Preparation of hydroxyapatite from animal bones

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This paper presents the method of obtaining hydroxyapatite from animal bones. Bone sludge and calcined products were characterized by X-ray diffraction (XRD), Fourier transformed infrared spectroscopy (FT-IR) and scanning electron microscopy (SEM). Calcium concentration was determined with titration, and phosphorus – spectrophotometrically. Making use of the AAS and ICP methods the content of microelements was determined. In all the products, hydroxyapatite was the only crystalline phase indicated. The FT-IR spectra confirmed that calcination removed the total of organic substances. Calcium and phosphorus contents were 38% and 18%, respectively, which corresponded to the Ca/P molar ratio of nonstoichiometric hydroxyapatite. The specific surfaces of products were measured by BET method. The volume of micro- and mesopores was determined.

Key words: hydroxyapatite, bone sludge, calcining, physicochemical properties

1. Introduction

Bones consist of organic (30%) and inorganic compounds (70%). Mineral parts of bones provide their stiffness and proper mechanical properties. The model compound corresponding to a mineral phase of bones is a nonstoichiometric hydroxyapatite (HAp), i.e. HAp whose molar ratio of calcium to phosphorus is different from 1.67. Biological apatites are the components of bones and also pathological tissue (urolith, tooth scale and mineralized soft tissue) [1]–[3]. Those apatites are nonstoichiometric; in enamel and dentine the Ca/P molar ratio exceeds 1.67. Due to its chemical and structural similarity to bone minerals, hydroxyapatite is a promising candidate for bone substitutes. Hydroxyapatite is not only a biocompatible, osteoconductive, non-toxic, noninflammatory and nonimmunogenic agent, but also bioactive, i.e. it has got the ability to form a direct chemical bond with living tissues [4]–[5].

There are a few methods of extracting hydroxyapatite from animal bones: thermal decomposition, subcritical water process and alkaline hydrolysis [5]–[6]. In the research, we applied pork bones after acid hydrolysis. The chemical treatment was carried out using a mild agent, i.e. a solution of lactic acid, and soft conditions, i.e. a temperature of 125–135 °C and the pressure of 0.26–0.30 MPa. Contrary to alkaline hydrolysis, this method eliminates the problem of hazardous alkaline waste management. The final product of the hydrolysis process using lactic acid is, apart from bone sludge used for hydroxyapatite extraction, a protein hydrolysate of high purity used in food industry [7].

The research was conducted with the aim to produce hydroxyapatite using the other types of bone sludge: from bovine bones as well as pork legs and bones not treated chemically. Both the type of bone raw material and the process conditions have an impact on the physicochemical properties (among others the Ca/P molar ratio and surface morphology) of the hydroxyapatite obtained [8].

2. Material and method

The preparation method was developed in the Institute of Inorganic Chemistry and Technology, Cracow University of Technology. Deproteinised and defatted bone pulp, called bone sludge, was applied as a raw material, being formed as a by-product in the deproteinisation process of bones, in which a protein hydrolysate was the main product. Hydroxyapatite was obtained in two-stage calcination process at the temperatures of 650 °C and 950 °C, respectively, for each stage. The material was calcined at the maximum temperature for 3 hours. The calcination process was conducted in a chamber kiln with electric heating in the air atmosphere. In all the tests, a sieve fraction of dimension below 0.063 mm was used.

The phosphorus content was determined spectrophotometrically with a Marcel Media UV-VIS spectrophotometer after former sample mineralization in the mixture of concentrated hydrochloric and nitric acids. This method consists in forming a yellow-colour phosphorus–vanadium–molybdenum complex and a photometric measurement of absorbance at a wavelength of 430 nm. Calcium content was determined with a complexometric titration method using disodium versenate. The method involved dissolving a sample in nitric acid, precipitation of phosphates as bismuth(III) phosphate(V) $BiPO_4$, and then determining the calcium content by complexometric titration with disodium versenate (EDTA) in the presence of a mixed indicator (calcein and thymolphthalein).

Phase composition was analysed using the X-ray method with Philips X'Pert diffractometer equipped with graphite monochromator using Cu Kα 1.54, Ni filter (40 kV, 30 mA).

The concentrations of microelements such as Cu, Co, Ni, Cr and Cd were determined after former sample mineralization in concentrated nitric acid by the AAS method with Analyst 300 Perkin Elmer Spectrometer, and those of heavy metals such as Pb, Hg and As were measured using the ICP (Inductively Coupled Plasma).

