IMMOBILIZATION OF PROTEOLYTIC ENZYMES ONTO SILICA NANOFIBERS

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Abstract: *Even in modern medicine, it is still necessary to remove necrotic tissue from burns in a very painful method – by using surgical instruments. It is possible to replace the surgical method with application of proteolytic enzymes directly to the wound, which removes the necrotic tissue completely painlessly. However, most enzymes are active only for a short time and under the specific conditions. The catalytic activity of enzymes can be increased e.g. by immobilization of enzymes onto the biocompatible silica nanofibers. The nanofibers must be functionalized by suitable reagents to form a ligand between silica nanofibers and amino groups of proteolytic enzymes. In our research, the nanofibers surface was modified by 3-Aminopropyl triethoxysilane firstly, than functionalization by succinic anhydride and N-Hydroxysuccinimide ester was done. As the next step, 7 different proteolytic enzymes were immobilized to the silica nanofibers surface. Proteolytic activity of enzymes was tested under conditions simulated skin burns environment (temperature of 37°C and pH 4.6).*

Keywords: *burns, immobilization, proteolytic enzymes, silica nanofibers.*

1 INTRODUCTION

Painless removal of necrotic tissue is called an enzymatic debridement. The necrosis occurs especially in deep skin burns; it appears in the second and the third degree burns, when the tissue is damaged and skin tissue recovery is not possible. Therefore, removal of the damaged tissue is necessary step before beginning of the granulation and re-epithelization phase of healthy tissue. Especially proteolytic enzymes are chosen for the enzymatic debridement for their ability to catalyze the proteolytic reactions (hydrolysis of the peptide bonds that link amino acids together in a polypeptide chain). In other words, proteolytic enzymes provide a digestion of necrotic tissue. For example bromelain [1], collagenase [2], or papain-urea [3] are already used in the treatment of burns, however, they are usually mixed in gels or ointments. Unfortunately, application of enzymes in this form may cause the infection formation by using wet wound dressing. Native enzymes are unstable, have a short lifetime, and high temperature and pH dependence. The solution of this problem is to immobilize the enzyme onto a suitable material. Silica nanofibers appear to be a very good supporting material for the immobilization of enzymes, because they are biocompatible, biodegradable and they have a number of active functional groups for covalent binding of enzymes.

The aim of the research is to develop a method for the immobilization of enzymes on silica nanofibers that can be used in healthcare (without toxic ligands). Furthermore, it was tested the proteolytic activity of seven immobilized enzymes to determine which enzymes would be the most suitable for these purposes. For this reason, proteolytic activity of enzymes was tested under conditions simulated skin burns environment and pH and thermal dependence were monitored.

2 EXPERIMENT

2.1 Material

Nanofibers were prepared from tetraethyl orthosilicate (TEOS, Sigma-Aldrich, 98 wt.%) and propan-2-ol (Penta CZ, p.a. 99.9 wt.%).

For functionalization of the nanofibers were used: ethanol absolute (Penta), 3-Aminopropyl triethoxysilane (APTES, 98 wt.%), succinic anhydride (SU, 99 wt.%), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, 98 wt.%), N-Hydroxy-succinimide (NHS, 98 wt.%), 4-Morpholineethane-sulfonic acid monohydrate (MES, 99 wt.%), Tris(hydroxymethyl)aminomethane (TRIS, 99,8 wt.%) (all from Sigma-Aldrich).

Trypsin from hog pancreas was purchased in Fluka, other proteolytic enzymes: protease from bovine pancreas, protease from *Aspergillus oryzae*, protease from *Bacillus licheniformis*, bromelain from pineapple stem, trypsin from bovine pancreas and α-chymotrypsin from bovine pancreas were from Sigma-Aldrich. For enzyme activity assay was used casein from bovine milk and trichloroacetic acid (TCA, 99 wt.%) were used, both from Sigma-Aldrich too.

