PRODUCTION OF HYDROLYZED BOVINE COLLAGEN WITH ORANGE JUICE CONCENTRATE

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(Manuscript received 19 March 2017)

Abstract

Hydrolyzed bovine collagen is a polypeptide composite made by further hydrolysis of denatured collagen or gelatin. The results indicated that molecular weights are within the range approximately 500 to 25000 Da. In hydrolysate, the molecular mass and the size of the molecules have been deliberately decreased by partially hydrolyzing the peptide bonds of the gelatin molecules. This will increase the hydrolyzed collagen dissolvable in cold water and does not gel anymore. The processes involved in processing hydrolyzed collagen are demineralization, extraction of gelatin from collagen, enzymatic hydrolysis to obtain hydrolyzed collagen, ion exchange, filtration, evaporation, sterilization and finally spray drying. In previous study a large number of studies focused on the enzymatic hydrolysis of collagen or gelatin for the production of bioactive peptide. This study aims to produce hydrolyzed bovine collagen with concentrate orange juice from low bloom gelatin which considered byproduct in gelatin manufacturing to be food supplement for arthritis. The result indicated that adding concentrated orange juice during producing of hydrolyzed collagen increased the nutritional value of collagen especially in vitamin C from 0 mg/100g. to 500 mg/100g. While protein content decreased from 91% to 88%. For sensory evaluation it was found that overall acceptability for hydrolyzed collagen with orange juice was higher than hydrolyzed collagen without adding orange juice 8.37 and 6.31 respectively. Amino acid profile showed that the hydrolyzed collagen was high in glycine content 339.6 mol/1000 mol and the Proline and Hydroxyproline were 103.6 and 80.7 mol/1000 mol respectively, this means that the Proline and Hydroxyproline percent were almost 18.4 % which are important for producing glucosamine in the human body in presence of ascorbic acid

Keywords: hydrolyzed collagen, orange juice, spray drying, Arthritis, glucosamine

INTRODUCTION

Collagen is an important biomaterial in medical applications due to its special characteristics, such as biodegradability and weak antigenecity. (Lee *et al.,* 2001) Thus collagen, as a new type of biomaterial, has been used in drug delivery systems (Friess 1998) and tissue engineering. (Pachence 1996) In addition, there are some intrinsic relationships between collagen and many diseases such as rheumatoid arthritis (Holmdahl *et al.,* 2002) and systemic sclerosis. (Tamby *et al.,* 2003). Gelatin

with high bloom degrees more than 100 degree can be used in food industries and more than 200 bloom degree used in drug capsules and can also be used as a biomaterial in biomedical applications, such as in drug delivery systems.(Einersona *et al.,* 2002) which are very different from the traditional capsule. Collagen hydrolysate is a polypeptide composite made by further hydrolysis of denatured collagen. It has been used in cosmetics and as a food additive. (Langmaier *et al.,* 2002, Langmaier *et al.,* 2001 and Morimura *et al.,* 2002) Many attempts have been made to utilize the limed split wastes in ways other than utilization as the source of commercial gelatins.
Collagen-rich animal by-products can be added value by enzymatic modification (Kida *et al.,* 1995; Morimura *et al.,* 2002) and over the past decade, collagen-rich discarded materials from the meat and food processing industries, have been found to be valuable sources of hydrolysates and peptides with bioactive properties such as antioxidant, antihypertensive/ACE-inhibitory activity, antimicrobial and immunomodulatory activities (Aleman et al 2011; Byun and Kim, 2001; Ding *et al.,* 2011; Ichimura *et al.,* 2009; Kim *et al.,* 2001; Yang *et al.,* 2009).

Orange juice is the most consumed fruit juice in Europe and around the world Galaverna, and Dall'Asta, (2014). It is obtained from the endocarp of the Citrus sinensis fruit. Several varieties of oranges are cultivated to make Orange juice, among which the main ones are Hamlin, Valencia and Pera. Orange Juice contains substantial amounts of several micronutrients such as vitamin C, folate and polyphenols (e.g., hesperidin which is a flavanone) Ohrvik, and Witthoft, (2008) and may therefore contribute significantly to their daily intakes. Data obtained from a representative sample of the French population showed that fruit juices contributed to 31% of the daily vitamin C intake of children and to 16% of the daily vitamin C intake of adults (Braesco et al. 2013). When applying the market share figures described above, orange juice would contribute to 15% of the daily intake of vitamin C for children and adolescents, and 8% for adults. The same data indicated that fruit juices and orange juice contributed to 10% and 5% of the daily intake of simple carbohydrates in children, and 5% and 2% in adults, respectively

MATERIALS AND METHODS

Materials

All process of this research were implemented with cooperate with El Amin for Gelatin Company 6 of October industrial city Giza Egypt. So all raw materials, raw bovine skin and low bloom gelatin and other chemical come from this company
Protease (pro type 2, 3×10^6/gm.) enzyme was purchasing from infoenzyme Germany.