The FT-IR investigations were carried out with a Scimitar Series FTS 2000 Digilab spectrophotometer in the range of middle infrared of 400–4000 cm^{-1}. A 0.0007 g sample was pressed with 0.2000 g of KBr. 16 scans and the resolution of 4 cm^{-1} characterized these measurements.

The effect of calcination temperature on the microstructure of samples was examined using an S-4700 Hitachi scanning electron microscope. Chemical analysis was carried out with an energy dispersive X-ray spectroscope (EDS) at 20.0 kV and 15.0 mA.

The specific surface of powders was evaluated by BET method using the apparatus ASAP 2405 Micromeritics Inc. USA. The surface and volume of pores were also tested.

3. Results

A general observation was that upon calcination at different temperatures the colour of samples changed. Bone sludge was yellow-brown in colour. After the first stage of the process the colour of sample was grey, and after the second stage it changed into white. The grey colour observed for the material obtained at 650 °C indicated an incomplete removal of organic compounds. The content of calcium and phosphorus in bone sludge and calcination products are shown in figure 1.
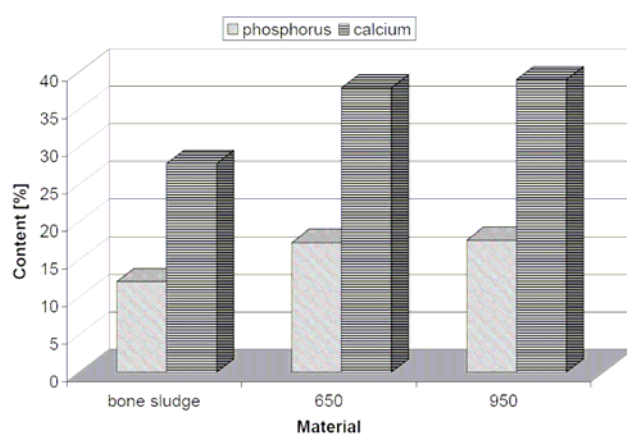

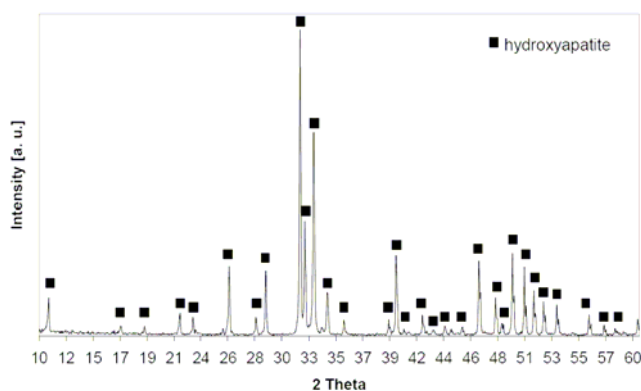Fig. 1. Content of calcium and phosphorus in bone sludge and calcination products

Fig. 2. X-ray diffraction pattern of sample obtained at 950 °C

Figure 2 presents an X-ray diffraction pattern of hydroxyapatite obtained at 950 °C. The X-ray analysis showed that hydroxyapatite was the only crystalline phase in both bone sludge and the calcination products.

Although the contents of all the chemical elements were the highest in a final product, they met the standards established [9] (see table 1).

Table 1. Contents of microelements and heavy metals
in bone sludge, intermediate and final products

Chemical element (ppm)	Bone sludge	Intermediate product (calcinated at 650 °C)	Final product (calcinated at 950 °C)	Standards established [9]
Cr	6.70	7.30	9.53	–
Cu	4.25	4.93	5.86	–
Ni	8.82	9.81	10.19	–
Co	5.42	6.23	8.43	–
Cd	2.28	2.97	3.46	4
Pb	0.033	0.025	0.152	25
Hg	0.00019	0.00092	0.0028	4
As	0.035	0.075	0.152	2

The FT-IR spectra of materials after the first and second stages of the process, i.e. at 650 °C and 950 °C respectively, are given in figure 3.
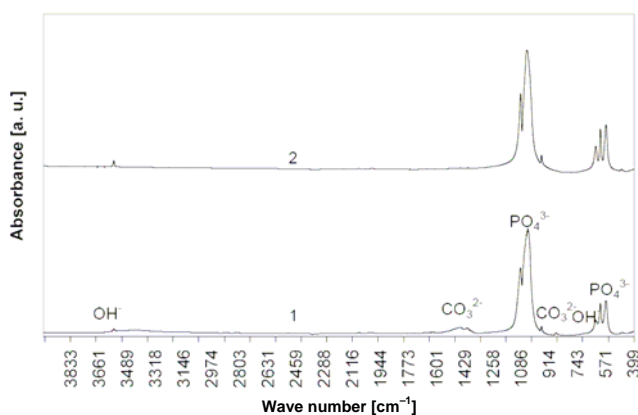

Fig. 3. Infrared spectra of calcination products
at temperature of 650 °C (1) and 950 °C (2)

Figures 4 and 5 show the representative SEM images of the calcination products of bone sludge, obtained at the temperatures of 650 °C and 950 °C, respectively.

