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Rheological properties of skin gelatin of Beluga Sturgeon (*Huso Huso*) from The Caspian Sea

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ABSTRACT

The effect of Pre-treatment condition has been studied for extracting gelatin from Beluga Sturgeon fish skin with acetic Acid (0.2 N and 2.0 N) and NaOH (0.2 N and 2.0 N) on gelatin extraction yield, melting point, viscosity and SDS-PAGE electrophoresis pattern of the solution. The results showed that extraction yield increased by increasing acid concentration or decreasing NaOH concentration ($p<0.05$). The samples treated with 2.0 N acid- 2.0 N NaOH solutions and 0.2 N Acid – 2.0 N NaOH solutions had the lowest and the highest melting point respectively. The values were respectively 17°C and 23°C. The results showed a significant correlation between the type of gelatin and its functionality.

Keywords: Pre-treatment, Gelatin, Rheological properties, Melting point

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INTRODUCTION

Gelatin represents a major source of good quality protein biopolymer with many applications in the food, pharmaceutical, photographic and cosmetics industries [1]. Collagen found in the skin and bone of animals and fish yields high quality gelatin when it undergoes thermal denaturing [2]. Gelatin is an important functional property of fish protein affecting the texture of fish products. By-products from fish processing are potential sources of collagen. The waste from fish processing after filtering can account for as much as 75 % of the total catch weight [3]. Production and utilization of fish gelatin not only satisfies the needs of consumers, but also serves as a means to utilize some of the by-products of the fishing industry [4]. The classical food, photographic, cosmetic and pharmaceutical application of gelatin is based mainly on its gel-forming properties. Recently, and especially in the food industry, an increasing number of new applications have been found for gelatin in products such as emulsifiers, foaming agents, colloid stabilizers, biodegradable film-forming materials and microencapsulating agents, in line with the growing trend to replace synthetic agents with more natural ones [5]. Commercial gelatin is almost extracted from porcine or bovine bones and skins using hot water extraction after acid or alkaline pretreatment. In extraction field, Hydrochloric acid, sulfuric acid, phosphoric acid, calcium hydroxide and sodium hydroxide used [6]. Compared to mammalian gelatins, fish gelatins have many different properties. The gelling and melting temperatures of fish gelatins are lower than those of mammals [7].The utilization of fish gelatin is partial. So far, the extraction of gelatin from fish scales is not usual, and only several fish gelatins were produced, e.g. Atlantic salmon *(Salmo salar*) [8] and rainbow trout *(Onchorhynchus mykiss)* [9] and Bigeye snapper (*Priacanthus tayenus*) [1], Dover sole (*Solea vulgaris*) [10] Nile perch (*Lates niloticus*) [11]. The objective behind this research was to optimize gelatin extraction from fish skin and characterize its rheological and physical properties.

MATERIALS AND METHODS

Preparation of materials

The Beluga Sturgeon (*huso huso*) was always caught from south of The Caspian Sea. Frozen fish skin was provided From Kian mahi Khazar Co., Babolsar, Iran. Then, the skins were washed by cold tap water in order to remove the attached meats and scales, also cut into pieces both in length and width about 3- 4 c.m. The pieces were thoroughly rinsed in excess cold water to remove superfluous materials and rinsed with tap water (1:6 w/v) at room temperature three times. Excess water was drained off and squeezed in a manual press, then stored at –18°C. The washed skins before use were allowed to thaw below 15°C and the adherent tissues of skins were scrapped manually by a scalper.

Pretreatment and gelatin extraction*:*

Gelatin was extracted using a slightly modified method of Sarabia *et al.* (2000) [12]. Based on preliminary experiments, thawed skins (weight, 50gr) were added to a flask containing four times as much as volume of different NaOH (0.2 –2 M) solutions for 60 min. at 7 °C. Alkaline pretreatment helps the removal of fat and non collagen proteins also prevent the effects of endogenous proteases on collagen. Treated skins were washed thoroughly with excess cold water, rinsed with cold distilled water, and then treated with acetic acid by the same previous condition. Skins were drained off using cheesecloth. Rinsing with distilled water was carried out between soakings. The above procedure was repeated for three times. Gelatin was extracted through adding four volumes of distilled water in a water bath (Memmert, WB14, Germany) at 16-18 hr. Finally, the extract was then filtered through Whatman filter paper (No. 4), collected, dialyzed against distilled water overnight at room temperature. Then, final protein concentration diluted to 2µgr/µlit. The solutions were mixed by sample buffer and lyophilized. Gelatin characterization has been done immediately after extraction or keeping in refrigerator (6-7°C) for maximum 24 hrs all three times. All of the solutions used in the above steps were kept at 4^{o}C.

