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Protease from Courgette (*Luffa Acutangula L* (*Roxb*)): Isolation, Purification, and Some Characteristics

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The Courgette or oyong (*Luffa Acutangula L.* (*Roxb*)) is member of *Cucurbitaceae* mainly used as vegetable. Beside used as vegetables, courgette aslo used as keratolytic agent. This fact is supposed that this vegetables contain protease. This research is succeed to purified courgette's protease by four step. That was precipitate by 70% ammonium sulphate saturation, purification using DEAE cellulose ion exchange chromatography and gel filtration chromatography using sephadex G-100 and G-75. Purified courgette's protease had 81,922 U/mg for specific activity and 34 kDa molecular weight. This enzyme had the characteristic such as activated optimally at 37°C, pH 7 and 10 minute duration time. This enzyme activity can decrease by PMSF and H_2O_2, its remarkable that courgette protease is serine protease and had the thiol group in its structure. The ability to digest food proteins materials like boiled meat and boiled white egg by courgette protease proves that the courgette protease enzyme is could be used in enzyme replacement therapy in mild digestion problem.

Key words: *Characteristic protease, Courgette, Luffa acutangula L (Roxb), protease purification, serine protease*

INTRODUCTION

Protease is one of hydrolases class enzyme, that is able to break peptide bonds on various types of proteins by hydrolysis and converting proteins into simple peptides or single amino acids. (Naidu 2011) Protease has many roles in physiology, it is also widely used for several objective. Proteases are widely distributed in nature, as in animal, both in plant and microorganism. In present study, the use of protease from plants is still focused to bromelain from pineapple (Ketnawa *et al.* 2011) and papain from papaya (Amri and Mamboya 2012). However, several studies were done on other sources especially from *Cucurbitaceae* class. It was realized in cucumber (Nafeesa *et al.* 2017), melon (Devi and Hemalatha 2014), pumpkin (Dąbrowska *et al.* 2013), Bitter gourd (Wang *et al.* 2008), and Chayote (Ratnayani and Lia 2011). some of them are use traditionally for cosmetics objectives.

The Courgette or oyong (*Luffa Acutangula L.* (*Roxb*)) is member of *Cucurbitaceae* mainly used as vegetable. Courgette is used in Indonesia beside as vegetable also as cosmetics agent, I.a as keratolytic agent in tradiotionals facial cure. Because of the fact that courgette is member of *Cucurbitaceae*, and because it is use as keratolytic agent, it is supposed that this vegetable contain protease.

The purpose of our study is to detect and isolate and characterize protease in the courgette.

MATERIALS AND METHODS

Material. The courgette were purchased from a farm near Jakarta and the species was determined in Indonesian Institue of Science (Center of Plant Conservation Botanic Garden). The chemicals are gelatin, KH_2PO_4, K_2HPO_4, NaCl, TCA, Sodium carbonate (Na_2CO_3), Folin Ciocalteus reagent, L-tyrosine, DEAE cellulose, sephadex G-100 and G-75, Tris-HCl, SDS, *Coomassie brilliant blue* R-250, glycerol, glycine, methanol, acetic acid, acrylamide, N, N' bisacryl, Ammonium persulphate, Calsium chloride ($CaCl_2$), Zinc Chloride ($ZnCl_2$), Magnesium Chloride ($MgCl_2$) and Cupro Chloride ($CuCl_2$), PMSF (Phenyl methyl sulfonyl fluoride), EDTA (Ethylendiaminetetra acetic), Iodoacetate, urea, β-mercaptoetanol and H_2O_2.

Methode.

Preparation of Crude Extract. 400 g of courgette were peeled, weighed and homogenized with a blender in 90 ml of 0.05 M pH 7 phosphate buffer. Then the homogenate was squeezed with flannel cloth and the juice obtained was centrifuged at 6,500 rpm in 4°C for 60 minutes.

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Assay of Protease Activity. One mililiter of sample was incubated for 5 minutes in 37°C then 1 ml of 1% gelatin substrate was added and the incubated continue for 10 minutes at the same temperature. The mixture was precipitated by addition of 2 ml 5% TCA and incubated for 10 minutes at room temperature. The end of this period the mixture was centrifugeted (6,500 rpm, 4°C, 10 minute). 1 ml supernatant was pippeted into a cup, followed by 5 ml of 4% Na_2CO_3 and 1 ml of Folin-Ciocalteu reagent and incubating for at 37°C. Absorbance was read at 660 nm. As blank we use the supernatant precipitate immiditially with TCA then followed by substrate and then the mixture centrifuge. Supernatant was preceed with the same treatment as a sample.

