Protease Activity In Flesh Leaves Of Bidens Pilosa

Fidele Benimana, Irina Yurievna Potoroko, Dr Uday Bagale, Maniriho Olivier, Karege Felicien

Abstract— Proteases catalyze the hydrolysis of peptide bonds of a protein; they are present in many organisms such as plants, animals and microorganisms. Proteases perform various medicinal functions in humans they participate in wound healing, blood coagulation, digestion process and also can be used in antibody therapy and protease haves many other applications like in detergents, leather industry, pharmaceutical industry and bioremediation processes. The present study aimed at determination of protease activity from traditional medicine plant *Bidens pilosa* and study of incubation pH effect, *Bidens Pilosa* were collected in Rwasave wetlands. The leaves were ground in a mortar in an appropriate buffer. The protease activity was assayed by hydrolyzing casein as substrate by incubation at two different pH (pH4 and pH 10), at 30oC. The breakdown of casein results in releasing tyrosine. The tyrosine was colororated with the Folin-Ciocalteu reagent and read in a spectrophotometer at 660 nm and the levels were calculated, thanks to standard curve. The protease activity was expressed in mmol/mg leaves/min of incubation. The present study, shows that absorbance increase when the concentration of tyrosine increase and protease activity reported in terms of mM/mg/min in 2 different pH condition and it was found *Bidens Pilosa* presents higher Protease activity in acid pH 4 in comparison with alkaline pH10 its protease activity were 8.2567×10-7 mM/mg/min and 5.15×10-7 mM/mg/min respectively. This means that proteases are sensitive to pH and pH affect protease activity. The conclusion of the study states that fresh leaves of *Bidens Pilosa* contain proteases as common biological activity used in medicinal property and proteases from *Bidens Pilosa* can be used in acidic medium pH 4 than in alkaline medium pH 10.

Keywords— Protease activity, Casein, Bidens Pilosa, pH, protein, substrate, temperature

1 INTRODUCTION

Proteases or peptidases are enzymes that perform breakdown of protein, the proteolysis. Proteases break down peptides bonds in polypeptides that form (Anshu *et al*., 2007; Kalpana *et al*., 2008).They differ from each other by the way in which they carry out this activity(Feijoo *et al,*. 2010). Proteases constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market (Riddhi *et al.,* 2014). For several physiological processes the action of the proteolytic enzyme is essential, e.g. in digestion of food proteins where proteases digest proteins into small fragment which can be absorbed by the body like amylase which degrade starch into small fragments (Devlin. 2005). And they cleave a specific site on a protein so they can either turn them on or off. This can be a part of a mechanism for signaling a physiological change. Also proteases help in the processing of proteins that are produced in larger forms such as the amyloid precursor protein. Proteases are very useful in many domains like in medicine, industrial biotechnology, pharmacy. In medicine proteases are used in wound healing, blood clotting and also can be used as antibody. In wound curing, considerable proteases are the matrix metalloproteinases and the serine proteases, eg elastase. By the way, different wound-related proteases work on different proteins. Among them are extracellular matrix and connective tissue proteins such as, gelatin, collagen, and elastin.

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In wound healing process, proteases break down damaged extra cellular matrix proteins and invading material in order that new tissue can form and wound closure can occur in an orderly fashion. But when the protease activity is too high the balance between tissue breakdown and repair is disordered (Baker *et al*., 2003). In medicine protease are used as antibiotic therapy because some pathogenic bacteria produce biofilms or exudates containing proteins in which some degree help the bacteria adhere to the host tissue or in some degree physically shield the bacteria or hinder the penetration of substance such as antibiotic administered with the intent, so protease digest those proteins(Mecikoglu *et al.,* 2006). Proteases do many varieties of functions and they play a big role in biotechnological applications. Peptidases is one of the three biggest groups of industrial enzymes and find application in washing dirts from clothes, food industry, leather industry, pharmaceutical industry and to remove contamination from waste. (Anwar and Saleemuddin, 1998; Gupta *et al*., 2002). the largest application of proteases is in laundry detergents, they help removing protein- based spot from clothing (Banerjee *et al*., 1999) In case of removing blood stain from cloth, it was seen that the protease can remove blood spots very easily without addition of any detergent . Proteases showed high capability for removing proteins and stain from cloth. The ability of proteases to work in the presence of solvents and detergents can be maximized exploited for this purpose. Saleemuddin and Anwar, 1997 showed the importance of protease from *Spilosoma obliqua* for removal of blood spots from cotton cloth in the presence and absence of detergents.

2 MATERIALS AND METHODS

2.1Materials

Refrigerator, test tube, centrifuge, mortar and pestle, buffer homogenizer, incubator, paper filter, pipettes, electric balance, pH meter, gloves, blouse, glasses, hygienic paper, volumetric flasks, graduated cylinder, spot plate, stirring rod, volumetric pipette, spatula, test tube rack, ring stand, Erlenmeyer flasks, weighing paper, clock.

