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Research Article

Proteolysis of water washed meat from *Catla catla* using bromelain and papain: Optimization of hydrolysis parameters

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ABSTRACT

Fish protein hydrolysate has gained much attention in the recent past due to their health promoting properties. The properties of hydrolysates are governed by the specificity of enzyme used, hydrolysis conditions employed and degree of hydrolysis (the extent to which the proteins are cleaved into peptides). In the present study, the effect of hydrolysis parameters on the extent of hydrolysis of water washed meat from *Catla catla* was studied for two enzymes namely bromelain and papain in order to know the optimum hydrolysis conditions. Optimization was carried by varying one variable and fixing three other variables constant at a time. The extent of hydrolysis was monitored by measuring the liberated tyrosine content at 280 nm. The SDS-PAGE pattern of total proteins from water washed meat of *C. catla* confirmed the presence of myosin and actin as major protein fractions. The temperature, pH and time to hydrolyze the washed catla meat using bromelain and papain was found to be 0.5:100, 50°C, 6.5 ± 0.2 and 60 min, respectively. The increase in E/S (Enzyme to substrate) for both bromelain and papain increased the extent of hydrolysis. However, one need to be careful in selecting the E/S ratio based on the cost of enzyme and desired degree of hydrolysis.

INTRODUCTION

Fish and fishery products represent a valuable source of nutrients of fundamental importance for diversified and healthy diets (FAO 2016). Apart from being highly nutritious, fish muscle proteins can be made use for preparing fish protein hydrolysates which comprises of bioactive peptides with valuable nutraceuticals and pharmaceutical potentials. Fish protein hydrolysates (FPH) are the mixture of amino acids and peptides obtained by digesting proteins from fish meat or fish processing waste using proteases (Elavarasan and Shamasundar, 2016).

The generation of bioactive peptides by proteolysis of food proteins, using exogenous proteases is a new and interesting approach. In fact, proteolysis of protein substrates, having different amino acid composition and sequences, by proteases having different specificities may generate numerous specific health beneficial peptides with different lengths and amino acid sequences. These peptides generally referred as bioactive peptides (Ren *et al.*, 2008). For deriving the peptides from fish proteins, proteolytic enzymes from microbial and plant origin have been widely used. By optimizing the conditions of hydrolysis, using different enzymes and substrates, a wide range of hydrolysates with desired physico-chemical functional and biological properties can be produced (Kristinsson and Rasco, 2000).The choice of enzyme for the preparation of protein hydrolysate will be critical as the properties of hydrolysates will depend on the nature of enzyme.

The global fish capture production is more or less stagnant. Aquaculture is driving the growth to meet the growing demand in total global fish production. Production of freshwater fishes has always been dominated by carps (71.9%, 24.2 million tonnes, in 2010) (FAO, 2012). Freshwater aquaculture has made a rapid development in Asia and dominated by the carp species. Freshwater aquaculture in Asia particularly in China and India is growing at faster rate. Indian major carps contribute about 15% of the total world aquaculture production and 88.5% of total aquaculture production in India (FAO, 2012). The Indian major carps include three species, namely *Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*. Value addition by the development of new products is one of the thrust areas that can be beneficial for the producer. The

preparation of fish protein hydrolysates from freshwater carps will strengthen the link between the production and utilization. With this rationale, the objective of the present investigation was to optimize the hydrolysis parameters for the preparation of hydrolysate using commercially available proteolytic enzymes such as bromelain and papain.

MATERIALS AND METHODS

Raw materials

Fish

The fish, *Catla catla*, used in the present study was harvested from culture ponds in Shivamogga, Karnataka. The fishes were iced in the ratio of 1:1 (fish: ice). The fish and ice were packed in an alternative layer in poly- urethane boxes and transported to the laboratory. The time taken to reach the laboratory from harvest centre to laboratory was about 5–6 h.

