IMMOBILIZATION OF PHENYLALANINE AMMONIA-LYASE ON HYDROXYAPATITE AND HYDROXYAPATITE COMPOSITES

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ABSTRACT. A new and efficient immobilization method of phenylalanine ammonia-lyase was obtained using hydroxyapatite (HAP) and hydroxyapatite - carbon nanotube, gelatin and chitosan - composites (HAP-CNT, HAP-GEL and HAP-CS) as support material. HAP and HAP composites were characterized by transmission electron microscopy (TEM), laser diffraction particle size analyzer and XRD measurements. The obtained nanobioconjugates were tested in the ammonia elimination reactions from L-phenylalanine (L-**1**). The reusability and the time-course profile of the immobilized enzyme preparation was also tested. While the biocatalyst obtained by using HAP-CS-15 as support material provided the highest conversion rate in the ammonia elimination reaction from L-**1** (98,1% compared to 98,5% obtained using the non-immobilized enzyme), the most stable biocatalyst proved to be HAP-PAL, which maintained more than 80% of its initial activity even after 5 cycles of reuse.

Keywords: hydroxyapatite, composites, carbon nanotubes, gelatin, chitosan, phenylalanine ammonia-lyase, immobilization, ammonia elimination

INTRODUCTION

Due to the increasing interest for natural resources and reduced pollution, biocatalytic routes are gaining more importance [1]. Enzymes are obtained from renewable sources, they are biocompatible, thus the enzymatic processes are cost effective and environmentally friendly [2]. However, their application on industrial scale is hampered by their instability and difficulty of retain and reuse [3]. These drawbacks can be overcome by immobilization, when biocatalysts with improved stability and reusability are obtained [4].

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Hydroxyapatite (HAP) is the principal mineral component of bone [5]. HAP has unique properties as biocompatibility, bioactivity, osteoconductivity and affinity to biopolymers [6]. Due to its biocompatibility and structural similarity with human hard tissue its widely used as inorganic biomaterial [7]. It founds applications in drug delivery [8], biomedicine [9] or medical devices [10]. Their use is however hampered by their poor fracture toughness and resistance. HAPs mechanical properties can be improved with materials such as carbon nanotubes [11], graphene oxide [12], gelatin [13], or chitosan [14].

Ammonia-lyases are acting on C-N bonds and catalyse the formation of α, β unsaturated bonds by elimination of ammonia from their substrates [15]. Phenylalanine ammonia-lyases (PAL EC 4.3.1.24, EC 4.3.1.25) are homotetrameric enzymes performing the non-oxidative deamination of L-phenylalanine into (E)-cinnamic acid [16] (Scheme 1). Cinnamic acid is the precursor of various phenylpropanoids, such as lignins, flavonoids and coumarins [17]. These derivatives are also widely used in cosmetics [18]. Several immobilized PAL preparates have been reported on various support materials [19] [20] [21]. Our previous successful immobilization of *Pc*PAL on carboxylated and aminated carbon nanotubes drove our interest to the immobilization of the same enzyme on hydroxyapaptite and its composites. These support materials could present a promising alternative, since they are cost effective and environmentally friendly.

In this study our aim was the immobilization of phenylalanine ammonia-lyase from *Petroselinum crispum* (*Pc*PAL) on HAP and HAP composites obtained with carbon nanotubes (HAP-CNT), gelatin (HAP-GEL) and chitosan (HAP-CH), and to test the stability and reusability of the obtained nanobioconjugates.There are several studies which reported the immobilization of enzymes on HAP and HAP composites [22],[23],[24], but according to our knowledge the immobilization of *Pc*PAL on these materials has not been investigated.

RESULTS AND DISCUSSION

The aim of the study was the immobilization of *Pc*PAL on hydroxyapatite (HAP) and HAP composites obtained by using carbon naotubes (HAP-CNT), gelatine (HAP-GEL) and chitosan (10, 15 and 20%) (HAP-CS-10, HAP-CS-15 and HAP-CS-20), and testing the activity and stability of the obtained immobilized enzyme preparations.

