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Extraction condition of protease frombroccoli (*Brassicaoleracea* var *italic*) and study of Biochemical Characteristic fromthe selectedsource

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ABSTRACT

Four species were tested for the Brassicaceae , *Brassicaoleracea* var *italic*, *Brassicaoleracea* var *botrytis*, *Brassicarapa* var *rapa* , *Brassicaoleracea* var *captata*.to investigate the presence of protease. The result showed that broccoli possessed a high level of protease ,which because it has a high specific activity of the enzyme ,so it was chosen as a source of enzyme extraction . the best condition to extraction protease from broccoli was by using sodium phosphate buffer (0.1M,pH 7.5) reaching an specific activity 70.769U/mg .the enzyme was extracted with different extraction ratios of 1:1, 2:1, 4:1, 6:1,8:1, 10:1(v:w) and the best ratio was 1:1(v:w) to specific activity 70.6 U/mg .the enzyme extraction in different concentration 0.05,0.1,0.2,0.4 the highest specific activity were 76.4when enzyme extract in 0.1M. finally the enzyme was extracted with different time intervals of 5,10,15,20 ,and the best time period for extracting was 5 min with specific activity 76.06 U/mg .the enzyme purified from broccoli by several step concentration by ammonium sulfate at saturation 75% the enzyme reached activity 30.2U/m,purified fold 1.63 and yield 74.60 .The second step was purified by DEAE-Cellulose column , the enzyme reached activity 20.1U/ml and yield 69.51.The optimum pH for the enzyme activity and stability were 7.5 for 30 minute and 7-8.5 respectively . The optimum temperature of the enzyme activity 35 C^0 for 30 minutes and that for stability was 25-40C^0 for 30 minutes.

Key words: protease, broccoli, extraction, Characteristic, purification

INTRODUCTION

Protease with serial number (E.C.3.4.21) are classified as hydrolysis enzymes, which stimulate the degradation of the peptide of protein in different location and in different condition. These enzymes play a major role in the metabolic processes of living cells . This effect has the effect of destroying biological active proteins as well as generating enzymes, effectively active compounds are ineffective (Mufti *etal*.,2006). As it can be divided depending on the type of amino acids that occupy the active site to four groups serine protease, cystine protease, metalloprotease and aspartic protease (Whitaker,1972).protease are found in different animal formation such as plants, animals, and microorganisms(Bayoumi and Babobil, 2011). Protease enzymes are also among the commercial enzymes because they have an industrial importance as they form a large proportion among enzymes marketed commercially and are used in various industrial, medical and nutritional fields (Gohal *etal*., 2006). The physiological processes in all life forms depend on protease such as food digestion , regulation of the formation and decomposition of clots, stimulating the programmed death pathway (apoptosis) of the cell, the process of germination, the formation of spores, stimulation of hormones, controlling the over active immune response (Guilloteau

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etal.,2005). As well as cell differentiation and growth(Devi and Hemilatha, 2014) . as well as its use in the food industries such as meat and bread ,as well as pharmaceuticals and medical the production of cosmetics as the protease ability to remove wrinkles (Mala *etal* ., 1998).the study aimed to detect the contend of enzymes ,especially protease ,in the plants of the Brassicaceae and study the optimal conditions for extraction ,such as the extraction ratio, type of buffer, pH , concentration of buffer , and extraction time and finally purification and characteristic enzymeQasim M T and Al-Mayali H K2019).

MATERIALS AND METHODS

Enzyme extraction:

The crude extract was prepared from the fruits of the selected plants belonging to theBrassicaceae. a weight of 50g of the fruits to which 50 ml of chilled and prepared sodium phosphate buffer at concentration (0.1 M ,pH 7.5),1:1 (weight/volume) is added and added to it with 0.5% PVP for the purpose of preserving the activity of the enzyme from inhibitory condition. The fruit was crushed in cold condition using the blender for 5 min ,then filtered with two pieces of gauze .discard the leach ate using a cooling centrifuge at 5000 rpm for 15 minutes , neglect the precipitate and take the leach ate ,which represents the enzymatic extract. Then the enzymatic activity in order to choose the appropriate source for the production of protease.