Yield of protein

The soluble protein concentration was determined by the Biuret method using a spectrophotometer at 540 nm. (PG Instruments, T80+UV-VIS, UK) has used Bovine Serum Albumin (Zistchimi, Tehran, Iran) as standard protein. The concentration of BSA was increased from 1 to 10 mgr/mLit in order to obtain a better linear relation for the standard curve.

The yield of protein (YP), mainly gelatin, extracted for skin was calculated using the following equation1: Yield (%) = [(C×V)/ M] ×100,

Where C= protein concentration in extracted solution (g/ml); V= volume (ml), M= weight of sample before extraction (g)

Determination of melting point

Melting point was measured using a slightly modified method from [13]. Solutions containing 3% (w/v) gelatin were prepared in thin wall (12 mm×75 mm) screw cap test tubes. The test tubes were filled to leave some headspace. Samples were matured at 7^{o}C for 16–18 hr in a refrigerator. In each tube strew mixture, were added 2-3 drops of chloroform – methyl red. The tubes were transferred into a cold water bath (4°C) and warmed gradually (about 0.2 ^{o}C/min). The temperature at which the gel method was applied for moving down purposes was recorded as the melting point. Moving down was recorded as melting point.

Measurement of gelatin viscosity

Distilled water was used to adjust the total protein concentration of the extracted gelatin solutions (0.5, 1, 2, and 3) % (w/v). A capillary viscometer of the Ostwald type, i.e., a Cannon–Fenske routine viscometer Number 200 Viscometer (Cannon Instrument Co., State College, PA, USA) has been applied to determine the viscosity (V, k) of 6.71 ml gelatin solutions at 30,40,50 ^{o}C in water bath. Each sample had at least three replications. The efflux time was recorded using a stopwatch. Viscosity can be calculated according to 2: V=k (t- θ) equation

SDS - polyacrylamide gel electrophoresis (SDS-PAGE)

The gelatin solution was dialyzed for 24-48h against distilled water using 12 kDa cut-off dialysis tubes. The concentration of gelatin solutions were adjusted to 2 mg/ml with distilled water and then a 3-fold-concentrated loading buffer containing ß-mercaptoethanol was added at 60ºC. Protein samples were heat-denatured for 5 min. at 90ºC and analyzed by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) according to Laemmli (1970) [14] with a 5% (w/v) stacking gel and 15% (w/v) separating gel. Vertical slab gel electrophoresis was carried out in a mini electrophoresis unit (Cleaver scientific Ltd) at 25±2°C. Equal amounts of proteins were loaded on to each lane of gel and run for 3 hrs at 50 mA in a 1 mm thick gel. After electrophoresis, the gel was stained with Coomassie

Blue R-250 dye in methanol: acetic acid water solution (5:1:4, by volume) and destained in methanol: acetic acid water solution without dye (1:1:8, by volume). The density of gel was determined by volumogram method on a photo documentation system (GENIUS Tech., USA) using GeneSnap software ver. 6.0.8. Approximate molecular weight of the sample was determined using wide-molecular-weight marker kit obtained from Fermentas (Fermentas Company, Germany).

Statistical Analyses

A completely randomized design through factorial experiment (2×2×3) with 3 replications has been applied. The means of the results were compared using Duncan's Multiple Range Test ($P<0.05$) by means of MSTATC software ver. 1.42 [15]. All experiments were conducted at least three replicates.