The immobilized enzymes were tested in the ammonia elimination reaction from L-phenylalanine, L**-1 (Scheme 1)**.
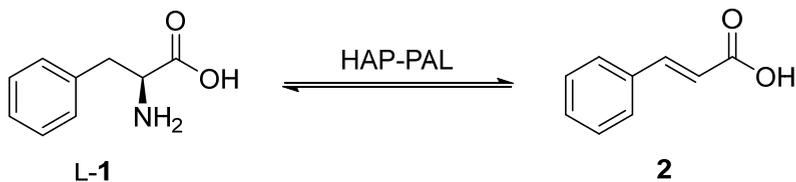
Scheme 1. PAL immobilized on hydroxyapatite and its composites for ammonia elimination reaction from L-phenylalanine

Preparation of HAP and HAP based nanocomposites

HAP and HAP composites were obtained by coprecipitation, the most straightforward and frequently used method for the preparation of these materials [25].

Even though being used on a large scale, HAPs poor tensile strength and fracture toughness makes it unsuitable for a wide range of applications. HAPs mechanical structures can be improved by using different additives, such as carbon nanotubes, gelatin or chitosan [26], [27]. Due to the CNTs excellent mechanical properties, HAP can be toughen and strengthen [28].

HAP-gelatin composites have high porosity and interconnectivity, great biocompatibility and good mechanical properties [29].

The polymeric composites of HAP have improved properties, including modulus, strength and stiffness compared to pure HAP [30]. The HAP/chitosan composites are good candidates for tissue engineering, bone and cartilage scaffolds [31].

Characterization of the support material

Particle size distribution

Several characteristics, such as chemical reactivity, opacity, texture, viscosity, density, porosity or material strength of materials are affected by the size of their particles [32]. The particle size and surface area greatly influence the properties of HAP. Depending on the growth conditions the particle size of HAP is usually between 10 and 500 nm [33].

The particle size of the obtained HAP and HAP composites was determined by laser diffraction particle size analyzer.

By adding the additives to the HAP a slight increase of the particle size can be observed. This could be due to the ease of aggregation of the particles in presence of polymers. The smallest particles are obtained by using carbon nanotubes as additive (**Fig. 1**).
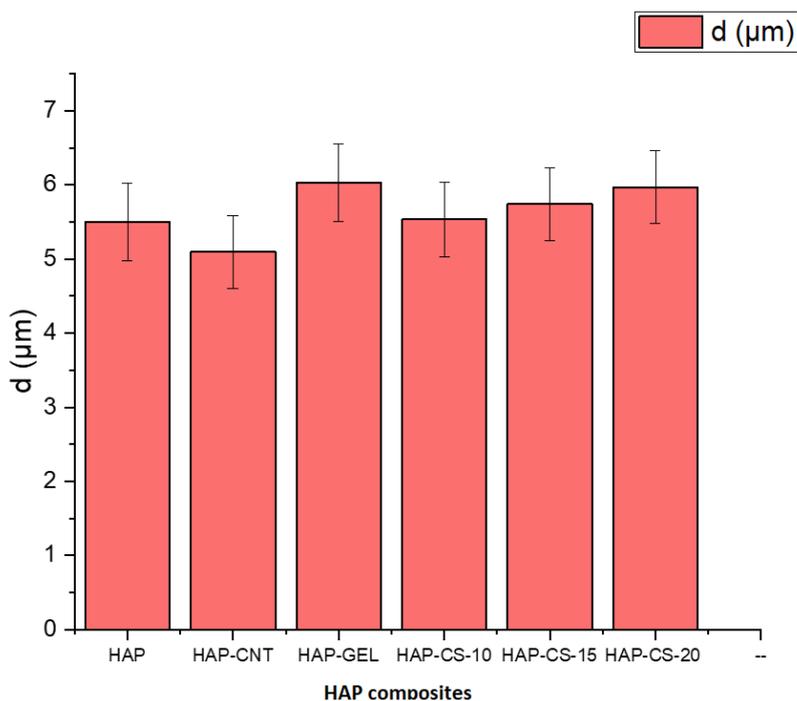
Figure 1. Particle size distribution of HAP and HAP-composites