Enzyme assay:

the method described by (Brock *etal*, 1982) , was used to estimate the activity of protease using 1 % casein as a base material and defined the activity alone as a quantitative enzyme that caused an increase in light absorption at a wavelength of 280nm at 0.01 ppm under standard condition. The enzymatic activity was calculated by equation:

$$\textbf{enzyme activity(U/ml)} = \frac{\textbf{absorption at a wavelength of 280 nm}}{\textbf{0.01}\times\textbf{0.2}\times\textbf{30}}$$

Protein concentration determination:

the method described by (Bradford, 1976) was used to estimate protein concentration using the standard curve for bovine serum albumin.

Extraction condition of enzyme:

Protease extraction:

Protease extract from four sources. These sources werebroccoli ,cauliflower cabbage and turnip All sources were washed with tab water then extraction of enzyme. The enzyme activity and concentration of protease were determination.

Extraction ratio:

A blended50 g of broccoliin different volumes of buffer extract (0.1 M phosphate buffer pH 7.5) with 0.5% PVP for extraction of protease. The extraction ratios were 1:1, 2:1, 4:1, 6:1, 8:1 and 10:1 (v:w). the enzyme activity and protein concentration was determination to determine optimum ratio to extraction.

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Type of extraction buffer:

broccoli was blended with different types of buffers extraction. These buffers are sodium acetate at concentration 0.1 at pH (4,4.5, 5, 5.5), sodium phosphate 0.1 at pH (6, 6.5,7,7.5), and Tris-HCl 0.1 at pH (8, 8.5 and 9) for extraction of proteasefrom broccoli. The enzyme activity and protein concentration was determination.

Effect of buffer concentration on protease activity:

The enzyme extraction at different concentration of sodium phosphate buffer (0.05, 0.1, 0.2, 0.4) to determine the optimum concentration. The enzyme activity and protein concentration was determination.

Extraction time:

After determine optimum buffer and concentration of protease, protease extraction in different time.Broccoliwas blended in solution extract (0.1 M phosphate buffer pH 7.5) at proportion 1:1 (v:w) using blender for different periods, 5, 10, 15,20minutes. The enzyme activity and protein concentration were estimate in each periodEnzyme.

concentration using ammonium sulfate

ammonium sulfate was added to a volume of enzyme extract to reach saturation of (20%,30%,40%,50%,60%,70%,80%,90%).the sediment was separated in a cooled central centrifuge at a speed of 10000xg for a period of 20 min. Discard the residue and dissolve the precipitate in a small amount of phosphate solution with a concentration of 0.1 M and pH 7.5 . the dialysis process was carried out against the phosphate buffer itself. To get rid of the ammonium sulfate residues, the enzymatic activity was estimated and the protein concentration measured in each of the concentrated precipitates

Ion exchange chromatography

The ion exchanger was prepared by DEAE- Cellulose according to the instructions of the equipped company (Whitman) , and the exchanger was packed in glass column to give dimensions(3.5x25) cm , and the balance process was carried out for the exchanger column with a buffer of sodium phosphate solution at concentration of 0.02 M and pH7.5 the speed flow is 60 ml/hour .

add the form

The sample enzyme solution was passed over the column surface gently and the column was washed with a buffer sodium phosphate buffer at a concentration of 0.02M pH7.5 to drop off the non-bound proteins .the effluent portions of the column were collected at a rate of 5 ml/tube. The enzyme was recovered from the column using a linear saline gradient from a buffer solution of sodium phosphate at a concentration 0.02 M pH7.5 with an addition of 0.5 M NaCl with a pH7.5 .The protein was followed up in the collected fraction by measuring their optical absorption at a wavelength of 280nm. Then the fractions near the apex of the activity curve were collected and the enzymatic activity and protein concentration in them were estimated.

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Characterization of protease

Optimum pH

The casein reaction solution was prepared at a concentration of 1%with different pH ranging from (4-9)distribute on tubes with 1.8 ml of casein base material and incubate the tubes at a degree of $37C^0$. Then 0.2 ml of purified enzyme solution were added to the tubes and incubated for 30 min , then the reaction was stopped and the enzymatic activity was estimated .

