Assiut J. Agric. Sci., (48) No. (6) 2017 (40-54) *ISSN: 1110-0486*
Website: http://www.aun.edu.eg/faculty_agriculture *E-mail: ajas@aun.edu.eg*

Characterization and Purification of Alkaline Proteases From Viscera of Silver Carp (*Hypophthalmichthys molitrix*) Fish

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Received on: 20/11/2017 **Accepted for publication on:** 26/12 /2017

Abstract

Proteases from viscera of Silver carp (*Hypophthalmichthys molitrix*) fish have been extracted and characterized. The alkaline proteases show optimum activity in 0.2M Tris-HCl buffer at pH 8.5 and 45°C using soluble milk casein as substrate. The crude alkaline protease lost about 35% or 51% of its specific activity if it was heated at 50°C or kept at pH 9 for 60 min., respectively. Purification of proteases purified by ammonium sulfate precipitation, gel-filtration using Sephadex G 50 column and ultrafiltration via polyamide membrane (30 KDa) led to increase its specific activity up to 20, 24 and 99 fold, respectively.

Keywords: *Silver carp, alkaline proteases, characterization, purification, specific activity*

Introduction:

Protease enzymes (e.g. trypsin, chymotrypsin, chymosin or cathepsin-like) normally found in all living organisms. They are specific for breakdown the peptide linkages to create peptides and amino acids. There are four types of proteases according to their active site, namely serine (EC 3.4.21), cysteine (EC3.4.22) aspartic (EC 3.4.23) and metallo-proteases (EC 3.4.24) (John *et al*, 2003). Proteinases from animal, plant, and microbial sources have been used for enzymatic protein hydrolysis (Qian and Kim, 2007). These enzymes could be used as processing aid to transform foods into finished products. Where they are selective and specific for their substrates, which permits to produce food products free from contaminants and less toxicity (Lara-Márquez *et al*., 2011) or to generate bioactive peptides from proteins (Jiang *et al*., 2014). Therefore, a group of different types of enzyme may be used as a commercial enzyme in manufacture food product. Moreover, proteases are a powerful tool for modifying the properties of food proteins. They modify the functional properties such as emulsification, fat-binding, water-binding, foaming properties, gel strength, whipping properties (Sawant and Nagendran, 2014), as well as bioactive peptides synthesis (Zhong *et al*., 2011). Thus, proteases are using on a large scale in the food industry, where it is estimated to represent about 40-65% of the total commercial enzyme preparations in the global market (Zhang *et al*., 2009). In the industrial view, proteases can use as pharmaceutical agents (e.g. contact-lens, enzyme cleaners, enzymatic deriders and reduce dental plaques) (Kumar and Suresh, 2016). Also, proteases find applications at various steps of leather processing such as de-haring (Dettmer *et al*., 2011), enzymatic soaking of salt-preserved buffalo hides (Deshpande *et al*, 2004) and for the recovery of silver from used X- ray film by decompose the gelatinous coating of X-ray films, from which silver is recovered (Gupta *et al*., 2002). Different alkaline proteases (alkaline proteases) produced by *Bacillus sp* or extracted from industrial fish-waste may be used as effectively ingredient in detergent powder industry since 1963. Nowadays, detergent enzymes account for 89% of the total proteases sales in the global market (Talita *et al*., 2009).

General, the amounts of fish processing waste is depending mainly on the type of final product, it could be accounted up to 80, 66 and 27% from surimi, fillet as well as beheaded and gutted fish (Chandrasekaran, 2016).

The most common fish processing operation includes three steps: (a) beheaded and removing skin, (b) removing the tail and fins (c) eviscerated and boneless fish fillet. Fish waste is generated from the unwanted parts of the fish which can generally be divided into two types: (a) Solid waste including heads, tails, fins, frames, offal (guts, kidney and liver) and skin. (b) Liquid waste including waste water from cleaning the fish and equipment (IFC, 2011). About 70% of fish is processed and resulting in 20-80% of fish waste according to the level of processing and type of fish (Zhou *et al*., 2011). The presence of endogenous digestive enzymes in fish has been reported in numerous studies (Kishimura *et al*., 2008; Jellouli *et al*., 2009 and Xiong *et al*., 2011). The current global fisheries production of 93.4 million tons (81.5 million tons from marine waters and 11.9 million tons from inland waters), is rising as a result of increases in aquaculture production. Nowadays, the aquaculture provided only 39% for human fish consumption and wild-catch fish present 61% (FAO, 2016).

The total catches of fish in Egypt about 1.34 million tons per year (GAFRD, 2012) which will be expected to increase more than 1.5 million tons (FAO, 2010).

