A TECHNIQUE TO IMMOBILIZE TRYPSIN ON STAINLESS STEEL IN ORDER TO PREVENT BIOFILMS FORMATION♦

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Abstract: The molecular mechanisms governing the formation of biofilms by the colonization of solid surfaces with living organisms such as bacteria, algae and eukaryotic cells, are poorly known, in spite of their importance in the medical devices, the food-processing industry and the marine equipments. The aim of this work was to explore different pathways to attach in a covalent manner commercial trypsin to stainless steel coupons to create a thin layer of active enzyme. Different reagents such as functionalized trialkoxysilanes compounds [γ-(aminopropyl)trietoxysilane] has been use to create a reactive molecular layer to attach proteins. The surfaces were characterized after each treatment by Fourier transform infrared reflection-absorption spectroscopy (FT-IRRAS technique) to

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determine the molecular composition of the films. Finally, the efficiency of the different ways immobilization technique have been assessed by enzymatic assays with selected substrate (N_α-benzoyl-L-arginine-ethyl-ester – BAEE), then on microbial cultures generating biofilms. The immobilized trypsin exhibited much higher relative activity than lysozyme in the similar conditions.

Keywords: *immobilization enzyme, trypsin, aminosilanization, FT-IRRAS technique, enzymatic activity*

INTRODUCTION

Immobilized enzymes are currently a topic of active research in enzyme technology for their enhanced stability over their soluble counterparts. In recent years, interest has increased in the potential utilization of this enzyme in food processing, enzyme reactors and peptide synthesis [1- 4]. Although trypsin being one of the least stable neutral proteases, usually it was used in some industrial applications. Its rapid autolysis in solution makes it difficult to control the reaction conditions and as a consequence, the catalytic efficiency of this enzyme decreases and the cost of its use increases. A few of practical applications of the immobilized enzymes have been reported, and the key problem that should be solved urgently was still the choice of an appropriate support material and a simple immobilization method [5-7]. These considerations claim researchers to provide new strategies to protect metallic surfaces from bacterial adhesion and subsequent biofilm growth. Many successful approaches to date are based on the construction of very thin films of poly(ethylene oxide)/poly(ethylene glycol) onto the metallic surfaces. These approaches were particularly efficient to inhibit protein adsorption and promising as regards bacterial adhesion on stainless steel substrates [8-16]. Minier et al. have devised another approach to prepare biocompatible surfaces of stainless steel which is the most currently encountered material in food, medicine, or marine environment [17]. This report is based on the controlled, covalent immobilization of hydrolytic enzymes that could prevent protein or polysaccharide adsorption or even have a bactericidal effect. They chose for this strategy lysozyme from hen egg white (HEWL). This readily accessible, low molecular weight enzyme cleaves the polysaccharidic component of cell walls of Gram (+) bacteria (muramidase activity) and this may have an inhibiting effect on bacterial adhesion and proliferation. Indeed some sanitizers already include proteases such as trypsin from bovine pancreas (TBP) and glycosidases such as lysozyme. It was demonstrated that treatment by a lysozyme solution of stainless steel coupons covered by a biofilm of *Bacillus flavothermus* removed it and had a protecting effect toward further reattachment of cells. Incorporation of lysozyme into polymeric packaging film structure induced a bactericidal effect against *Lactobacillus plantarum* and *Micrococcus lysodeikticus*. The key point in enzyme immobilization onto solid substrates such as stainless steel is to devise a strategy that preserves its activity. Thus, direct adsorption of TPB or HEWL may induce protein denaturation and subsequent loss of enzymatic activity. Release of enzyme from the surface may also occur in the long term. It was showed that controlled