Chemicals

Proteases namely bromelain (from pineapple stem, activity 3 – 7 U / mg of protein, 52 % protein) and papain (from latex of *Carica papaya*, *activity* ≥3U / mg), L-tyrosine, acrylamide, bis-acrylamide (N, N'-methylene–bis-acrylamide), 2-mercaptoethanol, trizma base (tris[hydroxyl methyl] amino methane), coomassie blue-G and protein molecular weight markerss (wide range, MW 200 kDa to 6.5kDa), TEMED (N, N, N^1, N^1-tetramethyl ethylene diamine) and bromophenol blue were purchased from Sigma–Aldrich (St. Louis, MO, USA).All other chemicals and reagents used in the present study were analytical reagent grade (AR) or guaranteed reagent grade (GR).

Preparation of substrate

Separation of meat from IMC

Fishes brought to the laboratory in iced condition were washed twice with chilled potable water. The fishes were beheaded and eviscerated to get dressed fish. Meat from dressed fishes was separated using reciprocatory meat bone separator (SG, Toyo Seikan Kaisha Ltd., Tokyo, Japan).

Water washing of meat

Separated meat was subjected to water washing using chilled potable water (4 ± 1°C). The quantity of water used for washing was 1:3 (meat: water) and the number of washing cycle was 1. The slurry was agitated for 3 min and allowed to settle for 7 - 10 min. Water was decanted and filtered through muslin cloth. The excess water was removed manually by squeezing the mince by placing between coarse cloths. Water washed meat was frozen at -35°C using air blast freezer (Armfield, Armfield Limited, Ringwood Hampshire, England) and stored at -20°C (Vest frost, Denmark) till further use.

Preparation of meat homogenate for hydrolysis

Water washed meat was mixed with water at meat to water ratio of 1:2 (w/v) and homogenized at 9000 rpm for 2 min using a homogenizer (ULTRA-TURRAX T25, IKA Labortechnik, Germany). The resultant mixture of water and meat was referred as meat homogenate.

Optimization of hydrolysis parameters

The parameters such as enzyme to substrate ratio (E / S), temperature, pH and time was optimized using the water washed meat of *C. catla (WMC)* as a substrate against two different proteases namely bromelain and papain by varying one parameter at a time and keeping other parameters constant. During the optimization, the extent of hydrolysis was measured by determining the liberated tyrosine content. The activity of the enzymes was expressed as µM of tyrosine / g of protein. The overall experimental flow is given in Fig.1.

Optimization of Enzyme to Substrate ratio

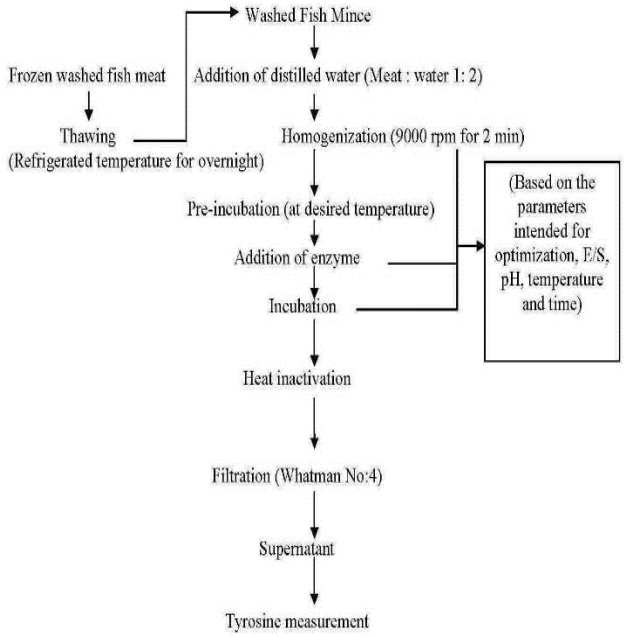

Fig. 1. The procedure used to optimize the hydrolysis conditions for hydrolyzing the washed meat of *C. catla* using different enzymes.