RESULTS AND DISCUSSION

Effects of different pretreatments on melting point of extracted gelatins

The melting point of gelatins in different pre treatment conditions are shown in Figure1. The amino acid composition of gelatins prepared by alkaline process differs from those by the acid process. In general, the alkali process possesses higher hydroxyl proline and lower tyrosine contents than either the acid process gelatins or the raw materials [16]. Gelatin melting point is a function of amino acid composition and average molecular weight. The samples treated with 2.0 N Acid- 2.0 N NaOH solution and 0.2 N Acid -2N NaOH solution had the lowest and highest melting point respectively. Thus, the values were 17°C and 23°C. Melting point increased as the result of a raise in NaOH concentration. But acid concentration had an adverse effect. The results showed that by decreasing acid concentration and increasing of NaOH, melting point increased considerably. Physical properties of gelatins are influenced more by extraction conditions than by imino acid composition [17]. These melting points in these samples are far higher than those reported for cod skin which was in the range 8–10 ^{o}C [18]. It is also known that fish gelatin has a lower melting point than mammalian gelatin [19]. Choi and Regenstein (2000) reported that the melting point increases with the maturation temperature [20]. The amino acid composition may also contribute to the melting point characteristics [19]. In Appearance aspect, Acid 0.2 N – 2.0 N NaOH solutions had haze mode and contain more impure material compared to other samples.

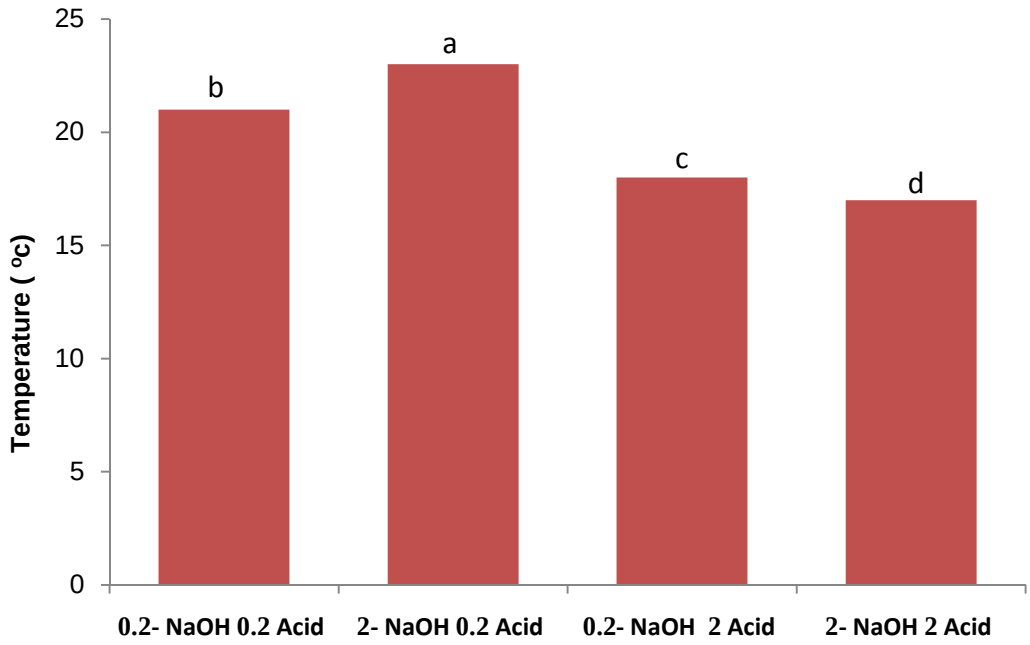

Fig. 1: The effect of pretreatment on melting point in extracted gelatins of beluga sturgeon (*huso huso*) skin fish

Yield of gelatin extraction

The functional properties of gelatin are related to their chemical characteristics and quality of gelatin for a particular application therefore depends largely on its rheological properties [20]. The effects of the extraction conditions on gelatin yield and the corresponding physical properties have been reported for the skins of many fish species [21]. These yields were lower than those reported by Grossman and Bergman (1992) for tilapia spp. [22], for lumpfish skin by Osborne, Voight, and Hall (1989) [23] and for cod skin (Gudmundsson and Hafsteinsson 1997) [18]. The results of the experiment showed that extraction yield increased by increasing in acid concentration or decreasing in