Assay of Protein Total Concentration. Determination of protein concentration is read by Warburg methode, which measure the absorption at 280 nm. As standard protein we used bovine serum albumin (BSA) concentration range from 0.1-0.5 mg/ml, distilled water as the blank.

Purification Protease Enzyme.

Protein Precipitation with Ammonium Sulphate. Two hundred milliliter were added ammonium sulphate crystals with a series of saturation (30, 50, 70, and 90%). The precipitates were collected after incubation at 4°C overnight and then centrifuged in 6,500 rpm, 4°C during 1 hours. The precipitate was redissolved in 0.05 M phosphate buffer pH 7 untill it reaches a volume of 30 ml. The solution was dialzsed with distilled water for 24 hours at 4°C. The protease activities and protein concentration were measured and the specific activity was calculated.

DEAE Cellulose Ion Exchange Chromatograpy. The dialysate with the highest specific activities was applied to DEAE cellulose coloumn (20 x 1.5 cm). The sample was eluted with 0.05 M phosphate buffer pH 7 (without NaCl) and followed by series the same buffer but containing a grading NaCl from 0.1-0.7 M. Fractions of 1.5 ml elution were collected and protein concentration was read at 280 nm. Fraction forming a peak were collected and assayed for protease specific activity. The fraction with the highest protease activity was lyophilized. (Asker *et al.* 2013).

Gel Filtration on Sephadex G-100 and G-75. One hundred milligrams of the highest protease activity from DEAE cellulose peak was applied to sephadex G-100 coulomn (20 x1.5 cm). Sample was eluted by 0.05 M phosphate buffer containing 0.4 M NaCl. G-100 peak with the highest specific activity was applied to sephadex G-75. (Gunarti 1997) The highest protease activity was lyophilized.

Polyacrylamide Gel Electrophoresis. Molecular weight was determined by non-denaturing electrophoresis and zymogram methode. Protein marker was used 10-250 kDa. (Nafeesa *et al.* 2017) Non denaturing electrophoresis was performed in polyacrylamide gel 10% at 90 V for 2 hours. Gelatin zymography was performed to test the proteolytic activity of the purified fraction with minor modifications polycarilamide gel 10% and co-polymerized with 0.5% gelatin. The sample were applied in non-reducing condition and run at 90 V for 2 hours in 4°C. The gel was washed with 2.5% Triton X-100 for 30 minute to remove SDS was incubated in 30 mM Tris-HCl pH 7.4, 200 mM NaCl, and 10 mM CaCl for 1 hours. Gel was stained with Coomassie brilliant blue R-250 for 30 minute and then gel was washed with de-stained solution. Protease activity's area was appeared as non-stained clear band on a dark blue background.

Effect of Temperature, pH and Incubation Time on Enzyme Activity. To determine the optimum of pH, temperature and incubation time, the enzyme was reacted with the gelatin substrate 1%. The optimum incubation time was determine by incubating the mixture of enzyme–gelatin substrate, and the mixtures were stopped after various time (0-2 hours). (Wang *et al.* 2008) The optimum pH was determine by incubating the mixture in various pH ranging from 4 to 10. The optimum temperature was studied by incubating the mixture in optimum pH and optimum incubation time with various temperature ranging from 4 to 90°C. (Wang *et al.* 2008; Nafeesa *et al.* 2017).

Effect of Metal Ion and Inhibitors on Enzyme Activity. To determine the effect of metal ion and protease inhibitors in protease activity. Protease was treated with $CaCl_2$, $ZnCl_2$, $MgCl_2$, and $CuCl_2$ as metals ion and Iodoacetate, urea and EDTA as protease inhibitors. Each concentration of metals ion and inhibitors was 1 and 5 mM. (Sulistyowati *et al.* 2016; Nafeesa *et al.* 2017).

Effect of oxidizing and reducing agents on enzyme activity. To determine the effect of oxidizing and reducing agents. Protease activity was treated with H_2O_2 as oxidant agent, and β-mercaptoetanol as redactor agent. Each concentration for reductor and oxidizers is 1 and 5 mM.

Determination of Km and Vmax Value for the Protease Enzyme. To determine the reaction rate (Vmax) and the Michaelis-Menten (Km) value, protease was reacted with gelatin substrate with a concentration range of 0.014-0.555 M in 0.05 M phosphate buffer solution pH 7. The result was made in Lineweaver-burk curve.