The effect of enzyme to substrate ratio (E / S) on the extent of hydrolysis of water washed meat of catla was studied separately by varying the E / S ratio while keeping other parameters like temperature, pH and time of hydrolysis constant. The details are given in Table 1. The meat homogenate from *C. catla* was prepared as described previously. The homogenate was transferred to conical flask (100 ml) and enzyme was added at different enzyme to meat ratio. Other parameters such as temperature, pH and time were 27 ± 2°C (ambient), 6.5±0.2 and 60 min respectively. After incubating the mixture at room temperature for 1 h, the hydrolysis reaction was terminated by keeping the mixture in a boiling water bath for 15 min. Reaction mixture was filtered using Whatman filter paper number 4 (Whatman Plc, Maidstone, Kent, UK) to remove the unhydrolyzed meat. Supernatant was collected and tyrosine was quantified by reading the absorbance at 280 nm (UV-VIS spectrophotometer; LaboMed, Inc.,Los Angeles, CA, USA) after making the appropriate dilution. Meat homogenate incubated with the addition of heat inactivated enzyme was served as control.

Optimization of temperature

Meat homogenate was prepared as mentioned early. The temperatures studied for different enzymes varied from 27°C to 70°C. The optimization of temperatures for different enzymes was carried out by keeping the E / S ratio, pH and time of hydrolysis as constant. The details are given in

Table 1. Prior to the addition of enzymes, the homogenate was pre-incubated at desired temperature levels for 5 min so as to equilibrate the temperature. After attaining the desired temperature, the enzymes at a given concentration was added and incubated for 60 min. The hydrolysis reaction was terminated by keeping the mixture in a boiling water bath for 15 min. Reaction mixture was filtered using whatman filter paper number 4 to remove the unhydrolysed meat. Supernatant was collected and tyrosine was quantified by reading the absorbance at 280 nm after making the appropriate dilution. Meat homogenate incubated with the addition of heat inactivated enzyme was served as control.

Optimization of pH

Meat homogenate was prepared as mentioned earlier. The range of pH used for different enzymes were 5 to 9. The pH of the homogenate was adjusted using either 1M HCl or 1M NaOH. The experimental details for optimizing the pH are given in Table 1. The hydrolysis reaction was carried out by adding the enzyme to the pre-incubated meat homogenate. Hydrolysis time for both the proteases was 60 min. The hydrolysis was terminated by keeping the mixture in a boiling water bath for 15 min. Reaction mixture was filtered using Whatman filter paper number 4 to remove the unhydrolysed meat. Supernatant was collected and tyrosine was quantified by reading the absorbance at 280 nm after making the appropriate dilution.

Optimization of Time

The time of hydrolysis chosen for different enzymes in the study varied from 15 to 60 min. The parameters such as E / S ratio, temperature and pH employed for the experiments are detailed in Table 1. The hydrolysis reaction was terminated by keeping the mixture in a boiling water bath for 15 min. The mixture was filtered using Whatman filter paper number 4 to remove the unhydrolysed meat. Supernatant was collected and tyrosine was quantified by reading the absorbance at 280 nm after making the appropriate dilution.

Analyses

Determination of protein content

Total nitrogen content of water washed meat from *C. catla* was estimated by Kjeldahl method as described in (AOAC, 2000). Crude protein content of washed meat was calculated by multiplying total nitrogen by a factor of 6.25.

SDS-PAGE pattern of proteins

Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried using the method described by Laemelli (1970). The total proteins were dissociated using sodium dodecyl sulfate (SDS). Electrophoresis was carried out using polyacrylamide gel slabs of 10×8 cm (length×width) in a vertical slab electrophoresis apparatus (Model mighty small II, SE 250/SE 260, Hoefer Pharmacia Biotech Inc., Halliston, USA). The concentration of acrylamide for separating gel was T%=10 and C%=2.6 while for stacking it was T% = 4 and C% = 2.6. For polymerization of the gel, TEMED was used as the initiator and APS as the catalyst. The gels were cast in a duel gel caster. The thickness of the gel was 0.75 mm. The number of wells in each gel was 10.

