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EDITORIAL

Production and Characterization of Glucosamine from Bovine Synovial Fluid.

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الخلاصة

يعتبر الجلوكوزامين((Glucosamine) من السكريات الطبيعية الاحاديه الامينيه وهو المكون الاساسي لماده الجلايكوزامينوجلايكان (Glycosaminoglycans)الموجوده فى الوسائد الغضروفيه والسائل السينوفي . ويلعب الجلوكوزامين دورا في تصنيع الغشاء المبطن للخلايا والكولاجين والعظام.

أجريت هذه الدراسة لإنتاج الجلوكوزامين من السائل السينوفي البقري . تم فصل الجلوكوزامين (0.73-2.60%) عن طريق التحليل المائي بواسطة حمض الكبريتيك وحمض الهايدروكلوريك المركزين. كما تم التعرف علي الجلوكوزامين المنتج بواسطة الاختبارات الكيمائية اللونية, كروماتوغرافيا الطبقه الرقيقة ، وكروماتوغرافيا السوائل عاليه الكفاءه ومطيافيه الاشعه تحت الحمراء وذلك بمقارنته مع الماده المرجعيه.

يمكن أن نستنتج ان للجلوكوزامين حوجه ماسه لأجسامنا ويمكن تناوله كمكمل غذائي لتحفيز تصنيع السائل السينوفي الذي يلين الغضروف ويكسب المفاصل صحة جيده، لذلك وفي هذا البحث وبعد الاستقصاء وجد ان السائل السينوفي البقري والمعالج بالتحليل المائي الحمضي ينتج كميه معتبره من الجلوكوزامين.كما ان الطرق المستخدمه والموصوفه للتحليل الكيميائي يمكن تطبيقها كميا ونوعيا لتحديد نسبه الجلوكوزامين في مختلف المنتجات المحتويه عليه.

Abstract

Objectives: Glucosamine is a natural aminomonosaccharide, which is a normal constituent of glycosaminoglycans in cartilage matrix and synovial fluid. Glucosamine has a role in the synthesis of cell membrane (building) lining, collagen, osteoid, and bone matrix.
The present work was undertaken to investigate bovine synovial fluid for the production of glucosamine. The method used for isolation of glucosamine included acid hydrolysis of the fluid.
Results: The produced glucosamine(0.73-2.60%) had been purified and characterized by different identification methods that include: chemical colour tests; thin layer chromatography; C_{18}- high performance liquid chromatography and infrared spectrophotometery compared to standard glucosamine.
We can conclude that, there is an essential need for every body to produce and/or to take glucosamine supplements to stimulate the production of synovial fluid which lubricates cartilage and keeps joint healthy. This led to the fact that reliable analytical methods should be carried for quality assessment of glucosamine commercial products used by consumers. In this research the investigated bovine synovial fluid was recognized to afford a considerable amount of glucosamine obtained by acid hydrolysis.
Conclusions: The adopted and the described methods of analysis could be applied in qualitative and quantitative determination surveys of different supplement products containing glucosamine.

Key words
Glucosamine production; Characterization; Glycosaminoglycans; Glucoproteins Boviane Synovial Fluid.

Introduction

Glucosamine occurs naturally in bacteria, plants, and animals synovial fluid; vitreous humor of the eyes; articular cartilage; umbilical cord; cornea and skin. The exoskeleton of crustaceans, cuticle of insects and cell walls of fungi also contain glucosamine as their main component is chitin (Nguyen, 2002).
Glucosamine in chitins-the main commercial source-is either pre-existent as glucoproteins or as glucosamine glucoside and/or formed in the process of hydrolysis by the interaction of carbohydrate residues and amino acids (Winifred, 1915).

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Bovine synovial fluid, the whole weight of complex is accounted for in terms of protein, acetyl glucosamine, glucuronic acid and mineral content (Ogston and Stanier, 1952).

The major methods used for glucosamine production are: Acid hydrolysis and enzymes hydrolysis, although, precipitation by centrifugation and ultrafilteration may some times precede acid or enzyme hydrolysis (Ogston and Stanier, 1950; Ogston and Stanier, 1952).

Material and Methods

Materials

Synovial Fluid

Bovine synovial fluid was obtained from local slaughter house. The fluid was carefully withdrawn from the knee joint with a syringe through puncture needle,15-30minutes after slaughtering.

