

In vitro tests of dense hydroxyapatite materials

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The paper presents the results of the calcining process of deproteinised and defatted bone pulp called bone sludge. The calcining process was performed in two stages. The first step of the calcining process was realized at the temperature of 600°C in a rotary kiln. In the second stage the obtained bone ashes were calcined at five different temperatures from 650°C to 950°C for 2 hours in a chamber kiln and in air atmosphere. The products of the calcining process were characterized by the XRD method. Calcium content was determined by titration whereas the contents of total phosphorus and acid-soluble phosphorus – by the spectrophotometric method. The X-ray analysis confirmed that hydroxyapatite is the main component of the calcining products. Calcium and phosphorus contents were kept at the level of 40% and 17.5%, respectively, which corresponded to the Ca/P ratio of not stoichiometric hydroxyapatite. *In vitro* studies, in the simulated body fluid, Ringer liquid and distilled water were realised. The measurements of pH value of SBF and Ringer fluid were realized. Additionally electrical conductivity as well as pH for distilled water were conducted. The goal of these tests was to evaluate chemical durability of dense hydroxyapatite materials.

Keywords: hydroxyapatite, dense materials, *in vitro* tests, incubation.

INTRODUCTION

Different kinds of materials are applied in repair and reconstructive medicine, of special importance being hydroxyapatite HA biomaterials, which substituted the destroyed or damaged bone tissues. It is possible due to particular properties of HAp, i.e.: chemical and phase composition, microstructure, high biocompatibility, bioactivity and bioconductivity¹. The hydroxyapatite material adapts in a human body and it does not demonstrate toxic properties. HAp ceramic does not induce sharp or chronic pathological states, however it is active in the regeneration process of tissue. High biocompatibility resulted from a chemical similarity of an implant to natural bone apatite²⁻⁵. The surface of HAp creates optimal conditions for the accretion of a new bone tissue. A bioactive material creates an environment compatible with osteogenesis (bone growth), with the mineralizing interface developing as a natural bonding junction between living and non-living materials. The connection between the bone and implant resulted from a biological and physicochemical process. Osteogenesis consists in direct, functional and structural adhesion of an implant to the bone surface²⁻⁵.

Dense hydroxyapatite implants have an application as surgical and stomatological implants. They are used as bone eye ground and in laryngology as middle ear implants. Moreover, dense HAp materials could be applied as PD (Percutaneous Device) implants, which are part of equipment, for example for dialysis. In hard tissue engineering, properties like bioresorption and biocompatibility are made use of, and hydroxyapatite has an application as a scaffold².

EXPERIMENTAL PROCEDURES

Preparation of hydroxyapatite

The preparation method was developed in the Institute of Inorganic Chemistry and Technology at the Cracow University of Technology. Bone sludge was applied as a raw material that was formed as a by-product in the deproteinisation

process of pork bones, the main product of which was a protein hydrolysate.

Hydroxyapatite was obtained in the calcining process in two stages. In the first stage the material was calcined at the temperature of 600°C in a rotary kiln with gaseous heating. In the next stage the unified material (sieve fraction below 0.15 mm) was calcined at the temperatures of 650°C, 700°C, 800°C, 900°C and 950°C, keeping the material at the maximum temperature for 2.0 hours in a chamber kiln with electric heating in air atmosphere. Two series of measurement were realised simultaneously.

The phase composition determined with the X-Ray method on Philips X'Pert diffractometer equipped with a graphite monochromator showed that hydroxyapatite was the only crystalline phase observed in the calcining products. However, the crystallisation degree was dependent on the calcining temperature and increased with the temperature of the process.

Table 1. Losses of weight at the time of the calcining process at different temperatures

	Loss of weight [%] of Hap obtained at				
	650°C	700°C	800°C	900°C	950°C
Series I	16.7	15.8	19.5	17.5	18.4
Series II	17.4	16.4	18.4	20.0	18.0

The general tendency was an increase of weight losses with the calcining temperature. It confirmed that all the organic compounds were removed from the samples. The decomposition of carbonate groups that are present in natural bone was observed at 800°C.

The content of total phosphorus, phosphorus soluble in 0.4% hydrochloric acid and 2.0% citric acid were defined by the spectrophotometric method according to the PN-88/C-87015 with Marcel Media spectrophotometer. The determination of total phosphorus content was done after former mineralization of the sample in a mixture of concentrated hydrochloric and nitric acids. Calcium was determined by the titration method according to the PN-R-64750: 1993.