NaOH concentration ($p<0.05$). High yields can only be obtained using neutral and acid pretreatments [24]. This lower yield could be either due to the loss of extracted collagen, through leaching, during the series of washing steps or due to incomplete hydrolysis of the collagen. The effect of acid and NaOH on the yield of the studied samples indicated that it increases by increasing acid concentration. NaOH concentration had a negative relation on yield and reduced the extracted gelatin content. Ando has remarked this reason serves a hydrolysis between amino acid fractions and amine groups [17]. NaOH dissolved soluble imino acid and helped purification of the extracted gelatin. Against NaOH, acid, concentration was more effective on breakdown crosslink and degradation hydrogen bands, it caused more extraction protein and imino acid fractions. Therefore increase of acid concentration, extracted gelatin protein content rose. Also despite of NaOH presence, by increase acid concentration raise skin swelling and this case affected the extracted solution volume. Regarding Fig. 1 and Fig. 2, we observed a negative relation between yield and the melting point within all samples. The sample by highest melting point had the lowest yield. In comparison with other pretreatment conditions, this case can be due to high efficiency NaOH on dissolved skin protein, a decrease in swelling or an increase in the lost protein in extracted gelatins.

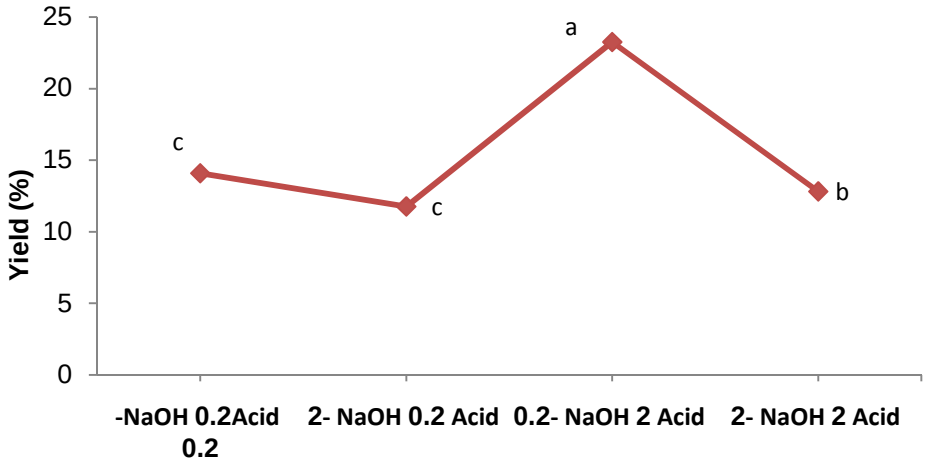

Fig. 2: The effect of pretreatment on yield in extracted gelatins of beluga sturgeon (*huso huso*) skin fish

Effects of different pretreatments on viscosity in different concentration and temperature of extracted gelatins

The most important physical properties of gelatin are gel strength and viscosity [8]. Viscosity is the second most important commercial physical property of a gelatin [16]. Gudmunsson and Hafsteinsson (1997) reported that the viscosity of the gel may be mainly due to the molecular weight distribution rather than the amino Acid composition of the gelatin [18]. viscosity of the gelatin of black tilapia can be considered to be in the mid-range and that of the red tilapia at the lower range since viscosities for commercial gelatin have been reported to be from 2.0 to 7.0 cP for most gelatins and up to 13.0 cP for specialized ones [25]. The viscosity of gelatins solutions is partially controlled by molecular weight and poly dispersity [26]. Minimum viscosity of gelatin has been noted to be in the range of pH 6–8 for many gelatins [16]. The pH effect on viscosity is minimum at the isoionic point and maximum at pH 3 and 10.5. Viscosity decreased by reducing of gelatin concentration and increasing of temperature. However, Acid concentration was more effective on viscosity than NaOH concentration ($p<0.05$). The hydrogen and hydrophilic band is of the most importance in viscosity changes .These cases cause a viscosity increase in gelatin solutions. In the study of the effect of different temperature and Acid concentration on viscosity on whole samples, it is observed that viscosity decreases against any Acid concentration, but in equal temperature, by increasing Acid concentration, viscosity increases. However, temperature as compared to Acid concentration on viscosity changes was even more (Figs. 3, 4, 5, 6). The effect of different temperatures and NaOH concentration on viscosity extracted solution has been investigated. In a different NaOH concentration, by increasing temperature, viscosity decreases. Low concentration NaOH not effective on reduces viscosity, but by an increase in NaOH concentration gradually decreases viscosity in all samples. By studying between electrophoresis pattern and viscosity changes in different pretreatment conditions presence of large HMW fractions in Acid 2 N- 0.2 N NaOH can be proved by increasing viscosity as compared to the other samples. Also increasing sodium hydroxide concentration causes lower viscosity. In a nutshell, we may conclude that different pretreatment conditions. Acid causes increasing viscosity by having a main role in demineralizing collagen and degradation peptides chains and establish protein fractions. Low concentration Acid can improved hydrolysis peptide bands and facilitate transforming collagen structure to gelatin. This changing was little and decreasing viscosity may be due to presence of low molecular weight peptide