Fish products for human consumptions include fresh, frozen, whole, fillet and other innovative products (Vannuccini, 2004). Most discards composed of head, intestine, skin, bones and viscera reflects the amounts of fish processing waste (Khoddami *et al*., 2009). In fish, adaptive changes in the activity of proteolytic enzymes have been reported in relation to diet. Activity of internal enzymes is influenced by nutritional conditions of fish where they live and the circumstances which adapt those (German *et al*., 2010).

Tilapia fish is popular in Egypt, whether it is wild-capture or farmed followed by Mullet fish. Although the consumer acceptance of carp fish is low, it represents the third rank of farmed fish (Mohamed *et al*., 2012). Tilapia represents 78% of total production of aquaculture while the mullet and carp represent 12 and 10%, respectively, (GAFRD, 2004).

Silver carp (*Hypophthalmichthys molitrix*) is a kind of fresh water aquaculture species. Huge amounts of silver carp by products (heads, skin, bone, scales and viscera) are produced during the fish processing. This became as resources of hazard environmental pollution resources wastes (Zhang *et al*., 2009). Viscera is one of the most important by products delivered digestive enzymes, especially proteases that have high activity over a wide range of pH and temperature conditions (Kumar *et al*., 2016). The environmental had effects condition and type of food in fish environment on the characteristics of the extracted enzyme especially the temperature and pH (Ben Khaled *et al*., 2011).

This work was performed to characterize of alkaline proteases extracted from the viscera of Silver carp (*Hypophthalmichthys molitrix*) fish. Also, effect of partial purification by different methods of the enzyme activity was studied.

2. Materials and methods:

2.1. Materials:

2.1.1. Fish sample:

Fresh Silver carp (*Hypophthalmichthys molitrix*) fish (average weight 500-900g for each specimen) were bought from privet Aquaculture Farm located at Trompat 7- Kafr El-Sheikh Governorate, Egypt. Fish were placed in an icebox and immediately transferred to the laboratory of Food Science and Technology Department, Faculty of Agriculture, Tanta University.

2.1.2. Chemicals:

Milk soluble casein was bought from Sigma (USA), Tris-HCl was obtained from LabaChemie (Mumbai-India), trichloroacetic acid (TCA) was purchased from SDFCL Fine-Chem Limited (Mumbai-India) and ammonium sulfate was obtained from ADWIC EL-Nasr pharmaceutical Chemicals Co. Sephadex G-50 was supplied from Pharmacia (Uppsala – Sweden).

2.2. Methods

2.2.1. Preparation of crude alkaline proteases:

Alkaline proteases were prepared from fresh Silver carp fish in the form of acetone extract powder. The fresh fish were eviscerated to separate viscera using a sharp knife. The separated viscera was defatted by 8 volumes of cold acetone (-18°C) for 30 sec in homogenizer (Hamilton Beach, southern Pines, North Carolina, USA) at high speed. The homogenate was filtered through filter paper (Whatman no. 40) and the residual material was washed several times with acetone. Finally the residue was washed up with 50 ml of diethyl ether and dried overnight at room temperature. The obtained powder was kept in brown glass and stored at -18°C for further analysis.

2.2.2. Protein assay of the prepared crude enzyme

Protein content of crude alkaline proteases preparation was calorimetrically determined according to Brad (1976) method using Coomassie Plus™ Protein Assay Kit (Thermo Scientific, Illinois, USA) and bovine serum albumin BSA (included in the kit) as a standard protein. Absorbance was measured at 595nm using visible Spectrophotometer (UV-Visible Spectrophotometer–UV 1901PC-Phenix- Chain).

2.2.3. Characterization of the prepared crud enzyme

2.2.3.1. Selection of optimum buffer:

Tow buffers namely sodium phosphate and Tris-HCl at 0.2M and pH 8 were prepared. Alkaline proteases activity was carried out according to the method of Caramori *et al*. (2011) with slight modification as follows:

Accurate one g of acetone powder was dissolved in 100 ml buffer to prepare 1.0% acetone powder (as crude enzyme). Added 2.0 ml 0.5% casein in 0.2M Tris-HCl buffer with 1.0 ml 1.0% acetone powder in 20-ml test tube. The mixture was incubated at 37°C in water bath for 10min. The reaction was stopped by adding 1.0 ml of 5.0% TCA, centrifuged at 10,000rpm for 15 min at room temperature. The supernatant was transferred to 10-ml measuring flask and the volume was brought to the mark with distilled water. Absorbance was measured at 280nm in Spectrophotometer (UV-Visible Spectrophotometer, – UV 1901PC- Phenix-Chain) against blank. Where, blank is carried out by incubated the substrate without enzyme then added 1.0 ml of 5.0% TCA.