Orange juice concentrate were obtained from El Maraow Company 6 of October industrial city Giza Egypt.

METHODS

Preparation of gelatin and collagen hydrolysate

Bovine limed split wastes were relimed with 3-4% lime and 0.5% NaOH for two weeks at room temperature. The relimed split wastes were washed and neutralized to pH 5.5-6 with 1.5% HCl. Gelatin (type B) was extracted about five times from 70 to 90°C at a 5°C interval. Collagen hydrolysate was prepared by hydrolysis of low bloom gelatin using 0.02% (w/v) protease (1:250) at 40, 45 and 50°C for different times from 60 to 240 min. The solutions were mixed with 10 ml orange juice concentrate (60% TSS) then the mixture were dried using a spray dryer (Buchi B-191, Switzerland). The concentrations of the samples were determined by the Biuret method. (Li et al 2003).

Hydrolysis determination by different methods

Sodium dodecyl sulphate-polyacrylamide gelelectrophoresis (SDS-PAGE)

Samples were mixed with 0.5 M Tris- hydrochloric acid (HCl) buffer (1% SDS, 10% glycerol and 0.01% bromophenol blue, pH 6.8) and then boiled for 5 min. 15µl treated samples were injected into 10% gel wells and run for approx. 120 min. The gel was stained with 0.25% Coomassie Brilliant Blue R-250 for 45 min and de-stained with 7.5% acetic acid/5% methanol solution until the bands were clear. Yuhao et al (2013).

Isoelectric points of samples

Collagen, gelatin and collagen hydrolysate solutions (0.05% w/v) were titrated with 0.25 M sodium hydroxide (NaOH) or 0.25 M hydrochloric acid (HCl) and the Zeta potentials at a given pH were recorded by a Zeta potential titration apparatus (Malven Zetaweight Nano ZS, UK). The titration temperature was 25°C and the decreasing pH intervals were 0.5 pH. The change of Zeta potential was plotted against the change of pH for the samples. The isoelectric points of the samples were determined at the pH value where the Zeta potential was zero.

Preparation of collagen and gelatin membranes

Collagen and gelatin were dried to form membranes at 30°C in silica gel desiccators. The collagen hydrolysate membrane dried under the above conditions was too fragile to be used in practice. So only the collagen and the gelatin membranes were chosen for assay.

Physical properties of membranes

The denaturation temperatures of the collagen and the gelatin membranes were determined by a differential scanning calorimetry apparatus (DSC) (Netzsch DSC 200PC, Germany). The membranes were conditioned in a humidistat containing saturated ammonium nitrites (NH4NO3) solution at 20°C for 24 hours before test. Approx. 2mg samples were sealed in aluminum pans and the empty pan was used as a control. The endothermal curves of the samples were recorded from 10 to 80°C at a heating rate of 5°C/min in a nitrogen atmosphere. After cooling with liquor nitrogen, secondary heating was run under the same conditions. The morphologies of the membranes were examined by SEM as in the fibril formation experiment. In addition, their tensile strength and elongation at the break were also determined by a strength tester (Gaotie GT-AI-7000S, China). (Li et al 2003).

Chemical analysis of collagen.

The chemical composition of collagen produced was determined according to the official methods of analysis AOAC (2010).

Microbiological evaluation.

Malt extract agar medium was used as a selective medium for the detection and enumeration of yeast and moulds according to Galloway, and Burgess, (1952).

Sensory evaluation

Sensory attributes of hydrolyzed collagen produced with orange juice were evaluated by 10 panelists according to Lawless, and Klein, (1991).

Statistical analysis

The results were analyzed by analysis of variance (ANOVA) using the procedure by statistical analysis system (costate) program. Significant differences were determined at the level P = 0.05. (Aurelie et al. 2016).

