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Analysis of bovine-derived demineralized bone extracts

Ramlingam Murugan · Seeram Ramakrishna · Koritala Panduranga Rao

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Abstract Demineralized bone factors are capable of stimulating bone regeneration through an osteoinductive mechanism and thus it has been recognized as a good bone graft. In this study, a kind of demineralized bone extracts (DBX) derived from bovine tibia by a chemical route. The extracts thus obtained were analyzed for their bio-chemical and physical properties using various techniques and results provided quite interesting insights into the demineralization process. There is no significant evidence of mineral phase associated with the connective tissue detected during chemical as well as physical testing, indicating the formation of DBX. This kind of bone extracts may used as a bone graft material and as a substrate for the growth of biomimetic apatites.

Introduction

Biomaterials derived from a biological origin are well recognized and much adopted by human owing to their satisfactory performance with living tissues. The deproteinated xenogeneic bone is one among them proposed as a bone graft material due to its structural and chemical similarity with carbonated hydroxylapatite, a mineral phase of calcified connective tissue [1, 2]. On the other hand, demineralized xenogeneic bone or demineralized bone matrix (DBM) has also gained much attention as a bone graft material. The rationale for using DBM is not only based on its ability to regenerate the bone in heterotopic and orthotopic sites through a process called osteoinduction [3], but also through an osteoconduction mechanism [4]. Osteoinduction is a process of making undifferentiated mesenchymal cells differentiates into osteoblasts, leading to formation of new bone. Osteoconduction refers to the ability of biomaterial to serve as a scaffold for the bone cells to attach, migrate, differentiate and proliferate in a controlled fashion, resulting in a new bone growth. DBM has a number of additional advantages that make it as a good bone graft, which includes biocompatibility, biodegradability, cost-effective, readily available in abundant quantity, sterilizable, and could be stored for a reasonable time [5, 6]. It is clinically superior to allogenic fresh or frozen bone because of demineralization process that destroys antigenic substances associated with a bone tissue, ensuing in less immunogenic than calcified allograft. Furthermore, complications encountered in harvesting autogenic bone tissue can be eliminated. There are numerous evidences available for the suitability of DBM as a bone graft extender, a bone graft enhancer, and a bone graft substitute [5–11]. It has been recently used as plates for orbital and craniofacial reconstructions [12].

As per literature survey, DBM can be derived from a variety of biological resources corresponding to a specific need, but the details on their physiochemical characterizations are limited. A complete analysis of any biologically derived material is of great importance regardless to field of application. The basics of physical, mechanical, chemical, biochemical, and biological characteristics should be well established before intending them for

R. Murugan (✉) · S. Ramakrishna
NUS Nanoscience and Nanotechnology Initiative (NUSNNI), Division of Bioengineering, Faculty of Engineering, National University of Singapore, 9 Engineering Drive 1, Singapore 117576, Singapore
e-mail: engmr@nus.edu.sg

K. P. Rao
Spark Biotech, 3720 Sunburst Lane, Naperville, IL 60564, USA

biomedical applications. Much research has focused on the kinetics of demineralization process or its mechanical strength with respect to processing conditions. Apart from this, bio-chemical and physical properties are also much influences the success rate of DBM upon implantation. With a view that analysis of fundamental properties may provide some interesting insights into the demineralization, the present study of processing and characterization, particularly bio-chemical and physical, of demineralized bone extracts (DBX) from the bovine bones was undertaken.

Experimental section

Preparation of DBX

The DBX was processed from the adult bovine tibia bones. Briefly, the bones were cleaned for macroscopic adherent soft tissues and bone marrows by washing with cold deionized water. They were sectioned into small chips in dimension of about 10 mm^3 under running water and defatted with absolute alcohol/dichloroethane (1:1). The extraction of the cleaned bone tissue was performed with frequent changes of absolute ethanol, dehydrated with anhydrous ether and air-dried for 12 h. After air-drying, they were fragmented in a liquid-nitrogen impacting mill to a cocktail particle size of ranging between 50 μm and 200 μm. The proteoglycans were removed using 6 M urea in a 0.05 M Tris buffer at pH 5.8 followed by demineralized by acid-extraction with 0.5 M of HCl for 3 days at room temperature. The residual acid was removed by a continuous washing with 0.1 M of NaCl solution and deionized water by centrifuging at 10,000 rpm for 10 min until the pH of the supernatant become same as the rinsing water and lyophilized. Finally, demineralized bone was extracted with absolute ethanol followed by ether and then evaporated under chemical hood.