A known amount of sample was mixed with 5% SDS. The mixture was homogenized at the speed of 9000rpm for 2 min using an Ultra-Turrax homogenizer. The homogenate was incubated at 85°C for 60 min, followed by centrifugation at 8000×g for 10 min at room temperature. The supernatants were mixed at 1:1 (v/v) ratio with the sample buffer (0.5M Tris-HCl, pH 6.8 containing 4% SDS, 20% glycerol and 0.03 mM of coomassive brilliant blue-G) and boiled for 3 min. The supernatant was stored in vials, labeled properly and stored at -20°C till electrophoresis was carried out.

The samples (75µg protein) were loaded into the wells of the polyacrylamide gel (10% running and 4% stacking). The run was carried out on a constant voltage mode of 30V using power pack (Model PS-3000, Hofer Pharmacia Biotech Inc., Halliston, USA) till the samples reached the end of staking gel. Further, the voltage was raised to 90V and the run was terminated when the dye front reached the bottom of the gel. A standard molecular weight marker of wide range was loaded into a separate well of the gel. After the run, the gel was stained in coomassive brilliant blue G-250 (0.025% in 40% methanol and 7% acetic acid) for 30-40 min. The gels were de-stained using acetic acid-methanol mixture (7% acetic acid and 2% methanol) till the protein bands were clearly visible. The molecular weight of the bands obtained in the sample was approximated by measuring the relative mobility of the standard protein markers.

Measurement of liberated tyrosine

The supernatant (150 µl) obtained after hydrolysis, was diluted to 3 ml using distilled water and the absorbance was measured at 280 nm using double beam UV-VIS spectrophoto meter. L-tyrosine was dissolved in 0.01M HCl such that different concentrations were obtained and concentrations were measured at 280 nm using double beam spectrophotometer. The tyrosine liberated in FPH was expressed as µM of tyrosine liberated / g of protein. Standard tyrosine curve is given Fig.2.

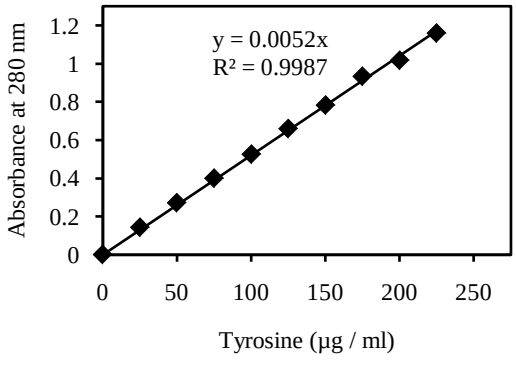

Fig. 2: Standard curve for tyrosine measurement. The standard curve was obtained by measuring the absorbance of tyrosine solutions at 280 nm

Statistical Analyses

The hydrolysis reaction for each experiment was carried out in triplicate and the average values for the liberated Tyrosine was used to plot the graph. The statistical programme given in Microsoft office excel was used to perform the calculation and plot the graph.

RESULTS AND DISCUSSION

Protein fractions in the substrate used for hydrolysis

In the present investigation the meat was subjected to single washing cycle and used for FPH preparation. The purpose of water washing was to remove the water soluble fractions, which include enzymes, pigments and other non-protein constituents. The water washing of mince also facilitates the reduction in fat content, which will help in better stability of hydrolysates during preparation and storage. The crude protein content of water washed meat from *C. catla* was 19.81 %.

Understanding the molecular weight of proteins in fish meat is vital as degradation of protein molecules by enzymes leads to generation of varying size of peptides. The SDS-PAGE profile of proteins from washed IMC was carried out and protein pattern is presented in Fig.3.