2.2.3.2. Determination the optimum pH:

The optimum pH was determined by measuring the activity of the prepared crud enzyme in 0.2M Tris-HCl buffer at various pH's (7.5, 8.0, 8.5 and 9.0), according to Yanez *et al*. (2005).

2.2.3.3. Determination of optimum temperature:

The optimum temperature was determined by measuring the activity of the prepared crud enzyme in 0.2M Tris-HCl buffer at optimum pH 8.5 and various temperatures (35, 40, 45 and 50°C) according to Yanez *et al*. (2005).

2.2.3.4. Determination of optimum substrate concentration:

Activity of the prepared crud enzyme at various enzyme concentrations (0.5, 1.0, 1.5, 2.0 and 5.0; p/p) (protein enzyme / protein substrate) using 0.2 M Tris-HCl at pH 8.5 and 45°C was determined according to Kalpana Dev *et al*. (2008).

2.2.3.5. Determination of thermo-stability:

Thermo-stability of crud prepared alkaline protease was evaluated by incubation the enzyme-substrate mixture (1:5 P_E: P_S) using casein (0.5% as substrate) at different temperatures (30, 40, 45, 50, 60 and 70°C) for 60 min. The activity was determined according to Ktari *et al*. (2014).

2.2.3.6. Determination of pH stability:

The pH stability was evaluated by incubated the prepared crud enzyme at various pH (8.0, 8.5, 9.0, 10 and 11.0) for 60 min. Residual activity was determined at 45°C using 0.5% casein as substrate according to Ktari *et al*. (2014).

2.2.4. Purification of the prepared crud enzyme:

2.2.4.1. Ammonium sulfate precipitation:

The crud alkaline protease was mixed with ammonium sulfate at 20%, 40% and 60% saturation. The mixture was incubated at 4°C for 24hrs. Then the mixture was centrifuged at 10,000 rpm at 4°C for 30min and discarded the supernatant (EL-Beltagy *et al*., 2005). The pellets were re-dissolved in 0.2mM Tris- HCl buffer at pH8.5 and dialyzed against the same puffer at 4°C in the refrigerator for 24hrs. After that enzyme activity was determined at 45°C using 0.5% soluble milk casein as substrate according to Kishimura *et al.* (2005).

2.2.4.2. Gel filtration:

Crude prepared alkaline protease was purified by gel filtration technique using Sephadex G-50 according to Atta (1986). The enzyme was loaded into Sephadex G-50 column (5cm long × 20mm) at 4°C using Tris-HCl buffer at pH 8.5 as an eluting solvent. Fractions were collected each 1.0 ml in 1.5-ml Eppendorf tubes. The collected fractions were subjected to protein assay and enzyme activity as mentioned above.

2.2.8. Ultrafiltration:

Crude prepared alkaline protease was purified by Ultrafiltration using polyamide membrane (cut off 30KDa). The proteases activity was determined in the filtrate at 45°C using 0.5% casein in Tric-HCl buffer as substrate as mentioned above.

3. Results and Discussions

3.1. Characterization of enzyme preparation:

3.1.1. Selection of optimum buffer:

The results indicated that optimal buffer for alkaline proteases activity was Tris-HCl, which was better than that of sodium phosphate buffer. This may be related to Tris-HCl is very freely soluble in water, inert in many enzymatic systems and has a high buffer capacity. This result agrees with those reported by Kishimura *et al.*(2007) and Klomkla *et al.* (2011).

3.1.2. Determination of optimum pH:

The highest significantly enzyme activity of crude alkaline proteases (2.5 U/mg) was achieved at pH 8.5 among the other tested pH's (Fig 1). Similar results for trypsin extracted from viscera of hybirid cat fish were reported by (Klomkla *et al.*, 2011). Similar results were obtained from trypsin activity extracted from the viscera of Monterey sardine Kishimura *et al.* (2006) or true sardine (Yanez *et al.*, 2005) and alkaline proteases extracted from digestive system of carp (*Catla catla*) (Khangembam *et al.*, 2012). In this reported, (Marcuschi *et al.*, 2010) pointed out that trypsin extracted from the digestive system of fish is normally active in the alkaline region (from pH 7-12) using BAPNA as a model of substrate.

This may be related to the activity of alkaline proteases normally does not work in the acidic side, whoever the protein of the enzyme could be denaturated in the acidic medium (Klomkla *et al.*, 2011).