Transmisson electron microscopy (TEM)

The morphology of the obtained composites was examined by transmission electron microscopy. As expected, in case of the HAP (**Fig. 2** (1)) a good homogeneity can be observed, property which characterizes this support. The carbon nanotube particles are integrated between the HAP crystals (**Fig. 2** (2)). The composites obtained by the addition of gelatin (**Fig. 2** (3)) and chitosan **Fig 2** (4), (5), (6)), loses the prism shape characteristic to HAP, instead they have a round shape. Thus, the gelatin and chitosan have a strong influence upon the morphology of the composites, they disable the formation of the crystals. The morphology and the size distribution in case of HAP-CS are evan. A difference between the HAP (**Fig. 2** (1)) and HAP coated by APTES (**Fig. 2** (7)) can also be observed on the TEM images, since APTES creates a silica coating of HAP.
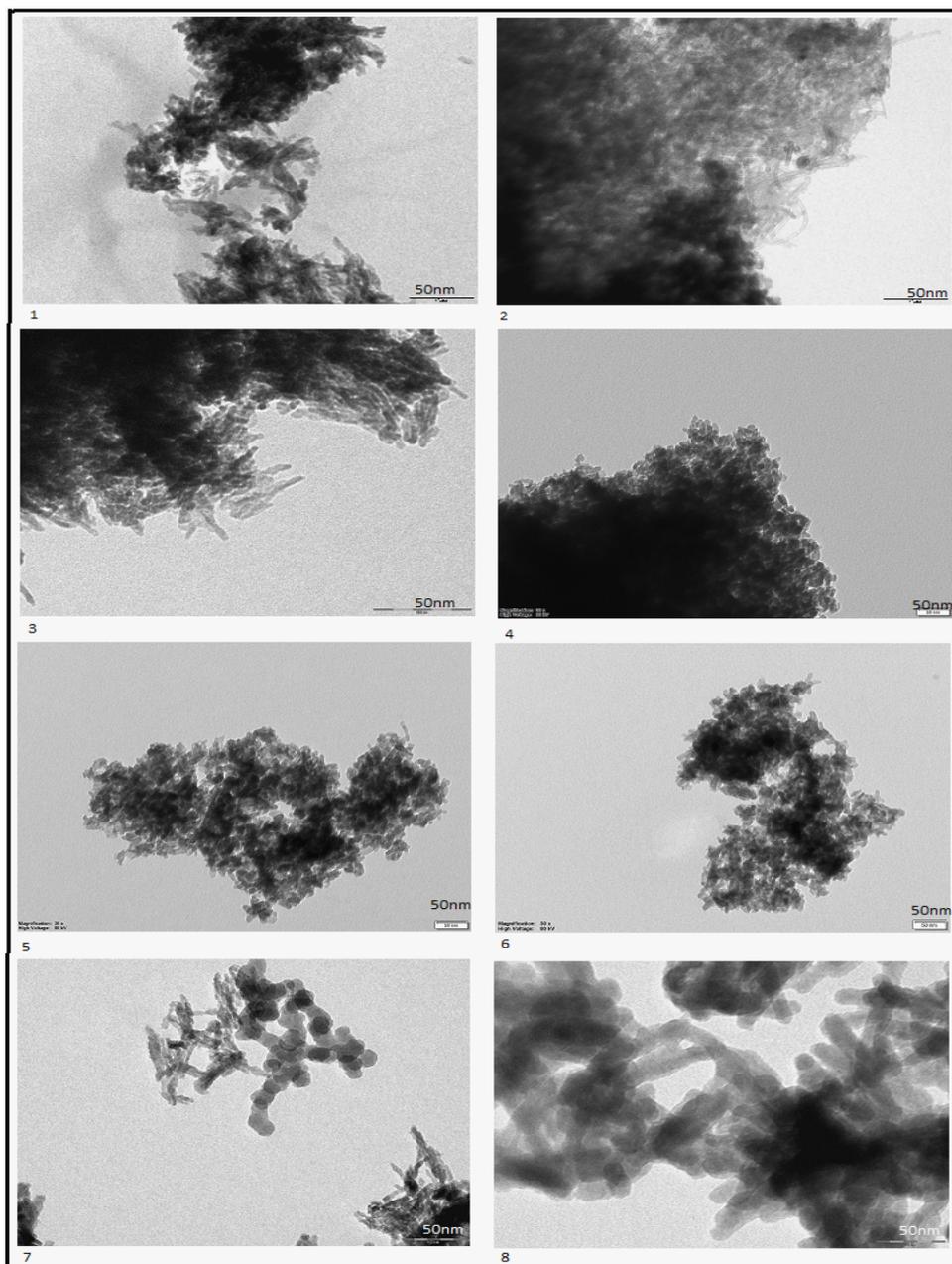
Figure 2. TEM images of: (1) HAP, (2) HAP-CNT, (3) HAP-GEL, (4) HAP-CS-10, (5) HAP-CS-15, (6) HAP-CS-20, (7) HAP+APTES, (8) HAP-GEL+APTES (The resolution of the images is 50nm)