RESULTS AND DISCUSSION

SDS-PAGE analysis

The electrophoresis patterns of the samples are shown in Fig. 1. Collagen displayed one b band (200 kDa) and two a bands (100 kDa for a1 and a2), which were the unfolding polypeptide chains of the triple helix ([a1(I)]2[a2(I)]). The molecular weight of type I collagen was about 300 kDa. In contrast, the molecular weights of gelatin and collagen hydrolysate were less than 300 and 50 kDa respectively and their molecular weight distributions were very wide. So the components of gelatin and collagen hydrolysate were more complex than those of collagen. Different preparative processes lead to different molecular weight distribution. Collagen was extracted in the acid solution containing pepsin, which only

attacked the non-triple helical domain of native collagen, whereas gelatin and collagen hydrolysate were prepared under severe conditions (above the denaturation temperature). Most of the triple helices of gelatin and collagen hydrolysate had been destroyed and parts of their peptide bonds were also broken out. Therefore, there were wide distributions and lower molecular weights for gelatin and collagen hydrolysate.

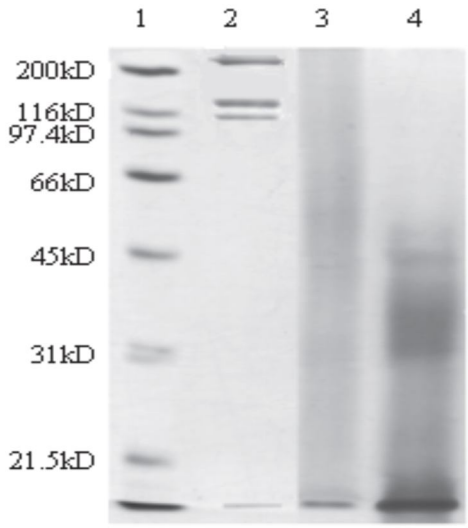

Fig. 1. SDS-PAGE analysis of molecular weight standards (lane 1), collagen (lane 2) gelatin (lane3) and collagen hydrolysate (lane 4) on10% gel. (KDa) means kilo Dalton

Isoelectric points of samples

Isoelectric point is an important parameter of proteins, which is related to the proportion of acid amino residues and base amino residues in protein. Gelatin and collagen hydrolysate were composites of different molecular weight polypeptides, and thus, the values of isoelectric points were the values of the system which included the various polypeptides and buffer. The isoelectric points of collagen, gelatin and collagen hydrolysate were 8.26, 4.88 and 4.56, respectively. The isoelectric point of collagen was in the basic range due to the acidic extraction, which kept side amide residues intact. In contrast, the isoelectric points of gelatin and collagen hydrolysate were in the acid range due to the higher density of carboxyl groups caused by the hydrolysis of side amide groups of samples under the strong base and high temperature preparative conditions (Yang *et al.,* 2009).

Effect of incubation temperature on the hydrolysis curve of gelatin

The collagenous domain is hardly digested by enzymes other than collagenase due to its stable triple helix but the denatured products such as gelatin and collagen hydrolysate are easily attacked by proteinases. Protease was used to test the samples for their resistance to enzyme digestion. The content of primary amines can be used as an index for the degree of hydrolysis of samples. The Protease digestion curves are shown in Fig. 2. The level of hydrolysis at 45°C was the highest compering to 40 and 50°C. Collagen hydrolysate was prepared by the hydrolysis of gelatin using Protease. Thus, there were only a few sites remaining for Protease to further attack under these mild conditions as a consideration of the denaturation of collagen. Collagen was more resistant to proteinase hydrolysis than the denatured products (gelatin and collagen hydrolysate) due to its specific triple helical structure (Aleman *et al.,* 2011).