Optimum pH and stability

Add 0.2 ml of purified enzyme solution to tubes containing 0.2 ml of buffer solutions prepared with pH 4-9 and incubated for 30 min , then placed in an ice bath ,with casein solution with pH 7.5 as a reaction substance , then incubated at a temperature of 30 C^0 for 30 min ,then stopped the reaction ,and the remaining enzymatic activity was estimated as %

Determining the optimum temperature

0.2 ml of enzyme was added to 1.8 ml of 1%casein with a pH7.5 as a substance for the reaction ,then incubated at different temperature ranging between(25-60)C^0 for 30 min , the reaction was stopped and the enzymatic activity was estimated.

Temperaturestability

incubate 0.4 ml of purified enzyme solution with different temperature ranging (25-60) C^0 for 30 min .immediately after wards, put the tubes in an ice bath and withdraw 0.2 ml of the enzyme solution .1%casien solution was added to the reaction solution with pH 7.5 and incubated at $37C^0$ for 30 min .then the reaction was stopped and the remaining enzymatic activity was estimated .

RESULTS AND DISCUSSION

Protease source:

shows in this table(1) that protease from broccoli gave highest specific activity 70.769U/mg, while cauliflower,cabbage and turnipwere 47.619,22.954 and 16.666 U/mg protein respectively. based on the result obtained broccoli was adopted as a source of protease extraction ,as a cheap and unscientific source in the country. In another study protease extraction fromleaves of papaya Carica Papay(Agrahari and Sharma., 2014). And extraction from*Morngaoleifera* leaves Banik,S *etal*., (2018) . and extraction from *Zahdi* dates *plam* seeds (*Phoenixdactylifera* L.)Al-Jumaily *etal*.,2009).

Table1. specific activity of protease from different source

Fruit	Activity U/ml	Protein concentration	Specific activity U/mg
broccoli	10.12	0.143	70.769

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cauliflower	10	0.21	47.619
cabbage	13.52	0.589	22.954
turnip	2.3	0.138	16.666

Extraction ratio:

The enzyme was extracted with a different extraction ratio of 1:1, 2:1, 4:1,6:1, 8:1, 10:1(v:w), Figure (1) shows that 1:1 (v:w) was the best ratio, with specific activity 70.6 U/mg, while 2:1 (v:w) ratio was 49.21 U/mg. Other ratios were 4:1, 6:1 and 8:1, 10:1 (v:w) with specific activity 32.18, 25.2,12and 6.1 U/mg respectively, because the enzyme was diluted. Rawaa and Nazar (2015) found that 1:1(v:w) best extraction ratio for protease extraction from *Citrilluscolocynthis* .while in another study found the best extraction ratio 3:1to extract protease from Seed Of *Phaseolusvulgaris* L (Al Mamouri,2011)

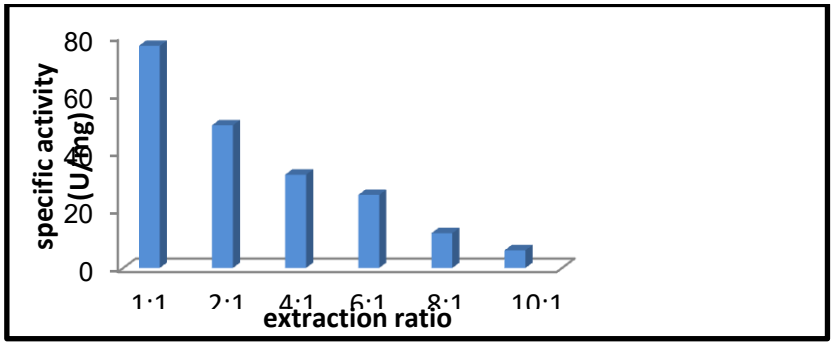

Figure1. The effect of extraction ratio of protease from broccoli

Effect types ofbuffer in enzyme extraction:

three different buffers were studies with varying pH range (4-9) in the extraction of protease from broccoli. the result shown in figure (2)showed the different in the specific activity of the enzyme according to the quality of the buffer and pH used . the optimal pH is 7.5 with specific activity 70U/mg. while the decrease in the specific activity in the acid and alkaline pH.the reason is that the acidic and alkaline buffer effect the stability of the enzyme and the decomposition of the plant parts (Chaplin and Chri ,2004) . The study was agreed with Rawaa and Nazar (2015) where it was found that the best pH 7.5 to extract the *Citrilluscolocynthis*while the best pHto extract the protease from *Cucumismelo*var *agrestis* 7.2(Devi and Hemathala.,2014).