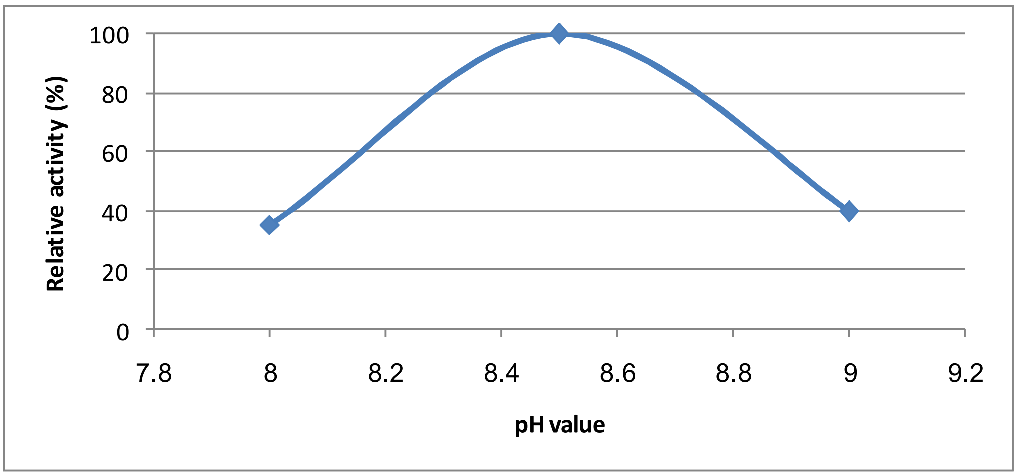

Fig (1): Optimum pH of crud alkaline proteases extracted from the viscera of Silver carp (*Hypophthalmichthys molitrix*) fish

3.1.3. Determination of optimum temperature:

The significantly highest activity of crude prepared enzyme (3.31U/mg) was found at 45°C. Similar results were obtained for alkaline proteases extracted from the viscera of Tilapia fish (EL-Beltagy *et al.*, 2005) but slightly higher than that of purified trypsin extracted from the digestive system of carp fish (Khangembam *et al*., 2012) and lower than that purified trypsin extracted from the viscera of hybrid cat fish (*Clarias gariepinus*) (Klomkla *et al.*, 2011) or pyloric caeca of jacopever (*Sebastes schlegelii*), elkhorn sculpin (*Alcichthys alcicornis*) (Kishimura *et al.*, 2007) and zebra blenny (*Salaria basilisca*) (Ktari *et al.*, 2012). These differences may be related to the variation in the environment conditions including the temperature and type of food (Rungruangsak-Torrissen, 2016).

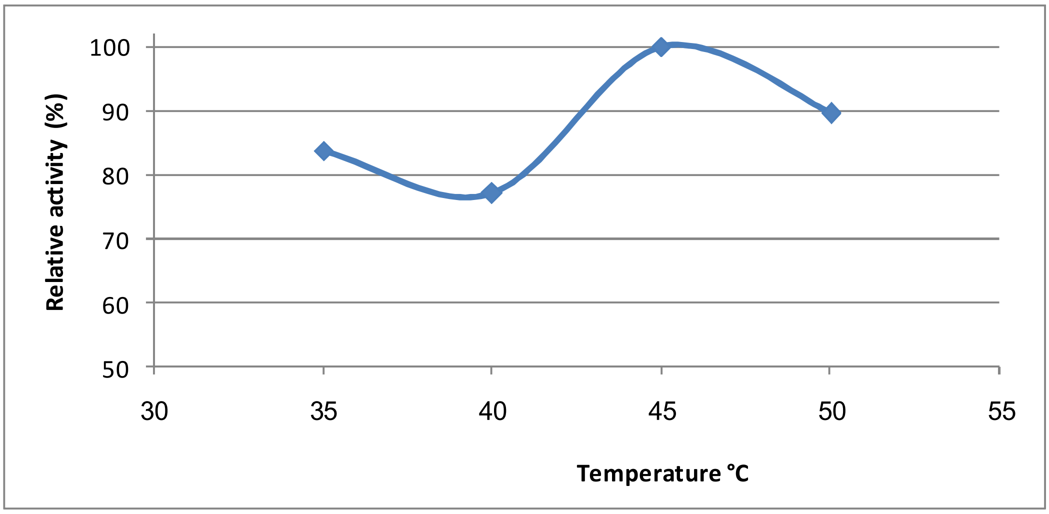

Fig (2): Optimum temperature of crud alkaline proteases extracted from the viscera of Silver carp (*Hypophthalmichthys molitrix*) fish

3.1.4. Determination of optimum substrate concentration:

Results of (Fig., 3) revealed that the highest significantly activity was 1:5 (p_E: p_S) among the other tested concentration (0.5:5, 1.0:5, 1.5:5 and 2:5 p_E: p_S). This result more than that reported by Dey and Dora (2011) for shrimp waste protein hydrolysis using microbial proteases (1:2%).