The Ability of Courgette Protease to Digest of Food Proteins. To determine the potential of protease to digest food protein. 0.5 g meat and egg were boiled with with 0.05 M phosphate buffer solution pH 7. Then the samples was incubate with crude and protease that had been purified for 5-60 minute. After incubation process was done, sample was weighed and tyrosine release was measured with activity protease procedure. The difference weight between before and after incubation and tyrosine release in medium must be recorded.

RESULT

Purification Protease. Crude extract were precipitate with ammonium sulphate with various saturation (Table 1). The result showed that the highest specific activity was measured in precipitate of 70% saturation. Therefore, the fraction of 70% saturation were used as starting material for following experiment.

Figure 1 shows the anion exchange using DEAE cellulose chromatography result. The highest activity was found in the fraction that eluted with phosphate buffer solution containing 0.4 M NaCl, which is found in the fraction number V. The highest specific activity from DEAE cellulose was applied to the gel filtration column with sephadex G-100 and G-75. Protease purification using sephadex G-100 resulted the two highest protein peaks. The highest specific protease activity was founded in fraction III presented in (Figure 2). Further molecular sieving chromatography using sephadex G-75 gave two highest peaks. The highest protease specific activity peak was founded in fraction II, which is presented in (Figure 3).

Figure 1 shows that from all of the three highest peaks, the peak with the highest protease activity is fraction number V with a protease activity 42,763 U/ml and total protein concentration is 1,105 mg/ml. Therefore, the highest specific activity is in fraction number 5 with a value of 42,763 U/mg.

Figure 2 shows that highest protease activity is in fraction number III, with the value of 63,715 U/ml and total protein concentration is 0.923 mg/ml. Therefore, the highest specific activity is 69,001 U/mg.

Table 1. Spesific activity after salting out and dialysis

Fraction (%)	Total protein concentration (mg/ml)	Protease activity (U/ml)	Specific activity (U/mg)
30	3,790	25,362	6,693
50	7,692	40,744	5,297
70	6,639	62,207	9,371
90	2,731	5,586	2,045

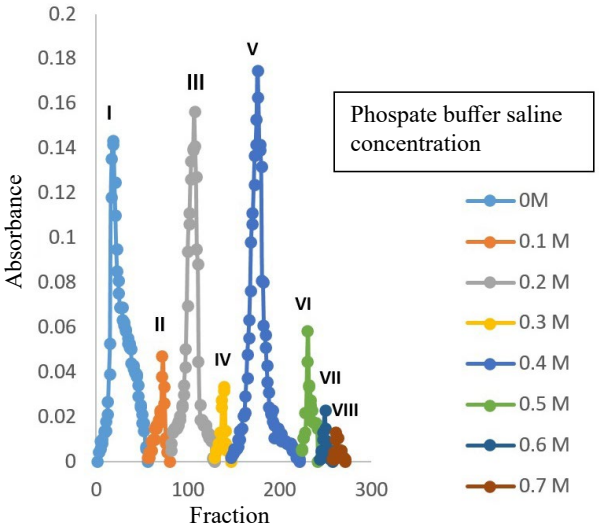

Figure 1. Fractionation using DEAE cellulose chromatography column using various buffer phosphate saline concentration

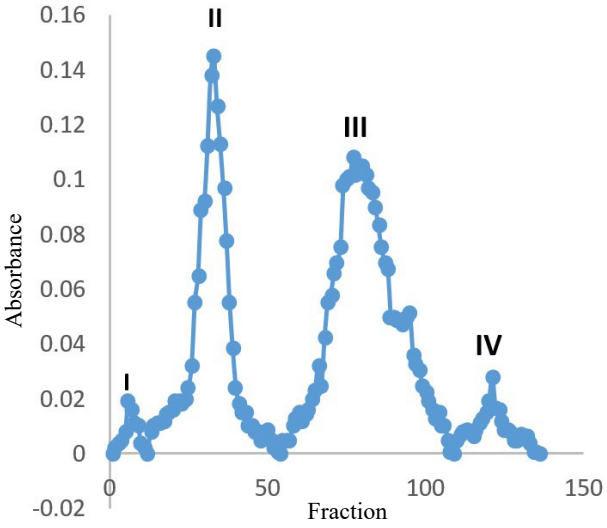

Figure 2. Fractionation with filtration gel column chromatography using sephadex G-100