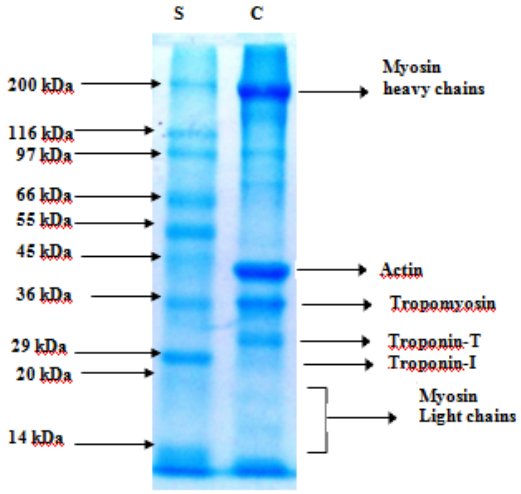

Fig.3: Protein Protein pattern of *C. catla* water washed meat. S: Standard Sigma Wide Range Molecular Weight Markers, C: Water washed meat of *C. catla*

The myosin heavy chain with a molecular weight of 200 kDa was more prominent among different protein bands obtained from *C. catla*. Myofibrillar proteins contribute to over 60% of the total protein content. The major components myosin, actin, tropomyosin and troponin C, I and T, many other components of the myofibrils called scaffold proteins accounts for less than 1% of total myofibrillar protein (Sikorski, 1994). Hydrolysis of washed meat with proteases will cleave different protein fractions of varying molecular weight sizes and yield the mixture of peptides.

Effect of hydrolysis conditions on extent of hydrolysis by Bromelain and Papain

Effect of E/S ratio

The extent of hydrolysis by bromelain as given by tyrosine value was found to be dependent on concentration of enzyme (Fig. 4A). Bromelain, at the E / S ratio of 2: 100 liberated 223.38µM of tyrosine / g of protein. Bromelain is a cysteine endo-peptidase has been used mainly for meat tenderization. The substrate spectrum is broad, extending from synthetic low molecular mass amides and dipeptides to high molecular substrates such as fibrin, albumin, casein, angiotensin II and bradykinin. Bromelain preferentially cleaves glycyl, alanyl and leucyl bonds. The source of bromelain enzyme can be from a stem of pineapple plant or from the fruit itself. The two proteases namely from stem and fruit are different with nomenclatures as well as specificity. In the present study the bromelain from the stem was used which is cysteine endopeptidease with EC number 3.4.22.32. The bromelain from fruit is an aspartic endopeptidase with EC number 3.4.22.3.

Table 1: Experimental conditions employed for optimizing the hydrolysis conditions

Enzymes	Hydrolysis parameters			
	E/S ratio (%)	Temp. (°C)	pH	Time of hydrolysis (min)
Optimizing the E/S ratio				
Bromelain	0.1 0.5 1.0 2.0	27	6.5 ± 0.2	60
Papain	0.5 1.0 1.5 2.0	27	6.5 ± 0.2	60
Optimizing the Temperature				
Bromelain	0.5	27 50 60 70	6.5 ± 0.2	60
Papain	1.0	30 50 70	6.5 ± 0.2	60
Optimizing the pH				
Bromelain	0.5	50	6.0 6.7 8.0 9.0	60
Papain	1.0	50	6.5 7.0 8.0	60
Optimizing the Time of hydrolysis				
Bromelain	0.5	50	6.7	15 30 45 60
Papain	1.0	50	6.5	30 60 90 120

Papain liberated 503 µM / g of protein at the E/S ratio of 2: 100 (Fig. 4B). The tyrosine liberated by papain at E / S ratio of 1:100 and 1.5:100 was almost constant after which at E / S ratio of 2:100 the increase in tyrosine was quite steep. It is generally accepted that concentration of enzyme is directly related with the activity of enzyme. Papain is a cysteine protease with wide specificity, cleaving peptide bonds of basic amino acids, leucine, or glycine. It also hydrolyzes esters and amides. Papain will digest most protein substrates more extensively than the pancreatic proteases. Papain consists of a single polypeptide chain with three disulfide bridges and a sulfhydryl group necessary for

activity of the enzyme (Carrey, 1997). The specificity of cleavage of the X-Y bond is: where X is a nonspecific amino acid, but arginine and lysine are preferred; and phenylalanine-XY bond where residues following phenylalanine are preferred; Y is a nonspecific amino acid residue. However, there are other factors which may impact this relationship such as temperature, pH and hydrolysis time (Quaglia and Orban1987; Shahidiet al. 1995). From the results it was evidenced that papain is more effective in hydrolyzing the water washed meat from *C. catla* than the bromelain.