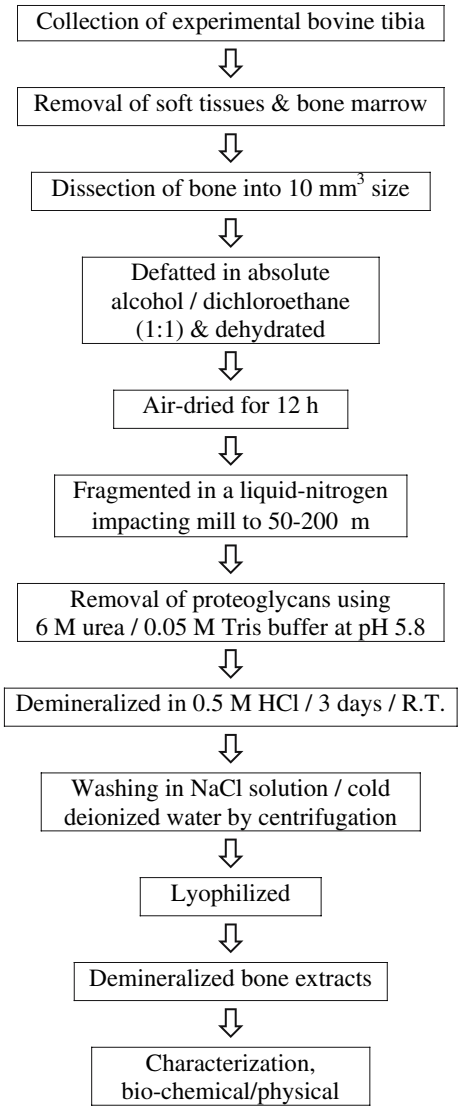
Bio-chemical and physical characterization

The biochemical analyses of DBX, such as hydroxyproline, collagen, and protein estimations, were carried out by the standard measures in which HCl-hydrolyzed samples were diluted with a known volume of deionized water to make a solution, homogenized and then insoluble residues were removed by filtration. The bone extracts thus obtained were subjected to analysis for hydroxyproline and collagen contents using Neuman and Logan method [13, 14] and protein content using Lowry method [15]. The surface morphology of DBX was studied by a scanning electron microscopy (SEM) (JSM 5600, JEOL, Japan) with a gold sputter-coating technique using JEOL JFC-1200 fine coater (Japan) to a thickness of 20–30 nm and examined with an accelerating voltage of 20 kv. The particle size and distribution of DBX were determined by a particle size analyzer (Malvern, UK) employing a laser diffraction technique. Before taking measurement, the samples were excited ultrasonically for 10 min to break-up loosely bound agglomerates. The characteristic chemical groups present in the DBX were identified by spectroscopic method using a Fourier transform infrared (FTIR) spectrophotometer (ThermoNicolet Avatar 360, USA). The transmissions were recorded within the range of 400–4000 cm^{-1} with 2 cm^{-1} resolution averaging 100 scans. The thermal behaviour of DBX was evaluated by differential scanning calorimetry (DSC) using a Perkin-Elmer DSC-7 (USA). The thermogram was recorded from 30 to 430 °C at a heating rate of 10 °C/min under nitrogen atmosphere (50 cc/min).