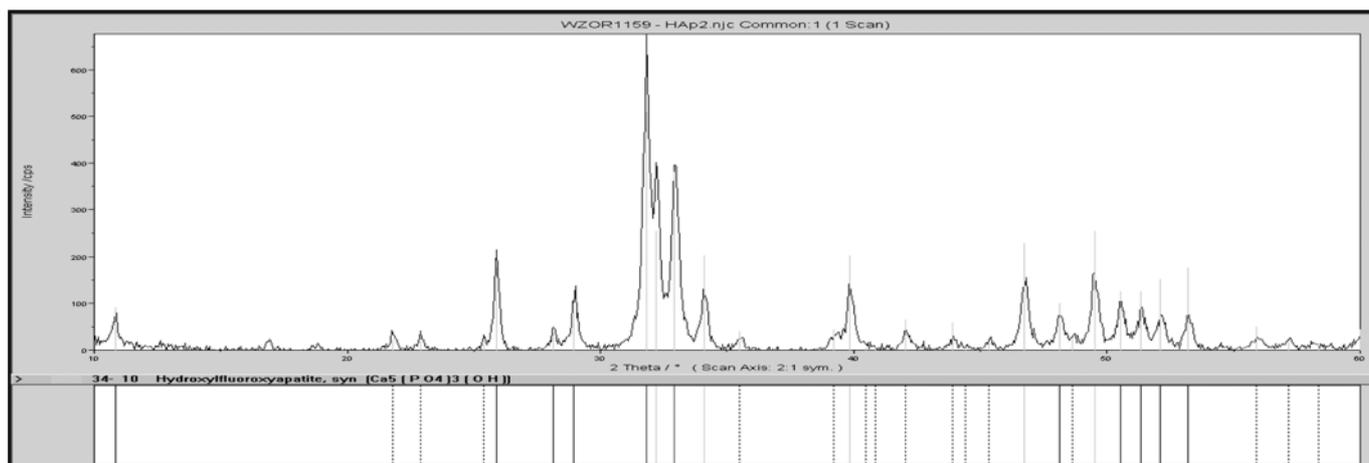


Figure 1. An X-Ray diagram of the calcining product obtained at the temperature of 650°C

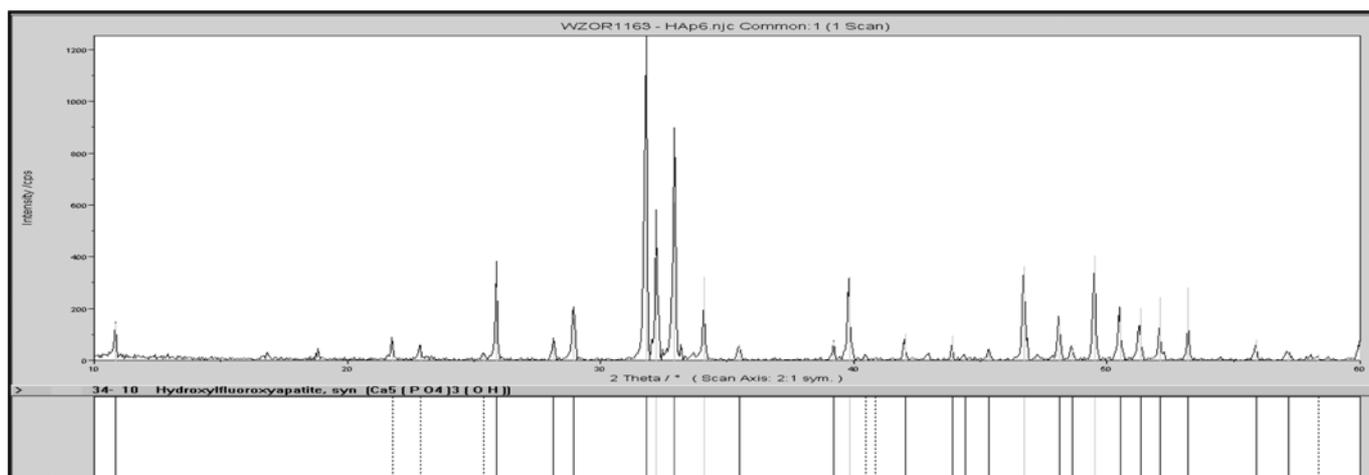


Figure 2. An X-Ray diagram of the calcining product obtained at the temperature of 950°C