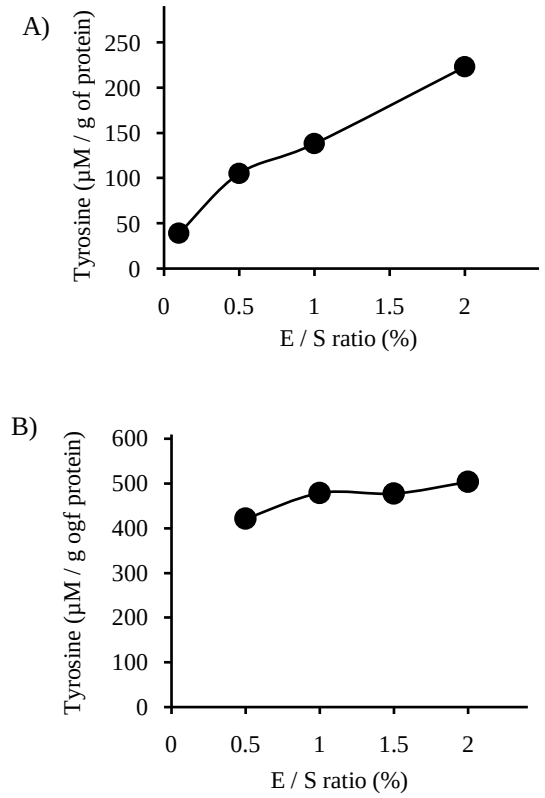

Fig. 4: Effect of enzyme to substrate (E/S) ratio on extent of hydrolysis water washed *C. catla* meat. (A): Bromelain; (B): Papain

Table 2: Optimum conditions for the preparation of FPH from washed meat of *C. catla* using Bromelain and Papain

Enzymes	Optimum hydrolysis parameters			
	E/S	Temperature (^{0}C)	pH	Time (min)
Bromelain	2%	50	6.5±0.2	60
Papain	1%	50	6.5±0.2	60

Effect of temperature

The optimum temperature for bromelain as well as papain to hydrolyse the washed meat of catla was found to be 50°C (Fig. 5A & 5B). It is evident that at 50°C, hydrolysis was higher than in the temperature regions of below or above 50°C as indicated by liberated tyrosine content. The temperature of the enzyme-substrate complex is a critical parameter in controlling the rate of reaction. The temperature of the system is a direct measure of kinetic energy of the molecule in the system. However, catalysis by enzymes does have an optimum temperature range wherein, the substrate is hydrolyzed at a faster rate. The greater the temperature of the system, higher the kinetic energy and has several effects on rate of reactions (Voet *et al*., 2006). In order to convert substrate into products there exist a) more energetic collisions, b) number of collisions per unit time will increase and c) the heat of the molecule in the system will increase (Palmer and Bonner, 2007). With increase in temperature of the system, the internal energy of the molecules in the system will increase. Internal energy of the molecules may include translational energy, vibrational energy and rotational energy. This type of energy may involve in chemical bonding as well as nonbonding interactions. Any enzyme to be active at a given temperature should not lose the structure wherein, the bonds that determine three dimensional structures should not be weakened (Nelson and Cox 2004).