Methods

Hydrolysis of Synovial Fluid with Concentrated Sulfuric Acid and/or Hydrochloric Acid

As described by Winifred (1915), 150 grams of the synovial fluid were treated with 100ml of concentrated sulphuric acid (a) and/or hydrochloric acid (b), and refluxed on water bath for four hours. The solution was then allowed to cool for another four hours, diluted with 200ml water and allowed to stand for 24 hours. Sulphuric acid was removed by barium carbonate, while excess hydrochloric acid was removed by vaccum evaporation. The precipitated barium sulphate was filtered. The brown filterates decolourized by heating with charcoal and then filtered. The resulting solutions (a and b) were concentrated at room temperature and partioned in butanol separately. Butanol fraction which proved to contain no glucosamine was rejected, while small amount of ammonia solution (just to make alkaline) was added to the water fraction of both (a) and (b) solution mixture separately. After hydrolysis, decolourization, concentration and partioning, the obtained water fraction (a) and (b) solution was examined for glucosamine using chitosan test, Fehling's and Benedict's solution. Furthermore, fraction (a) obtained by sulphuric acid hydrolysis which afforded sufficient product was subjected to column fractionation, thin layer chromatography (TLC) tracing, C_{18} – HPLC and FTIR analytical methods for qualitative and quantitative determination of glucosamine.

Confirmatory Colour Tests

The produced hydrolyzed solution mixtures (a) and (b) were examined separately for glucosamine qualitative chemical colour tests with chitosan (potassium hydroxide, 90% alcohol, iodine potassium iodide and dilute sulphuric acid); Fehling's ($CuSO_4$. $5H_2O$ + Sodium potassium tartarate); Benedict's(Na citrate ,Na carbonate and $CuSo_4$) and ninhydrin.

Column Chromatography

Glass columns of 2.0cm wide were used. Columns were prepared using 12 grams silica gel of mesh size (0.063 – 0.2 mm) for column chromatography by using dry packing method. The water fraction of the hydrolyzed product (a) was dissolved in water (1-2ml) and added to the top of a dry-packed column. Flash column chromatography elution commenced with 50ml of water and water/ethanol (10ml) mixture in a decreasing manner as shown in Table 1. Fractions of 10 ml were collected and analyzed by TIC using butanol, acetic acid and water (BAW) in a ratio of (3:1:1) as solvent system, ninhydrin and iodine vapour were used as locating reagents.

Table 1: Column chromatography of the water fraction of the hydrolyzed Bovine synovial fluid.

Fraction number	Eluent	Amount of eluent used
1 – 5	Water	50ml

6	Water/ethanol (7:3)	10ml
7	Water/ethanol (5:5)	10ml
8	Water/ethanol (3:7)	10ml
9	Ethanol	10ml

Similar fractions were mixed together. TLC results showed four distinct pink spots one of which (fraction 5 and 6) was corresponding to glucosamine standard sample (Rf = 0.51).

Thin Layer Chromatography (TLC)

TLC plates were prepared using silica gel G type 60 with $CaSO_4$ as binder. A slurry of silica gel in water was applied by means of a spreader to glass plates, previously cleaned with ethanol. A layer of 0.25mm thick was used routinely and the plates were activated by heating at 100-110ºC in an oven for an hour.

The produced hydrolyzed solution mixtures (a and b) together with the standard glucosamine were Co-chromatographed on TLC plates, using butanol, acetic acid and water (BAW) in a ratio of (3:1:1) as solvent system, ninhydrin and iodine vapour were used as locating reagent.

C_{18}-High Performance Liquid Chromatography (C_{18} - HPLC)

The produced hydrolyzed solution mixture (a) and glucosamine standard sample were co-chromatographed on C_{18}-HPLC column ODS 4.6 mm× 150mm 5 mic (HPLC Sykam; S3210UV/visible detector; Germany). Acetonitrile and water (10:90) were used as mobile phase at flow rate of 1ml/min at ambient temperature. UV detector was operated at 240nm.

In this method 500mg of the dried sample product (fraction 5 and 6) was dissolved in 250ml of the mobile phase, filtered and sonicated for 10 minutes. 250mg of the standard glucosamine sulphate was dissolved in 100ml mobile phase, filtered and sonicated for 10 minutes. 20µl from each was injected into the HPLC system and the peaks monitored by UV absorbance at 240nm. Results are shown in Fig 1.