XRD measurements

The composites were also analyzed by x-ray diffraction. There is no significant difference between the phase composition and crystallinity of HAP, HAP-GEL, HAP-CS and HAP-CNT. The peaks marked with star are characteristic for the hydroxyapatite. In case of all composites other phases besides HAP can not be identified (**Figure 3**).
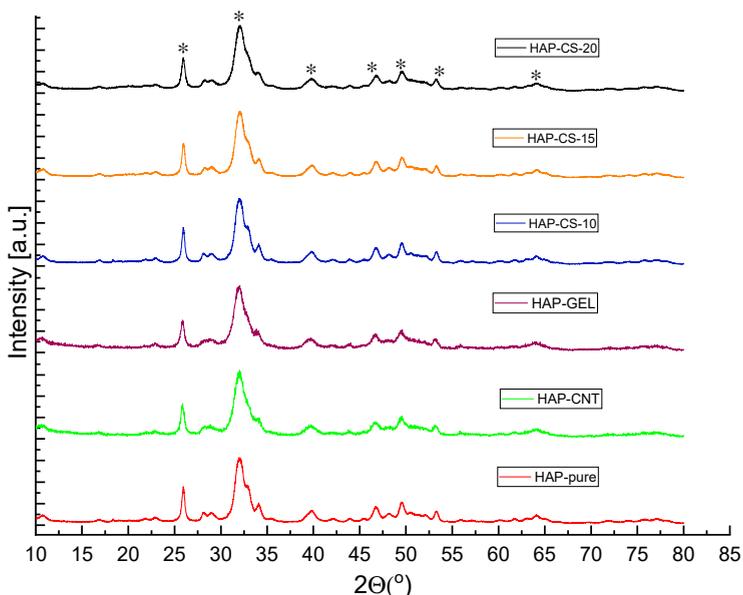

Figure 3. XRD measurements of HAP and HAP composites

Immobilization of *Pc*PAL on hydroxyapatite and its composites

For the immobilization of the enzyme on the HAP and its composites obtained with carbon nanotubes and gelatin first a surface modification of the support material was achieved using aminopropyl triethoxy silan (APTES), followed by the addition of glycerol diglycidyl ether a crosslinker which was recently successfully used for the stable, covalent attachment of *Pc*PAL on the surface of carbon nanotubes [21]. The enzyme was immobilized on the surface of the modified support material (**Scheme 2A**). For the HAP composites obtained with chitosan the direct linkage of the enzyme on the support material

