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Isolation and caracterization of ficin enzyme from *Ficus septica* Burm F stem latex

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Abstract

This research aims to isolate and characterize the ficin enzyme from *Ficus septica* stem latex. Ficin from *Ficus septica* stem latex was isolated using column chromatography. Then enzyme activity was tested at different temperature (40°C, 50°C, 60°C, 70°C) and pH (6.0, 7.0, 8.0) levels. Ficin enzyme activity of joint treatment with variations in temperature and pH was analyzed using two-way ANOVA with a factorial pattern followed by Least Significant Difference (LSD) test. The results showed that temperature treatment significantly affects enzyme activity. However, the treatment of pH and the interaction between temperature and pH did not significantly affect the ficin enzyme activity. There was no significant difference in ficin activity at the incubation temperatures of 40°C and 50°C, as well as 60°C and 70°C. However, comparing the incubation temperatures of 40°C and 50°C with treatment 60°C and 70°C showed a significant difference in ficin enzyme activity. In the treatment of incubation at pH 6, 7 and 8 for ficin enzyme activity showed no significant difference. We concluded that the *Ficus septica* plant latex contained ficin enzyme with an optimum temperature of 60°C and optimum pH of 6, 7, and 8.

Keyword: *Ficus septica* Burm F, ficin enzyme, temperature, pH

Introduction

The ficin enzyme (EC 3.4.22.3) is a protease enzyme that can be obtained from the latex of the ficus plant group. Based on its chemical properties, the ficin enzyme is classified as a sulfhydryl protease enzyme, which is an enzyme that has a sulfhydryl group (SH) on the active side (Pourmorad *et al*., 2011). Protease enzymes from plants have been widely used in industry, food, medicine, biotechnology, and pharmacology (Siota and Villa, 2011). Safriani *et al*., (2011) reported that crude ficin enzymes from *Ficus hispida* L can be used in the extraction of virgin coconut oil.

Protease can be produced by plants (43.85%), bacteria (18.09%), fungi (15.08%), animals (11.15%), algae (7.42%), and viruses (4–41%). Plants are the largest source of protease enzyme (up to 43.85%). Plant protease plays a very large role in industry and has not been fully replaced by microbial enzymes. An enzyme produced from animal tissue is relatively expensive and its availability depends on the demand for the animal so that the source of the enzyme in the market is limited. Protease enzymes can be produced from fresh fruit of *Carica papaya* latex after scratches on its skin. On the other hand, with the increasing use of protease in the food industry, the availability of protease enzyme is insufficient. Therefore, it is necessary to find other sources of protease (Witono *et al*.,2006). The latex from the ficus group has a proteolytic activity. There are more than 1.800 species of *Ficus* and over 800 types of *Ficus carica*, with each variety having a specific proteolytic activity (Pourmorad *et al*., 2011).

Ficus septica is found in the tropics, such as Indonesia and its surrounding areas. The plant is very abundant in Indonesia, and

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usually used as a hedge plant. *Ficus septica* plant contains protease enzyme, antifugal, antibacterial, anticancer, and alkaloids. Ficin enzyme has a structure in which the sulfhydryl group containing these groups works as a proteolytic. Besides having proteolytic activity, ficin also has a function as a preservative (Pourmorad *et al.,* 2011).

This research aims to isolate and determine the characteristics of the ficin enzyme obtained from the latex of the *Ficus septica* stem. This research is also expected to encourage the discovery of a new source of protease enzyme in *Ficus septica* stem latex. That is because the enzyme needs a protease that is increasing both for industrial, food, and health. The isolation of ficin from *Ficus septica* stem latex beacuse stem contain more latex than leaves and roots of *Ficus septica* plant.

Material and Methods

This research took place in March–May 2015 in the Chemistry and Biology laboratory, Semarang State University. The sample used in this research is the *Ficus septica* young stem latex from Sekaran village in Gunungpati Subdistrict, Semarang.