Infrared Spectroscopy

IR spectra of the produced compound (fraction 5and 6) and reference glucosamine sulphate were recorded on FTIR spectrophotometer (Thermo Nicolet FTIR spectrophotometry, USA), using KBr disk of IR spectroscopic grade (Central Drug House Laboratory Reagents).Results are shown in Fig. 2 and Fig. 3.

Optical Rotation

The optical activity of glucosamine sulphate isolated was measured on polarimeter (Bellingham, Stanley Limited, England).

Results and Discussion

Glucosamine and it's derivative N-acetylglucosamine are synthesized in all organisms, including bacteria, yeast, fungi, plants and animals. In humans, glucosamine and N-acetylglucosamine are precursors of the disaccharide units in glycosaminoglycans which are necessary to repair and maintain healthy cartilage and joint function (Deng, 2004).

Chitin hydrolysis is considered as the major natural commercial source of glucosamine (Deng, 2004; Zhu et al, 2005).However, on hydrolysis, bovine synovial fluid and hyaluronic acid from different origin give remarkable amount of the aminosugar glucosamine (Ogston and Stanier, 1952; Curtain, 1955; Johnston, 1955; Preston et al., 1965).

Among hydrolysis procedures, acid hydrolysis of synovial fluid and/or hyaluronic acid is the most common preferable mechanism for production of glucosamine (Zhu et al., 2005). Therefore from results of numerous experiments, isolation of definite glucosamine was a strong evidence. However, glucosamine hydrochloride was readily formed in a total yield of 3.4% when Boletus edulis fungi had been hydrolyzed by Winifred (1915) with concentrated hydrochloric acid as described earlier in the method, and who concluded that glucosamine is derived from a complex nature of glucoproteins, carbohydrate and amino

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acid residues.Likewise, for synovial fluid being a glucoprotein its acid hydrolysis can follow the same pattern of reactions as postulated by Winifred (1915) can be summerized as follows:

Glucoprotein $\xrightarrow{\text{acid hydrolysis}}$;ugar + an acidamide → ammonia + carboxylic acid

OR

Glucoprotein $\xrightarrow{\text{acid hydrolysis}}$ aminosugar + an acid

Hyaluronic aciu ⟶ glucuronic acid + N-acetylglucosamine

N-acetylglucosamine ⟶ acetic acid + glucosamine hydrochloride and/or glucosamine sulphate.

Therefore, it can be concluded that degradation of hyaluronic acid could be achieved under acid conditions to give a mixture of products one of which is the aminosugar, glucosamine which is known to be resistant to further acid hydrolysis (Kaye and Stacey, 1950).

In this study, chemical tests for the presence of glucosamine in the produced hydrolyzed mixtures (a) and (b) was confirmed when its characteristic identifying colour tests were carried out where both mixtures readily reduced Fehling's solution,gave positive chitosan test, produced yellowish brown precipitate with Benedict's and gave four distinct pink coloured spots with ninhydrin on comparative TLC chromatogram where the dominant spot of which corresponds to glucosamine standard with Rf value = 0.51.

When the obtained solution mixtures of (a) and (b) are partioned separately in butanol, glucosamine appeared as dominating compound in water fraction while butanol layer was found to be devoid of and/or containing very low amount as confirmed by comparative TL

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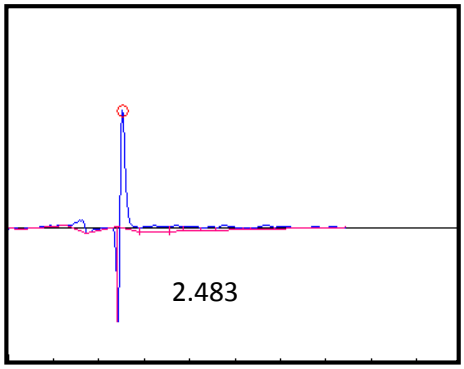
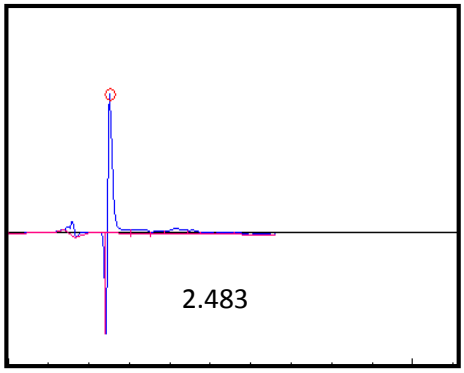


a.



b.