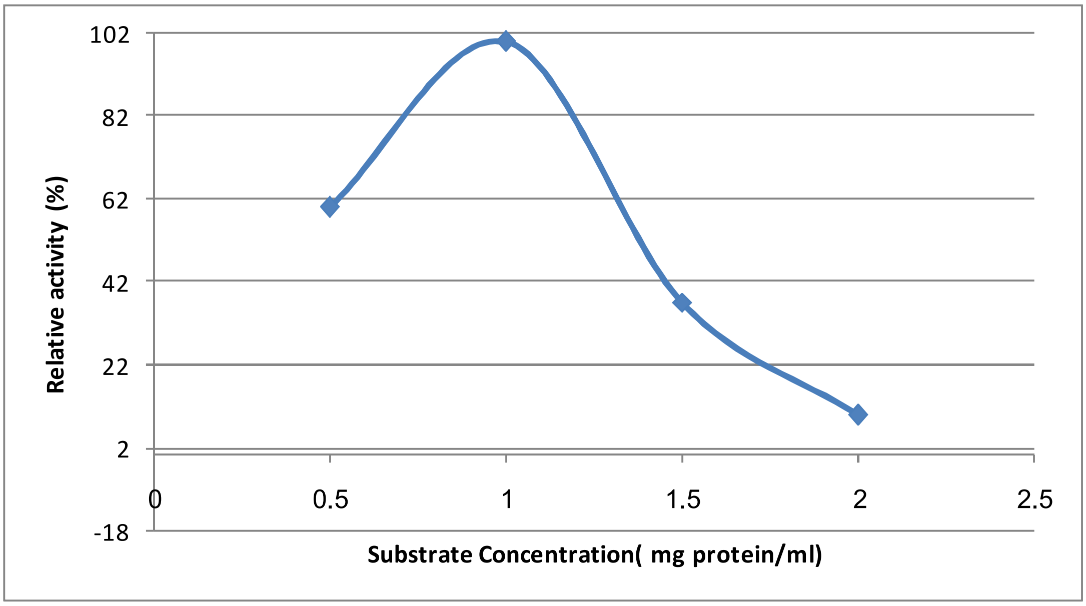

Fig (3): Relative activity of alkaline proteases extracted from the viscera of Silver carp (*Hypophthalmichthys molitrix*) fish as affected by substrate concentration

3.1.5. Thermo-stability:

The thermo-stability of alkaline proteases prepared enzyme was achieved at 40 to 50 ° C. This result is in agreement with Kishimura *et al.* (2006) for trypsin extracted from the viscera of true sardine. Also, Barkia *et al*. (2010) found that purified trypsin was highly stable below 40°C and begin to inactivate at higher temperatures. These differences in thermal stability of enzyme stabilization could be related to the effect of temperature on the unfolding of aboenzyme (protein moite of enzyme) and change the active center, thus enzyme-substrate binding become difficult at high temperature than 40°C. As a result, enzyme turns to in active one.

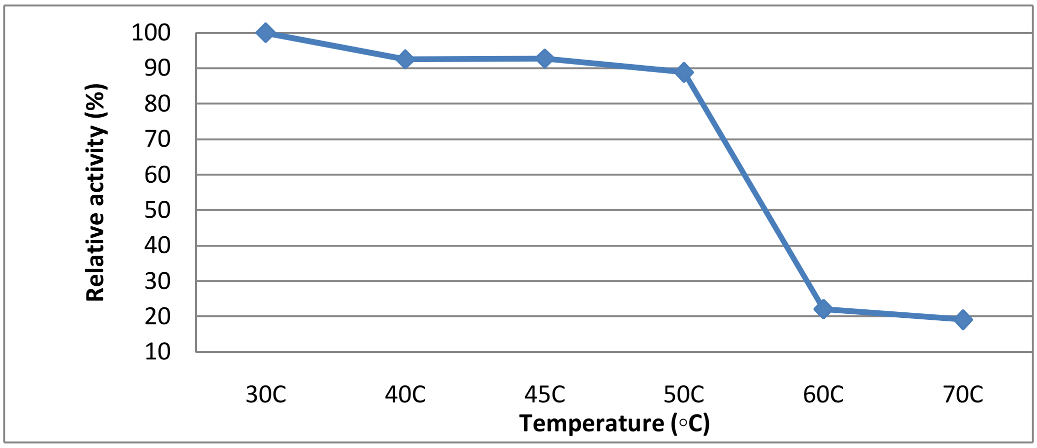

Fig (4): Thermo-stability of crud alkaline proteases extracted from viscera of Silver carp (*Hypophthalmichthys molitrix*) fish

3.1.6. The pH stability:

Maximum relative activity of prepared alkaline proteases was (0.40U/ml) achieved at pH8.5 for 30min, which is not significantly different than that at pH 9.0 for 30 min (0.36U/ml) (Fig.5). On the other hand, relative activity of the prepared enzyme was significantly droped to 0.26 at pH 11.