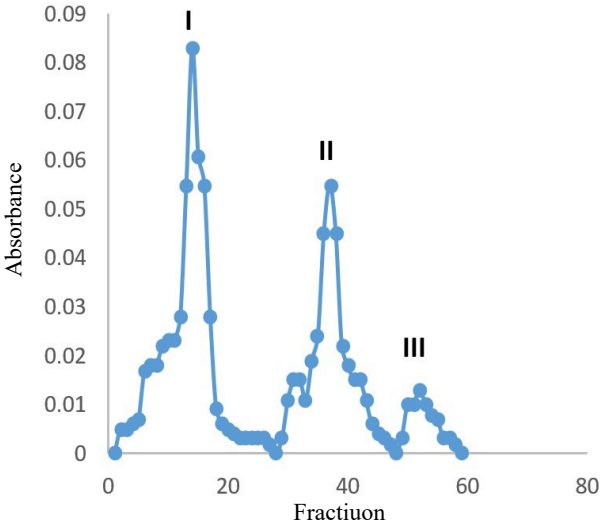

Figure 3. Fractionation with filtration gel column chromatography using sephadex G-75

Figure 3 shows that there is the highest protease activity at peak II with an activity of 53,903 U/ml and a total protein concentration of 0,684 mg/ml. therefore the specific activity obtained is 81,922. from this figure it can be stated that there was an increase in specific activity after purification.

Protein Weight Determination. Purified courgette protease from sephadex G-75 chromatography (G) had a single band of 34 kDa. The single band was resulted from standar curve semilog with the equation y = -1,9012x + 5,328. Protein weight determination was obtained using non-denaturing electrophoresis gel. The results of the gelatin zymogram show that there is a specific zone in the same position with a single band obtained in non-denaturing electrophoresis gel. Molecular weight of purified protease are presented in Figure 4.

Optimum Temperature, pH and Duration of Incubation Time. Figure 5 shows that courgette protease is active in the pH range 6-8. However the optimum pH at 7 with the highest activity is 144,661 U/ml.

Figure 6 shows that courgette protease had the highest activity at 37°C with activity value is 56,356 U/ml. While 40 and 50°C the activity is slowly decrease.

Figure 7 shows that optimum duration incubation time is 10 minute with the activity is 47,898 U/ml.

Effect of Metal Ions and Protease Enzyme Inhibitors on Protease Enzyme Activity. There were no siginificant influences of the addition of various metal ions on enzyme activity. Some compound had various influences on enzyme activities. Urea, iodoacetate and EDTA practically had no influence at all. These suggest that the enzyme is a single polypeptide, neither a cysteine protease nor a metalloprotease. However PMSF had a clear inhibition, which suggest that the enzyme was a serine protease. The results were presented in Table 2.

Effect of Reducing and Oxidizing Agent to Enzyme Activity. Addition of H_2O_2 reduced the activities to 40% of control, whereas reducing agent as mercaptoethanol practically had no influence. The data suggest that the enzyme have reduced cysteine moiety for maintaining the proper structure. On the other hand, the enzyme did not contain intramolecular -S-S- bridge, as indicated by no influenced of mercaptoethanol. The results of this experiment are presented in Table 3.

Determination of Vmax and Km. A lineweaver-burk curve was constructed for determining Km and Vmax. The equation obtained was y = 0.0002x + 0.0059 the calculated Km was 0.0339 M/ml and Vmax was 164,491 U/M/minute. The result was served in Figure 8.

The Ability of Protease Enzyme to Digest of Food Proteins. The aim of this experiment was to see if the courgette protease could be used in enzyme replacement therapy in mild digestion problem. Using boiling meat and boiling white egg. The result were evaluated in 2 ways by weighing periodically the incubated boiling meat and boiling white egg, and by reading tyrosine release into the medium at the same period. Figure 9 is presenting the weight reduction result and table 4 shows that protease could digest both food materials, and the