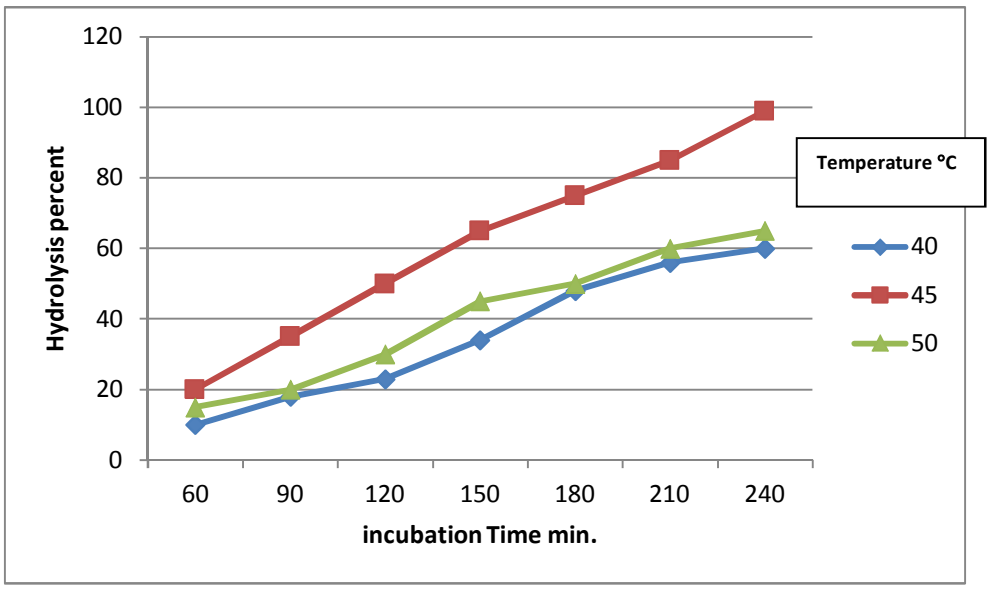

Fig. 2. Protease hydrolysis curves for gelatin to produce hydrolyzed collagen

Physical properties of membranes

The thermo-physical properties of the collagen and the gelatin membranes were examined by DSC. Fig. 3 shows the thermograms of the collagen and the gelatin membrane. The peak temperature of the collagen membrane was about 50°C similar to that of the gelatin membrane (62.5°C), but its peak area (enthalpy) was markedly larger than that of the gelatin membrane in the first scan. In the second run, the endothermal peaks of the collagen and the gelatin membrane both disappeared and only exhibited a weak step baseline, showing the glass transition. The thermal behaviors of collagen and gelatin are related to the moisture content and the thermal history (preparative conditions).

The collagen membrane presented a larger peak area than that of the gelatin membrane in the denaturation process due to the greater presence of collagen triple helices. In addition, the DSC traces in the first scan corresponded to the helix-coil transitions during the heating of the samples، while those in the second scan corresponded to the characteristics of completely amorphous biopolymer due to the elimination of the triple helical structure. Therefore، there were no endothermal peaks in the second scan. ((Gomez *et al.,* 2011)