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Fig. 1: C_{18} – HPLC chromatogram.
a. Standard, glucosamine sulphate b. Sample (Test), glucosamine sulphate

In flash column chromatography, the water fraction which is rich in glucosamine and obtained by sulphuric acid hydrolysis (a), was further fractionated on silica gel for column chromatography. Separation was initiated with water, water/ethanol mixed solvent with gradual increase in ethanol content was used to perform further separation. Glucosamine containing fractions 5 and 6 were eluted from the mixture with water and water/ethanol (7:3), yielding 0.73 – 2.60% yellowish - white product which readily reduced Fehling's solution,gave positive chitosan test, produced yellowish brown precipitate with Benedict's and gave a pink coloured single spot with ninhydrin on comparative TLC chromatogram (Rf = 0.51). Permanent rotation of the compound in water, $[\alpha]_D = +70.0 - +73.5^o$, which corresponds with that determined by Winifred (1915).

In reversed – phase C_{18}-HPLC comparative analysis performed at UV 240nm and acetonitrile and water (10:90) were used as mobile phase, a peak of the same retention time (2.483minutes), appeared in chromatograms of both tested solution and the standard (Fig. 1). The substance was undoubtedly glucosamine sulphate, which was further confirmed by FTIR analysis.

The yield of glucosamine obtained from bovine synovial fluid (0.73 – 2.60%) seems to be remarkably higher compared with the amount (0.012 – 0.028%) obtained by Ogston and Stanier, (1952) and the amount (0.037 – 0.043%) obtained by Curtain, (1955), while chitin of B.edulis yield was 3.4% glucosamine, as reported by Winifred, (1915) from a similar process of hydrolysis.

Infrared spectra of glucosamine sulphate sample and standard were obtained using FTIR instrument (Thermo Nicolet FTIR spectrophotometer, USA) with frequency range $4000 - 400\,cm^{-1}$, (Fig. 2 and Fig. 3).

It was noted that compounds containing C-H, N-H and O-H group have absorption bands in the frequency ranges as: C – H, $3100 - 2850\,cm^{-1}$; N – H, $3500 - 3150\ cm^{-1}$; O – H, $3600 - 3200\,cm^{-1}$ (Gunstone and Sharp, 1970; Shriner et al., 1980; Dudly and Felming, 1980).

Fig.2 and Fig.3 show that, characteristic and diagnostic bands of glucosamine are visible at $3347\,cm^{-1}$, $1382\,cm^{-1}$, $1126\,cm^{-1}$ and $615\,cm^{-1}$. Literature agrees in assigning $3347\,cm^{-1}$ band to $-NH_2$, -OH and C-H stretching vibration. In addition lower IR finger print region of the isolated glucosamine was found to be identical to that of the reference sample. Moreover, as the $-NH_2$, -OH and C-H stretching bands are aligned, thus appear as a characteristic broad band from ($3600\text{-}2750\,cm^{-1}$) in the spectrum. Band at $1126\,cm^{-1}$ represents the free amino group ($-NH_2$) at C_2 position of glucosamine. Peak at $1382\,cm^{-1}$ represents the –C-O stretching of primary alcohol group ($-CH_2OH$) and peak at $615\,cm^{-1}$ representing –N-H bending band (Gunstone et al., 1970; Shriner et al.,1980; Dudly and Felming,1980;Saraswathy et al.,2001;Guo et al.,2002;Tanveer et al.,2002; Davika and Varsha.,2006).

In short, infrared spectra for both the isolated and the standard sample reproduce those shown in literature confirming thus that the isolated compound was indeed glucosamine sulphate.

Conclusions

It can be concluded that, there is an essential need for every body to produce and/or to take glucosamine supplements to stimulate the production of synovial fluid which lubricates cartilage and keeps joint healthy. This led to the fact that reliable analytical methods should be carried for quality assessment of glucosamine commercial products used by consumers. In this research the investigated bovine synovial fluid was recognized to afford a considerable amount of glucosamine obtained by acid hydrolysis. Moreover the adopted and previously described methods of analysis could be applied in qualitative and quantitative determination surveys of different supplement products containing glucosamine.

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