Isolation of ficin enzyme from **Ficus septica** *stem latex (Pourmorad et al., 2011)*

In this research, the ficin enzyme was collected from the young stems of *Ficus septica* at 5 am. *Ficus septica* stem latex was placed into a microtube containing 1.5 ml phosphate buffer (pH 7), 6 mM EDTA, and 6 mM cysteine. Then, the *Ficus septica* stem latex was centrifuged at a speed of 11,000 rpm for 30 minutes at 4°C (Walti1938). The *Ficus septica* stem latex was fractionated using column chromatography (3 cm × 70 cm) over silica gel and eluted using hexane. For the fractionation, the solvent polarity was increased using chloroform and methanol with certain comparison (Table 1.).

Table 1. Comparison of solvents used to separate *Ficus septica* stem latex fractions using column chromatography

Fraction	Eluent
1–25	$CHCl_3$-CH_3OH 95:5
26–34	$CHCl_3$-CH_3OH 90:10
35–50	$CHCl_3$-CH_3OH 80:20
51–55	$CHCl_3$-CH_3OH 70:30
55–70	$CHCl_3$-CH_3OH 50:50
71–101	$CHCl_3$-CH_3OH 40:60
102–200	CH_3OH

Preparation of standard tyrosine curve (Hasibuan et al., 2014)

A standard tyrosine solution was diluted to 200, 400, 600, 800, and 1000 ppm. Then each solution's absorbance was measured using a spectrophotometer with a wavelength of 280 nm. Based on the tyrosine absorbance data of the solution at various concentrations, a tyrosine standard solution curve was made between tyrosine concentration against absorbance by Beer–Lambert law. Following this, we devised a tyrosine standard curve regression equation expressed as $y = ax + b$, provided y is absorbance and x is the concentration of tyrosine.

Determination of the optimum temperature and pH for ficin enzyme activity (Gagaoua et al., 2014; Perello et al., 2000)

Determination of the optimal temperature and pH for protease activity in *Ficus septica* was performed at various temperatures (40°C, 50°C, 60°C, 70°C) and pH (6.0, 7.0, 8.0) with a 20 minute incubation. Then a solution of enzyme activity was tested at every level of temperature and pH. Optimum temperature and pH was then concluded based on which solution showed the largest protease activity.

Protease activity test (Pourmorad et al., 2011)

A stock solution was prepared by taking 1 g of casein in 100 ml of a sodium phosphate buffer (0.1 M at pH 7) containing EDTA (0.001 M) and merkaptoetanol (0.007 M). The suspension was heated for 15 minutes in boiling water. 1 ml of crude ficin enzyme that had been diluted with a buffer

poured into the tube in the waterbath. Then 1 ml of casein solution was added to the tube with an interval of 30 seconds and incubated at various temperatures (40°C, 50°C, 60°C, and 70°C) and pH levels (6, 7, and 8) that has been set for 20 minutes. Furthermore, the reaction was stopped by adding 3 ml trichloroacetic acid (5%) into the tube. The solution was mixed until homogenous in the waterbath, left to settle for 1 hour, and then centrifuged at 4500 rpm speed for 15 minutes. Solution absorbance was measured using a spectrophotometer at a wavelength of 280 nm. Absorbance data were plotted on a tyrosine standard curve to calculate the activity. Proteolytic enzyme activity was calculated by the following formula:

$$\text{Protease activity} = \frac{[\text{Tyrosine}]\times V}{p \times q \times Fp}$$

Specification:
[Tyrosine]: concentration of tyrosine is formed
v: volume of the sample in each tube (mL)
q: incubation time (minutes)
p: enzyme volume (mL)
Fp: dilution factor

Results And Discussion

Isolation of ficin enzyme from Ficus septica *stem latex*

Ficin enzyme isolated by column chromatography had the highest activity (164,52 U/ml) at fractions 102–200 with a methanol eluent (Table 2). Indicating that this fraction of proteins was the most numerous and most of these proteins were ficin enzyme. Ficin enzyme activity calculation results in fractions 1–25, 26–34, 35–50, 51–55, 55–70, 71–101, and 102–200 are presented in Table 2.