covalent chemisorption of HEWL may lead to a strongly attached enzyme, the challenge being to keep its lytic activity. Goradia et al. [5] studied the immobilization of hydrolytic enzyme trypsin onto various mesoporous silicates (MPS). They prepared MPS by using cationic surfactants having average pore diameters in the range of 28 – 300 Å. They found that enzyme purity strongly influenced loading trypsin adsorbed on MPS was found to be desorbed more readily by polyethylene glycol than by ammonium sulfate suggesting that hydrophobic-hydrophilic interactions were important. Immobilized trypsin showed 10 – 20 times more activity and stability (for 4 – 6 weeks at 4 or 25 °C) and was successfully used up to 6 cycles [4]. In 1995, the new route has been described in the literature [18] for the covalent immobilization of proteins onto inorganic materials by aminosilanization of these substrates with γ-(aminopropyl) triethoxysilane (APTES) so as to graft a thin layer of reactive amino groups. In aqueous solution, APTES quickly hydrolyzes to yield the highly reactive silane triol APS that may condense to form polysiloxanes. Silanization of minerals occurs by hydrogen bonding, and metalloxane M-O-Si bonds are formed upon film drying. Total number of amine groups was determinate using Bromophenol Blue (BPB). This derivatization technique has been used for the measurement of total amine groups on glass surfaces, PE powders and PS beads. Piperidine is used in a second step to release BPB molecules in solution (figure 1).

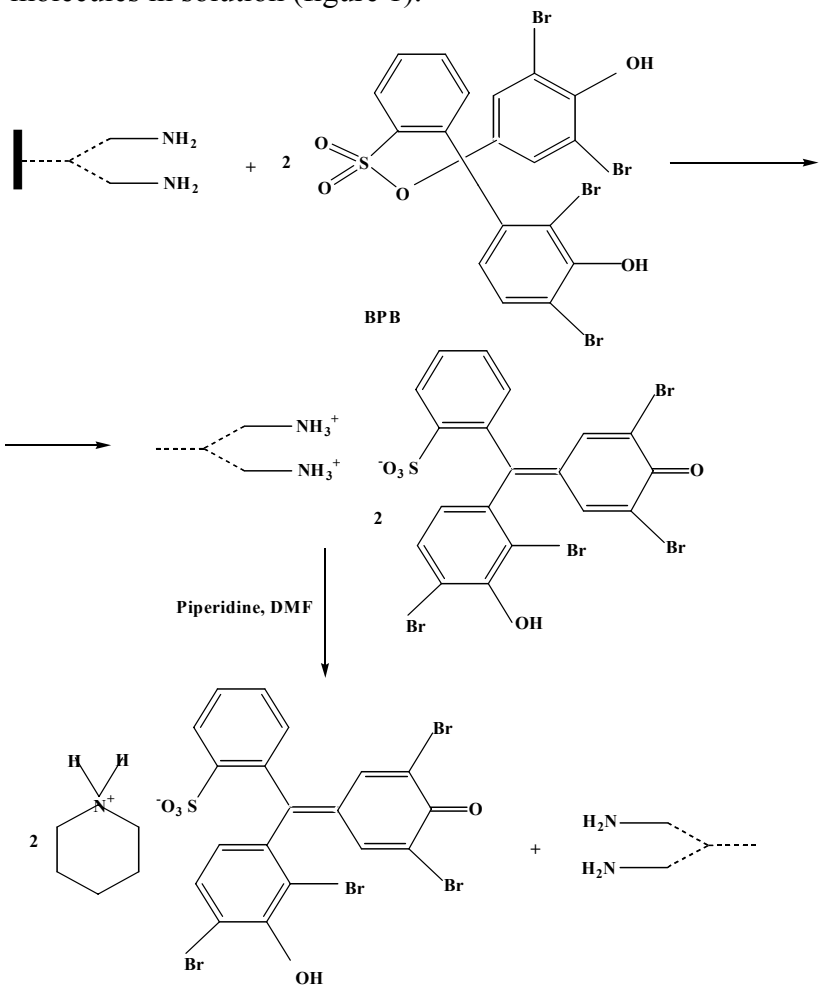

Figure 1. *Determination of total amine groups using BPB*

Silanization step is followed by cross-linking of the proteins using the dialdehyde like glutaraldehyde (GA) (figure 2). This manner has applied in various kinds of proteins including catalytically active enzymes being immobilized on silicon, titanium oxide, gold and some metallic alloys [19-20]. Another possibilities of trypsin's attack on the stainless steel was to surface modification by the adsorption of branched poly(ethylenimine) (PEI) from water. After this step, it followed the same route for the immobilization of trypsin like was described above (figure 3).