Table 1 Characteristic of immobilized enzymes

Designation	Name	Source	Optimum pH	Optimum temperature	Reference
P1	Protease type I	bovine pancreas	6.5-7.5	35-55°C	[6]
P2	Protease type XXIII	*Aspergillus oryzae*	4.5-5.5	55-60°C	[7]
P3	Protease type XXIV	*Bacillus licheniformis*	6-5-8.5	65-70°C	[8]
BR	Bromelain	pineapple stem	6.5-7.5	55°C	[9]
T1	Trypsin type I	bovine pancreas	7-9	40°C	[10]
T2	Trypsin type V-S	hog pancreas	7-9	40-45°C	[11]
CH	α-Chymotrypsin type II	bovine pancreas	7-9	40°C	[12]

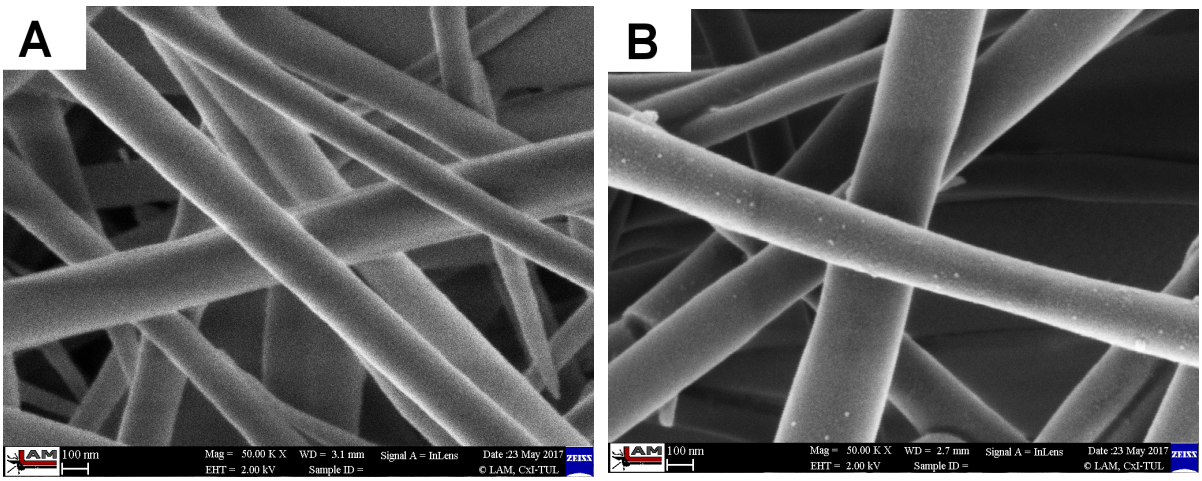

Figure 1 SEM images of non-modified silica nanofibers (A) and silica nanofibers with immobilized trypsin from hog pancreas (B)

2.1 Preparation of silica nanofibers

Silica nanofibers were prepared according to [4] by sol-gel method and subsequently electrospun on the needleless NanospiderTM device (Elmarco). Silica nanofibers were electrospun from free liquid surface under standardized conditions [5]. The nanofibers were thermally stabilized at 260°C for 2 hours.

2.2 Immobilization of enzymes onto nanofibers

For immobilization of proteolytic enzyme, it is necessary to functionalized silica nanofibers surface. As a silanization reagent, 3-Aminopropyl triethoxysilane (APTES) was chosen. For carboxylation of APTES amino groups, succinic anhydride was used.

The final step was the reaction of mentioned chemicals with N-Hydroxysuccinimide ester created using N-(3-Dimethyl-aminopropyl)-N′-ethylcarbo-diimide hydrochloride and N-Hydroxysuccinimide. After functionalization of silica nanofibers, 7 different proteolytic enzymes were covalently bonded to the nanofibers surface. Full characteristics of used enzymes are in Table 1.