component. By increasing Acid concentration breakage collagen bands and changing collagen conformation structure accelerates. Also micro component serve hydrolysis make interaction together and thus viscosity increases. NaOH decreases viscosity by reducing molecular weight collagen extracted establish intrusive structure and avoid formation crosslink inter chain. Hence increasing NaOH concentration can decrease viscosity.

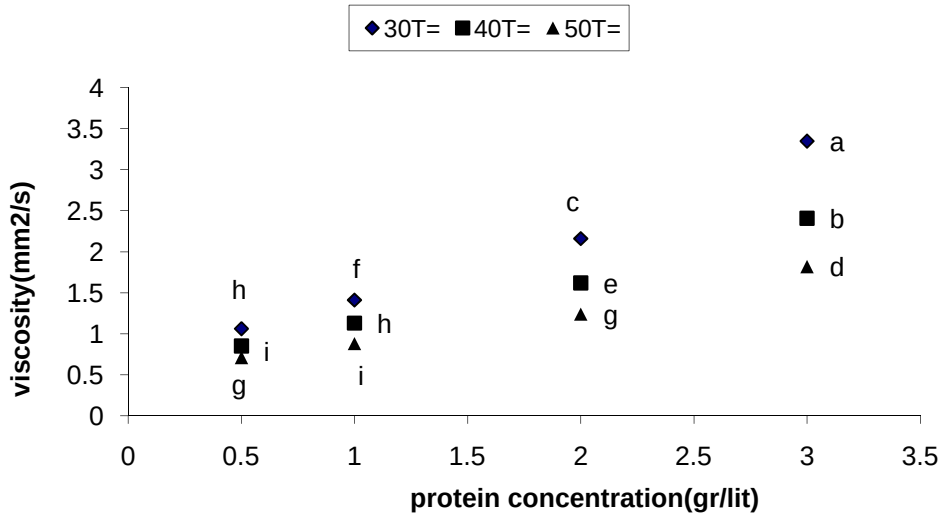

Fig. 3: viscosity of gelatin aa s function of temperature and gelatin concentration (treated with Acid 0.2 N – NaOH 2.0 N)