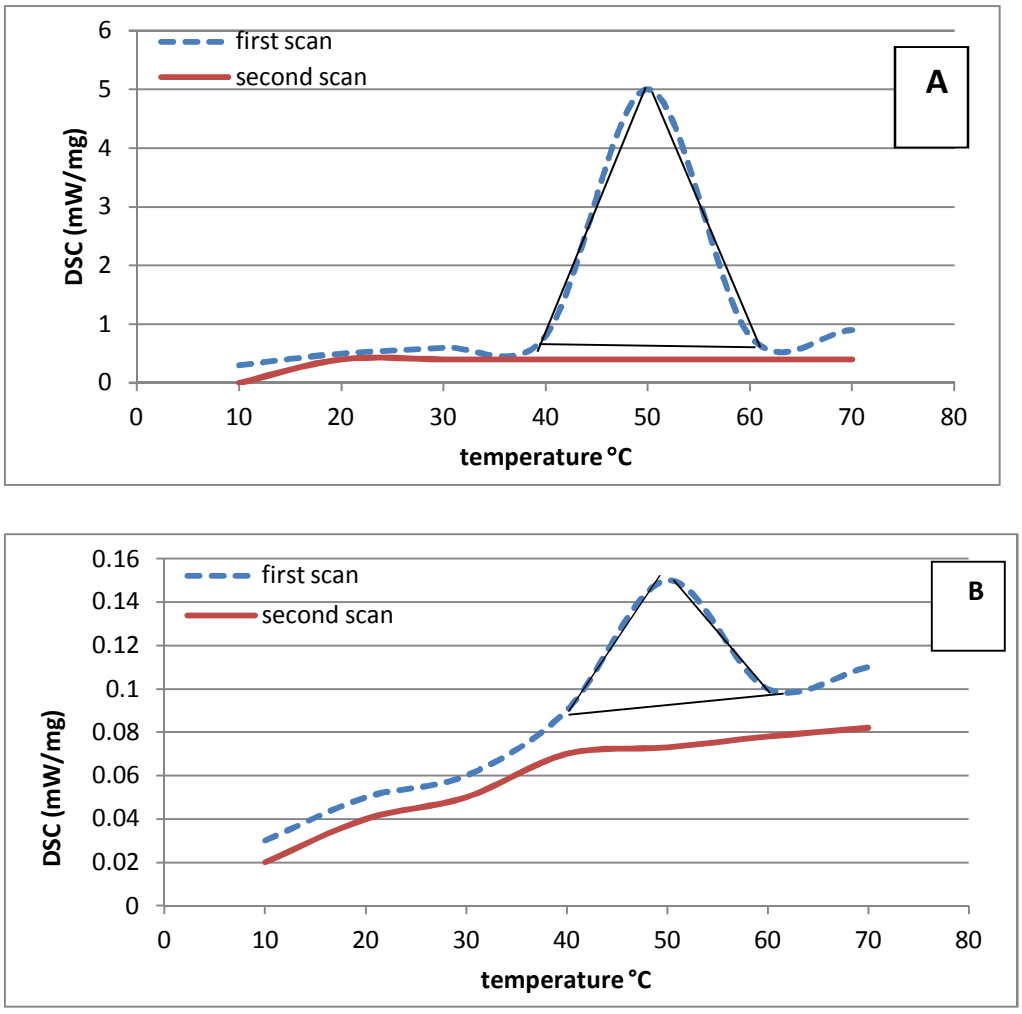

Fig. 3. Thermograms of the collagen membrane A, and the gelatin membrane B determined by DSC.

Surface morphologies of the collagen and the gelatin membrane.

The surface morphologies of the collagen and the gelatin membrane were greatly different from each other (Fig. 4). There were obvious fibril networks with a

rough membranous structure for the collagen membrane. In contrast, the gelatin membrane without fibril networks appeared only as a smooth structure on the surface. In addition, the tensile strengths and elongations at the break for the collagen and the gelatin membrane were 58 ± 5N/mm2, 7 ± 0.5% and 36 ± 4 N/mm2, 3 ± 0.5%, respectively. The differences of the mechanical properties were caused by the different structures of the membranes. There were more rigid native triple helices and fibril networks in the collagen membrane than in the gelatin membrane, which played important roles in elevating the mechanical properties of the collagen membrane.

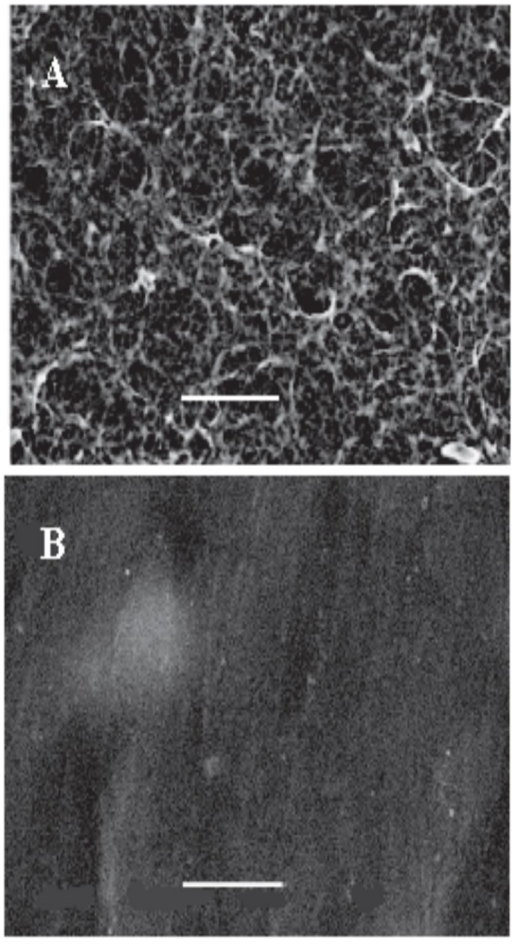

Fig. 4. Surface morphologies of the collagen membrane **A,** and the gelatin membrane **B** observed by SEM. Bars 5 mm

Physiochemical composition of hydrolyzed collagen with concentrated orange juice

The physiochemical composition of hydrolyzed collagen with adding concentrated orange juice was determined and the results are shown in table 1. From the table it could be observed that adding concentrated orange juice during producing of hydrolyzed collagen caused to increase the nutritional value of collagen especially in vitamin C from 0 mg/100g. to 500 mg/100g. which is very important to forming glucosamine by reaction with collagen amino acid especially proline anino acid and hydroxyproline amino acids in the human body (Asghar 1982).