was achieved by using GDE (**Scheme 2B**). For control experiments also the adsorption of the enzyme on the surface of HAP was achieved. The obtained products were characterized by reproducibility (immobilization reactions were performed in duplicate) and high immobilization yields (**Table 1**.)
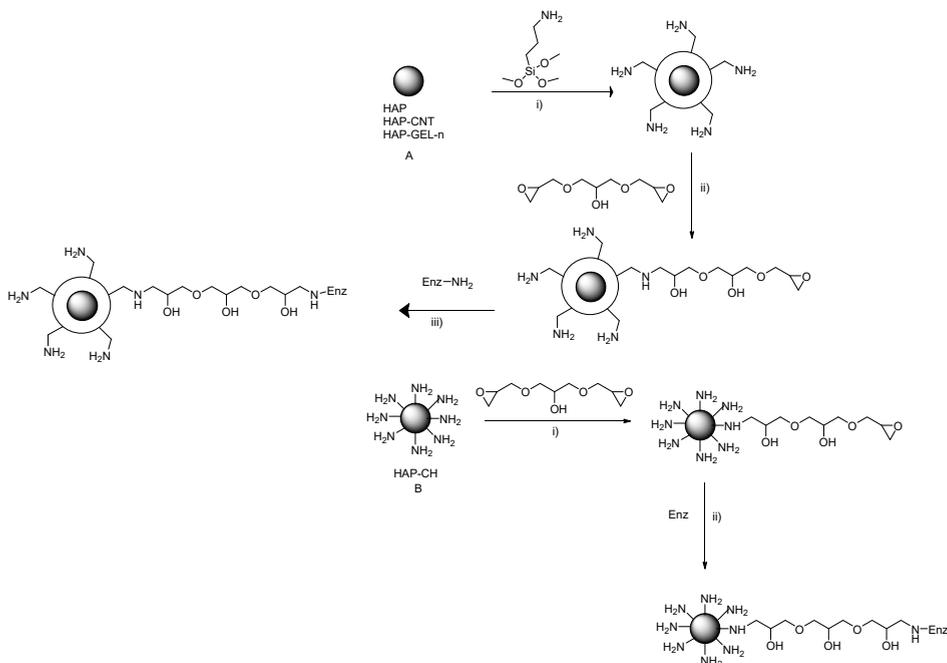
Scheme 2. Immobilization of *Pc*PAL on **A**. HAP, HAP-CNT and HAP-GEL, *i*) APTES in EtOH, *ii*)GDE in EtOH, *iii*) *Pc*PAL in Tris buffer, 0.1M, pH 8.8 and **B** HAP-CH, *i*) GDE in EtOH, *ii*) *Pc*PAL in Tris buffer, 0.1M, pH 8.8

Table 1. Immobilization yield of the enzyme preparations

SUPPORT MATERIAL	IMMOBILIZATION YIELD (%)
HAP	99
HAP-CNT	99.9
HAP-GEL	99.7
HAP-CS-10	91.9
HAP-CS-15	99.6
HAP-CS-20	92.4
HAP-ADS	99.6

## Biocatalytic behavior of the obtained enzyme preparation in the ammonia elimination reaction from L-phenylalanine

The biocatalyst obtained by adsorption and by the covalent attachment of *Pc*PAL on the HAP and HAP composites were tested in the ammonia elimination reactions from L-phenylalanine. The highest activity was achieved using the enzyme preparation obtained by HAP with 15% chitosan as support material (**Table 2**, entry 6), where the conversion was comparable with the conversion of the reaction catalyzed by the native enzyme (**Table 2**, entry 1), followed by HAP (**Table 2**, entry 2) and HAP-GEL (**Table 2**, entry 4). The lowest enzyme activity was achieved using HAP-CNT as biocatalyst (**Table 2**, entry 3). In order to further increase the conversion rates, different chitosan content was used as additive in the HAP. But by increasing or decreasing the chitosan content above or under 10%, the enzyme activity decreased dramatically (**Table 2,** entry 5 and 7). The adsorbed enzyme preparation also showed high enzymatic activity (**Table 2**, entry 8).