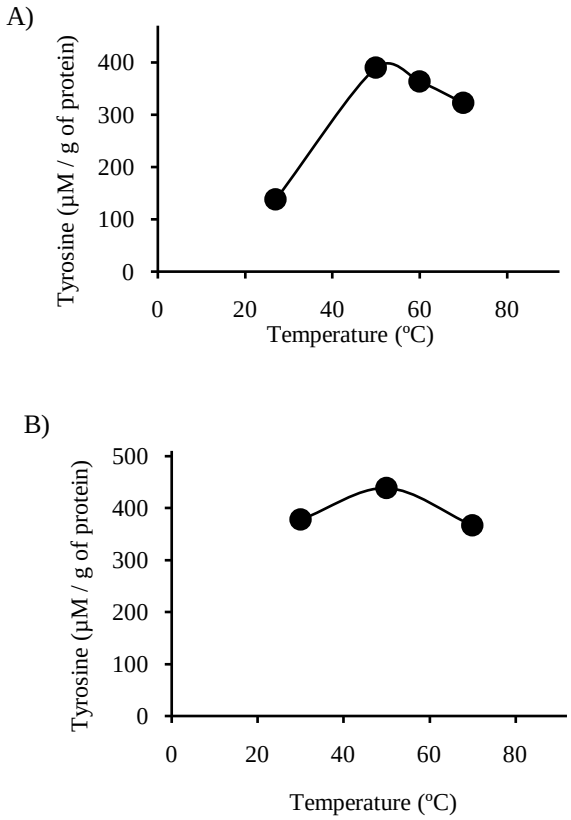

Fig. 5: Effect of temperature on extent of hydrolysis of water washed meat from *C. catla* (A) Bromelain and (B) Papain

Effect of pH

The optimum pH for bromelain enzyme was determined. The different pH chosen for the reaction were 6, 7, 8 and 9 and the results are presented in Fig. 6A. Other variables such as E / S ratio, temperature and time were kept constant at 0.5:100, 50°C and 60 min, respectively. With increase in pH, tyrosine liberated during hydrolysis decreased. The decrease was more significant between pH 7 and 9. At pH 6 and 7, the liberated tyrosine was almost constant with values ranging between 417 and 418 µM / g of protein. The bromelain enzyme reported to have a wide spectrum of pH optima between 5.5 and 8.0 (Taussig and Batkin1988). The effect of pH on the hydrolysis of washed catla meat using papain was profiled. The activity of papain

as revealed by liberated tyrosine was lower at pH 8 as compared to pH 7.

The liberated tyrosine content at pH 7 was 449 µM / g of protein (Fig.6B). At pH 7, the papain had maximum activity. The optimal pH for papain activity was reported to range from 6.0 to 7.0 (Carrey 1997; Price and Stevens 1989). The enzyme activity is affected by pH. The pH outside the optimum range can induce structural changes in enzyme thereby altering the activity. The pH affects on state of ionization of acidic or basic amino acid residues (Voet *et al.*, 2006). If the state of ionization of residues in the proteins (enzymes) is altered then the ionic bonds that are responsible for maintaining three dimensional structures will be altered. This will lead to altered protein recognition. In other words, enzymes become inactive. Apart from changing the shape of the molecule, pH may also change charge properties of the substrate preventing substrate to bind to the active site of enzyme (Palmer and Bonner 2007). Most of the enzymes are active in the pH range of 5.0-9.0. This is due to the fact that, proteins function in environment that reflects this pH. Further, a large number of amino acid residues have pKa values in the range of intracellular pH. As a result, a change in pH can protonate or deprotonate the residues thereby changing the chemical nature (Nelson and Cox 2004). The pH shows similar trend like temperature. The enzymes are active at specific pH and activity decrease at extreme ranges (Adler-Nissen1986).

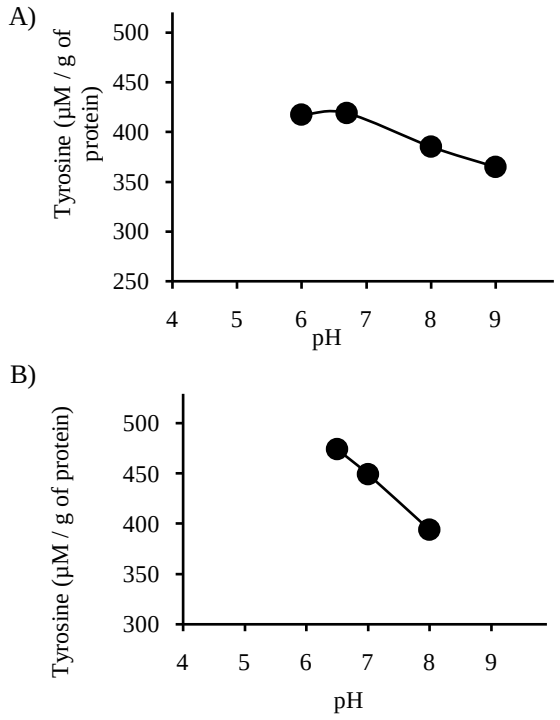

Fig. 6: Effect of pH on extent of hydrolysis of water washed meatfrom *C. catla*.(A): Bromelain; (B): Papain