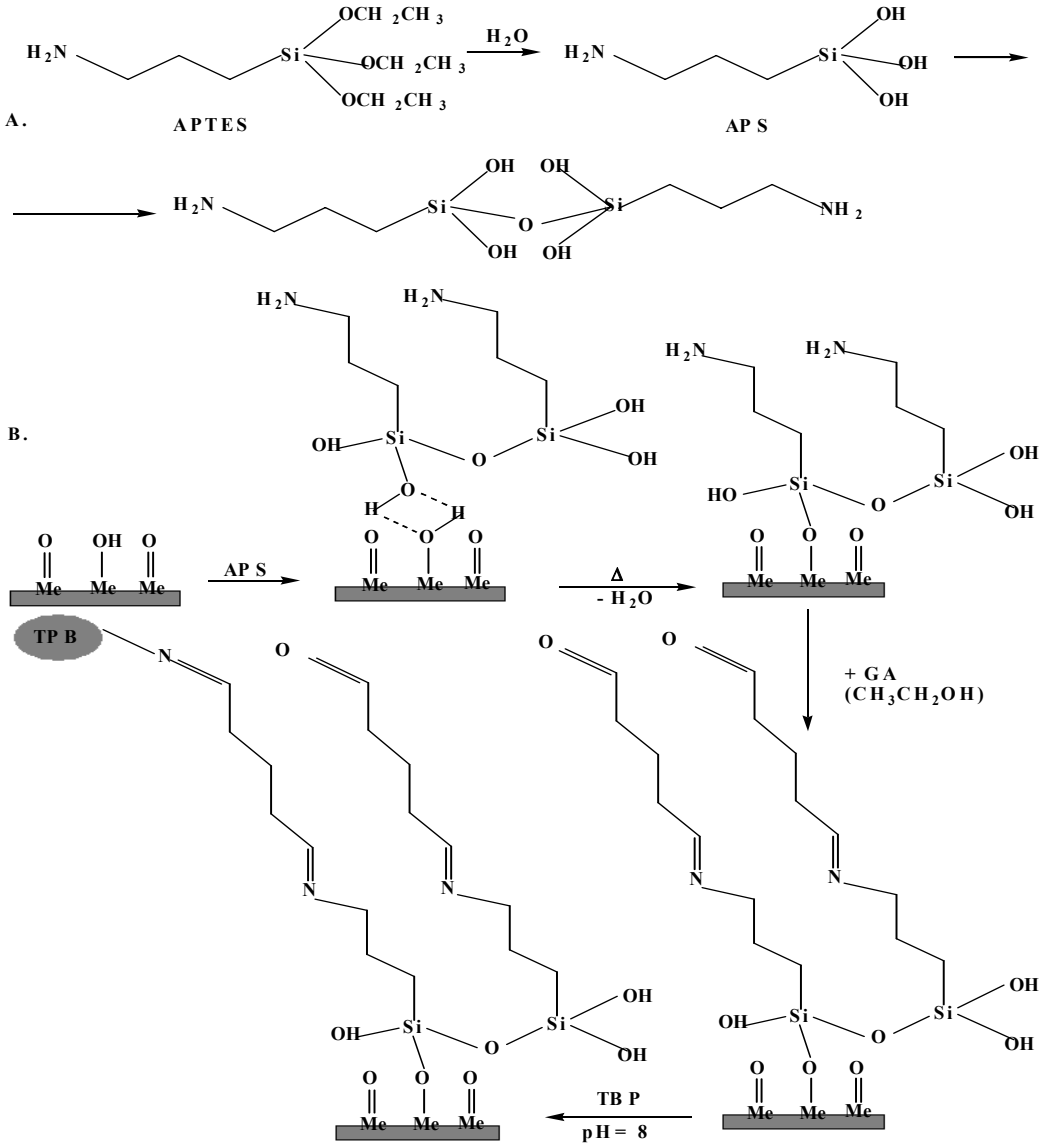

Figure 2. *General scheme for covalent immobilization of trypsin onto stainless steel*