Results and discussion

The present study employs an elegant processing method for the demineralization of bovine bones. A schematic representation of a processing condition involved in the demineralization is given in Scheme 1. The processed DBX was subjected to biochemical testing for determining its hydroxyproline, collagen, and protein contents. The results of biochemical analysis are listed in Table 1, showing 2.94% of hydroxyproline, 21.93% of collagen and about 25% of protein contents. These data are well in accordance with a standard data of protein components associated with the bone tissue [14]. The SEM micrograph of DBX is shown in Fig. 1, indicating an irregular and coarse surface morphological structure owing to chemical processing condition. It shows that there is no indication of mineral phase related to a bovine bone on the surface of DBX, suggesting a completion of an adequate demineralization process.

The observation of particle size and distribution is an important factor for the biologically derived materials as they involve in the biological performances upon implantation. Perhaps, osteoinduction may depend on the particle size of biological implants. It is also noticed that the particle size less than 100 μm may cause a considerable reduction in the osteoinductive capability of demineralized bone factors [16]. Hence, the particle size and distribution of DBX was examined and illustrated in Fig. 2. The particle size distribution of DBX indicates that the particles are widely distributed in the range between 40 μm and 200 μm with a maximum number of particles (>60%) having 100–150 μm sizes. The average particle size of the DBX is found to be 120 μm. As the DBX has a mean particle size quite higher than recommended one, it could serve as a good osteoinductive agent upon implantation.



Scheme 1 A representative chart of a process involved in the demineralization

Table 1 Biochemical analysis

Biochemical factors	Total amount in bone tissue (%)
Hydroxyproline[a]	2.94
Collagen	21.93
Proteins	24.86

[a] contains 29.4 μg/mg of bovine bone tissue

Furthermore, it may help to avoid migration of particles beyond the implanted site to surrounding tissues, resulting in a good intact with host tissue

Figure 3 represents a FTIR spectrum of the DBX, showing all the absorptional peaks characteristically to proteinic components of the bony tissue. It shows absorption peaks pertaining to amide I at 1660 cm^{-1}, C–N