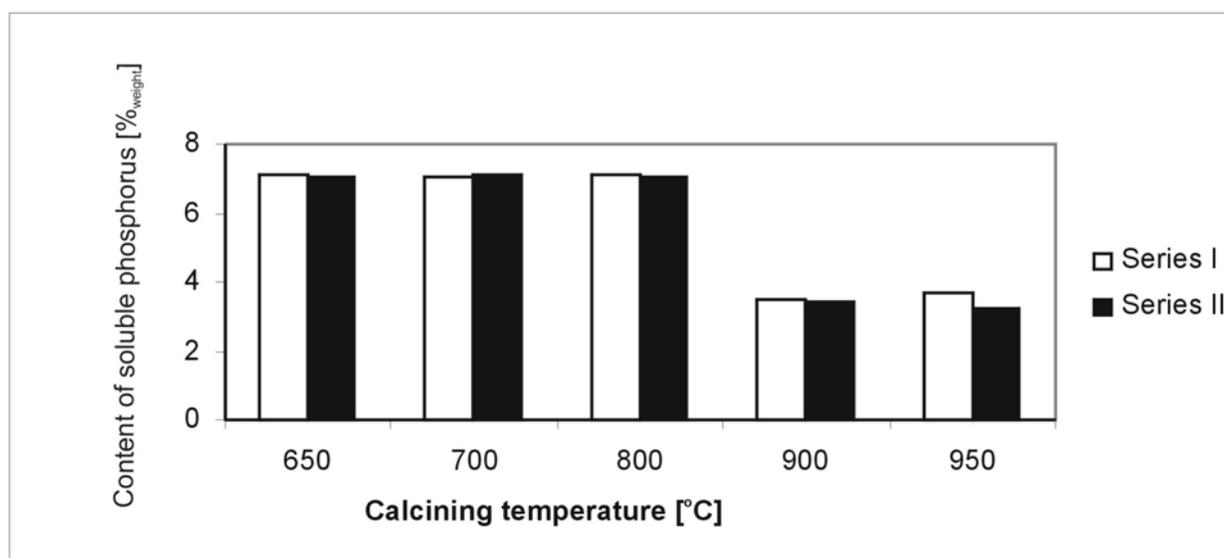


Figure 3. The content of phosphorus soluble in 0.4% hydrochloric acid in hydroxyapatite obtained at temperatures 650 – 950°C

The amount of phosphorus was approximately 17.5%_{weight} while the quantity of calcium was in the range from 39.48%_{weight} to 41.72%_{weight}. The analyses confirmed that the content of calcium and phosphorus increased with the process temperature.

The content of assimilated phosphorus was in the range from 3.25 to 7.12%_{weight}. For the samples received at lowest temperatures the amount of phosphorus soluble in hydrochloric acid was higher. The content of phosphorus decreased with the calcining temperature. It could be a result of sintering

of hydroxyapatite grains at the highest temperature and difficult migration inside the samples, as well as the absence of carbonate groups.

Figure 4 shows two tendencies – a decrease in the content of assimilated phosphorus up to obtaining hydroxyapatite at 800°C, and the subsequent increase.

Preparation of dense hydroxyapatite materials

After the calcining process the samples were boiled out in distilled water for 30 minutes in order to remove a small