2.2Reagents Preparation

Reagent to be used are: 1M sodium hydroxide, Folin and Cioucalteu'sreagent,1% Casein, 2% Sodium Carbonate, 5% Trichloroacetic acid and 0.1M citric acid, Sodium potassium tartrate, 1% copper sulfate, tyrosine, 0.1M potassium phosphate monobasic, distilled water, sodium 0.2M dihydrogenophosphateanddisodium monohyrogenophosphate.

Buffer for protease extraction:
Our process was done on pH4 and pH10 scales (acidic and basic pH). On one side, the citrate phosphate buffer was used to provide acidic buffer which was prepared by adding citric acid 0.1M to disodium monohyrogenophosphate 0.2M.
On other side basic pH was obtained by adding disodium monohyrogenophosphate 0.2M to sodium dihydrogenophosphate 0.2M.

Casein preparation :
Potassium phosphate monobasic (6.8g) was dissolved in one liter of distilled water to yield 0.05M and pH value equals to 7.5 By this solution, 1% of casein solution was prepared by mixing 10g of casein into one liter of potassium phosphate monobasic solution. The dissolution was completely by gently heating till 850c and will be kept at 45oc before its use.

Alkaline and acidic solution preparation:
During this process both alkaline and acidic solution was used. In this research 5% trichloroacetic acid was needed and it was prepared by dissolving 4.07g in 100ml of distilled water.
An alkaline reagent was prepared by mixing 2% sodium carbonate (2.7g of sodium carbonate in 100ml of distilled water) and 1% copper sulfate (1g of copper sulfate in 100ml of distilled water). Sodium hydroxide was used (1M of NaOH) and was prepared by dissolving 4g in 100ml of distilled water.

Tyrosine preparation
Standard tyrosine 1.1mM was diluted. The vials containing the following volume (ml) 0.05, 0.10, 0.20, 0.40, 0.80, 1.6 of tyrosine was filled to 2ml of water producing the following concentrations in mM respectively: 0.0275, 0.055, 0.11, 0.22, 0.44 and 0.88

Protease activity assay in *Bidens pilosa*
To each pH solution 200ml were used to homogenize 20g of grinded leaves of *Bidens pilosa*. The solution was kept for 10 minutes before adding casein solution. 1ml of the enzyme solution was kept in the test tube then 4ml of casein were added and 2ml of enzyme solution as blank solution in other tube in which there was no addition of casein solution. After adding casein, the solution was mixed and was incubated with blank solution on 30oC. 30 minutes after incubation, 5ml of 5% trichloroacetic were added to the tube, even in blank to stop the reaction by precipitating the residual casein. The precipitation took place and the aliquot was filterd using whatman paper. To 1ml of filterd aliquot, 5ml of the alkaline solution was added, which was prepared by mixing 2% of sodium carbonate, 2.7% of sodium carbonate and 1% of copper sultate. The content was alkalinized by adding 2ml of sodium hydroxide. After ten minutes 0.5ml of folin ciocalteu's phenol reagent diluted three times were added to each tube. The content was then incubated according to 30o C about 30 minutes. The intensity of color was measured using spectrophotometer at 660nm. The blank prepared to each pH was used to set the baseline of spectrophotometer. During the assay of *Bidens pilosa* tubes of tyrosine with known concentration were processed. To each tube 5 ml of sodium carbonate were added even in the blank solution. After that, 2ml of sodium hydroxide were added to alkalinize the mixture. 0.5ml of folin ciocalteu's phenol reagent diluted three times were added to each tube followed by incubation on 30oc during 30 minutes .the absorbance formed was measured on the spectrophotometer as it was done on sample tube after using tyrosine blank solution as the baseline

3. RESULTS

3.1. Action of Bidens pilosa's proteases on casien

After adding Folin-Ciocalteu's phenol reagent the dark color was formed. This was showing of the free tyrosine production from casein. When the protease we were testing digests casein, the amino acid tyrosine was liberated along with other amino acids and peptides fragments. Folin and Ciocalteu's phenol or folin's reagent reacts with free tyrosine to yield a blue colored chromophore, which was quantified and measured as an absorbance value on the spectrophotometer (Carrie Cupp-Enyard, 2008) The dark color intensity of tyrosine was measured by the spectrophotometer set at 660 nm. The table below summarizes the absorbance data of the difference between the tyrosine absorbance and blank without tyrosine.