3 RESULTS AND DISCUSSION

3.1 Visualization and characterization of nanofibers

Visualization of nanofibers was taken by scanning electron microscope Carl Zeiss ULTRA Plus. Samples were gold-dusted in advance and observed in the form of secondary electrons SE1 (Figure 1). According to the procedure described above, silica nanofibers with specific weight ~40 g.m^{-2} and mean fiber diameter 222±97nm were produces. The histogram of the nanofibers diameter is shown in Figure 2.

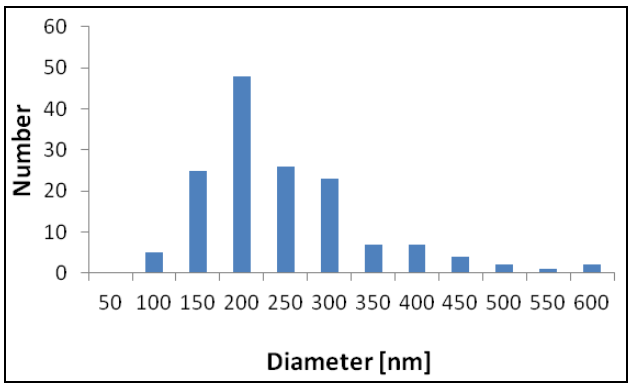

Figure 2 Histogram of the nanofibers diameter

3.2 Enzyme activity assay

Protease activity assay was measured by casein as a substrate. When proteolytic enzyme catalyses a digestion of this substrate, the amino acid tyrosine and peptide fragments are separated from casein. Free tyrosine reacts with Folin & Ciocalteu reagent to produce a blue coloured chromophore. The chromophore can be spectrophotometrically quantified as an absorbance value at wavelength 750 nm. 1 unit of activity is defined as the amount of tyrosine equivalents in micromoles released from casein per minute. In testing of immobilized enzyme, the activity units refer to mg of nanofibers. Activity of three types of proteases, bromelain, 2 types of trypsins and α-chymotrypsin immobilized on the nanofibers was tested at 37°C and pH value of 4.6 (Figure 3). By analysis of variance (one-factor ANOVA test) was found that the measured enzyme activity values differ from one another at a significance level of 5% – F (6.42)=232.18 p=2.32. By sequential testing of the measured enzyme activity values (2-sample t-test, 5% significance level) it was found that the activity values of BR, P1 and CH enzymes do not differ from each other and activity value of P2, P3 and T1 do not differ from each other too. Thus, the lowest activity was measured for the T2 enzyme, the higher activity was measured for the enzymes P1, BR and CH and the highest for P2, P3 and T1.

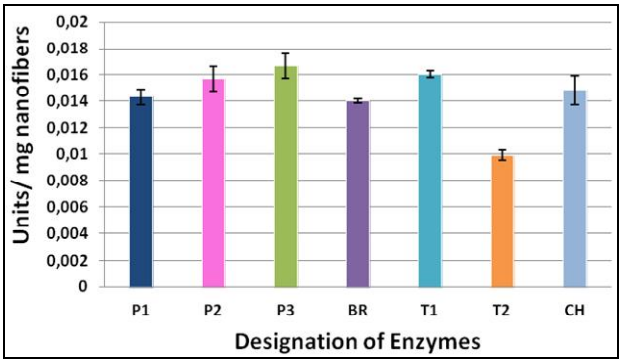

Figure 3 Proteolytic activity of protease from bovine pankreas (P1), protease from *Aspergillus oryzae* (P2), protease from *Bacillus licheniformis* (P3), bromelain from pineapple stem (BR), trypsin from bovine pancreas (T1), trypsin from hog pancreas (T2) and α-chymotrypsin from bovine pancreas

The highest activities were detected for P3 enzyme (protease from *Bacillus licheniformis*) and T1 enzyme (trypsin from bovine pancreas). P2 (protease from *Aspergillus oryzae*) and CH (α-chymotrypsin from bovine pancreas) had high proteolytic activity too. The immobilized enzymes activity was compared with the activity of free enzymes under the same conditions. Activity of enzymes in percent was determined as a ratio of proteolytic activity of immobilized enzyme to proteolytic activity of soluble enzyme (Figure 4).