Table 2. Conversion of the reactions catalyzed by the immobilized
*Pc*PAL on HAP and HAP composites (rt, 22h)

ENTRY	ENZYME PREPARATION	CONVERSION (%)
1	PCPAL	98.5
2	HAP	87.9
3	HAP-CNT	77.7
4	HAP-GEL	80.8
5	HAP-CS-10	49.5
6	HAP-CS-15	98.1
7	HAP-CS-20	53.97
8	HAP-ADS	72.6

Next, the *reusability* of the best performing enzyme preparations (HAP-PAL, HAP-CS-15-PAL, HAP-GEL-PAL, HAP-CNT-PAL, HAP-ads-PAL) was tested in repeated batch cycles. In case of all preparations the enzyme activity showed a decreasing tendency. The most stable enzyme preparation in the reusability test was the one obtained using HAP as support material, followed by the HAP-CS-15. In case of the HAP-GEL the first three cycles show a stability in the enzyme activity, followed by a drastic decrease.

The less efficient biocatalyst proved to be the one obtained with HAP-CNT. The enzyme adsorbed on the surface of the support material showed a drastic decrease at the second reuse, the obtained conversion being only 14%. This could be due to the leaking of the enzyme from the support material when immobilized by adsorption (**Figure 4**).
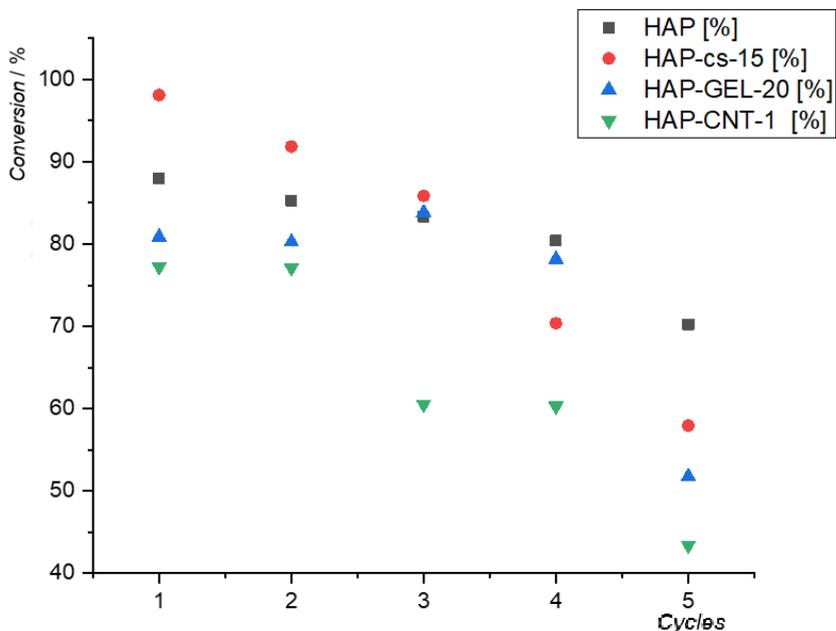
Figure 4. Reusability of the best performing immobilized enzyme preparations (rt, 22h)

Time-course profile

The time course profile of the ammonia elimination reactions from L-**1** mediated by the two best performing enzyme preparations (HAP-PAL and HAP-CS-15-PAL) was investigated. Samples were withdrawn from the reaction mixtures after 1, 2, 4, 7, and 24 hours. In both cases an almost linear increase of conversion can be observed, however, in case of HAP-PAL the maximum conversion (91%) was achieved faster (**Figure 5**).
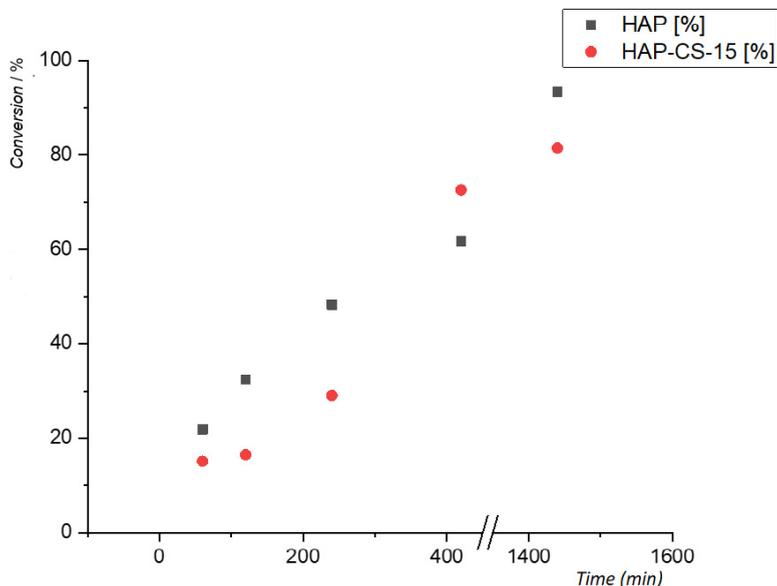
Figure 5. Time course profile of the reactions catalyzed by HAP-PAL and HAP-CS-15-PAL