This result is lower than that at the pH at ability alkaline proteases extracted from the viscera of Giant cat fish (*Pangasianodon gigas*) (Vannabun *et al*., 2014) and Red scorpion fish (*Scorpaena scrofa*) (Younes *et al*., 2015) which was pH 12. On the other hand, the obtained result is higher than that of trypsin extracted from monterey sardine (*Sardinops sagax*) (pH 7.0 ~ 8.0) as reported by Yanez *et al*. (2005)

Enzyme stability is related to protein net charge at a particular pH. The differences in optimal pH and pH stability are attributed to the net charge of the active center which is affected by the pH of the reaction environment (Robinson, 2015).

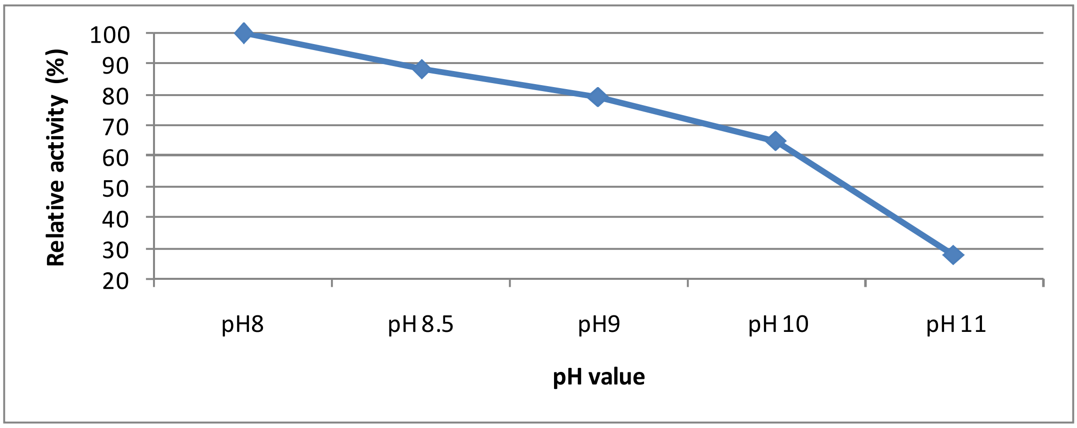

Fig. 5 the pH stability of crude enzyme extracted from the verscera of silver carp (*Hypophthalmichthys molitrix*) fish

3.2. Purification of crude enzyme

3.2.1. Ammonium sulfate precipitation:

Specific activity of crude alkaline prepared proteases was augmented after precipitation the enzyme using a saturated ammonium sulfate at 20%. The specific activity was also, increased to reach the significantly highest value after using ammonium sulfate solution saturated at 40%. After that, the specific activity was dropped to 22% when the ammonium sulfate solution was saturated at 60% (Fig.6). This result is close to Ben Khaled *et al*, (2011) they showed that higher specific activity of trypsin isoforms extracted from viscera of sardinelle (*Sardinella aurita*) fish preparation was detected by ammonium precipitation at 20–70% (w/v).