Curiously, the immobilization of trypsin on stainless steel never took place in this manner. After studies regarding the covalent and controlled immobilization of HEWL to stainless steel AISI 316L coupons, new attempts were done with trypsin from bovine pancreas. All stainless steel coupons were previously etched by warm sulfochromic acid treatment. There were also unsuccessful attempts for reduction with $NaCNBH_3$ of

formed Schiff base after glutaraldehyde fixation. Metallic substrates were characterized after each treatment by Fourier transform infrared reflection-absorption spectroscopy (FT-IRRAS) to get a thorough insight into the molecular features of the thin organic films. Finally, preliminary enzymatic activity assays were performed on the modified stainless steel coupons and those revealed indeed some immobilized lytic activity.

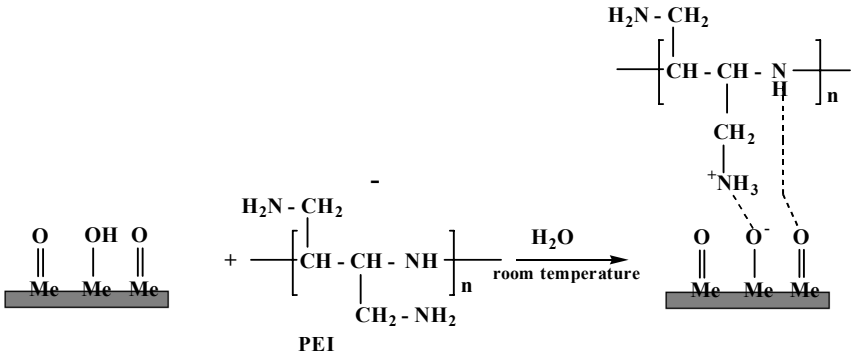

Figure 3. *Reaction scheme for the surface modification of stainless steel with PEI (poly(ethylenimine))*

MATERIALS AND METHODS

γ-(Aminopropyl)-triethoxysilane (APTES), PEI (branched, MW 25 kDa) glutaraldehyde (GA) 25% in water (w/v), and Bromophenol Blue were obtained from Aldrich reagent or used as available. Trypsin from Bovine pancreas (MW = 23 kDa, 223 amino acids, 12 cysteine by 6 S-S, specific activity 10000 U/mg protein), crystallized three times, and N_α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE substrate) were purchased from Sigma. Sulfuric acid 95% (analytical grade), potassium dichromate were purchased from Prolabo and sodium cyanoborohydride ($NaCNBH_3$) was purchased from Fluka. Phosphate buffered saline (pH = 8.0) was prepared in the laboratory. AISI 316 L stainless steel coupons (1.2 × 1.2 × 0.1 cm; area: 3.36 cm^2) were purchased from Goodfellow. They were mechanically polished on one side to 0.25 μm with SiC paper and diamond paste until a mirror finishing was obtained. Sulfochromic acid was prepared by dissolution of 6 g of $K_2Cr_2O_7$ per 100 mL of H_2SO_4.

Methods

Cleaning of stainless steel coupons

After being polished, the coupons were successively ultrasonically washed 5 minutes in acetone, 5 minutes in ultrapure water, 15 minutes in hexane, and 15 minutes in ultrapure water at 50 °C. This treatment was previously shown to form a stable mixed Cr and Fe oxide and hydroxide passivation layer on top of the substrates [17]. These coupons will be referred to as SS.

Pretreatment of Coupons

SS coupons were further cleaned ultrasonically in acetone for 5 minutes and then dipped in warm acetone (50 °C) for another 15 mins. Coupons were then etched by

sulfochromic acid at 60 °C for 10 minutes and extensively washed with ultrapure water and dried with a flow of nitrogen. They were kept in a desiccator under vacuum until use (no longer than 2 days). The resulting coupons will be named SS-SC.