Figures 6 and 7 present the distribution of chemical elements on the product surfaces. These images demonstrate a very good homogeneity of the ash surfaces analyzed. The distribution of chemical elements in both cases is almost uniform.
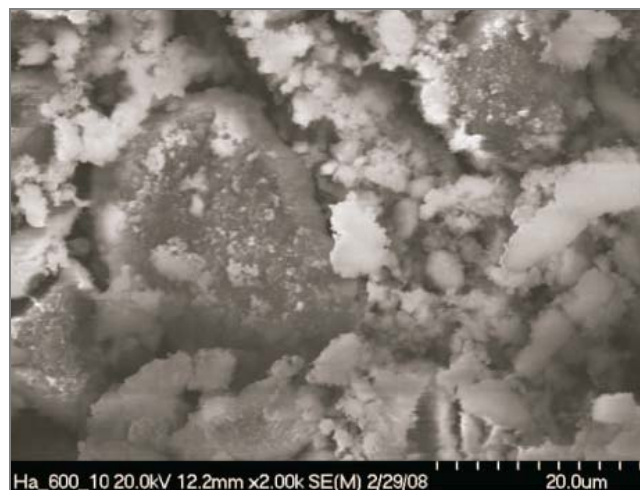

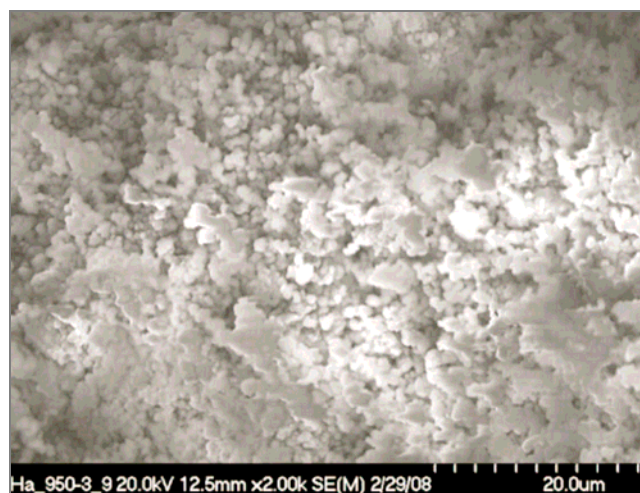Fig. 4. SEM of ash surface obtained at 650 °C
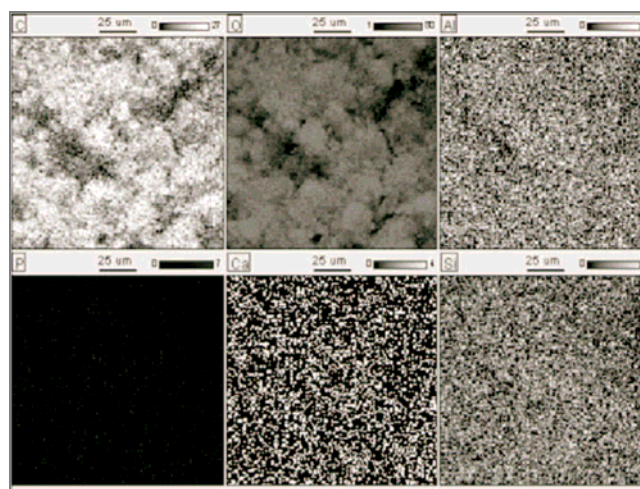

Fig. 5. SEM of ash surface obtained at 950 °C

Fig. 6. SEMs of ions in ash
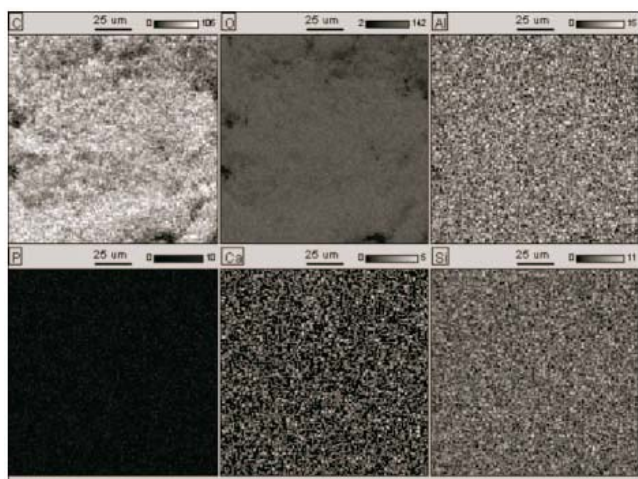after preliminary calcination at 650 °C

Fig. 7. SEMs of ions in ash
after preliminary calcination at 650 °C

The specific surfaces of the powders obtained were characterized by BET method. The surface and volume of pores were also analysed. The results are presented in table 2.