CONCLUSIONS

This study demonstrates that hydroxyapatite and its composites obtained with carbon nanotubes, gelatin, and chitosan can be used as support material for the immobilization of phenylalanine ammonia lyase. The support materials were obtained by coprecipitation method and were characterized by transmission electron microscopy, laser diffraction particle size analyzer and XRD measurements. The obtained nanoparticles were further used for the immobilization of phenylalanine ammonia lyase. The nanobioconjugates were tested in the ammonia elimination reaction from L-phenylalanine. The most durable immobilized enzyme preparations were HAP-CS-15-PAL and HAP-PAL. The highest conversion rates were obtained with HAP-CS-15 (98,1%), while in the reusability studies HAP-PAL had the best performance, it maintained more than 80% of its initial activity even after 5 cycles of reuse. Although these enzyme preparations show lower catalytic activity then other *Pc*PAL preparations previously obtained, by further optimization procedures hydroxyapatite could be a promising alternative as support material for the immobilization of PAL.

EXPERIMENTAL SECTION

Materials

Enzyme

Phenylalanine ammonia-lyase from parsley (*Pc*PAL) was overexpressed in E. coli and purified according to the method described by Dima et al[34]

Chemicals

Single walled carbon nanotubes were purchased from Chengdu Organic Chemicals Co. Ltd (Chengdu, China). All other reagents were of analytical grade (Merck, Germany) and used without further purification. The technical grade solvents were dried and/or freshly distilled prior to use.

Equipments

Transmission electron microscopy (TEM) was performed using an automatic H-7650 TEM, Hitachi (Tokyo, Japan) with accelerating voltage 40-120 kV, zoom 200x-600000x, by dipping a holey-carbon TEM grid into a suspension of the immobilized enzyme. Sample was imaged using an Olympus KeenView G2 camera, transmission electron microscope operating at 120kV.

For the Bradford protein assay, an 8453 UV-Vis spectrophotometer, Agilent (Santa Clara, CA, USA) was used.

Ultrasonications during enzyme immobilization were performed in a Transsonic 460/H ultrasonic bath, Elma Schmidbauer GmbH (Singen, Germany) at 100W, 40 kHz.

The shaking and incubation of enzymatic reactions was performed using a Titramax 1000 instrument, equipped with a heating module.

The particle size distribution was measured with a Shimadzu WING SALD-7101 (Japan).

The XRD analysis was performed on a Shimadzu XRD 6000 (Japan) using CuKα radiation at 40Kv, 30mA, at λ=1.542 Å. X-Ray images were prepared for each material. The red lines indicates the caraceristic signs of the HAP crystals 25,8 32,05 39,84 46,78 49,53 53,13 63,97. The spectrum was identified by the ICPDS 09-0432 code in database.

The enzymatic reactions were monitored with an Agilent (Santa Clara, CA, USA) HPLC.