Aminosilanization of Coupons

A 0.12% (v/v) solution of APTES in a 3:1 EtOH/H_2O mixture was prepared in a freshly silanized beaker and stirred for 10 minutes. SS-SC coupons placed in separate silanized glass reactors were covered with 5 mL of this solution for 1-3 min, removed, and dried in a flow of nitrogen. Coupons were then cured for 1 h at 100-150 °C in air and then washed for 2 minutes in water to remove physisorbed material. The resulting coupons will be named SS-SC-SIL.

PEI adsorption onto the SS surface

Various concentrations of PEI in water from 0.1% to 1% were prepared. After cleaning of the SS coupons, they were immediately immersed in PEI solution for 2 – 3 h at room temperature to minimize the effects of hydrocarbon contamination from atmosphere. After this treatment, the surfaces (SS-$PEI_{(x)}$, where x is the concentration of PEI solution used) were thoroughly rinsed 3-4 times with water and air dried. The resulting coupons will be named SS-SC-$(PEI)_x$.

Immobilization of TPB

SS-SC-SIL or SS-SC-$(PEI)_x$ coupons were dipped overnight in a solution of GA prepared by mixing 1 volume of the 25% stock solution with 4 volumes of absolute ethanol and then washing with water to yield SS-SC-SIL-GA or SS-SC-$(PEI)_x$-GA coupons. They were then dipped overnight in a 1 mg/mL solution of TBP in 0.1 M phosphate buffer (pH 8.0) at 4 °C and then washed with the buffer solution (pH 8.0) or/and 5M guanidine solution for 15 minute and dried with a flow of nitrogen. For comparison, SS-SC, SS-SC-SIL and SS-SC-$(PEI)_x$ coupons were directly treated with TBP in the same conditions and washed similarly.

Schiff base reduction

In parallel, after 2 h from starting of immobilization of trypsin on metallic surfaces was added 5 mL of 0.045 M $NaCNBH_3$ solution in phosphate buffer (pH 8.0) containing 0.6M Na_2SO_4 in different samples.

Surface Analyses

Fourier transform infrared reflection-absorption spectra (FT-IRRAS) were recorded on a Magna 550 FT-spectrometer (Nicolet) equipped with a Veemax variable angle specular reflectance accessory set at incidence angle of 80°. Six hundred scans were co-added at 8 cm^{-1} resolution and ratioed by the background spectrum of a SS-SC coupon.

Surface Amine Groups Assay

The surface concentration of the total amine groups was determined by a colorimetric assay using Bromophenol Blue (BPB). The SS coupons were reacted with 0.03 M bromophenol blue in dimethylformamide (DMF) solution [17]. The samples were then washed with ethanol until removal of the free or loosely physisorbed BPB and dried by air. In a second step, the coupons were immersed in 20% (v/v) of piperidine in DMF

solution in order to release the covalently bonded molecules (figure 3). The concentration of BPB was evaluated by measuring the absorbance of the solution at 605 nm. The molar extinction coefficient was determined by preparing different standard solutions of BPB in piperidine/DMF solution ($\varepsilon = 1.23\times10^5$ L.mol^{-1}.cm^{-1}).

Enzymatic Activity Assay

The lytic activity of the stainless steel coupons was measured using the following procedure inspired from the classical trypsin assay. TBP-treated coupons placed in separate reactors were covered with 2 mL of a 0.085 % (w/v) suspension of N_α -benzoyl – L – arginine-ethyl-ester-hydrochloride in 67 mM phosphate buffer, pH 7.6. The reactors were placed on a rocking table, and the suspensions were gently stirred. They were withdrawn every 60 minute, and their turbidity was measured spectroscopically at 253 nm (Uvikon 860 spectrometer, Kontron). Two control experiments, whereby the turbidity of a stirred bacterial suspension alone or in the presence of a SS-SC coupon was monitored, were carried out to measure non-enzymatic bacterial lysis (autolysis). Plots of optical density versus time were generated and slopes measured. By definition, one unit produces an $\Delta A_{253\text{nm}}$ of 0.001 per min at pH 7.6, 25 °C, using a 0.085% (w/v) suspension of N_α-benzoyl-L-arginine-ethyl-ester-hydrochloride in a 3.2 mL reaction mixture [21]. The enzymatic activity of the coupons was calculated using the following equation:

$$enzimatic-activity = \frac{[\Delta A_{253nm}(SS-immobilizedTBP)-\Delta A_{253nm}(SS-\mathrm{Ref})](df)}{0.001\times0.2} \qquad (1)$$

where: *df* – dilution factor (10^{-2});
0.001 – the change in A_{253} nm/minute per unit of trypsin at pH 7.6 at 25^0C in a 3.2 mL reaction mix;
0.2 – correction factor results from the product of the volume correction factor and the substrate concentration correction factor.

RESULTS AND DISCUSSION

Characterization of formed silane film

SS coupons were pretreated with a harsh oxidant (sulfochromic acid) as in a previously described procedure [17]. After the silanization step, coupons were washed with ultrapure water; the coupons were subjected to FT-IRRAS analysis. The IR spectrum of bulk APTES was recorded and compared to the IRRAS spectrum of the APS film for recorded bands (figure 4). Band assignments were done based on handbook values (table 1) [22].

Similarity and some differences between the IR spectrum of pure APTES and APS film were observed. All the vibrations corresponding to the ethoxy groups ($\nu(CH_3)$as = 2974 cm^{-1} , $\delta(CH_3)$ = 1390 cm^{-1}, ν(C-O) as (Et-O) = 1167 cm^{-1}, and ν(Si-O-C)s = 956 cm^{-1}) are missing from the APS film IR spectrum, indicating that APTES was fully hydrolyzed when adsorbed onto the SS-SC surface. These observations are in complete agreement with previously reported results [17-18]. A very weak band at 930 cm^{-1} is attributed to the stretching Si-OH vibration. That is assigned according to previously

published data indicates that silanols are also present in the film. The formed film structure and the mode of binding of APS to SS appeared to be in agreement with previously published data, for example: hydrolysis of Si-O-Et bonds of APTES to generate Si-OH that further partially condenses into polysiloxanes and metalloxanes.

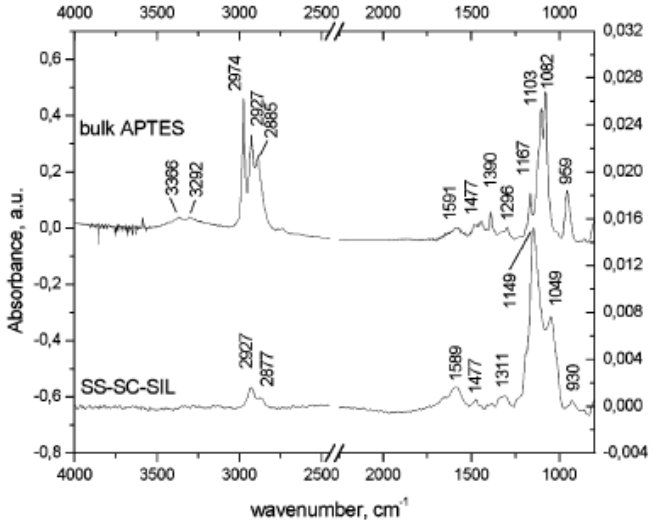

Figure 4. *IR (thin film) spectrum of APTES and IRRAS spectrum of SS-SC-SIL coupon*

The number of total amino groups on the surface of treated coupons measured by this way showed a weak reproducibility from 0.01 to 0.8 molecules/nm^2 and was surprisingly lower than the precedent values found for primary and secondary amines. It has been determinate maximum values between 0.168 nmol NH_2/cm^2 for silanized surfaces and 0.465 nmol NH_2/cm^2 for PEI adsorption onto the SS surfaces, comparative with 0.64 nmol NH_2/cm^2 founded for glass.