Table 2. Surface parameters of calcination products

Parameter	Material after the first stage of process (calcinated at 650 °C)	Material after the second stage of process (calcinated at 950 °C)
BET surface area (m^2/g)	44.1026	2.6868
Total adsorption surface area of mesopores (m^2/g)	44.8185	3.3124
Total adsorption surface area of microspores (m^2/g)	7.4745	0.6250
Total adsorption surface volume of mesopores (cm^3/g)	0.309106	0.006160
Total adsorption surface volume of micropores (cm^3/g)	0.003374	0.000283
Average pore diameter (nm)	20.54	7.536

Typical EDS spectra representing various elements found in the calcination products are shown in figures 8 and 9.
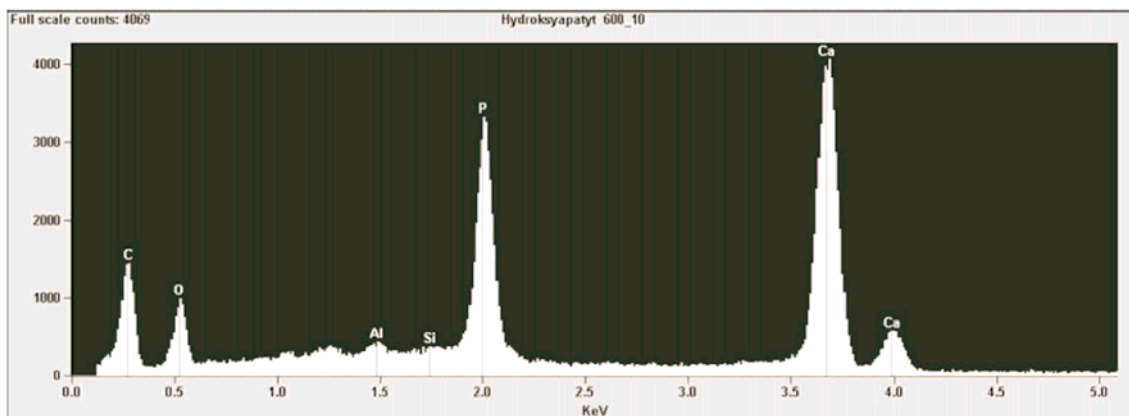

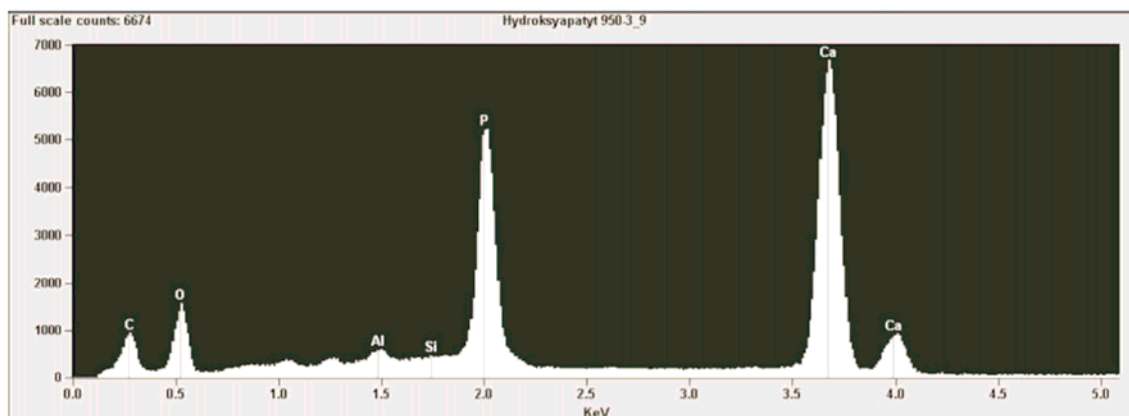Fig. 8. Chemical composition of ash obtained after a preliminary calcination

Fig. 9. Chemical composition of ash obtained after the second stage of calcination

4. Discussion

The colour of the samples depended on the calcination temperature: those obtained at lower temperature are darker because of the traces of organic matter, which are not present in the brighter samples obtained at higher temperature.