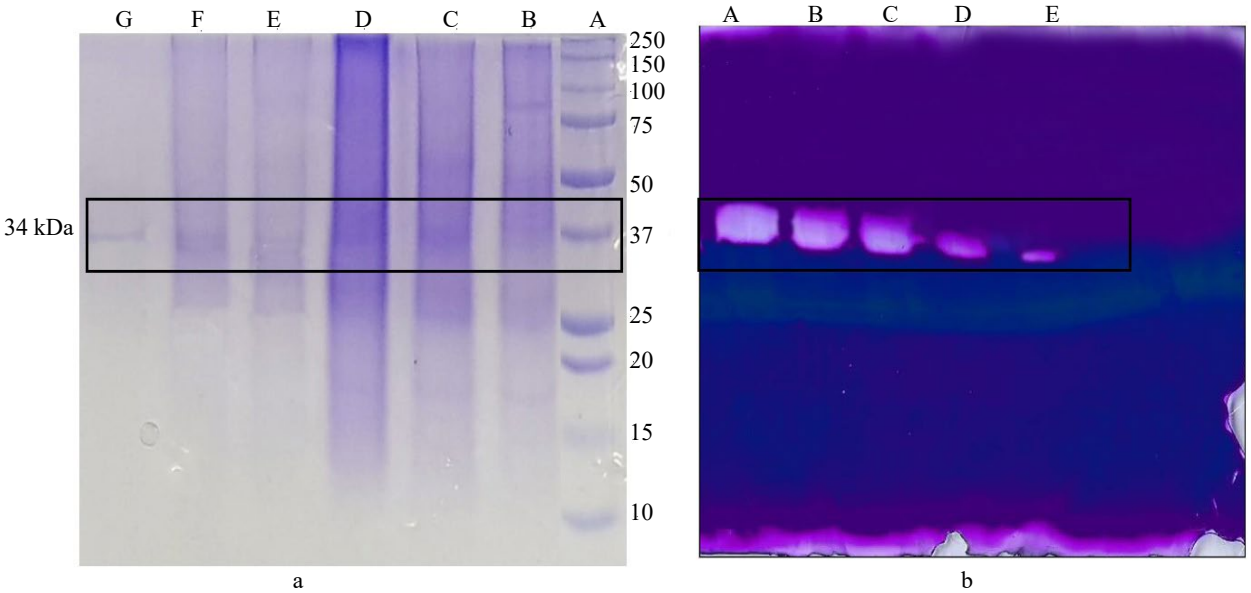

Figure 4. Protein weight determination using electrophoresis. (a) non denaturan electrophoresis result. A protein marker, B Crude, C Dialysate 70%, D fraction V DEAE Cellulose, E and F. Fraction III sephadex G-100, G Fraction II sephadex G-75. (b) zymogram electrophoresis result A Crude, B Dialysate 70%, C fraction V DEAE cellulosa, D Fraction III sephadex G-100, E Fraction II sephadex G-75

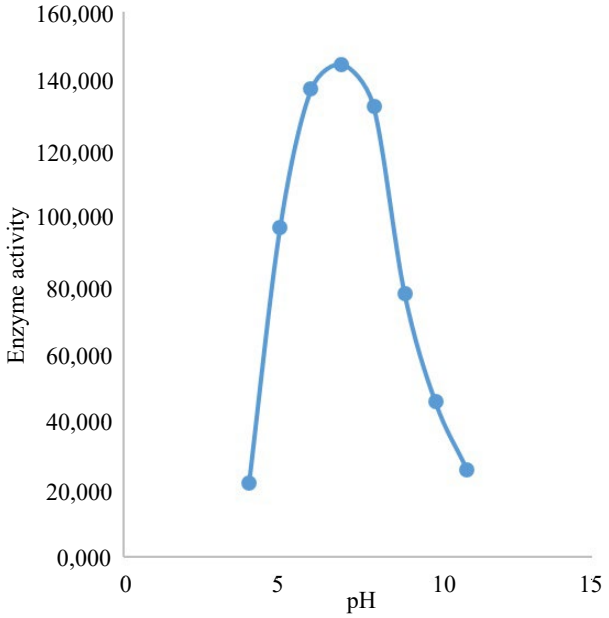

Figure 5. Effect of pH on protease enzyme activity. Activity is presented in enzymes units

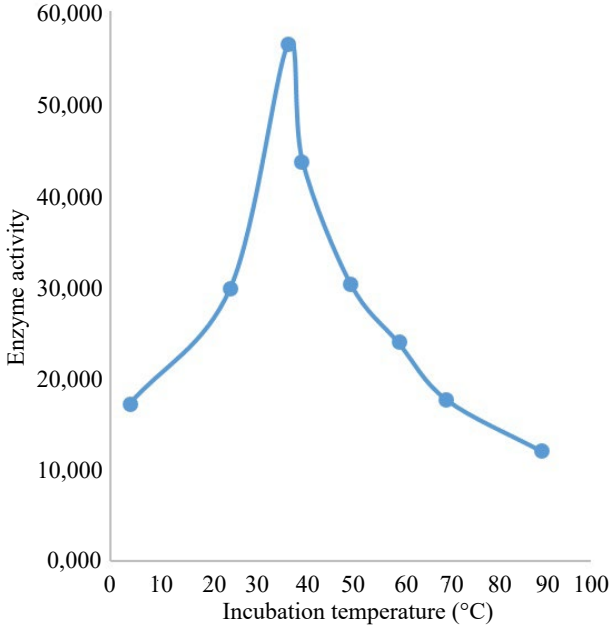

Figure 6. Effect of temperature on protease enzyme activity. Activity is presented in enzyme units

