Technical Notes: Heparin Sodium Extraction from Broiler Intestines Based on Acid/Base Methodology

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Abstract: Heparin sodium is the raw material of heparin, a blood coagulant commonly used in medical treatment. Heparin is clinically used to prevent blood vessel thrombosis. In the past, heparin sodium was mainly extracted from porcine intestines. Here, an acid/base methodology was modified and used to extract heparin sodium from broiler viscera. Trypsin was added to smashed chicken's intestines at pH 8.5. D208 resin was then applied to adsorb heparin, followed by a process through a series of acid/base changes. Crude heparin sodium product, recovered by drying, was purified with permanganate oxidation followed by centrifuging. Final heparin products were harvested from the precipitation. From 0.2 kg raw materials of a mixture of chicken's intestines, 23.80 g crude heparin was obtained, while 31.40 mg heparin was isolated and recovered after purification. Accordingly, 119.00 g crude heparin and 159.00 mg purified heparin can be harvested from 1 kg raw materials. The yield of heparin isolated from broiler intestines approximates that from porcine. This study indicates that chicken can serve a new source of pharmaceutical heparin, especially given an outbreak of African swine fever in Asia.

INTRODUCTION

Heparin, a highly negatively charged sulfated polysaccharide[1], exists in the mast cells of organisms, especially in tissues of hearts, livers and intestines[2]. Although, its function in the organism remains unclear, heparin has a good anticoagulant effect and is one of the commonly used anticoagulants in clinical practice. It is used to prevent vascular thrombosis[1]. Common extraction sources are pigs or cattle. Pig small intestine mucosa is often used as raw materials commercially[3], although, viscera of bovine[4, 5], dromedary[6], ovine[7] and turkey[8] were also applicable for heparin extraction. Sole usage of pig viscera as raw materials makes the heparin quality stable. With a massive scale of pig husbandry, China has become the biggest supplier of heparin. Nevertheless, the outbreak of African swine fever caused a sharp decline in the number of pigs, a similar case

occurring in bovine due to the outbreak of bovine spongiform encephalopathy in the 1990s[9], inevitably impacting the global heparin industry[10], especially considering the safety of heparin as a pharmaceutical. Obviously, over-reliance on pigs as a single source as raw materials for manufacturing heparin can cause an unbalance between demand and supply. To avoid the pandemic impacts on the heparin supply, an alternative for pig has lets needs to be explored.

Broilers are common edible livestock in Asia and are the world's largest number of birds. A staggering amount of chicken is consumed worldwide and annually. Nevertheless, at the same time, huge amounts of chicken offal waste are also left, causing environmental burdens and pollution. Without recycling, most inedible internal organs such as the trachea and lungs are discarded. Due to the change of dietary habits, chicken entrails are less and less eaten in Asia while the demand for chicken meat is getting higher and higher. Inevitably, piling viscera requires waste disposal. Ironically, the UN Sustainable Development Goals (SDGs) do not recognize industrial wastes as trash anymore. Instead, most wastes are recyclable to reduce the environmental loads. Recycling of poultry viscera is not only environmentally friendly but offers extra commercial benefits, especially considering of saving budget and energy for disposing of wastes. Besides, given a short life cycle of broilers, even pandemic disturbance exists, high recovering rates would compensate for the supply loss faster than pigs, making poultry viscera a perfect alternative to pig intestines.

Extraction and purification of heparin from offal is an old industry. Quite many methods, including chemical, chemoenzymatic and biotechnological approaches have been developed[11]. Basically, there are two methods of visceral treatment during the heparin extraction. The first is chemical methods that digest proteins and remove impurities in acidic or alkaline environments at high temperatures where as heparin may be damaged. Enzymatic hydrolysis is the other that breaks down a variety of proteins using relevant enzymes. Common proteases are trypsin, chymotrypsin, papain or subtilisin-like enzymes. Liu *et al.*[12] proposed a method of homogenizing the internal organs and separating the crude fat before drying to reduce the amount of enzyme protein used. Their method also increases the number of centrifugation steps in the process to reduce the impact of residues on the extraction of heparin such as tissues microorganism. This method can increase the shelf life and reduce the volume after the preparation of the internal organs, thereby reducing the cost. Here, we modified the acid/base method, combining the enzymatic hydrolysis method, visceral homogenous degreasing and resin adsorption. One of the benefits is that the acid/base method reduces the content of microorganisms in the extraction process. At the same time using resin adsorption not only saves the process of centrifugation time and centrifuge equipment but also simplifies the process complexity.