The calcium and phosphorus concentrations in bone sludge and in the material after preliminary calcination at 650 °C were lower than those in the material after the second stage of the process (at 950 °C) due to the presence of organic substances in both raw material and intermediate product. The calcium to phosphorus ratio in the samples obtained at 950 °C proved to be higher than the stoichiometric value of 1.67 and amounted to 1.70.

The analysis of the phase composition of the products obtained due to bone sludge calcination at 650 and 950 °C was carried out following a material exposure time of 3 hours. In all the materials obtained, only hydroxyapatite occurs in a crystalline phase, as indicated by X-ray method.

FT-IR spectrum (figure 3, (2)) of the calcination product exhibited only the characteristic absorption peaks of hydroxyapatite. The absorption bands corresponded to particular bonds which is in conformity with [10]–[11]. The second spectrum testified to the absence of bands corresponding to the vibrations of C–H and C–C bonds of organic compounds. It was confirmed that a total organic matter was removed after the second stage of the process. The vibrations of O–P–O bonds corresponded to the absorption bands within the wave number ranging from 570 to 636 cm^{-1}. The most intensive bands in the range of 1047–1095 cm^{-1} corresponded to asymmetric stretching vibrations of P–O, whereas the maximum of absorption at the wave numbers of 964 cm^{-1} came from the symmetric vibration of P–O. The low-intensity bands at 1427 and 885 cm^{-1} observed for the material obtained in the preliminary calcination could correspond to the stretching vibrations of CO_3^{2-}. The band with high wave number range corresponded to the vibration of O–H bonds.

SEM investigations confirmed that the products obtained at given temperatures demonstrated a diversified propensity for agglomeration. The biggest propensity for agglomeration was revealed by the ashes obtained at a temperature of 950 °C. The preliminary calcination of material led to the ash composed of various size grains. Most of them were large, irregularly shaped grains of a diameter approaching 30 μm. In a hydroxyapatite obtained at 950 °C, there were identified regularly shaped grains resembling a sphere, with a diameter of approximately 1 μm. It could be noticed that particular grains were sintered.

EDS analyses revealed that inorganic phases of bones were mainly composed of calcium and phosphorus as the major constituents with some minor components such as C, O, Na, Mg and Si. The relative intensity of peaks was comparable in both cases with one exception. The peak corresponding to carbon in an intermediate product was higher than that corresponding to final ash. This could result from a large amount of organic compounds in hydroxyapatite from a preliminary calcinination.

The BET research showed that the surface of the material after the preliminary calcination was nearly twenty times larger than the surface of hydroxyapatite obtained at 950 °C. This could be caused by a carbon residue in the material obtained at 650 °C. The reduction of a specific surface and average pore diameter confirmed that the grains of powder were sintered. The average diameters were calculated and proved to be 20.54 nm and 7.54 nm for the material from the preliminary calcination and after the second stage of the process, respectively.

5. Conclusions

Two stages of the calcination process of bone sludge at the temperatures of 650 °C and 950 °C confirmed that it is possible to obtain hydroxyapatite by thermal treatment of deproteinised bone waste. The calcination products contained hydroxyapatite as the only crystalline phase identified with the X-ray method. The Ca/P molar ratio of hydroxyapatite confirmed its nonstoichiometry resulting from the appearance of carbonate groups and a small amount of microelements.

On the basis of the investigation results it could be inferred that hydroxyapatite produced from bone sludge has a great potential to be used as a viable and economical biomaterial for clinical application. Hydroxyapatite obtained from animal bones can potentially be used as a biomaterial for both surgical and stomatological implants. HAp may be applied in orthopaedics, dentistry, orthognathic surgery, ophthalmology, laryngology and traumatology [2], [12]. In medical practice, HAp bioceramics are used as powders, granules, solid and porous materials, composite constituents or coatings on various foundations [13]–[15]. Powdered or granular hydroxyapatites prove to be useful in dentistry for the treatment of pulp (as a tooth cement additive), pathological tooth abrasion or post-extraction bone defect. They are also applied

to seal up canals and to treat perforated root canals; they are useful in forming tooth cleaning aids for conservative dentistry [2], [16]. Porous matrix materials can be used for post-extraction alveolar fillings in order to delay the atrophy of alveolar process bones. In addition, they serve as medicine vehicles and bases for cell cultures [17], [18]. Condensed matrix materials are applied as bone implants of an orbital bottom. They may also be used in otolaryngology as middle ear implants [2], [12], [17]–[19].

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