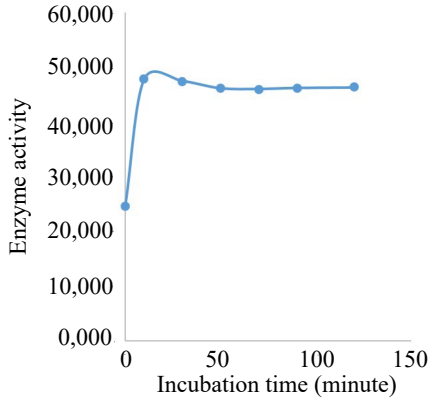

Figure 7. Effect of duration incubation time on protease enzyme activity. Activity is presented in enzyme units

Table 2. Enzyme activity after incubation with various metal ions and protease inhibitors

Metals ion	Relative activity (%)	Inhibitor	Relative activity (%)
Control	100	Control	100
ZnSO4 1 mM	95	Urea 1 mM	97
ZnSO4 5 mM	97	Urea 5 mM	98
$CaCl_2$ 1 mM	97	Iodoasetat 1 mM	97
$CaCl_2$ 5 mM	88	Iodoasetat 5 mM	98
$MgCl_2$ 1 mM	90	PMSF 1 mM	33
$MgCl_2$ 5 mM	94	PMSF 5 mM	15
$CuCl_2$ 1 mM	92	EDTA 1 mM	96
$CuCl_2$ 5 mM	98	EDTA 5 mM	114

Table 3. Enzyme activities after incubating with reducing and oxidizing agents

Oxidizing agent	Relative activity (%)	Reducing agent	Relative activity (%)
Control	100	Control	100
H_2O_2 1 mM	50	merkaptoetanol 1 mM	91
H_2O_2 5 mM	41	merkaptoetanol 5 mM	100

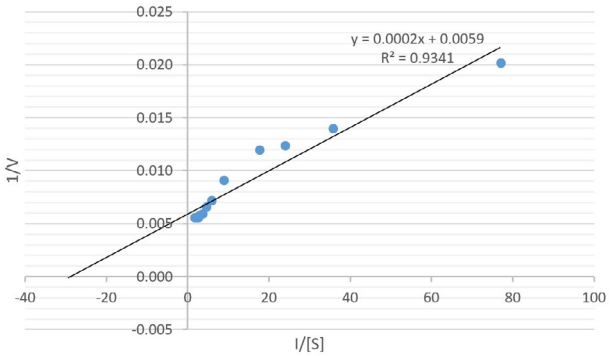

Figure 8. Lineweaver-burk curve

purified enzyme was more effective than crude enzyme.

DISCUSSION

Courgette protease isolation was carried out using ammonium sulfate with various saturations, namely 30%, 50%, 70%, and 90%. The highest protease activity was found in precipitates 70% saturation. This resembled with melon protease, which was precipitate 70% saturated (Devi and Hemalatha 2014). However, other member of *Cucurbitaceae* were precipitate in other saturation. Proteases from bitter gourd, cucumber, and chayote each precipitated in 40%, 90%, and 50% (Wang *et al.* 2008; Ratnayani and Lia. 2011; Nafeesa *et al.* 2017). Fruits of other types such as pineapple require ammonium sulfate with 80% saturation to precipitate proteases in the test solution (Silvestre *et al.* 2012). Our result and melon protease suggested that the protease was probably have a small size, smaller than bitter gourd and chayote. We decided