Effect of hydrolysis time

For optimizing the time of hydrolysis using bromelain enzyme, four different time intervals *viz.* 15, 30, 45 and 60 min were employed. Other conditions such as E / S ratio, temperature and pH were maintained at 0.5:100, 50°C and 6.5 ± 0.2, respectively. The extent of hydrolysis was found to be proportional to time of reaction. At the end of 60 min, the liberated tyrosine was found to be maximum reaching a value of 416 µM / g of protein (Fig. 7A). The time of incubation to achieve higher degree of hydrolysis with papain was standardized. The results revealed that the hydrolysis rate was constant beyond 60 min of incubation. At 60 min the liberated tyrosine was 464µM / g of protein. While at 120 min it was 470 µM / g of protein (Fig.7B). The time of hydrolysis or the incubation time at a given set of conditions is to be determined for the optimum production of the product. The longer the enzyme is incubated with its substrate, the greater the amount of product will be formed. However, the rate of formation of the product is not a linear function with the time of incubation. Different enzymes may have different optimum time due to the difference in the rate of cleavage of peptide bonds. A shorter hydrolysis time with desired degree of hydrolysis will allow more throughputs thereby reducing the cost of production (Adler-Nissen1986).

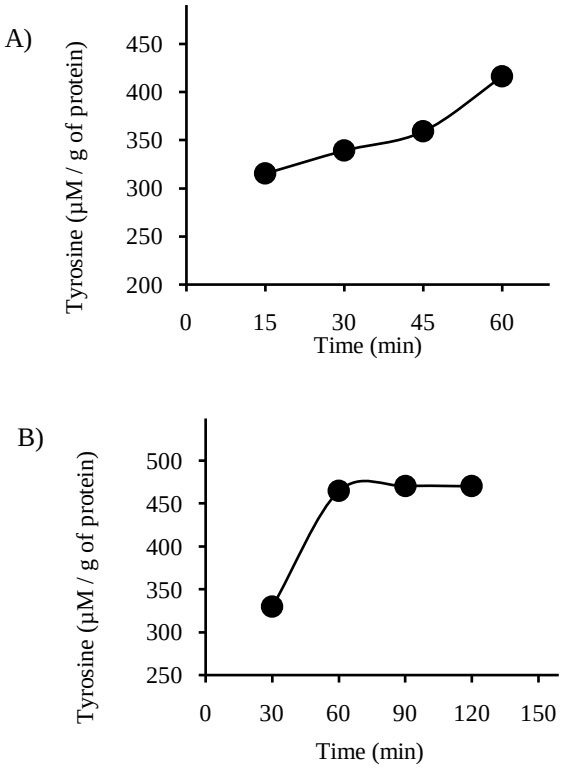

Fig. 7: Effect of time on extent of hydrolysis of water washed meatfrom *C. catla.* (A) Bromelain; (B) Papain

The optimum hydrolysis conditions for bromelain and papain are summarized in Table 2. The optimum pH and Temperature were 6.5±0.2 and 50 °C, respectively for both bromelain and papain. However, one should consider the operational cost and extent of hydrolysis while fixing the E/S ratio and time of hydrolysis.

CONCLUSION

Enzymes such as bromelain and papain were studied to hydrolyse the water washed meat of *C. catla* for their hydrolysis conditions with reference to extent of hydrolysis. The temperature, pH and time to hydrolyze the water washed catla meat using bromelain and papain was found to be 0.5:100, 50°C, 6.5 ± 0.2 and 60 min, respectively. The optimum E/S ratio for bromelain and papain was 2 and 1%,

respectively. Papain was more effective in hydrolyzing the catla meat compared to bromelain.

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