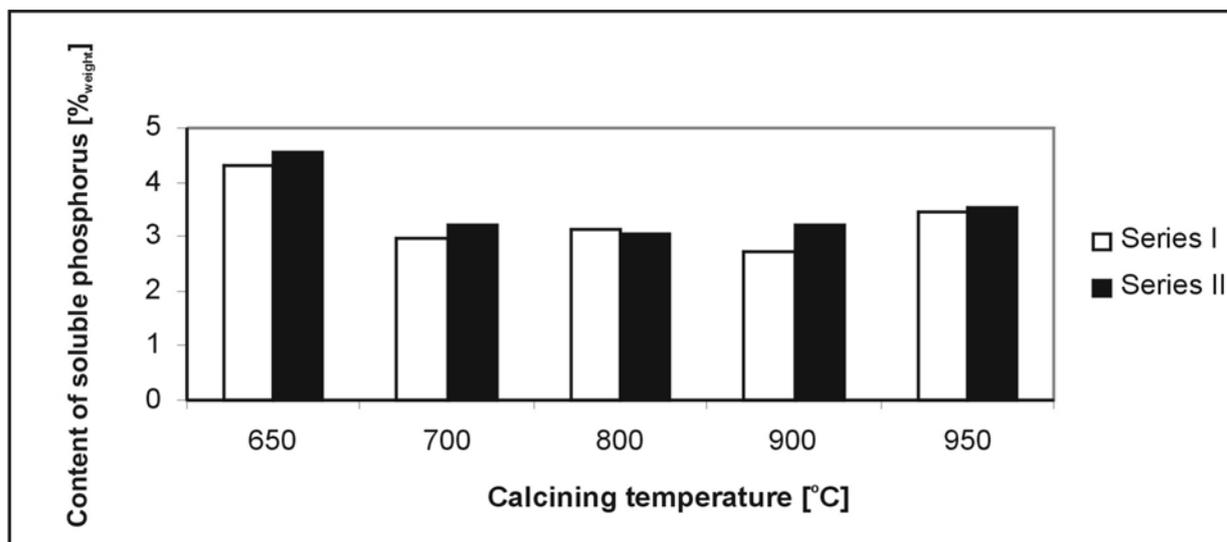


Figure 4. The content of phosphorus soluble in 2.0% citric acid in hydroxyapatite obtained at temperatures 650 – 950°C

amount of calcium oxide formed during the thermal treatment. Next the samples were dried to constant mass with a weight-dryer at 105°C. The powdered hydroxyapatite was pressed into cylindrical shape samples. Each sample had a diameter of 10mm and the weight of approximately 1.1g. Then the cylindrical samples were sintered in a chamber kiln with electric heating in air atmosphere at 900°C.

The *in vitro* researches were realised for 45 days in three types of liquid: SBF (simulated body fluid), Ringer fluid and distilled water, at the temperature of 37°C. Five samples were put into each plastic container and 40 ml of a proper physiological fluid were poured in. Changes of weight, pH and electrical conductivity of distilled water were measured. For the first seven days the measurements were made daily and then once a week. The final result was an average from five measurements. A fluid was exchanged after each measurement.

In vitro tests

Three physiological fluids were applied as an environment: Simulated Body Fluid (SBF) and Ringer fluid (solution of calcium, potassium and sodium chlorides in proper ratio), which simulated the natural human environment. Distilled water was applied in order to evaluate the degradation process of the hydroxyapatite samples. A comparison of SBF with the human plasma is shown in Table 2.

The chemical stability and biological activity were evaluated on the basis of changes in the pH value and electrical conductivity of distilled water. Electrical conductivity and the pH measurements were determined on Elmetron CX device equipped with a silver-silver chloride electrode and conductometer.

For all the samples an increase in the pH value in the initial period of incubation in a simulated body fluid was observed. Further analysed extracts showed a negligible tendency of decreasing the pH value with the incubation time. It concerned all the hydroxyapatite samples regardless of the

Table 2. A comparison of ions concentration and the pH value of SBF with the human plasma⁶

	Ion concentration [mmol/dm ³]								pH
	Na ⁺	K ⁺	Mg ²⁺	Ca ²⁺	Cl ⁻	HCO ₃ ²⁻	HPO ₄ ²⁻	SO ₄ ²⁻	
Human plasma	142.0	5.0	1.5	3.5	103.0	27.0	1.0	0.5	7.2 – 7.4
SBF	142.0	5.0	1.5	3.5	147.8	4.2	1.0	0.5	7.4