Tyrosine Concentration(mM)	Absorbance
0.0275	0.005
0.055	0.03
0.11	0.083
0.22	0.16
0.44	0.262
0.88	0.474

Table 1:Concentration of tyrosine and its absorbance

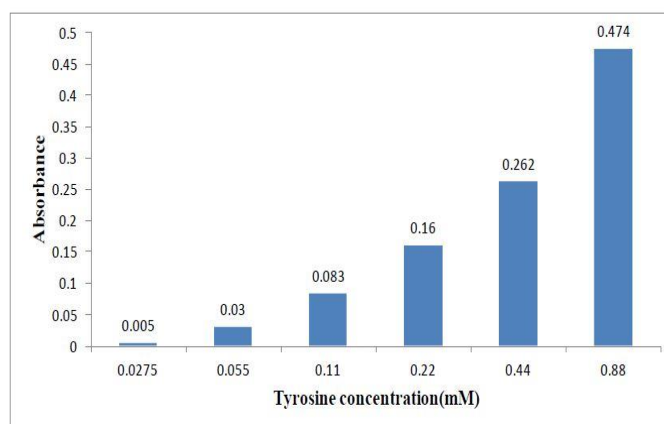

Figure 1: *Graphic curve showing the level of tyrosine vs the Absorbance*

The above results, are the corresponding standard curve was made using Microsoft excel. These concentrations (mM) were: 0.0275, 0.055, 0.11, 0.22, 0.44, and 0.88 respectively

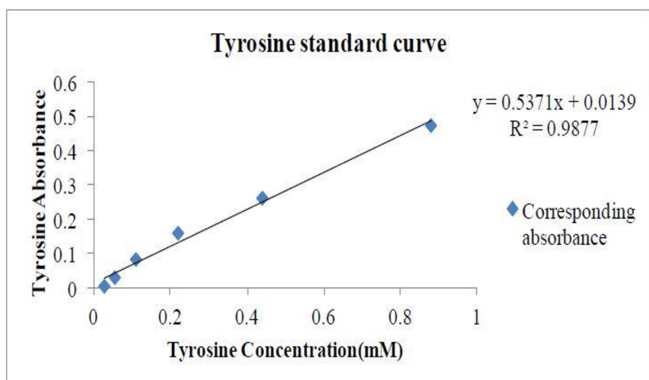

Figure 2: The tyrosine standard curve

The above graph shows the tyrosine standard. As the line is linear, this means that the photometry was good; this shows that the variation of optical density depend on the concentration of tyrosine, when tyrosine concentration increase also its absorbance increases. The **R** (correlation coefficient that shows the strength of the relationship between the two variables, this help the determination of linear regression between concentration and absorbance of tyrosine) was calculated using Microsoft excel and its value square is **R2=0.9877**

3.2. Results of absorbance obtained for fresh leave

The absorbance of the dark color formed by the fresh leaves was measured on the spectrophotometer and it's represented by the table below.

Buffer pH	30 degree celicius
4	0.28
10	0.18

Table 2: Absorbance formed by fresh leaves

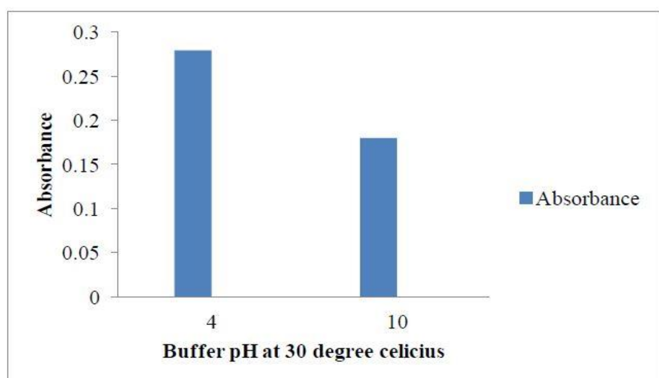

Figure 3: Absorbance of fresh leave vs Buffer pH

These results are the average of two experiments for fresh leaves. Each experiment was done using two tubes for each pH buffer.
The results obtained from the study were presented in the table format for buffer of pH4 and pH10.
The results presented in table 2 indicate that all samples of fresh leaves analyzed are associated with significant amount of protease activity.

3.3. Calculation of tyrosine released during this assay

The tyrosine standard curve equation , y = 0.5371x + 0.0139 where y stands for absorbance of tyrosine and x for concentration for concentration was used to find out the concentration of tyrosine released during this assay.