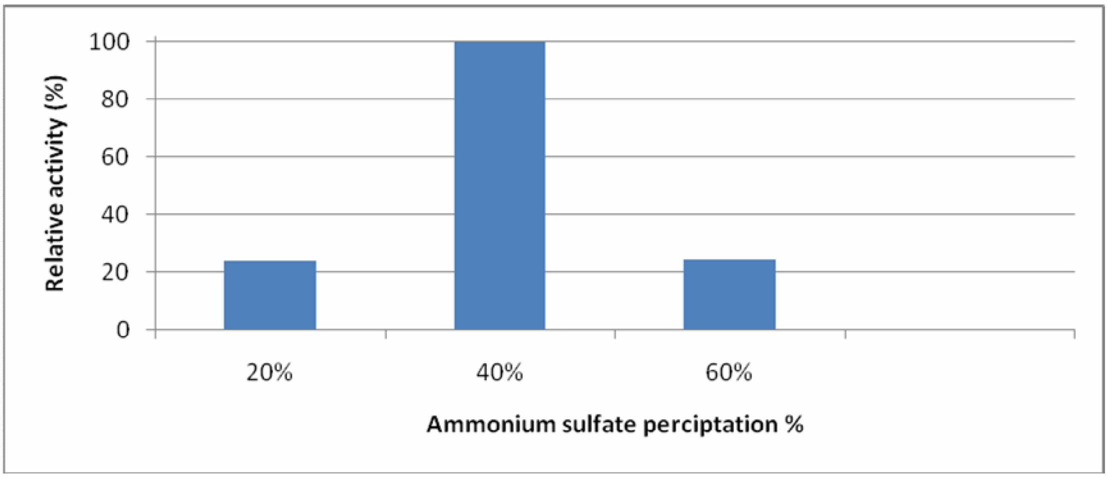

Fig (6): the relative activity of alkaline proteases extracted from the verscera of Silver carp (*Hypophthalmichthys molitrix*) fish as affected by ammonium sulfate precipitation

3.3.2. Gel-filtration:

Purification by gel-filtration technique using Sephedx G-50 column was applied. Tris-HCl buffer at pH 8.5 was used as eluted solvent. The results show that three fractions only have active enzyme. The significantly highest active fraction (fraction no 3) has 0.203 U/mg followed by fraction no 4 (0.11U/mg), while the first fraction (first one ml) does not show active enzyme, where the enzyme activity is binging from the fraction no 2. These result is in agreement with (Ben Khaled *et al.*, 2011).

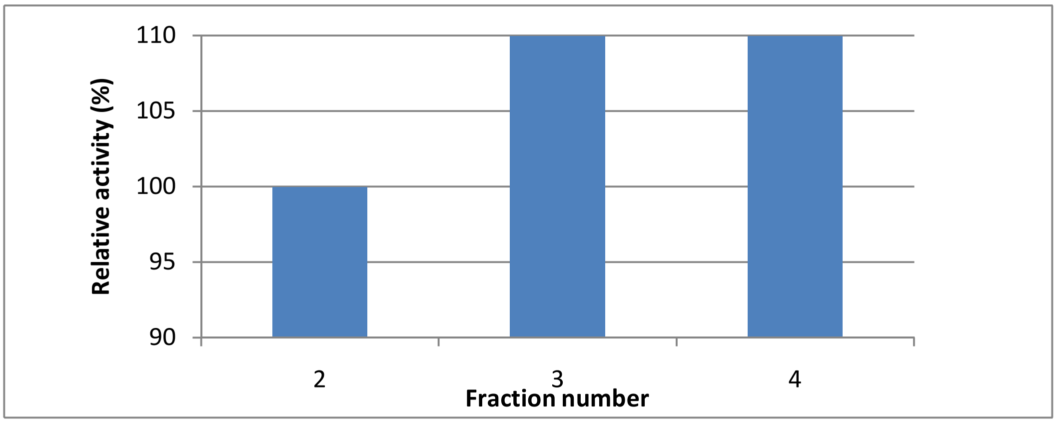

Fig (7): the relative activity of alkaline proteases preparation extracted from the viscera of Silver carp (*Hypophthalmichthys molitrix*) fish as affected by gel-filtration.

3.3.3. Ultrafiltration via polyamide membrane (30 KDa):

As shown in Table (1) and Fig.(8), the relative activity of alkaline proteases extracted from the viscera of silver carp was gradually increased according to the purification steps. Where, the significantly highest relative activity of the enzyme was obtained by ultrafiltration technique (139 U/ml) followed by Gel-filtration on the Sephadex column (21 U/ml) and the lowest one was detected in the crude enzyme (4.4 U/ml).