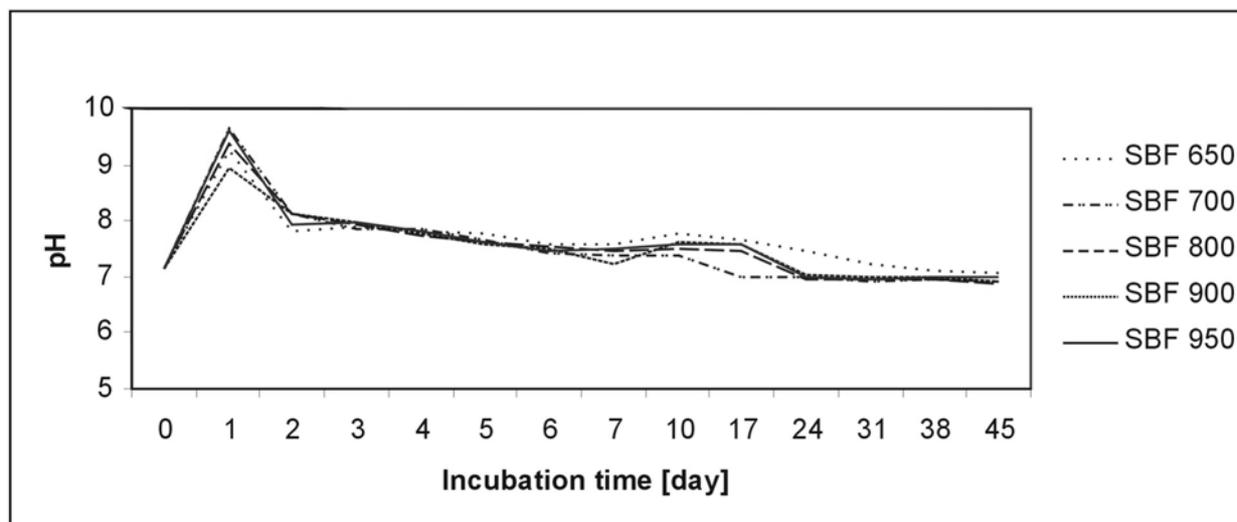


Figure 5. Changes in the pH value of SBF

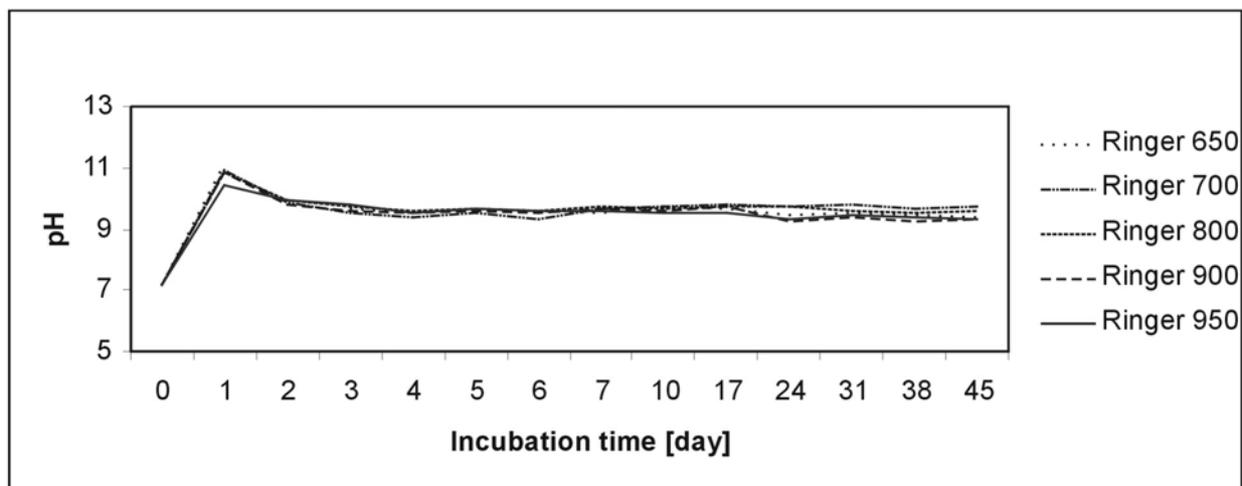


Figure 6. Changes in the pH value of Ringer fluid

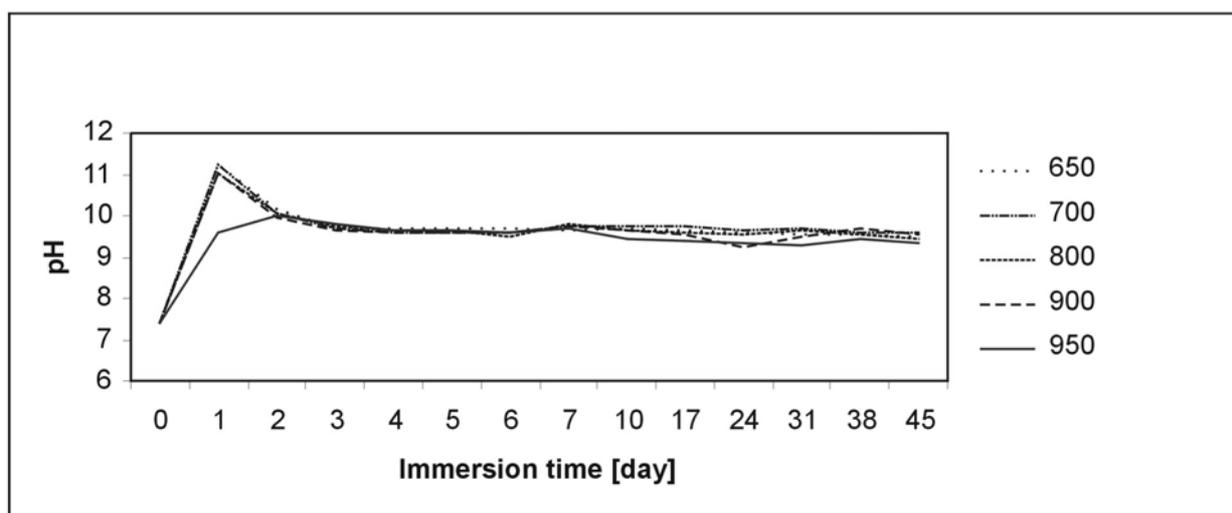


Figure 7. Changes in the pH value of distilled water

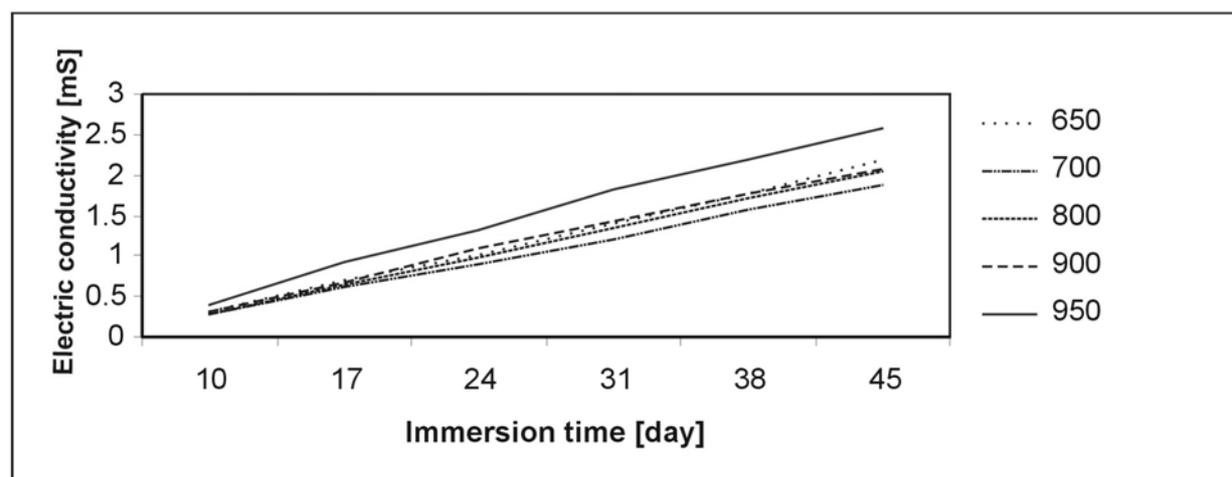


Figure 8. Changes in the electric conductivity of distilled water

calcining temperature. After a rapid growth of the pH of SBF up to 9.00, a decrease up to 7.00 was observed in the final days of immersion. The sudden increase of pH value at the beginning of *in vitro* tests may be connected with free calcium oxide that was incompletely removed from samples. For the following two weeks of incubation the pH value was stabilized at the level of 7.50 – 8.00. The highest pH value in comparison with pure SBF could be caused by selective desorption of calcium ions from hydroxyapatite into the solution during incubation. For all the samples immersed in

a simulated body fluid changes of the pH value were similar, which suggested that the calcining temperature did not influence significantly the *in vitro* behaviour of hydroxyapatite.

Samples immersed in the Ringer fluid showed the behaviour similar to that of the samples incubated in SBF. The pH value increased in the first period of the *in vitro* test up to ~11.00. In the second period of incubation the pH decreased and remained at this level ~9 up to the end of the investigations. The pH value of pure Ringer liquid was 7.185. Also in this case the free calcium oxide is probably respon-