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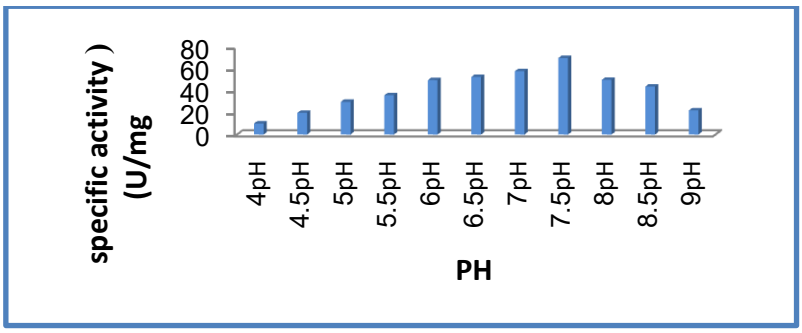

Figure2. Effect types of buffer in enzyme extraction protease from broccoli

Effect of buffer concentration in protease activity:

The results in figure (3) showed the sodium phosphate buffer (0.1M, pH7.5) was best extraction buffer with specific activity 76.4U/mg, while other buffer with different concentration were given low specific activity. the study came in agreement withDevi and Hemaltha (2014) , as it found that the optimal concentration of the buffer is 0.1M when extracting the protease from the*Cucumismelo* Var *agrestis*.while the optimal concentration of buffer is 0.2 Al-Mamouri,2011.when extraction protease from*PhaseolusvulgarisL*. .

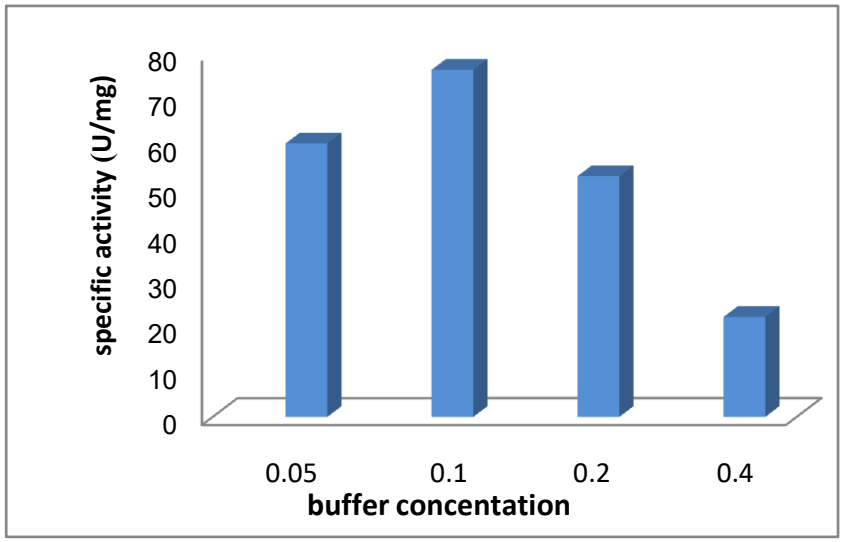

Fig. 3. Effect buffer concentration in enzyme extraction protease from broccoli

Extraction time:

the result shown in figure (4)indicate the relationship between the extraction time and the specific activity of the protease and found that the best time of extraction of protease from broccoli fruits is 5 min with specific activity 76.06 U/mg and the specific activity reached 40.3 U/mg with period of 10 min .while the specific activity reached 18.1 and 9.3 U/mg at the extraction time periods 15 and 20 respectively . the reason is due to the fact that the buffer solution in thetime period of the mentioned may disengage the enzyme from the components of the medium fully or when using a time period higher than that gets dantarase for the enzyme or loss of some accompanying substances enzyme (co-factors) (Kayani *etal*., 2011). The time required to extract the enzyme varies according to plant .the study came in agreement with Rawaa and Nazar (2015) , as it found that the highest peroid time is 5 min when extracting the protease from the *CitrilluscolocynthisL*.