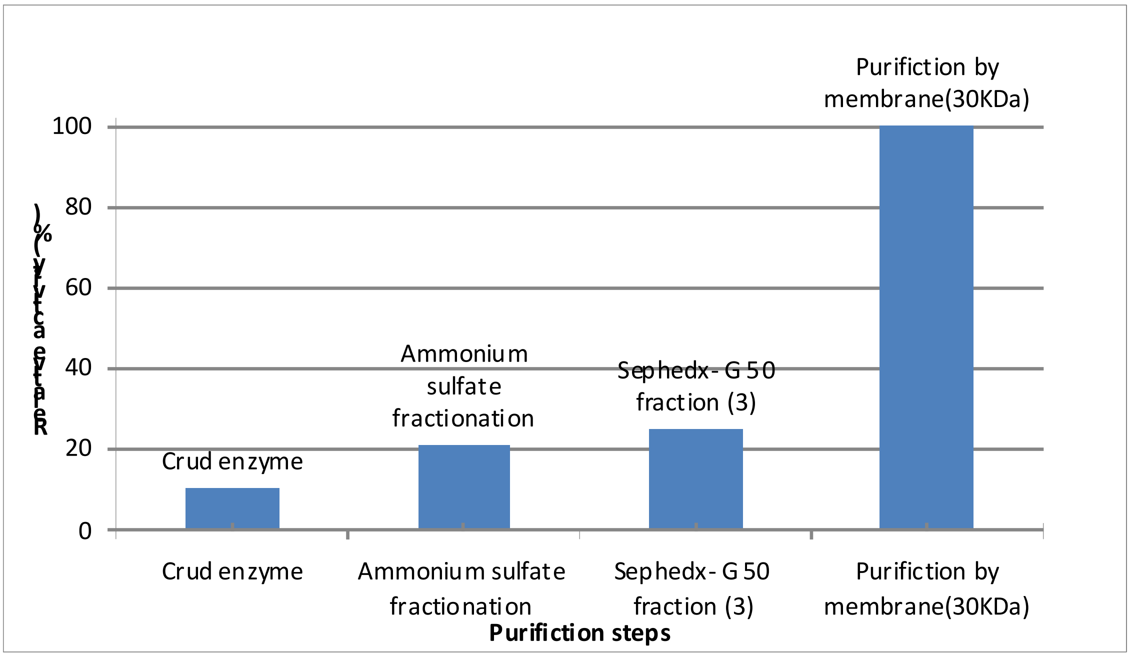

Fig (8): The relative activity of alkaline proteases extracted from the verscera of Silver carp (*Hypophthalmichthys molitrix*) fish as affected by different purification techniques

Table (1). Alkaline proteases enzyme activity as affected by purification method.

Purification phase	Activity (U/ml)	Protein (mg/ml)	Sp Act. (U/mg)	Pure. Fold
Crude enzyme	4.4±0.43	116.5±0.68	0.04^{d}±0.00	1
$(NH_4)_2SO_2$ (40% preceptation	22.1±0.59	28.0±0.80	0.79^{c}±0.02	20
Gel Filtration on Sphadex-G50	21.0±0.48	22.7±0.75	0.92^{b}±0.04	24
Polyamide Ultrafiltration(30 KDa)	139.6±0.45	37.3±0.63	3.74^{a}±0.05	99

M± SD = Means and standard deviation of three successful trails
Sp.Act = specific activity U/mg protein
Pure fold = Purification fold

Conclusion:

Viscera of Silver carp (*Hypophthalmichthys molitrix*) fish could be use as suitable source of proteases enzymes. These enzymes show high specific activity at pH 8.5 and temperature 45°C. Purification with ammonium salfat precipitation, gel-filtration by Sephedx G50 fraction and ultrafiltration via polyamide membrane (30KDa) increase its specific activity up to 20, 24 and 99fold, respectively. Consequently, fish viscera from silver carp (*Hypophthalmichthys molitrix*) manufactures offal's could be used as a raw material to extract alkaline proteases enzyme that may be use in different technological uses.

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Assiut J. Agric. Sci., (48) No. (6) 2017 (40-54) *ISSN: 1110-0486*
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استخلاص وتنقية الأنزيمات البروتينيه من أحشاء سمك المبروك الفضى

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الملخص

تم استخلاص الانزيمات البروتينية من أحشاء سمك المبروك الفضى و درست خواص الأنزيم (افضل بفر- تقدير الأس الهيدروجيني الأمثل- تقدير درجة الحرارة المثلي- تقدير تركيز الأنزيم الأمثل....الخ)، كما اجريت عمليات تنقية الأنزيم الخام بأستخدام التقنيات المختلفة (الترسيب باملاح كبريتات الأمونيا و السريان على عمود جيل G50 واستخدام غشاء 30 كيلودالتون) و قد بينت النتائج المتحصل عليها أن أعلي نشاط للأنزيم كان باستخدام بفر Tris-HCl عندالأس الهيدروجيني 8.5 (pH8.5) و درجة حرارة 45 م° باستخدام الكازين كمادة تفاعل. كذلك فقدت الانزيمات 35٪ و 51 ٪ من نشاطها عند التحضين على درجة حرارة 50°م و أس هيدروجيني 9(pH 9) لمدة 60 دقيقةعلى التوالى. أما بالنسبة لتنقية الأنزيم فقد اظهرت النتائج أن الترسيب بأملاح الأمونيوم والترشيح بالجل تزيد نشاط الأنزيم الخام الي 20 و 24 ضعفا على التوالى. أما تنقية الأنزيم بواسطة الترشيح الفائق باستخدام غشاء البولي اميد يزيد نشاط الأنزيم الي 99 ضعفا.