The color of the product is changed from creamy color to yellow color also the flavor become orange flavor duo to adding orange juice. On another hand the protein content was decreased from 91 to 88 %. The results showed that, the turbidity was increase from 10 NUT in control to be 18 NUT in produced collagen with orange juice concentrate. For pH it found that by adding orange juice concentrate decrease the pH from 6.5 to be 4.3 due to the acid content of concentrated orange juice

For microbiological parameters the result showed that the hydrolyzed collagen with adding concentrated orange juice was similar to the specifications of the standard with total bacterial conut less that 100 cfu/g., anaerobic bactria less than 10 cfu/g. while for salmonella and E.Coli it was absent in 25 g and 10g. respectivlly.

Table1. Physiochemical composition of hydrolyzed collagen with concentrated orange juice on dry weight bass

Analysis	UNITS	SPECEFICATIONS of standard*	Product **
Physical and chemical properties:			
- Viscosity (sol. 20% - 25 °C)	mPa.s	5.5-7.5	5.8
- Turbidity	NUT	10	18
- Flavor		Gelatin	Orange
- Color		Creamy	Yellow
- Ash	%	3	2.4
- Moisture	%	2	2.87
- pH value		5.0-6.5	4.3
- Protein	%	91	88
- Bulk Density	g/L	0.40-0.55	0.54
- Particle Size	µm	150-300	230
- Vitamin C	mg	0 mg	500 mg
- Fiber	g	0.32g	1.04 g.
Heavy metals	ppm	≤200	130
Microbiological parameters			
- Total bacterial count	CFU/g	≤1000	≤1000
- Salmonella sp in 25 g		Absent	Absent
- E.Coli in 10 g		Absent	Absent
- Anaerobic bacteria		≤10	≤10

* Standard of hydrolyzed collagen (Gelita Germany 2010).

** hydrolyzed collagen with adding orange juice concentrate.

Sensory evaluation of hydrolyzed collagen with concentrated orange juice

The sensory evaluation of hydrolyzed collagen with concentrated orange juice comparing with hydrolyzed collagen as control was evaluated and the result tabulated in table 2. From the same table it could notes that the sensory evaluation value for hydrolyzed collagen with concentrated orange juice was higher than that produce without adding orange juice concentrate in all attributes. That may be due to the orange enhance the sensory attributes of the final products.

Table 2. Sensory evaluation of hydrolyzed collagen with concentrated orange juice

Samples	Sensory attributes for collagen					LSD*
	Color	Odor	Flavor	Solubility	Overall acceptability	
Collagen hydrolyzed	6.25±0.20	6.33±0.21	5.14±0.18	7.57±0.23	6.31±0.20	0.20
Collagen hydrolyzed with orange	8.41± 0.26	8.26±0.24	8.12±0.22	8.29±0.23	8.37±0.25	0.24

- Standard deviation

Amino acids composition for hydrolyzed collagen

The Amino acids composition for hydrolyzed collagen were analyzed and the result are shown in fig (5) from the amino acid profile was found that the hydrolyzed collagen that produced in this study was high in glycine content 339.6 mol/1000 mol and the Proline and Hydroxyproline were 103.6 and 80.7 mol/1000 mol respectively, this mean that the Proline and Hydroxyproline percent were almost 18.4 % which are important for producing glucosamine in the human body in presence of ascorbic acid Morimura *et al.,* (2002), and Asghar, (1982).

