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Original Research Paper

Characterization of demineralization behavior of bovine bone granules related to particulate properties

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ABSTRACT

As a fundamental study on the preparation of partially demineralized bone matrix, the demineralization of bovine bone granules was investigated. Bone granules were obtained by pulverizing with a highspeed blade mill and classified by sieving into three fractions. The lengths along major and minor axes of the individual granules were measured by image analysis to calculate the circle equivalent diameter and the aspect ratio as particulate properties. The bone granules were dissolved in nitric acid, and the calcium concentration in the solution was measured to characterize the degree of demineralization in terms of the calcium content in the demineralized granules. Some weak correlations between the degree of demineralization and the particulate properties were found. The demineralization rate was fitted to a first order rate equation, which was modified by considering the sphere equivalent diameter of individual granule, the characteristic aspect ratio of each fraction and the effective surface area of the mineral component to improve the approximation accuracy.

1. Introduction

Demineralized bone matrix (DBM) is a powdery biomaterial prepared by *in vitro* dissolution of bone in an acid solution to remove mineral components. This procedure is referred to as demineralization. It is known that the implantation of DBM promotes the osteoinduction or bone regeneration [1]. Some researchers have investigated the effect of calcium amount remained in DBM on the osteoinduction and concluded that the best result was obtained under an optimum amount of calcium remained in DBM [2–6]. This means that such partial demineralization is preferable to the complete demineralization.

The results of implantation of DBM are known to depend on the size of the granules. However, the optimum size range is different from paper to paper [7–12]. We have been considering the reason for such difference. Doctors and dentists usually use two sieves to obtain bone granules over a desirable size range. In general, this mesh size range of sieve used is convinced to agree with the granule size range. If they are inconsistent with each other, it may become one reason for the differences among researchers described above.

In the previous paper [13], we have proposed a method of characterizing the size and size distribution of irregularly shaped bovine bone granules. By combining sieving and image analysis, it was found that the granules of smaller particle size have a tendency to reveal larger aspect ratio. That is, even if some specimens obtained by sieving powders pulverized under different conditions have similar average size, they may show different demineralization behavior. We have reported also that bone granules pulverized have different shape although the size is similar [13]. The most common particle sizing methods at present are laser diffraction and dynamic light scattering. Conventional sieving and gravity/-centrifugal settling are also used in practical works. These output the average size and size distribution of a powder as an assembly of a lot of granules, but the characteristics of an individual granule cannot be known by them. Electrozone sensing is also popular and can measure the size of an individual granule, whereas it cannot provide any information on the shape. Image analysis is the most suitable way to analyze irregularly shaped granules, because it can characterize the size and shape of an individual granule.

Researches on the degree of demineralization with regard to the particulate properties of bone granules have not been found in the

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Nomenclature

a	length of major axis [μm]
Av(X)	average of X
b	length of minor axis [μm]
c	height [μm]
C	calcium concentration [mol/L]
CV(X)	coefficient of variance for X [–]
d	characteristic diameter [μm]
d_e	equivalent sphere diameter [μm]
D	average particle size [μm]
k	rate constant
K	normalized rate constant [–]
n	amount of substance of calcium [mol]
m	mass [g]
r	aspect ratio [–]
R	average aspect ratio [–]
S	specific surface area [m^2/m^3]
S_e	effective surface area [m^2/m^3]
SD(X)	standard deviation of X
t	demineralizing time [h]
V	volume of a granule [cm^3]
X	arbitrary amount
α	normalized calcium content [–]
ϕ	shape factor [–]
ϕ'	modified shape factor [–]
θ	volume ratio [–]
ρ	density [g/cm^3]

Subscript

g	granule
M	mineral
P	protein
∞	complete demineralization

literature. In this study, bovine bone granules were dissolved in an acid, and the demineralization behavior was discussed with considering the particulate properties. When doctors and dentists carry out the animal experimentation to investigate the effectiveness of partial demineralization, DBM with the same degree of demineralization should be used. The purpose of this study is to propose the approach for predicting the conditions under which DBM with the desired degree of demineralization can be prepared.

2. Experimental

2.1. Pulverization

Bovine bone was cut into blocks of ca. 20 mm × 20 mm × 10 mm in size. The pulverizer used was a high-speed blade mill (Hayasaka Rikoh Co., Ltd., Sapporo, Japan). It is constructed of a crushing blade and a vessel, both of which are made of stainless steel.

One of the bone blocks was pulverized for a pulverizing time of 60 or 120 s at a revolution speed of 8000 rpm. The bone granules obtained were classified into three fractions with sieves of 500, 710, 1000, and 2000 μm. These fractions were referred to as Fractions I, II, and III, respectively. This size range from 500 to 2000 μm in mesh size was based on the good results of osteoinduction conducted by two of the authors [12].

2.2. Demineralization

Fifty milligrams of the granule of each fraction was put into 50 mL of 2% nitric acid and kept at 303 K with a thermostatic shaker reciprocating at 130 rpm for up to 24 h. The solution was drawn at given intervals. The bone granules demineralized, *i.e.* DBM, was filtered, washed, and freeze-dried. Part of the dry DBM was ground with a mortar and a pestle, and it was demineralized again for 24 h. The calcium concentration of this solution was measured to know the calcium content which had been remained in the DBM obtained by the demineralization for 24 h.

2.3. Characterization

The bone granules were observed under an optical microscope (STZ-40TBITb, Shimadzu Rika Corp., Tokyo, Japan). The lengths a and b as the major and minor axes of the smallest circumscribed ellipse of each granule were analyzed with free image analysis software, ImageJ [14]. The aspect ratio r_{ab} was defined as

$$r_{ab} = \frac{a}{b} \tag{1}$$

The characteristic diameter d was obtained as an equivalent circle diameter from

$$\frac{\pi}{4}ab = \frac{\pi}{4}d^2. \tag{2}$$

Some granules were put on a double-sided tape stuck on a slide glass plate and observed from the horizontal direction to measure the height c. Another aspect ratio r_{ac} was defined as

$$r_{ac} = \frac{a}{c} \tag{3}$$

The density ρ was measured with a densimeter (EW-300SG, TGK, Tokyo, Japan). The elemental analysis was performed with an electron probe microanalyzer (EPMA) (EPMA-1610, Shimadzu Corp., Tokyo, Japan) to examine the characteristic X-ray image of CaKα. The calcium concentration in the solution was measured by inductivity coupled plasma-atomic emission spectroscopy (ICP-AES) (SPS1500, Seiko Instruments Inc., Chiba, Japan).

The amount of substance of calcium in bone granules before demineralization is

$$n_\infty + n_{g,\infty}. \tag{4}$$

The former is calculated from the concentration in the solution obtained by the demineralization for 24 h, and the latter is obtained from DBM granules demineralized again for 24 h. The amount of substance of calcium remained in DBM during demineralization can be expressed as

$$n_\infty + n_{g,\infty} - n, \tag{5}$$

where n is the amount of substance of calcium in the solution for any demineralizing time. The normalized calcium content α was defined as

$$\alpha = \frac{n_\infty + n_{g,\infty} - n}{n_\infty + n_{g,\infty}} \tag{6}$$

This value ranges from 1 to 0, which corresponds to 0 to 100% of the degree of demineralization.

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