a higher inhibition degree was observed than the ones used with AchE. The LOD and LOQ were estimated to be 1.3 ppm and 4.5 ppm, respectively.

Molecular docking studies

Molecular docking was used to rationalize the interaction between cyantraniliprole and papain. The active site of papain is composed of an ion pair between cysteine (Cys-25) and histidine (His-159) called the catalytic dyad; the other amino acids that play important roles in their stabilization are asparagine (Asn-175) and glutamine (Gln-19) (Harrison et al., 1997). To validate the molecular docking approach, the inhibitor of papain (named *N*-acetyl-phenylalanyl-2-amino-propionitrile) was docked in the cavity of papain and its results were compared with Reddy et al. (2002). It was found that the *N*-acetyl-phenylalanyl-2-amino-propionitrile bound in papain with more or less the same position as co-crystalized benzyloxycarbonyl-phenylalanylalanyl-chloromethyl ketone (ZPACK) (Fig. 5A) and it formed H-bonding with Gln19, Gly65, Gly66 and Asp158 (Fig. 5B), which was similar to those observed by Reddy et al. (2002), indicating the validation of the current docking method.

As presented in Fig. 5A, the natural substrate, BAPNA, cyantraniliprole and *N*-acetyl-phenylalanyl-2-amino-propionitrile occupied the same orientation at the cleft between domains and bound at the same site of the catalytic pocket with binding energies of -87.98 kcal/mol, -77.33 kcal/mol and -75.23 kcal/mol, respectively. Their hydrogen bonding in the cavity of papain is also displayed in Fig. 5. It can be noticed that all three substrates (BAPNA, cyantraniliprole and *N*-acetyl-phenylalanyl-2-amino-propionitrile) formed hydrogen bonds with Gly66. Additionally, Fig. 6 demonstrates that both BAPNA and cyantraniliprole effectively fit in the catalytic dyad of papain and interacted with key amino acids, including Gln19, Cys25 and His159. These results could provide valuable guidance at a molecular level for the development of papain and papain substrate as a biosensor for the quantification of cyantraniliprole.

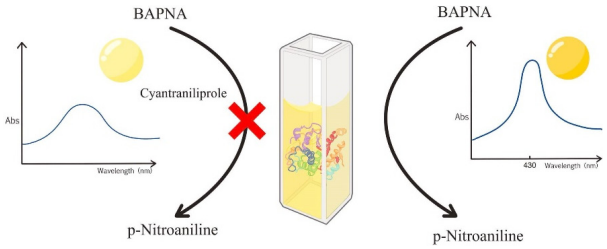

Fig. 4 Strategy for measurement of papain inhibition using cyantraniliprole, where Abs = Absorbance

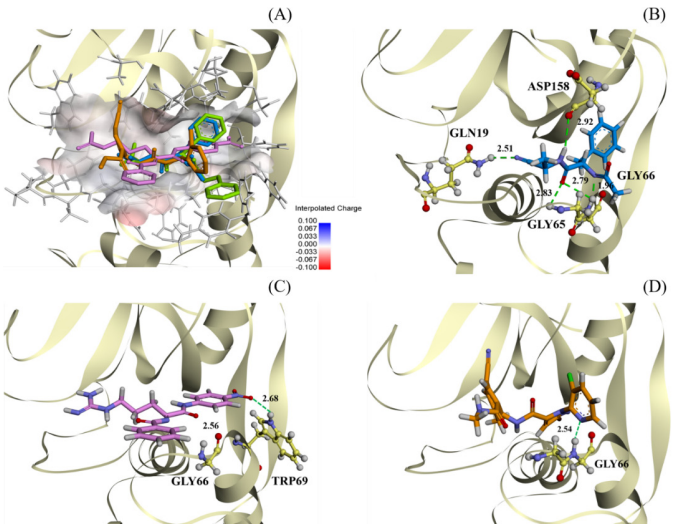

Fig. 5 (A) Schematic view of binding position of *N*-benzoyl-L-arginine-*p*-nitroanilide (BAPNA, pink), cyantraniliprole (orange), N-acetyl-phenylalanyl-2-amino-propionitrile (blue) and benzyloxycarbonyl-phenylalanylalanyl-chloromethyl ketone (ZPACK, green) in cavity of papain and H-bonding between them; (B) N-acetyl-phenylalanyl-2-amino-propionitrile in the cavity of papain; (C) BAPNA in the cavity of papain; (D) cyantraniliprole in the cavity of papain

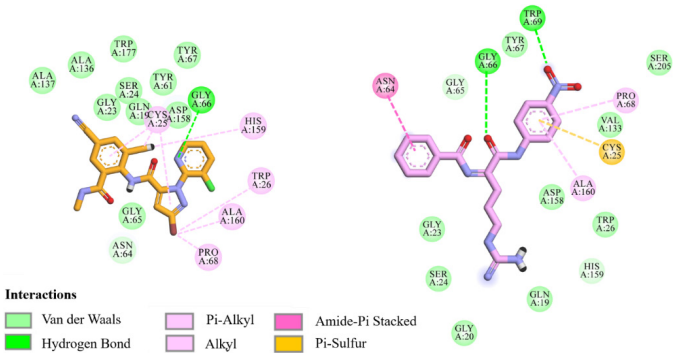

Fig. 6 2-Dimensional binding interaction of *N*-benzoyl-L-arginine-*p*-nitroanilide (BAPNA, pink, right) and cyantraniliprole (orange, left) in binding pocket of papain

In summary, the current work represents the first investigation of papain inhibition by cyantraniliprole. The results indicated that the inhibition of papain by cyantraniliprole is orders of magnitude higher than that found for AchE. Although the sensitivity of the biosensor still needs to be improved further, this finding has shed light on new opportunities to determine cyantraniliprole as a low-cost enzyme-based biosensor. Molecular modeling studies might help predict suitable mutations in the papain structure for this purpose. Future work will be focused on reaching a lower LOD for the analysis of cyantraniliprole in agricultural products.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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