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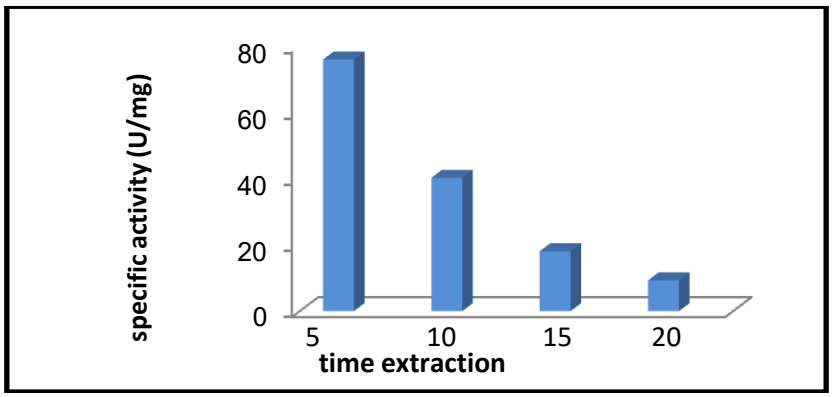

Figure 4. Effect of extraction time for protease extraction from broccoli

Enzyme purification

Enzyme concentration

A series of experiments were conducted to determine of the optimal sequence of purification steps for the protease from broccoli in order to purify and study its properties , the result showed that the best saturation rate with ammonium sulfate was 75% as it gave the highest specific activity (201)U/mg protein table(2).studies have focused on using ammonium sulfate or Organic solvents in the enzyme precipitation process in a step of the purification steps ,as it has a role in the concentration of the form and the disposal of some contaminated proteins**.** Andalso considered one of the most used substance in the deposition of enzymes because it is inexpensive and does not affect the pH of the solution and has a high solubility in water and does not affect the concentration of enzyme (Kornberg,1990).

Ion exchange chromatography

Ion exchangechromatography was performed as a second step in the purification process using the ion exchange (DEAE-Cellulose), which is a negatively charged exchanger . the pHand the optimal ion concentration of the sodium phosphate buffer are confirmed at a concentration of 0.02M and pH 7.5 and at this number the protease enzyme proteins is attached to the column gel and then the enzyme is recovered .by the action of a saline gradient solution ,it is known that the proteins that carry changes on their surface increase their ability to adsorb to surfaces with opposite charge and may be so tensile that it is difficult to separate them (Pohl, 1990).the enzyme solution was added to the ion –exchange column , and the protein were recovered using a sodium chloride salt gradient at a concentration of (0-0.5).the specific activity reached 201U/mg protein and fold 2.61 and yield 69.51. in other study enzyme reached specific activity 918.78 U/mg and purification tim11.33 and yield 68.29 when enzyme purification using DEAE-Cellulose from dates plam seeds (*Phoenixdactylifera L.*)Al-Jumaily*etal*.2009

Table (2) purification of protease from broccoli

step	Volume ml	Activity U/ml	Protein mg/ml	Specific activity U/mg	Total activity U	Time purification	Yield %

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Crude enzyme	100	10.12	0.143	76.769	1012	1	100%
Ammonium sulfate concentration 75%	25	30.2	0.24	125.83	755	1.63	74.60
DEAE-Cellulose	35	20.1	0.1	201	703.5	2.61	69.51

Characterization of protease

The optimal pH

The optimal pH for the activity of protease was 7.5 if given the highest activity 20.1U/ml figure (5),these results , it was clear that the protease was approaching the pH from other sources ,as most sources indicate that the optimal pHfor activity is 7.5 when using casein as a base material Yang *etal*.2000,(Chopra,1985) as for the mineral protease purified from the seeds*Holarrhenaantidysenterica*, the optimum pH was 7.5(Hidayatullah *etal*.,2008).while in other study found the optimal pHwas 7.6 when enzyme purified from *Ficusresigiosa*(Kumari *etal*.,2012),and the optimal pH was 7.5 when enzyme purified from*Citrullus colocynthis* Fruits(Rawaa and Nazar,2015).