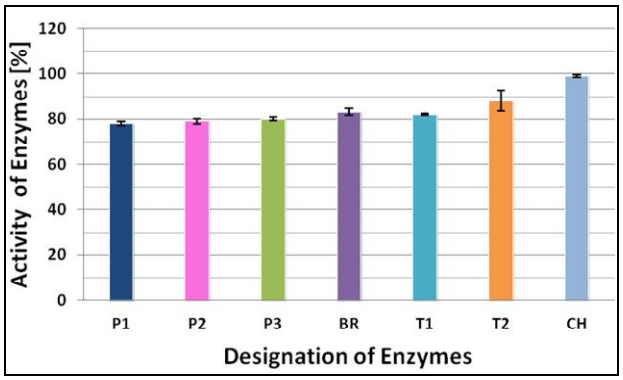

Figure 4 Immobilized enzyme activity to soluble enzyme activity ratio in percent

The ratio of proteolytic activity of immobilized enzyme to proteolytic activity of soluble enzyme was at least 76%. The results show that α-chymotrypsin has a relatively high activity compared to other proteolytic enzymes; and its activity did not decrease after immobilization onto silica nanofibers. Therefore, less concentration of the α-chymotrypsin enzyme would be required to maintain efficiency in comparison with other tested enzymes. 98% efficiency of the enzyme immobilized onto the nanofibers is a great success and it is a sign of high quality of enzyme binding and selection of ligands because the amount of immobilized enzyme is significantly limited by the number of functional groups on the nanofibers surface.

The highest success rate of α-chymotrypsin immobilization is very closely related to the temperature at which the tests were performed (37°C). Table 1 shows that the α-chymotrypsin has an optimum temperature at 40°C. But proteases and bromelain have its optimum temperature at higher temperatures. This can be one of the reasons why ratio of proteolytic activity of immobilized α-chymotrypsin to proteolytic activity of soluble α-chymotrypsin is almost 100 %.

Proteolytic activity of protease from *Aspergillus oryzae* (P2), bromelain from pineapple stem (BR), trypsin from bovine pancreas (T1) and α-chymotrypsin from bovine pancreas was measured at three different temperatures: 4, 23 and 37°C. The results are shown in Figure 5 (pH value was 4.6 too). Effect of temperature on enzyme activity was observed most in the case of protease from *Aspergillus oryzae* (P2). Conversely, bromelain seemed to be temperature independent in this temperature range. An analysis of variance (one-factor ANOVA test, significance level of 5%) found that temperature dependence was demonstrated in the case of P2, T1 and CH enzymes. This was not confirmed for the BR enzyme.

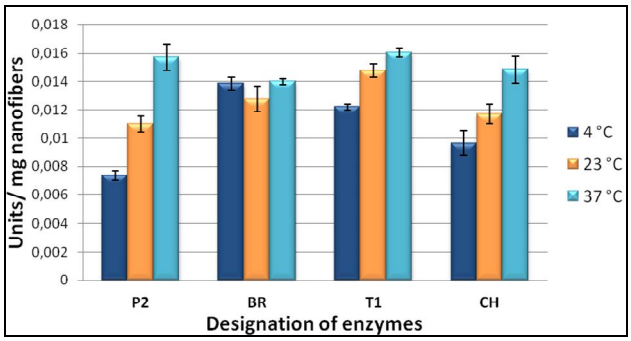

Figure 5 Activity of immobilized protease from *Aspergillus oryzae* (P2), bromelain from pineapple stem (BR), trypsin from bovine pancreas (T1) and α-chymotrypsin from bovine pancreas at the different temperatures

4 CONCLUSION

Suitable enzymes for immobilization onto silica nanofibers appear to be the α-chymotrypsin from bovine pancreas, protease from *Aspergillus oryzae*, bromelain from pineapple stem and trypsin from bovine pancreas. These enzymes were selected for further testing – the long-term stability of silica nanofibers with immobilized enzymes and cytotoxicity tests.

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