MATERIALS AND METHODS

Materials: Broiler intestines of *Gallus domestics* were obtained from Zhong-Lun slaughterhouse in Tainan, Taiwan, right after sacrifice. Intestines were washed with tap water and transported to the laboratory.

Acetone (>99%), Hydrochloric Acid (HCl), sodium hydroxide (NaOH), sodium chloride (NaCl), potassium permanganate ($KMnO_4$), hydrogen peroxide (H_2O_2), sodium sulfite (Na_2SO_3) and ethanol (EtOH) were purchased from Sigma-Aldrich. Trypsin was purchased from Gibco, Thermo Fisher Scientific Inc. All chemicals were used as received without further purification.

Acid/base extraction: Homogenizer was used to smash chicken's intestines and then the tissue fluid was squeezed out through gauze. After elevating. The reaction remained at 55°C for 2 h. D208 resin was applied to adsorb heparin and immersed in 5M NaCl for 3 h under regular agitation to ensure the completion of heparin decomposition. Furthermore, the resin was removed by filtration and with pH tuned to 3.5, followed by adding NaOH to adjust the pH to 10. Ethanol was added to the reactions and kept at room temperature for 12 h to precipitate heparin. Crude heparin sodium product was collected by drying out at 60°C. The process above is a traditional extraction method so-called acid/base extraction[12], nevertheless with disadvantages of contamination with residue chemicals, causing the impurity of heparin. Further purification is therefore required.

Chemical degradation analysis: After conducting acid/based extraction, the crude heparin sodium was dissolved in 2% NaCl. NaOH was used to adjust the pH of the solution to 8. Then, permanganate oxidation was performed at roomtemperature by adding 0.2 M $KMnO_4$ till the purple color persists which served as an indicator of the titration endpoint. Na_2SO_3 addition was made immediately until the purplecolor of $KMnO_4$ had disappeared. NaOH was then used to tune the pH to 10 after filtration. Next, 4% H_2O_2 that serves as an oxidizing agent was added to complete the second oxidation process at 25°C. HCl was used to acidify the solution to pH 6, followed by adding ethanol. Precipitation by centrifuging at 10,000 rpm for 10 min followed to remove the supernatant. Then, the solvent was removed by rotary evaporation to obtain the final heparin products. The procedure for crude heparin extraction is illustrated in Fig. 1.

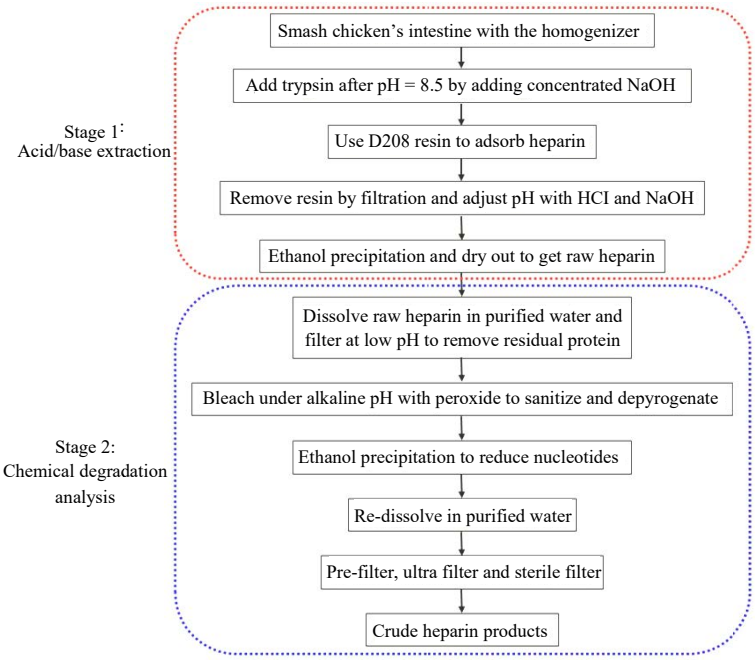

Fig. 1: The brief procedures of heparin extraction

Crude heparin quantification: The heparin amount was determined by the average ofthree separate purification processes. From per unit of raw materials we could obtain a certain number of crude products and then the products were divided into three parts for confirming if the purification steps were operated in a uniform manufacture process. The raw material we put into the extraction process accounted for 0.20 kg and the eventual quantified heparin amount per kilogram of raw materials was calculated by multiplying 5 and the weight of the products which was the average of three purification results.