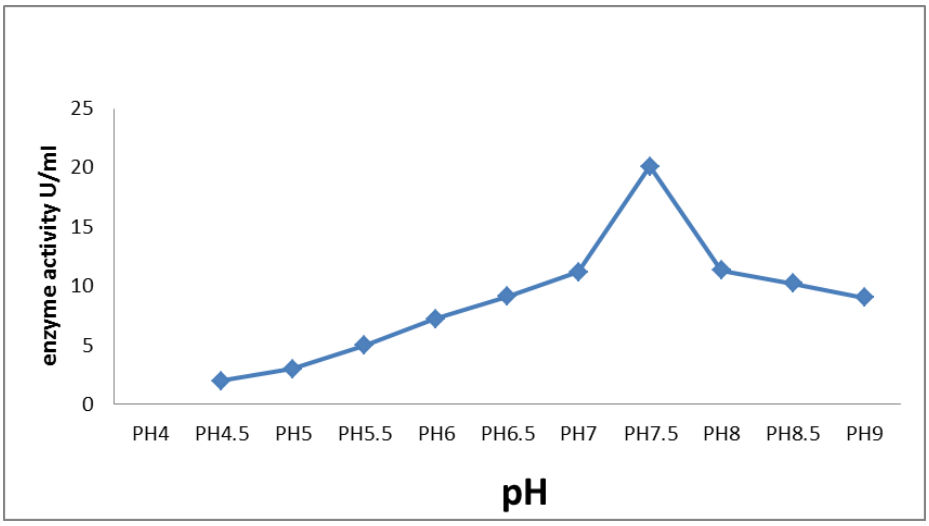

Figure 5.Optimum pH for the enzyme activity from broccoli

Optimal pH of enzyme stability

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The result of this study showed that the purified protease from broccoli possesses stability towards the pH at neutral values (7-8.5) figure(6) ,while decrease in the activity of the protease in the pHof the low acid values may be due to the effect of the acidity of the medium in the composition of the enzyme protein and the alteration of the groups present in the active site, and that an increase in the pH above the optimum leads to the protein deformation ,a change in the composition of the active site ,and a loss of the enzyme activity Segal, (1976) . this result was agree with (Rawaa and Nazar,2015), that found the optimal pH of stability was(7-8.5) when enzyme purified from *Citrulluscolocynthis* .and the optimal pH of stability of enzyme purified from Zahdi dates plam seeds (*Phoenixdactylifera* L.)was 7.5 Al-Jumaily *etal*.2009.

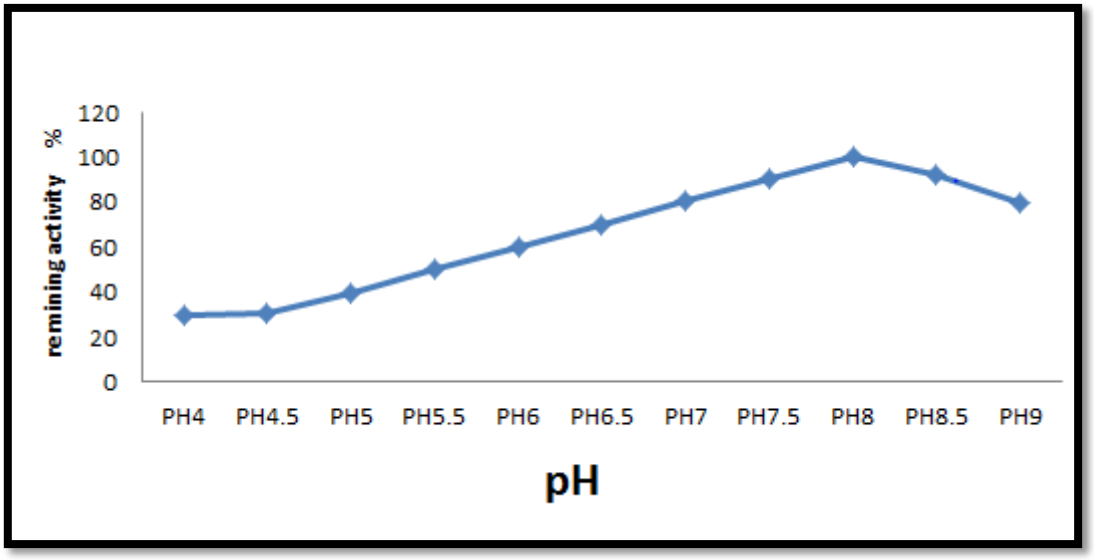

Figure 6. optimal pH of enzyme stability from broccoli

Optimal temperature of protease activity

The effect of different temperatures on the activity of protease purified from broccoli was studies and increase in activity was observed at a temperature of $35C^0$ (figure 7) this is due to the fact that the speed of the enzymatic reaction increases with the increase in the temperature within a certain extend due to increased motor energy for the parts and the reason for the low activity returns to enzyme conservation due to the effect of heat on the tertiary structure of the protein and the change of the active site composition (Segel, 2000). These results are in agreement with what has been reported by a number of researcher that the optimum temperature for protease is $35C^0$(Al-Jumaily and Welad,2005).

