

Investigation on the conditions of hyaluronic acid extraction from rooster combs by protein precipitants

M.T. Chen & X.Y. Lin

Department of Bioindustry Technology, Da Yeh University 112 Shan Jeau Rd., Da Tsuen, Changhua, Taiwan
515.

*E-mail: michen@mail.dyu.edu.tw.

Abstract

Many pharmaceutical HA products have been prepared from umbilical cord, synovial fluid, skin and rooster combs as well as microbial fermentation. At present, most of HA is prepared from rooster combs. The HA extract has been purified using precipitant to remove impurities and obtain desirable purity of product. Because of the precipitant used in this purification procedure is a high toxic organic solvent---chloroform and higher cost, the aim of this study is to search for other protein precipitants to replace of chloroform, and to investigate on the effect of different precipitants on the yield and quality of HA extracted from rooster combs.

Introduction

Many pharmaceutical HA products have been prepared from umbilical cord, synovial fluid, skin and rooster combs as well as microbial fermentation. At present, HA is prepared from rooster combs most commonly. The process of the HA extracts has been purified using precipitant to remove impurities and obtain desirable purity of product. Because of the precipitant used in this purification procedure is a high toxic organic solvent---chloroform and higher cost, the aim of this study is to search for other protein precipitants to replace of chloroform, and to investigate on the effect of different precipitants on the yield and quality of HA extracted from rooster combs.

Materials and methods

Rooster combs were collected from the local poultry slaughterhouse and placed at 4°C for preparing.

HA extraction procedure: The procedure described by Balazs (1979) are modified to extract crude HA from combs. The combs are cut into pieces by 2mm x5mm and soaked in ethanol:chloroform solution (combs:ethanol:chloroform=10:40:1) to dehydrate and remove impurities such as blood. The mixtures are centrifuged at 8000rpm, 15min (Hermle Z323) to obtain the crude HA extracts.

The crude HA is purified by three different procedures(see flow chart) modified from the methods of Balazs (1979) and Nimrod et al.(1988). 1%cetyl-pyridinium chloride(CPC), 100% hexane, 1% ammonium sulfate are used as precipitants to compare with the deproteinizing efficient of chloroform. And the crude HA extract is just adjusted pH to isoelectric point to precipitate proteins as the control sample.

Hexuronic acid assay: Purity of crude HA extract is expressed as the content of glucuronic acid of HA. The method of Bitter et al.(1962) is used to determine the content of glucuronic acid A modified colorimetric method is used for the estimation of N-acetylamino sugars. Quantification of HA confirmed by using HPLC assay according to the method described by Jamal et al. (2002). The dried HA is dissolved in concentrated

sulfuric acid and adjusted to pH 5 with NaOH for HPLC analysis.

Viscosity measurement: 0.1mg/ml of HA solution sample is used to measure its viscosity by Brookfield DV-E viscometer.

Results and discussion

Yield% of crude HA extract: Figure 1 is showed the effect of precipitants on the yield% of crude HA extract. It indicated that the yield% of the control is the highest among the treatments, then followed by CPC, (NH₄)₂SO₄, Chloroform and Hexane in descending order. CPC is one of quaternary ammonium compound possesses positive charge, therefore it can bind H.A. which possesses negative charge to form a complex compound precipitated under low salt concentration (low ionic strength). Therefore, CPC can efficiently bind with H.A. and resulting in a higher yield% than other deproteinizing agents. Again, although, chloroform can efficiently deproteinize during extraction process, but it can not bind with water soluble H.A. due to its lipophilic property. Thus, its yield% can not be higher than CPC.

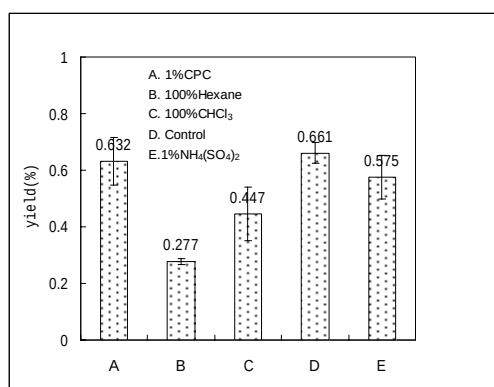


Figure 1. Effect of deproteinizing agents on yield% of crude HA extracts.

Effect protein precipitants on purity of crude HA extracts: Figure 2 it can be found that the purity of crude HA extracts from chloroform precipitation is the highest among all samples, then followed by the control, CPC, Hexane and (NH₄)₂SO₄ in descending order. The control sample is prepared without deproteinizing agent, and only adjust the pH to isoelectric point to remove the impurities, though its yield% is lower than that of chloroform but still higher than the products prepared with other precipitants. In order to confirm the purity measured by glucuronic acid content, HPLC is also employed. From the HPL chromatography (Figure 3), it indicated that sample of chloroform has highest purity, and then followed by the control, CPC and (NH₄)₂SO₄, in descending order. They are 0.176, 0.141, 0.138 and 0.108 mg/ml, respectively. The repression equation $y = 2E + 0.6x - 5576.7$, $R^2 = 0.9943$. The trends in the purity of the products from different precipitants are in the agreement with the measurement of glucuronic acid (Figure 2).