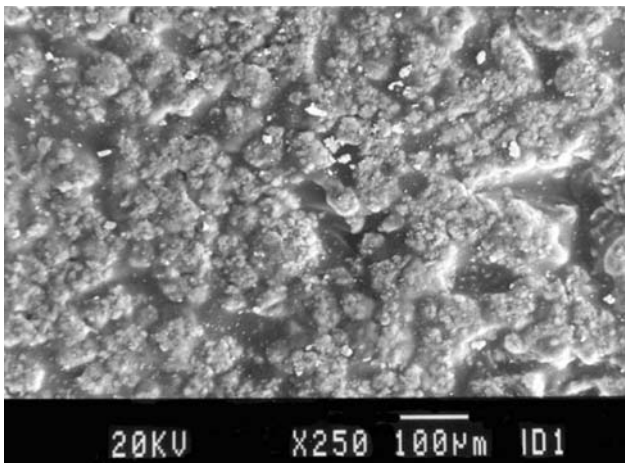


Fig. 1 SEM photograph of DBX, showing its surface morphology

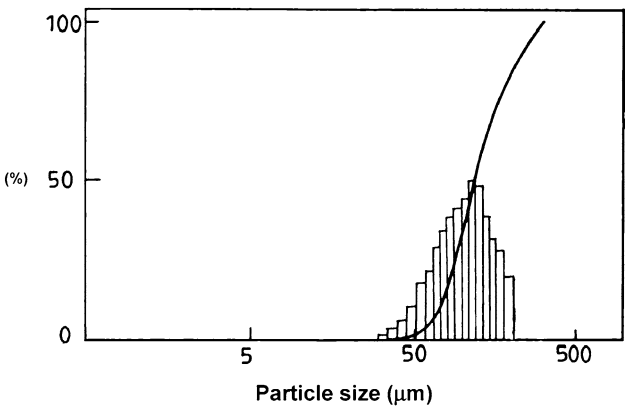


Fig. 2 Particle size distribution cure of DBX

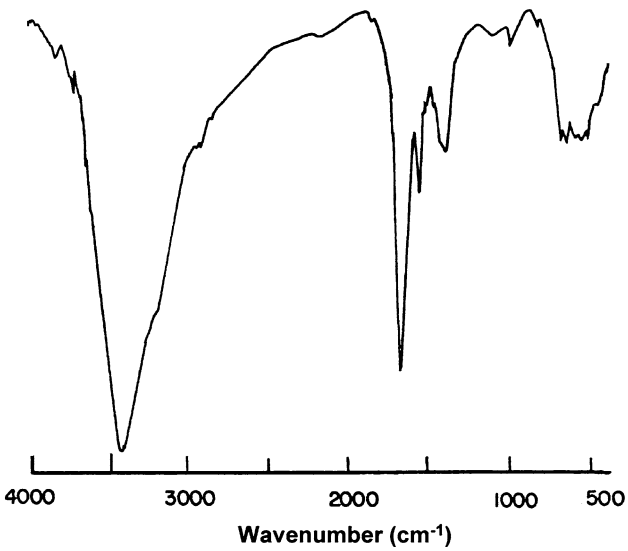


Fig. 3 FTIR spectrum of DBX

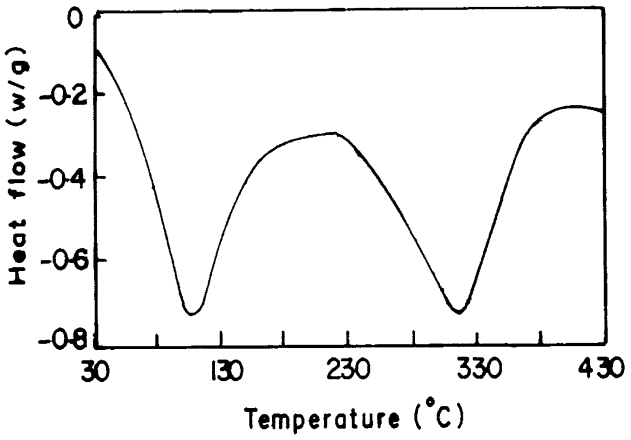


Fig. 4 DSC thermogram of DBX

stretching band at 1530 cm^{-1}, C–O stretching mode of primary alcoholic group at 1400 cm^{-1}, free primary amino group (-NH_2) at 1220 cm^{-1}, and amide V at 660 cm^{-1} owing to the existence of bone proteins or organic matrix. There is an overlapping peak related to N–H and O–H groups noticed at 3480 cm^{-1}, which may be attributed to the presence of collagen and adsorbed water molecules. All these peaks noticed are only responsible for the protein contents associated with the DBX. Apart from this, the spectrum does not show any absorptional peaks correspond to bone mineral phase, suggesting a good deal of demineralization process.

The DSC was used for studying the thermal behavior of DBX as it undergoes chemical and physical changes during heat treatment. As this method directly measures the heat capacity, we used it to determine the crystallization, glass transition and melting feature of existing proteinic components pertained to DBX. Figure 4 shows a DSC thermogram of DBX, indicating two endothermic peaks each at 105 and 313 °C. The curve shows a continuous endothermic weight loss starting from 30 °C to 150 °C for first instance followed by 230–360 °C for second instance. The first weight loss could be attributed to the dehydration process and the glass transition of collagen molecules associated with the DBX, which is in good agreement with a reported data [17]. The weight loss found at 313 °C could be attributed to the decomposition of denatured collagen and other macromolecular proteins associated with the bone organic matrix, which may be described as an irreversible melting [18]. There is no significant weight loss pertaining to decomposition or melting of mineral phase detected in the DSC thermogram, indicating an evolution in demineralization and encouraging the result of FTIR analysis.

Conclusions

In conclusion, this study provides a detailed processing condition of demineralized bone extracts form the bovine tibia. The demineralized extracts were systematically analyzed for their bio-chemical and physical properties. The spectroscopic and thermal analyses indicated the demineralization process at a reasonable limit as they do not show any characteristic sign of inorganic elements associated with bone minerals. The particle size and distribution of processed DBX lay within the optimal level required for a better osteoinduction. This kind of DBX may be used as a bone graft material and as a substrate for the growth of biomimetic apatites.

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