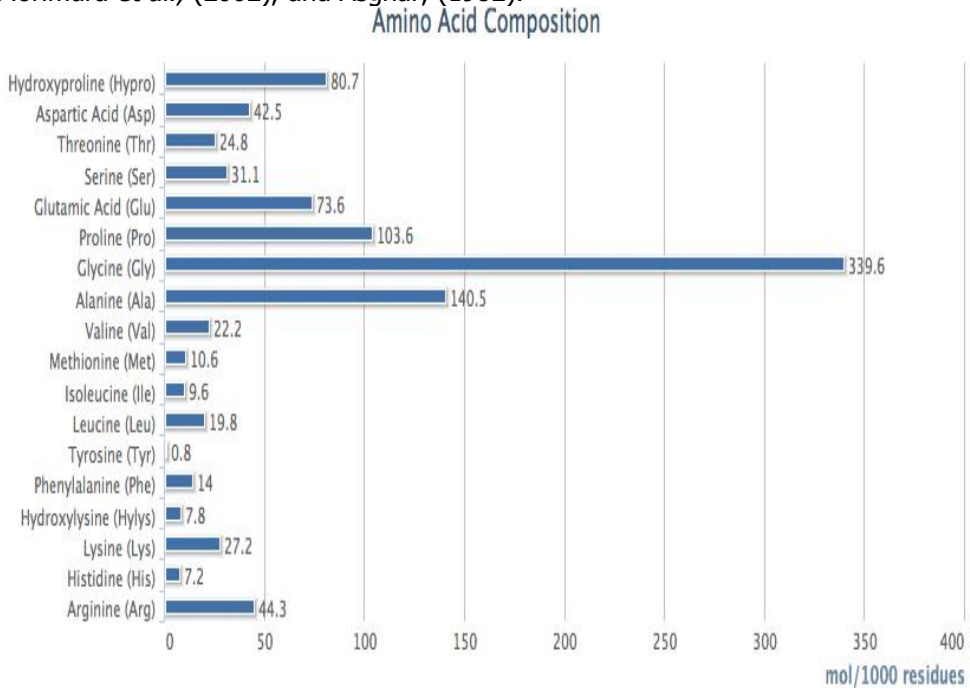

Fig 5. Amino acids composition for hydrolyzed collagen

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انتاج الكولاجين البقري المحلل مع عصيرالبرتقال المركز

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يعتبر الكولاجين البقري المحلل مركب بولي ببتيد يتم انتاجه من الكولاجين المعقد او الجيلاتين. وقد اوضحت النتائج ان الوزن الجزيئي للكولاجين يتراوحما بين 500–25000 دا (Da.) في الهيدروليسات، يتم خفض الكتلة الجزيئية وحجم الجزيئات من خلال تحليل جزء من الروابط الببتيدية لجزيئات الجيلاتين. وهذا يجعل الكولاجين المتحلل يذوب في الماء البارد ولا يكون هلام (الجيلي). العمليات التي تتضمنها خطوات انتاج الكولاجين المتحلل هي نزع الاملاح، الاسخلاص للكولاجين لاالجيلاتين، ثم التحلل الإنزيمي للحصول على كولاجين متحلل، يليه الترشيح بالتبادل الأيوني، ثم التركيز و التعقيم وبالنهاية التجفيف بالرزاز. وتهدف هذه الدراسة الى انتاج كولاجين بقري محلل مع اضافة مركز عصير البرتقال من الجيلاتين المنخفض البلوم والذي يعتبر ناتج ثانوي من صناعة الجيلاتين ليكون مكمل غذائي لسلامة المفاصل.

واظهرت النتائج انه بأضافة مركز عصير البرتقال اثناء انتاج الكولاجين المحلل ادي الى زيادة القيمة الغذائية للكولاجين وخاصة فيتامين سي الذي ارتفعت نسبته من 0 الى 500 ملجم/100 جم. بينما انخفض محتوي البروتين من 91% الى 88%. بالنسبة للتقيم الحسي وجد ان القابلية العامة للكولاجين المضاف اليه مركز عصير البرتقال كانت اعلى عن تلك التي لم يضاف اليها مركز عصير البرتقال 8,37 و 6.31 على التوالي. اوضح تفريد الاحماض الامينية ان الكولاجين المحلل كان مرتفع فى محتواه من الحامض الاميني جليسين 339,6 مول/1000 مول وكان محتواه من كلا من الحامض الاميني برولين والهيدروكسي برولين 103,6 و 80,7 مول/ 100 مول على التوالي، وهذا يعني ان نسبة كلا من الحامض الاميني بورلين والهيدروكسي برولين تقريبا 18,45 % وذلك له اهمية في انتاج مادة الجلوكوزأمين فى جسم الانسان فى وجود فيتامين سي.

الكلمات الدالة: الكولاجين المحلل– مركز عصير البرتقال – التجفيف بالرزاز– خشونة الركبة– الجلوكوزامين