Table 2. Ficin enzyme activity results following column chromatography with different eluents

No	Eluent	Fraction	Protease activity Unit/ml
1	$CHCl_3$-CH_3OH 95:5	1–25	5,74
2	$CHCl_3$-CH_3OH 90:10	26–34	13,51
3	$CHCl_3$-CH_3OH 80:20	35–50	18,91
4	$CHCl_3$-CH_3OH 70:30	51–55	27,70
5	$CHCl_3$-CH_3OH 50:50	55–70	35,47
6	$CHCl_3$-CH_3OH 40:60	71–101	131,75
7	CH_3OH	102–200	164,52

Determination of the optimum temperature and pH for ficin enzyme activity

Quantitative testing of ficin enzyme activity was conducted by statistical calculation. The homogenity test results showed that ficin enzyme activity during the temperature and pH treatments was homogeneous. Based on the test results of two-way ANOVA with a factorial pattern 3 × 4, the temperature treatment significantly affected the ficin enzyme activity. However, the pH treatment and interaction between temperature and pH did not significantly affect ficin enzyme activity.

Further test results based on Least Significant Difference (LSD) column (temperature) indicate that there was no significant difference between the incubation temperatures of 40°C and 50°C in the terms of ficin enzyme activity, nor did it differ significantly at 60°C and 70°C. On the other hand, a significant difference in ficin enzyme activity was found between the incubation temperatures of 40°C/–50°C and 60°C/–70°C (Table 3).

Table 3. Ficin enzyme activity at different temperatures and pH during incubation

pH	Temperature (°C)			
	40	50	60	70
6	405.31	390.12	756.65	716.57
	404.34	433.12	667.13	764.66
	452.20	386.56	687.17	649.77
Average	**420.61**[a]	**403.26**[a]	**703.65**[b]	**710.33**[b]
7	441.53	451.23	725.25	791.38
	428.59	442.50	689.17	744.62
	361.34	481.30	713.22	799.39
Average	**410.48**[a]	**458.34**[a]	**709.21**[b]	**778.46**[b]
8	387.53	417.92	743.28	731.94
	373.30	415.01	697.87	761.32
	375.57	399.82	792.72	739.27
Average	**378.80**[a]	**410.91**[a]	**744.62**[b]	**744.17**[b]

[a,b] Indicates the test results of LSD differed significantly ($p<0.05$).

The ability of the proteolytic ficin enzyme to the substrate is expressed in tyrosine units, which is the amount of enzyme required to liberate 1 mg of tyrosine per minute per mg of powder in experimental conditions. Addition of cysteine in the enzyme preparation of ficin from *Ficus septica* stem latex was to increase the activity of ficin enzymes. Ficin enzymes have active thiol groups (S-H) which will form disulfide bonds (S-S) with the amino acid site (S-H). Cysteine reduces the disulfide bonds to form a thiol or sulfihidril back, so it can increase the effective breakdown of the peptide protein bonds (Iswanto *et al.*, 2009). Ficin enzyme activity was tested with a spectrophotometer at a wavelength of 280 nm. A wavelength of 280 nm is the wavelength that receives a strong affinity with amino acids that have an aromatic ring such as cysteine. tryptophan and tyrosine (Layne, 1957).

Optimum ficin enzyme activity in this research occured at 60°C, showing that the ficin enzyme is resistant to high temperatures (thermophylae). According to the results of Gagaoua *et al.* (2014), the optimum temperature and pH of ficin in Ficus carica is 60°C and pH 6.5, respectively. Perello *et al.* (2000) showed that the optimum ficin enzyme activity of *Ficus pumila* is at 60°C, with decreasing activity at 75°C.

The speed of a chemical reaction increases in accordance with increasing temperatures during incubation because it can accelerate the thermal motion of molecules and parts of molecules that would increase energy in sufficient quantities to enter a state of transition. Ficin enzyme stability was indicated by decreasing enzyme activity after incubation for 20 minutes. At high temperature, ficin decreased its activity, possibly because of it enzyme incurred damage resulting in loss of catalytic activity (Soeka *et al.*, 2011). The process of inactivation of the enzyme at extremely high temperatures lasted through two stages, starting with the partial opening of the secondary structure, tertiary and quaternary enzyme molecules due to rupture covalent bonds and hydrophobic bonds. Further, enzyme primary structure changes because damage of certain amino acids by heating (Masfufatun, 2009).