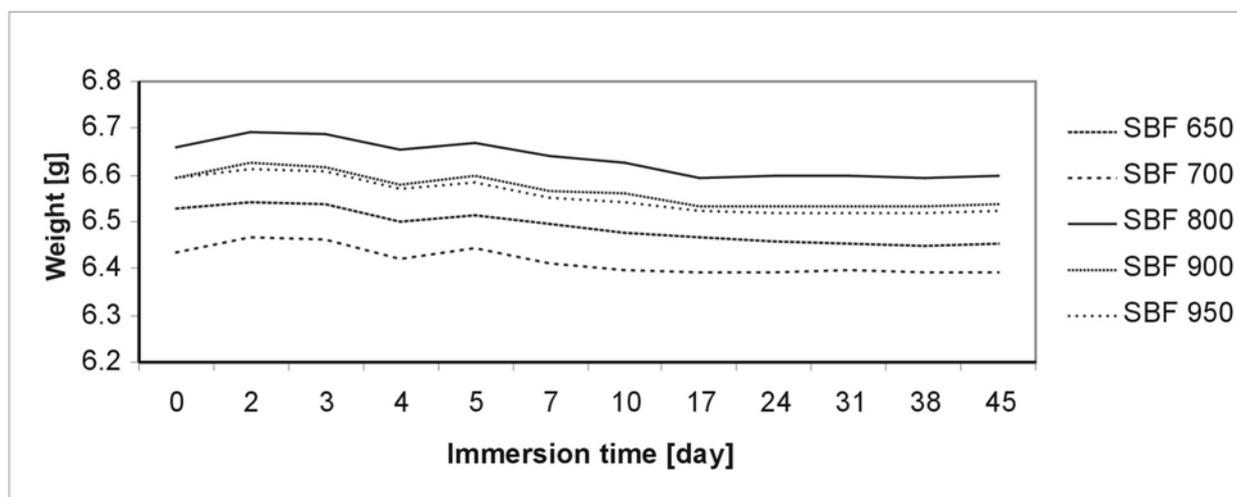


Figure 9. Changes in the mass of the samples immersed in SBF

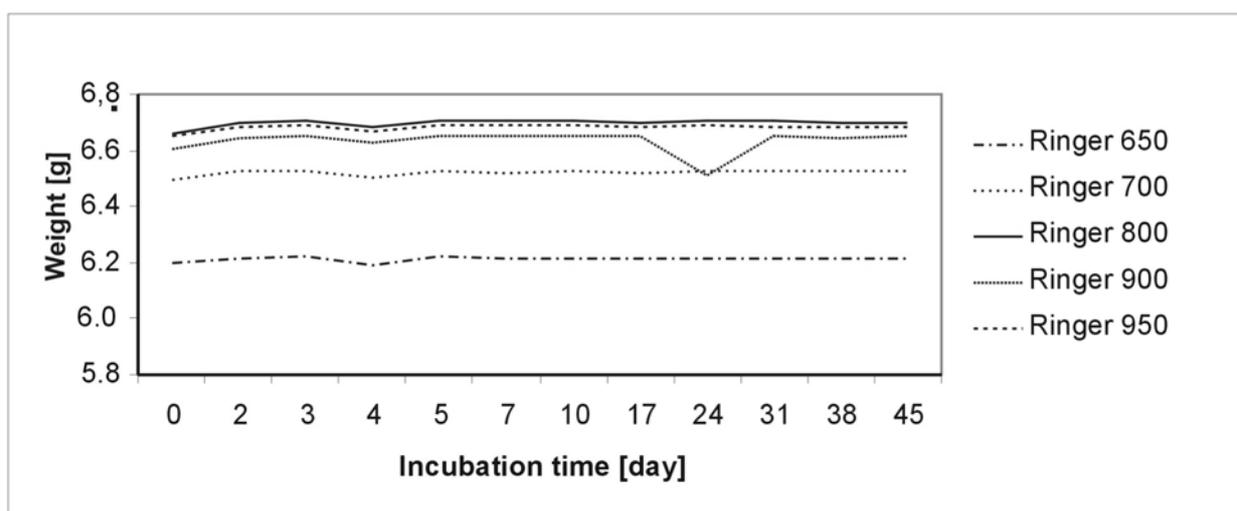


Figure 10. Changes in the mass of the samples immersed in Ringer liquid

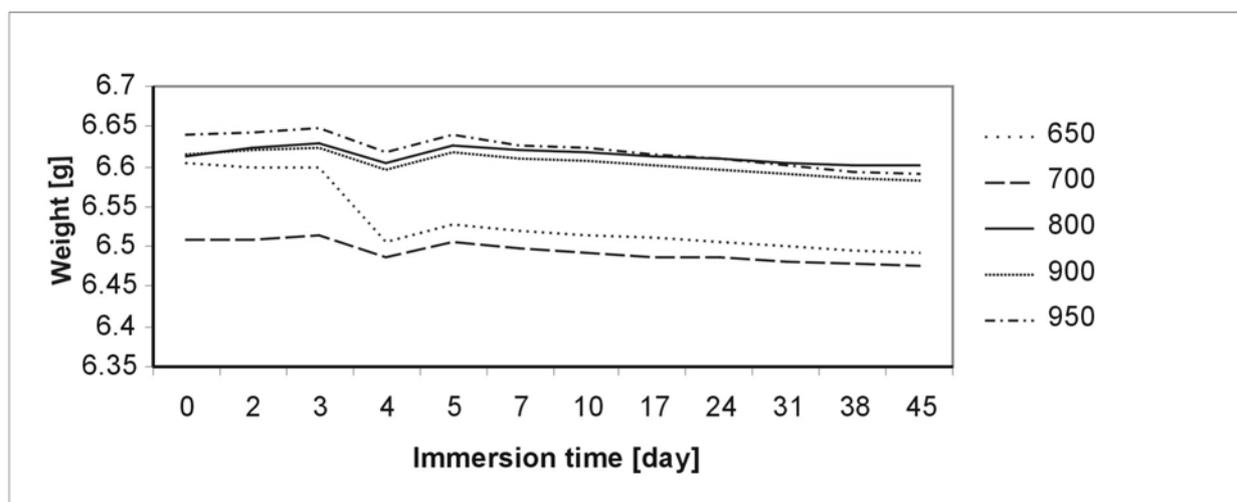


Figure 11. Changes in the mass of the samples immersed in distilled water

sible for the increase of pH value in the first day of immersion. On the next days the pH value was still at the increased level. It is caused by the calcium ions, released gradually from the hydroxyapatite surface into the solution. On the basis of pH measurements in physiological fluids it was confirmed that all the hydroxyapatite dense materials had bioactive properties independent of calcining temperature. Figure 7 shows changes of electric conductivity of distilled water in dependence on immersion time.

The analysed water extracts showed a similar tendency, independent of the calcining temperature of hydroxyapatite samples. At the beginning a distinct growth of the pH value up to approximately 11.00 was observed. An interpretation of this phenomenon was the same as in the case of SBF and Ringer, i.e. free calcium oxide. Within the next days of immersion the pH value stabilized at the level of above 9.00 and caused desorption of calcium ions from hydroxyapatite into the solution during the immersion.

These measurements confirmed that all the hydroxyapatite samples were gradually degraded. The largest propensity to degradation was exhibited by hydroxyapatite samples obtained at 950°C. An increase in electrical conductivity was characterised by linearity, it was the result of a progressive degradation process.

The changes of the mass measurements of the samples immersed in SBF indicated the loss of the masses of all hydroxyapatite materials independent of the calcining temperature. An interpretation of this effect required additional tests. It could be a result of an improper ratio between the liquid volume and a specific surface. Instead of the growth of the mass, which resulted from growing hydroxyapatite on a sample surface, we observed an inverse effect, i.e. the degradation of a hydroxyapatite surface and release of ions into the SBF solution.