RESULTS AND DISCUSSION

Heparin yield from broiler intestines: From 0.2 kg raw materials of a mixture of chicken's intestines, 23.80 g crude heparin was obtained while 31.40 mg heparin was isolated and recovered after purification. Accordingly, 119.00 g crude heparin and 159.00 mg purified heparin can be harvested from 1 kg raw materials. The number of heparin products extracted from various animal's organs includingdromedary, porcine, turkey and bovine were compared as shown in Table 1.

Application of acid/base extraction methodology: Apparently, the heparin amount after purification of broiler intestines in this work approximates that of porcine, while much lower than that of dromedary, turkey and bovine origins. Such differences may stem from extraction procedures that utilize various enzymes, purification approaches and reaction conditions (e.g., duration, temperatures). Nevertheless, heparin purification procedures are often deemed as industrial secrets, leading to the limited information available inthe public domains[11]. Here, the broilerheparin extracted presents a similar amount compared to the one from porcine which has been well analyzed and manufacturedvia different approaches.The heparin yield from broiler intestines reveals that the optimized methodology combining advantages of traditional acid/base extraction and chemical degradation analysisis applicable to the business operations.

Heparin is a crucial and primary material for anticoagulant and antithrombotic drugs[13]. Today, around the world, the elderly population that suffers from cardiovascular diseases with a great portion has been expanding yearly, leading to a big growth of heparin demand and price surging of the heparin related medicines. In India, heparin companies have already restarted their producing lines[14]. In the US, pharmaceutical associated industries have expanded the storage of crude heparin to ensure the industrial chains and medicine supplies. Obviously, heparin has become some strategic material that are critical for the national security.

For the heparin market worldwide, over 60% of supplies depend on China. Unfortunately, certain batches of heparin are highly associated with anaphylactoid-type reactions, seriously leading to death in some cases. These reactions were traced to contamination with semisynthetic Oversulfated Chondroitin Sulfate (OSCS) over the extraction process[15]. Events related to

Table 1: Comparison of crude heparin sodium isolated from intestines of various animals

Intestines resources	Weight of raw materials (kg)	Crude heparin per kilogram (g/kg)	Heparin amount after purification (mg/kg)	References
Broiler	0.20	119.00	159.00	This work
Dromedary	-	-	400	Warda *et al*.[6]
Porcine	1.00	19564	178.59	Lee *et al*.[2]
Turkey	0.20	-	307	Warda *et al*.[8]
Bovine	0.10	-	342-527	Sarwar *et al*.[5]

the OSCS adulteration of heparin are summarized on the US Foodand Drug Administration (FDA) website. Apparently, both heparin quantityand quality are required to match the medical standards.

OSCS, a kind of glycosaminoglycan (GAGs) with a unique structure and unnatural sulfonation pattern is one of the major pollutants. OSCS does not belong to a natural product arising from animal sources, whereas likely deriving from artificial errorsover the synthetic process. However, the detection of heparin contaminants and impurities is challenging primarily because they differ from heparin at sulfo group position, degree of sulfation and sugar epimers[16]. The characterization of OSCS contaminantsin heparin products relies heavily on the analysis of Nuclear Magnetic Resonance spectroscopy (NMR) which determines the specific individual functional groups with high accuracy.

CONCLUSION

This study provides evidence for showing inedible broiler viscera suitable for heparin manufacturing as a new source. African swine fever largely threatened the supply of pharmaceutical heparin. A method modified from acid/base extraction can reduce microorganism contents. By adding a procedure of resin adsorption, the whole process can be simplified, likely reducing the manufacturing costs. Recycling the viscera “trash” for heparin extraction not only saves the budget for waste disposal and lowers down the environmental burdens but also creates extra agricultural values.

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