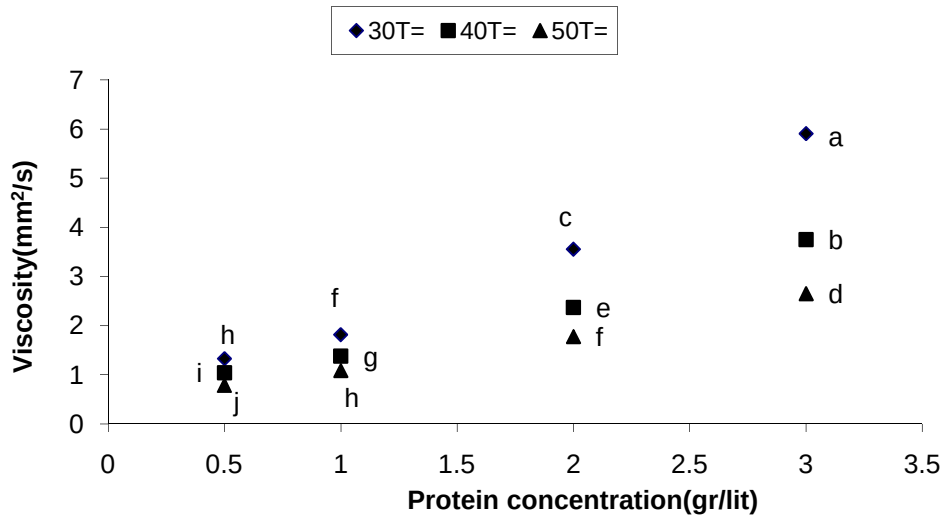

Fig. 4: viscosity of gelatin as a function of temperature and gelatin concentration (treated with Acid 2.0 N – NaOH 0.2 N)

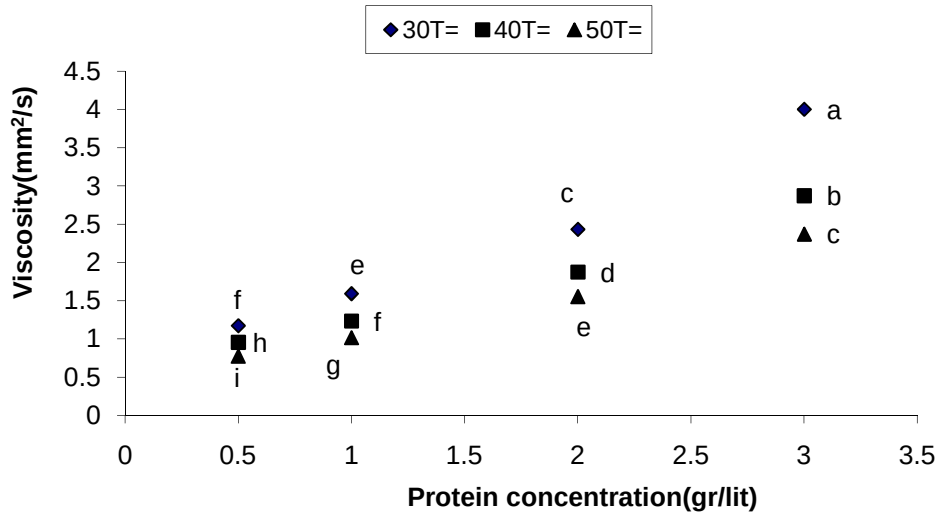

Fig. 5: viscosity of gelatin as a function of temperature and gelatin concentration (treated with Acid 0.2 N – NaOH 0.2 N)

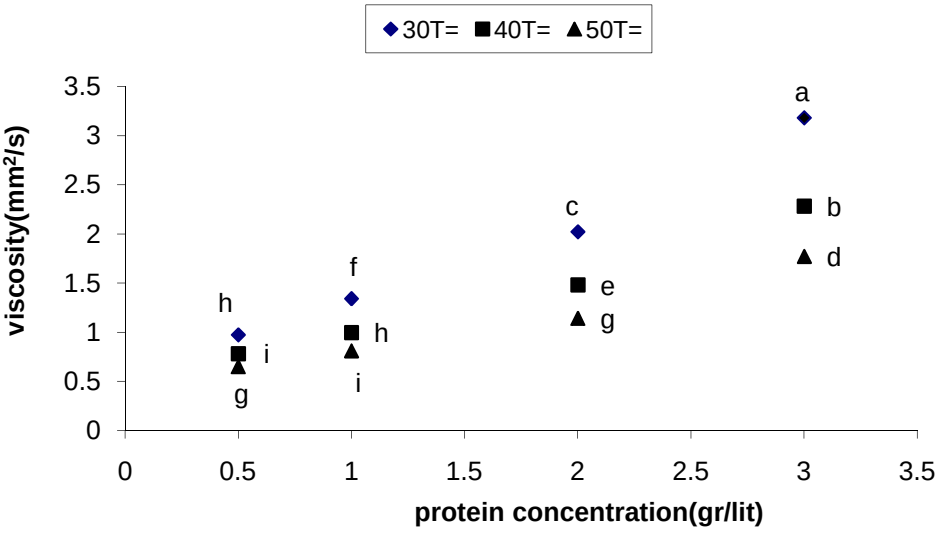

Fig. 6: viscosity of gelatin as a function of temperature and gelatin concentration (treated with Acid 2.0 N – NaOH 2.0 N)