Methods

Preparation of HAP and HAP composites

Pure HAP and HAP-based NCs were prepared as previously described [35]. The used precursors were $Ca(NO_3)_2$ as a source of Ca^{2+} and $(NH_4)_2HPO_4$ as a source of PO_4^{3-}. An aqueous solution of 0.9 mol/L $(NH_4)_2HPO_4$ was added

dropwise with a feed rate of 25 ml/min, using a peristaltic pump, into a stirred aqueous solution of 1.5 mol/L $Ca(NO_3)_2 \cdot 4H_2O$, at room temperature. The appropriate quantities of additives, 1% carbon nanotube, 20% gelatin, were added to the $Ca(NO_3)_2$ solution while chitosan (10/15/20%) was added to the $(NH_4)_2HPO_4$ solution. The pH was adjusted to 11 by adding 25% ammonia solution under constant stirring at room temperature. The suspension was matured for 22 h, filtered and washed.

Immobilization of the enzyme on HAP and HAP composites

Immobilization of PcPAL on HAP, HAP-GEL and HAP-CNT

HAP and HAP composites (HAP-GEL, HAP-CNT 20mg) were added to a solution of APTES (120 mg in 5 mL ethanol, **Scheme 2A** step *i*)). The obtained suspension was sonicated to avoid bundled HAP formation, and incubated at room temperature for 1 hour shaking at 1300 rpm. After 1 hour of incubation the reaction mixture was filtered and GDE (100mg in 5mL EtOH) was added (**Scheme 2A step** *ii*)). The reaction mixture was incubated for 4 hours at room temperature shaking at 1300 rpm. After activation the sample was filtered. To the GDE activated support *Pc*PAL (2 mg in Tris buffer, 0.1M, pH 8.8) was added and the reaction mixture was shaken at 1300 rpm for 4 hours at room temperature (**Scheme 2A** step *iii*)). After the immobilization the resulted biocatalyst was filtered and washed with distilled water (3x10 mL). The amount of PAL immobilized on the support material was determined from the total mass of the enzyme in the solution before the immobilization and in the unified filtrates after the immobilization (measured spectrophotometrically using the Bradford method).

Immobilization of PcPAL on HAP-CS

HAP-CS (20 mg) was added to a solution of GDE (100mg in 5mL EtOH) shaking at 1300 rpm at room temperature for 4 hours (**Scheme 2B** step *i*)). The activated HAP-CS was filtered and *Pc*PAL (2 mg in Tris buffer, 0.1M, pH 8.8) was added (**Scheme 2B** step *ii*)). The reaction mixture was incubated for 4 hours at 1300 rpm at room temperature. The reaction mixture was filtered and the obtained enzyme preparation was washed with distilled water (3x10 mL). The amount of PAL immobilized on the support material was determined from the total mass of the enzyme in the solution before the immobilization and in the unified filtrates after the immobilization (measured spectrophotometrically using the Bradford method).

Immobilization of PcPAL on HAP-CS by adsorption

HAP (20 mg) was added to a solution of *Pc*PAL (2 mg in Tris buffer, 0.1M, pH 8.8). The reaction mixture was incubated for 4 hours at 1300 rpm at room temperature. The reaction mixture was filtered and the obtained

enzyme preparation was washed with distilled water (3x10 mL). The amount of PAL immobilized on the support material was determined from the total mass of the enzyme in the solution before the immobilization and in the unified filtrates after the immobilization (measured spectrophotometrically using the Bradford method).

*Ammonian elimination reaction from L-**1** mediated by the obtained enzyme preparations*

HAPs-PAL (1 mg) was added to a solution of L-phenylalanine (**1**, 0.8 mg, 4.7 mM) in Tris buffer (0.1 M, pH 8.8, 1 mL). The resulting mixture was shaken (1250 rpm) at room temperature for 22 h. The reaction mixture was filtered and analyzed by high performance liquid chromatography.

Reusability of the immobilized enzyme preparations

For the recycling experiments the reactions were performed as described at the previous section. The enzyme was recovered from the reaction mixture after each cycle by centrifugation at 13400 rpm for 1 min and washed with buffer solution (3 x 1 mL) and reused in the following experiment.

The time course profile of the reactions

The time course profile of the reaction was obtained by performing the ammonia elimination reactions with reaction times of 1, 2, 4, 7 and 24h.

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