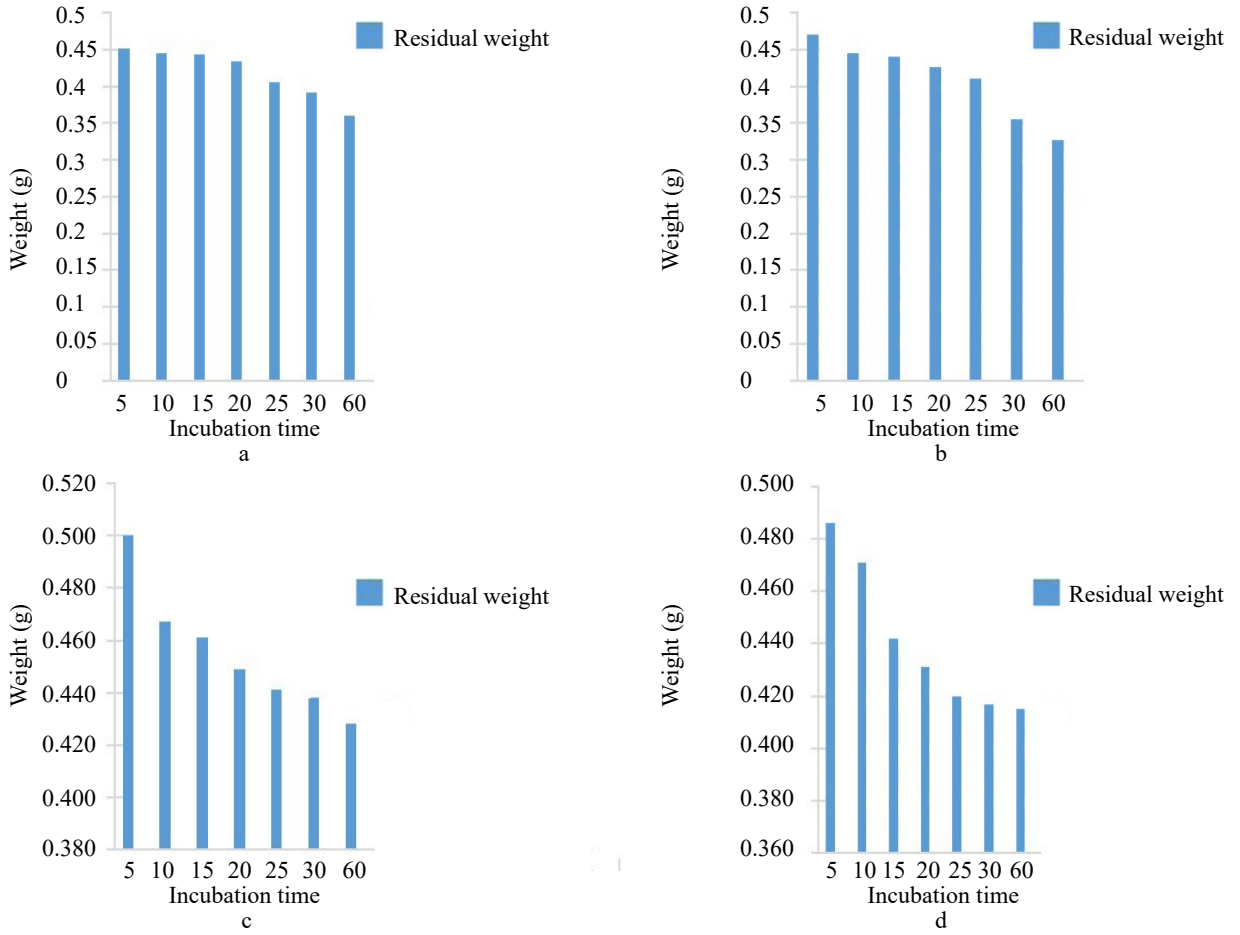

Figure 9. Results of depreciation the weight of boiled meat and eggs after incubation with crude and purified protease from courgette. (a) weight reduced of boiled meat after incubation with crude, (b) weight reduced of boiled meat after incubation with purified protease, (c) weight reduced of boiled white egg after incubation with crude, (d) weight reduced of boiled white egg after incubation with purified protease

Table 4. Free tyrosine concentrations in boiling meat and boiling white egg medium various time of incubation (µg/ml)

Time (Minute)	Boiled beef (crude)	Boiled beef (pure protease)	Boiled white egg (crude)	Boiled white egg (pure protease)
5	149,102	121,463	90,491	80,907
10	242,528	135,213	125,676	87,852
15	237,574	148,963	129,102	99,287
20	237,343	170,769	172,620	99,333
25	251,509	170,954	172,667	138,361
30	258,083	172,991	182,250	104,704
60	256,741	177,667	199,380	113,824

to use the 70% as a starting material for following process.

Courgette protease purification using two types of chromatography, that are DEAE cellulose ion exchange chromatography and gel filtration chromatography using sephadex G-100 and G-75. Research using two types of sephadex was also carried out on the purification of proteases from melons (Devi and Hemalatha 2014). The results obtained from gel filtration chromatography produced the higher specific activity and more purer than cellulose DEAE. The disadvantage of using two types of chromatography is make the sample more dilute. this is proven by zymogram (Figure 4b) which shows a smaller specific zone when the protease is successfully purified. Proteases that have been purified from gel filtration chromatography using sephadex G-75 produce a single band with a molecular weight of 34 kDa after separation using non-electrophoresis denaturation (Figure 4).