Effects of different pretreatments on SDS-PAGE electrophoresis patterns of extracted gelatins

The gel strength, viscosity, setting behavior and melting point of gelatin depend on their molecular weight distribution and the amino Acid composition [27]. The molecular weight distribution of gelatin depends to a large extent on the extraction process [24]. Pretreatment is an important step in preparing collagen for a successful gelatin extraction. The degree of conversion of collagen into gelatin is related to both pretreatment and the extraction processes, in which, pH, temperature, and time are three major factors for both processes. Physical properties of gelatins are influenced more by extraction conditions than by imino Acid composition [28]. In this research, Acid or NaOH concentration during the pretreatment step was studied. The most important physical properties of gelatin are gel strength and strength and viscosity [30]. Commercially, gelatin with a high viscosity and gel strength are preferred and most expensive while a reasonable yield of protein is necessary for efficiency of commercial production and economic viability [31].The electrophoretic pattern gelatin solution in different conditions pretreatment has been studied (Fig 7). α-Chain was observed in SDS-PAGE pattern of all samples. In addition, increasing Acid concentration observed higher molecular weight chains. The densest α-chain and β-chain was obseverd in sample 0.2 N Acid – 2.0 N NaOH and sample 2.0 N Acid - 0.2 N NaOH respectively. β to α ratio increase at higher Acid and lower alkaline concentration whereas Acid 2.0 N- NaOH 2.0 N solution extracted LMW chains. In Acid 0.2 N- NaOH 2.0 N sample viscosity value showed a little decrease that confirms the presence of low dense chain .Acid 0.2 N - NaOH 0.2 N sample had an intermediate behavior in electrophoresis pattern studies.

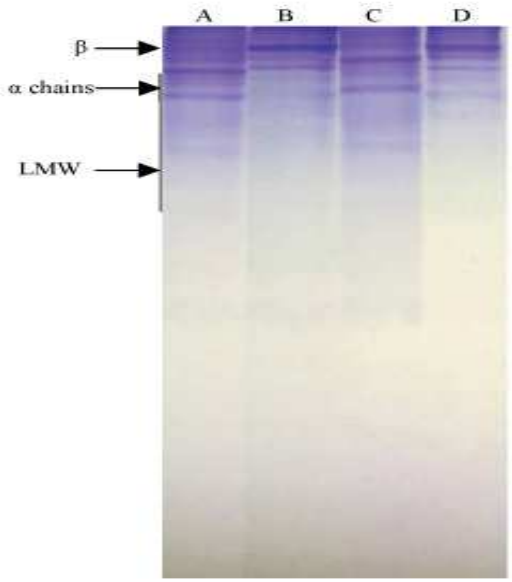

Fig.7 :SDS-PAGE of different gelatin fractions (A). Acid 2.0 N - NaOH 2.0 N (B). Acid 2.0 N - NaOH 0.2 N (C). Acid 0.2 N - NaOH 2.0 N (D) . Acid 0.2 N - NaOH 0.2 N.

CONCLUSION

The effects of Acid and alkaline pretreatment on the melting point, gelatin extraction yield, viscosity and SDS-PAGE electrophoresis pattern were investigated. In all solutions, the observed positive relation between viscosity and SDS-PAGE pattern. Acid 2 N – NaOH 0.2 N sample showed the highest yield of protein and HMW chains also a observed reasonable viscosity. Also Acid 2.0 N – NaOH 2.0 N sample showed a lower viscosity and molecular weight than other solutions. It seems that by increasing Acid and NaOH concentration can decrease rheological properties such as viscosity. The relation between viscosity and SDS-PAGE electrophoresis pattern resulted in samples by high viscosity showed HMW fractions. Finally , we cannot find sample that contain best physical and rheological properties .It seems extracted gelatins by low melting point have many application in food industry such as ice cream production, dessert and dairy products.

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