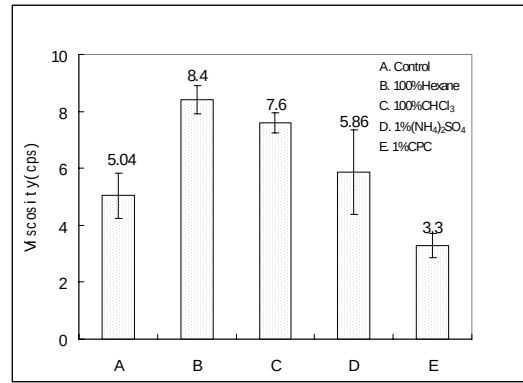


Figure 2. Effect of deproteinizing agents on the purity of crude HA extracts.

Viscosity of crude HA extracts: Figure 4 indicated that the viscosity of the crude HA extract from Hexane is the highest among all treatments, the chloroform, the control, (NH₄)₂SO₄, CPC. The lowest in viscosity of extracts with (NH₄)₂SO₄, and CPC is due to the polymer chain contracting firmly from an opening structure when salts presented in the extract solution. Thus, the salt concentration is higher the viscosity is lower. This finding may be caused by salt ions and denser electrons interaction (Piasrcik et al., 1995).

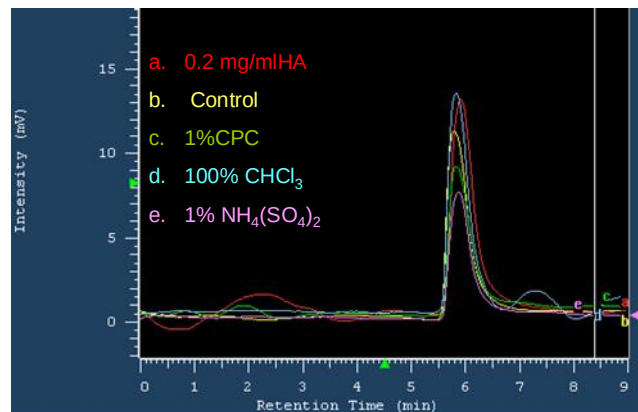


Figure 3. The purity of crude HA extracts from different deproteinizing agents confirmed by HPLC analysis.

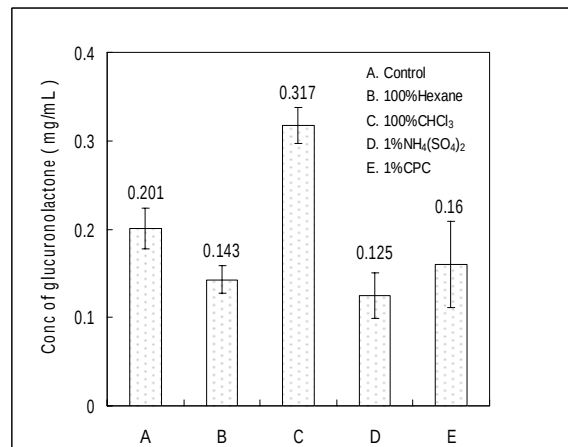


Figure 4. Effect of deproteinizing agents on viscosity of crude HA extracts.

Conclusions

1%CPC and 1% (NH₄)₂SO₄, were used as deproteinizing agents or precipitants to remove the proteins and other impurities existed in crude HA solution in this experiment. Highest yield% was obtained because of these two deproteinizing agents possess positive charge in the solution which can bind HA. But purity was lower due to their binding to HA firmly, then thus lowering the viscosity of the products. The purity and yield% prepared by using Chloroform as a deproteinizing agent were higher, but it has higher toxic and more polluted to environment. In addition, we used concentrated sulfuric acid to dissolve the sample for the content of HA determination by HPLC, it was more efficient and lower cost than the enzymatic method. It suggested that it is a convenient method for HA purity analysis.

References

- Balazs, E.A. 1979. Ultrapure hyaluronic acid and use. US. Pat.4141973.
- Bitter, T. and Muir, H. M. 1962. A modified uronic acid carbazole reaction. *Anal. Biochem.* 4:330.
- AFraser, J. R. E., Laurent, T. C. and Laurent. 1997. Hyaluronan: its nature, distribution, functions and turnover. *J. Int. Med.*, 242:27-33.
- Jamal, A. A., Yahya Meratani, Reinhard H. H. Neubert. 2002. New approaches for quantifying hyaluronic acid in pharmaceutical semisolid formulations using HPLC and CZE. *J. Pharmaceutical and Biomedical analysis.* 30, 913-919.