Table 2 shows the effect of metal ions and protease inhibitors on protease activity. Metal ions on protease activity especially metalloproteinase is acting as electrophiles in enzymes, metal ions play a role to help attach the substrate to the enzyme and act as electron donors in oxidation-reduction reactions (Riordan *et al.* 1977). Courgette protease apparently was not a metalloproteinase, as indicated by no influence of metal ions on the action (Table 2). Iodoacetate had also no influenced, which suggested that courgette enzyme was not a cysteine protease. It is well known that iodoacetate can react with -SH-group and replace the hydrogen with iodoacetate. If the -SH- group is found in active site like cysteine

protease, the enzyme activity will be reduced and it was not found in our observation. Several plant proteae, like bromelain and papain are cysteine protease (Amri and Mamboya 2012; Fadhilah *et al.* 2018). However, several studies regarding protase *Cucurbitaceae* that have been reported, no fruit of *Cucurbitaceae* is belonging to the cysteine protease was found. PMSF had a remarkable decrease of our enzyme. This fact suggested that courgette protease was probably a serine protease, as PMSF is well known as serine protease inhibitor (Sharma and Radha 2011). Some *Cucurbitaceae* fruits that have been reported include serine proteases such as melon, pumpkin, cucumber and chayote. (Ratnayani and Lia 2011; Dąbrowska *et al*. 2013; Devi and Hemalatha 2014; Nafeesa *et al.* 2017).

The effect of oxidizing and reducing agent to courgette activity are show in Table 3. From this result known that courgette protease can oxidized by H_2O_2, its was demonstrated that courgette protease had the free thiol group in its side chain amino acid. H_2O_2 can oxidizing thiol group so that release H_2O and –S-S- bridge, this condition would make the activity of enzyme reduce (Zeida *et al.* 2012). Mercaptoethanol is act to reducing agent because could make the cleavage of disulfide bridge in protein structure, its can effect to native protein structure. In our obervation, mercaptoethanol had no influence to make a reducing activity protease, its known that courgette protease had no disulfide bridge in their structure (Scigelova *et al.* 2001).

The Km and Vmax value were determined using a gelatin substrate in various concentrations of 0.014-0.555 M. Lineweaver-Burk line equation was constructed and gave an equation y = 0.0002x +0.0059. which resulted Km value of 0.0339 M/ml and Vmax value of 164,491 U/M/minute. Courgette Protease had a Km value greater than the cucumber protease as much as 2.1 x 10-4 M/ml (Nafeesa *et al.* 2017) Km is reflects the enzyme affinity to the substrate. The smaller Km value shows that the greater the affinity. Vmax is indicated the maximum capacity of an enzyme in catalyzing the substrate. (Sadikin 2002; German *et al.* 2011).

Courgette protease enzymes had the ability to digest food proteins properly. This was demonstrated by experiments with food protein materials in the form of beef and boiled egg whites which incubated with courgette crude as well as purified enzymes. Both form enzyme worked on both food materials and purified enzyme worked better than raw enzyme. These result were demonstrated by weight reduction and the increase of trypsin release in supernatant of medium (Figure 9). Purified courgette's protease can reduced beef meat as much as 0.173 g or 36.4% for 1 hours.The other research was reported that papain and bromelain can digest beef meat as much as 25.46% and 26.81% in less than 24 hours. (Istrati *et al.* 2012) In this case the courgette protease is better than bromelain and papain in digesting food proteins. Trypsin and pepsin had the ability to beef meat as much as 80% for 2 hours (Wen *et al.* 2015). This report is show that trypsin and pepsin is better that courgette protease in digesting food protein materials (Wen *et al.* 2015). The ability of courgette protease to digest food protein is also demonstrated by increase tyrosine release in supernatant of medium (Table 4). The greater of reducing sample's weight is more tyrosine release into supernatant. Courgette's crude can release tyrosine in medium as much as 256,741 µg/ml after reacting with boiled beef, while purified curgette protease is able to release tyrosine to the medium as much as 177,667 µg/ml. Both of these results indicate that courgette proteases better digest boiled beef proteins compared to boiled egg whites which only release tyrosine at 199,380 µg/ml by crude and 113,824 µg/ml by purified courgette proteases.

From all the experiments of this research, it can be concluded that the courgette protease is a serine protease and can be active optimally at neutral pH, 37^{o}C, 10 minute duration time and had lower Km value than cucumber Km. H_2O_2 had influence to make courgette activity was decrease. Its mean that courgette protease has thiol group in its structure. The ability to digest protein better than bromelain and papain, but is not better than pepsin and trypsin. This shows that courgette proteases could be used in enzyme replacement therapy in mild digestion problem but still need to be studied further about their application as proteases which act like digestive proteases. Finally, to get better result from courgette's protease we should more explore about the purification technique and characteristic to get purified protease with high concentrate and to know more about the type of courgette protease.

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