The samples incubated in the Ringer fluid showed an insignificant tendency of a mass increase because of chlorides precipitation from the solution and confirmed bioactivity. All the samples exhibited a similar activity in the Ringer liquid independently of the temperature of hydroxyapatite preparation.

The samples immersed in distilled water exhibited a tendency of a mass decrease. It was connected with the degradation process and the transition of hydroxyapatite ions into water. Fractures appeared on the sample's surface during incubation.

SUMMARY

On the basis of the *in vitro* tests it was confirmed that it is possible to produce hydroxyapatite from bone waste from meat industry for medical applications. The tests proved that the calcining temperature had influence on calcium and phosphorus content in hydroxyapatite. On the grounds of the *in vitro* study it was stated that it is possible to use hydroxyapatite from bone waste as a material for bone implants and also in stomatology.

The analysed physiological fluids contained HAp samples that after sudden increase of pH showed a tendency to decrease with the immersion time. It concerned all the hydroxyapatite samples independently of the preparation temperature and applied fluid. A rapid growth of the pH value resulted from the free calcium oxide formation, which was incompletely removed from the samples after the preparation process. The highest pH value in comparison with pure physiological fluid for all the immersed time could result from the selective elution of calcium ions into the solutions. All the dense samples exhibit bioactivity properties.

The analyses of electric conductivity of distilled water showed in all the cases an increase in conductivity. The measurements confirmed that dense hydroxyapatite materials were characterised by a similar propensity to degradation.

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