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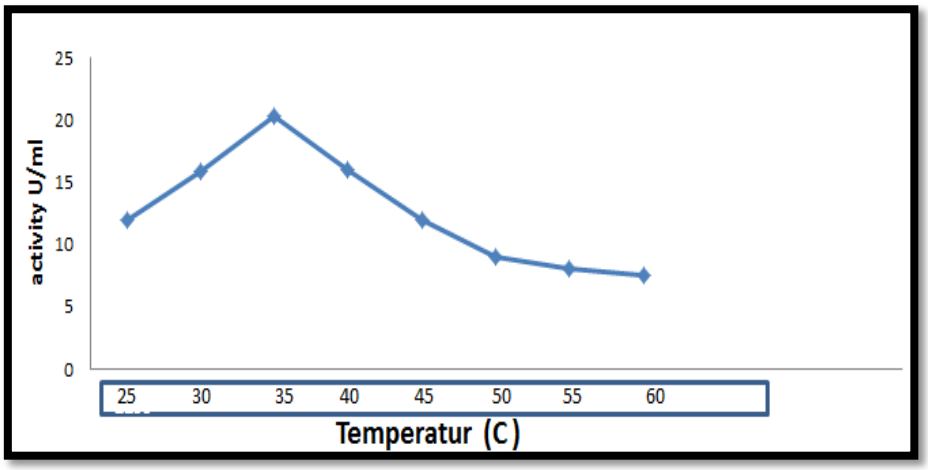

Figure7. optimal temperature of enzyme activity from broccoli

Effect temperature in enzyme stability

the results of this study indicated that the protease produced from the fruits of the broccoli retained its full activity when incubated at temperature (25-40) C^0 and for 30min then the enzymatic began to decrease with high temperature and loss of its activity at 70% limits when lap by $60C^0$.figure (8). The sensitivity of the enzyme to heat is related to some of the enzyme characteristics ,such as its molecular weight and extend to which it contains sulfur bonds. The composition of the surrounding medium contributes to the increase or decrease of the enzyme sensitivity to heat such as the pHand ionic strength (Segel, 2000) and (Whitaker, 1972).

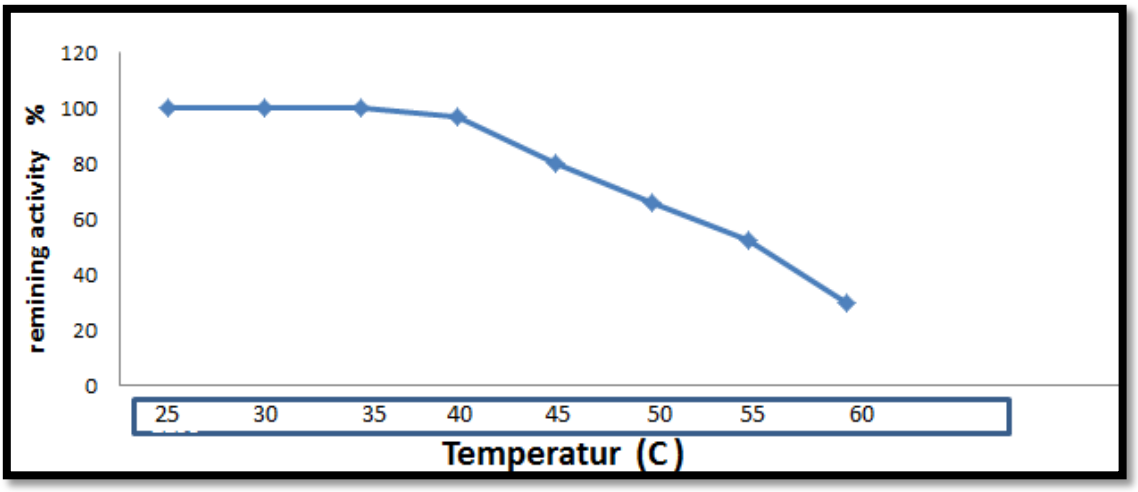

Figure 8. effect temperature of enzyme stability from broccoli

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