Buffer pH	Tyrosine concentration(mM)
4	0.495
10	0.309

Table 3: Concentration of tyrosine released during the assay at pH 4 and pH 10

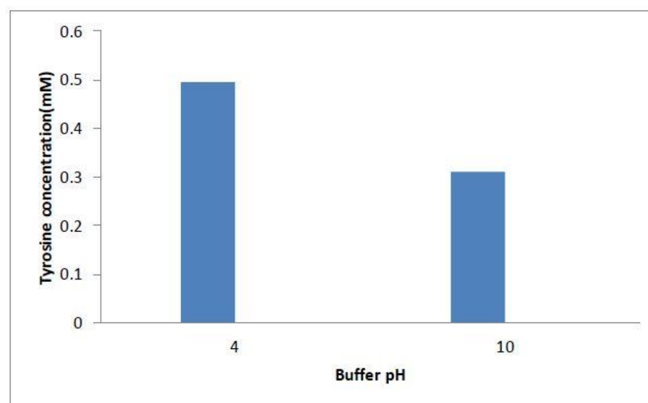

Figure4: Concentration of tyrosine released during the assay at pH 4 and pH 10

3.4. *Bidens pilosa* protease activity is expressed in terms of mM/mg of fresh leaves per time unit of incubation

Buffer pH	Protease-activity $\frac{mM/mg}{min}$
4	8.2567×10^{-7}
10	5.15×10^{-7}

Table 4: Protease activity of *Bidens Pilosa* (mM/mg/min)

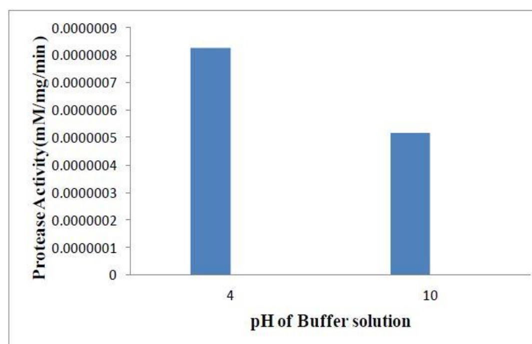

Figure5: Protease Activity against pH of Buffer solution

4. DISCUSSION

This research was aimed to determine the protease and protease activity of *bidens pilosa*. Proteases break down casein then liberate free tyrosine which was quantified in order to calculate protease activity. The research was carried out at 30 degree celicius of incubation, protease activity was measured in acidic buffer pH 4 and in basic buffer pH10. The highest protease activity was found at pH 4 which was 8.2567×10-7 mM/mg/min and the lowest protease activity was found at pH10 which was 5.15×10-7 mM/mg/min . In this research, there was a protease in *Bidens Pilosa* and there was a difference of protease activity among pH buffer used.

4.1. The effect of pH on protease activity

Enzymes including proteases have three dimensional structures which determine the activity of those enzymes so when the three dimension structure of enzymes change means that its activity also have been affected. Proteases like other enzymes are sensitive to pH and have specific range of pH under which they act in (Byrne, 1999). The pH normally denaturates (alter) the three dimension shape of protease by splitting its ionic and hydrogen bonds, this leads to slow or end up the protease activity. Proteases have optimum pH (the pH at which enzymatic reaction works at its maximum rate). If pH increases or decreases much beyond this optimum, the ionization of groups at the reactive site and on the substrate may change, effectively slowing or inhibiting the formation of enzyme substrate complex. At extreme pH, the bonds which maintain the tertiary structure hence the active site are disrupted and the enzymes are irreversibly denaturized (Byrne, 1999).During this research, only two pH were taken into consideration because of few materials and reagents availability (pH10 and pH4) and there was a difference in protease activity among those pH. This means that the activity of our protease was favorable at pH4 an acidic medium than at pH 10 a basic medium. Enzymes in order to work well they must form strong enzyme substrate complex means that the active site of enzymes must fit to its substrate like lock and key. pH may affect this strong enzymes substrate complex by splitting ionic and hydrogen bonds. In this study, acidic buffer pH 4 has low effect on protease extracted from *Bidens Pilosa* compared to pH 10.

5 CONCLUSION AND RECOMMENDATION

This research was aimed to study the protease activity of *Bidens Pilosa* to compare acidic and alkaline incubation conditions. During this research we extracted proteases from fresh leaves of *Bidens Pilosa* using buffers. The activity of protease was calculated by using casein as substrate. From the results, fresh leaves of *Bidens Pilosa* showed the protease activity. Fresh leaves of *Bidens Pilosa* have high protease activity in acidic buffer pH4 (8.2567×10-7 mM/mg/min) compared to alkaline buffer pH 10(5.15×10-7mM/mg/min) at 30 degree celicius. This study clearly showed that fresh leaves of *Bidens Pilosa* can be used as medicinal plants. This study showed that all proteases enzymes isolated from leaves of *Bidens Pilosa* have higher activities in both acidic and alkaline conditions. This study shows that the research on *Bidens Pilosa* has to be encouraged and continued because it is an interesting plant that can be used in many fields of our daily life. Researches can refer to protease activity in consideration other parameters like acidity, temperature, alkalinity, minerals. Also deep researches on other pH are needed because our study focused only on two pH. It is also necessary to continue the researches on *Bidens Pilosa* in order to improve the traditional medicine, further studies are necessary in order to clarify which part of *Bidens Pilosa*(roots, stem, flowers) has more protease activity than others because this research focused only on fresh leaves.

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