Protease activity increases along with increasing temperatures untill the optimum temperature is reached, after which a further increase in temperature will cause decreased protease activity. At lower temperatures than the optimum temperature, the enzyme activity is also low. That is because lower activation energy is available. The kinetic energy needed to the conditions of active complex level, both of the molecules enzyme and substrate molecules. Hames and Hooper (2000) stated that temperature affects the rate of enzyme catalysis reactions in two ways:

Increased temperatures will increase the thermal energy of substrate molecules, which will increase the rate of enzyme reactions. Increased temperature also affects the conformational changes of the substrate so that the substrate actively experience barriers to entering the active site of the enzyme and causes a fall in the activity of the enzyme.

Increasing molecule's thermal energy also increases the likelihood of breaking the non-covalent interactions (hydrogen bonding, van der Waals bonding, hydrophobic bonding, and electrostatic interactions) that keep the three-dimensional structure of enzymes together, leading to enzyme denaturation. However, even small changes in an enzyme's shape can affect the active site's structure and result in a decrease in enzyme activity.

The structure of the enzyme molecule composed of amino acids containing a hydrogen bonds in the polypeptide chain. If the temperature around the enzyme increases, there is the possibility that this bond will be stretchable and so broken. Chemical bonds contained in the inner structure of the enzyme molecule are important factors in supporting the stability of the ficin enzyme tertiary structure. The deciding

factor thermal stability enzyme towards thermal is the non-covalent forces to protein molecules which retain secondary and tertiary structure. This force is reflected by hydrogen bonding, electrostatic forces, and hydrophobic interactions. Non-polar groups contained in the amino acid will interact with one another. The increase also due to the nature of thermostable disulfide bridges that can maintain an active conformation of the enzyme (Sebayang, 2006).

The enzyme has an optimum pH characteristic, which can produce the maximum activity in catalyzing the reaction. Like other proteins, the enzyme structure consists of the amino acid polymer that has a positive, neutral, or negative charge. It causes the performance of the enzyme to be affected by the presence of H^+ ions (acid) and OH^- (base) (Ngili, 2010). The effect of pH was tested at pH 6.0, 7.0, and 8.0, and the optimum enzyme activity of ficin in this research was at pH 6–8. It shows that at pH 6–8 ficin enzyme from *Ficus septica* stem latex was able to work optimally to bind to the substrate and form an complex in order to produce maximum results. In this research the pH 6–8 is an optimal conditions for enzyme to bind with substrate, because pH determines the conformation of an enzyme based on the circumstances of the amino acid at the active center (Herdyastuti, 2006). The more distant the pH from this optimum, the lower the activity. It could be because the enzyme is a protein composed of amino acids, the effect of pH is associated with acid-base properties owned by the protein. An extreme pH may cause denaturation of the protein enzyme, which can change the three-dimensional spatial arrangement of the protein molecules in the enzyme (Witono *et al.*, 2007). Determination of the optimum pH of ficin from *Ficus septica* stem latex in this research was consistent with the results of Gagaoua *et al.* (2014), who stated that the optimum ficin enzyme activity in *Ficus carica* is occured at pH 6.5. Perello *et al.* (2000) showed that the optimum ficin enzyme activity of *Ficus pumila*is was at pH 7–8.

Changes in pH affect the activity of enzymes and can change the structure or amino acid residues charged to substrate binding functions. Varying pH levels can also cause conformational changes of enzymes, because the charged group (-NH3 or -COO-) away from the bound substrate region serves to maintain the tertiary structure of the enzyme. The tertiary structure of the enzyme will change the charge at different pH levels. This will lead to the disruption of ionic bonds and the folding of enzyme so that the enzyme changes conformation and enzyme activity is decreased (Masfufatun, 2009). The optimum pH measurement of enzyme is necessary to adjust the use of enzymes. The enzyme activity at optimum physiological pH can be utilized in various fields, for example for the treatment of a disease (Sajuthi *et al.*, 2010).

Ficin enzyme has very low activity at very acidic pH levels (pH 3–6). That is because functional groups in the active site are disrupted by the excessive H^+ ions. At pH 7–9. the enzyme activity is relatively maximum because the enzyme reaches the expected degree of ionization. At pH 10–12, enzyme activity is decreased due to an excess of OH^- ions (Hames and Hooper 2000).

Conclusion

We conclude that ficin enzyme can be found in *Ficus septica* Burm F. stem latex and has an optimum temperature of 60°C and optimum pH of 6, 7 and 8.

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