Immobilization of trypsin from bovine pancreas

To covalently attach TBP to the amine-containing silane layer, a cross-linker is needed. The glutaraldehyde provides a convenient means to cross-link proteins via their free amino groups (lysine, arginine and the N-terminal group) to aminated solid substrates. SS-SC-SIL coupons were treated by an ethanolic solution of GA, and surface analysis was done by FT-IRRAS. A slight change of the IRRAS spectrum of the SS-SC-SIL-GA coupon was also observed (figure 5). The δ (N-H) band from 1589 cm^{-1} disappeared while two new bands were now observed at 1640 and 1545 cm^{-1}. One band may be assigned to the C=N stretching vibration while the other might, probable, correspond to the symmetric δ (NH_3^+). There was expected that the C=O stretching vibration of the aldehyde group to exist at ca. 1730 ±10 cm^{-1}, but in all situations it was absent from the spectrum. The following hypothesis might be to explain this observation. No free aldehyde groups exist in the organic film and the double linkage between carbon and oxygen might be oriented parallel to the metal surface and this was not detectable by IRRAS. Considering the flexible structure of GA, two neighboring amines may have reacted to form a cyclic diimine. This structure may however still be reactive toward other amines as the Schiff base condensation is known to be a reversible process (figure

5). The SS-SC-SIL-GA coupon was treated with a buffered solution of trypsin at slightly basic *pH*, as this condition should favor condensation between the surface aldehydes and the protein amines. The resulting SS-SC-SIL-GA-TBP coupon was analyzed by IRRAS. The functionality group spectral range of the IRRAS spectrum of the SS-SC-SIL-GA-TBP coupon displayed two typical bands at 1659 and 1558 cm^{-1}, assigned to the amide I and amide II vibrations of the polypeptide backbone of TBP (figure 5). There were recorded similar values for SS-SC-PEI-GA-TBP coupon (1660 and 1554 cm^{-1}).

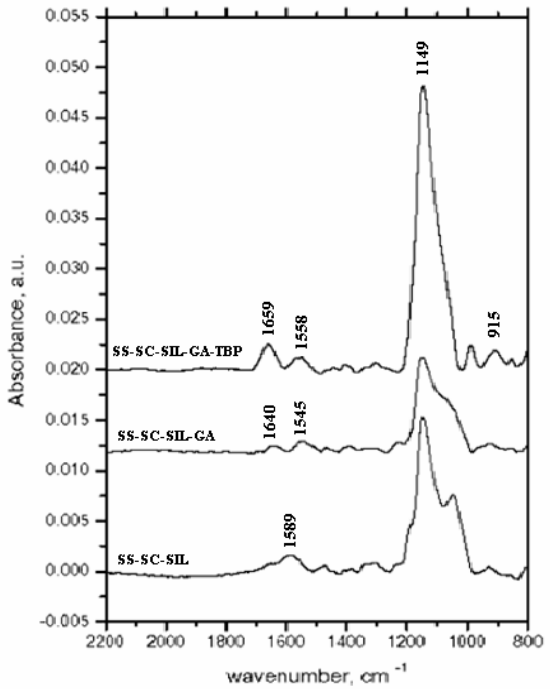

Figure 5. *FT-IRRAS spectra of SS-SC-SIL; SS-SC-SIL-GA and SS-SC-SIL-GA-TBP*

Enzymatic Activity Assay

Trypsin is a member of the serine protease family. Trypsin consists of a single chain polypeptide of 223 amino acid residues. The amino acid sequence of trypsin is crosslinked by 6 disulfide bridges. Autolysis of β-trypsin (the native form of trypsin which is cleaved at Lys^{131} – Ser^{132}) results in α-trypsin which is held together by disulfide bridges. Trypsin cleaves the peptides on the C-terminal side of lysine and arginine amino acid residues. Also, trypsin hydrolyzes ester and amide linkages of synthetic derivatives of amino acids such as benzoyl-L-arginine ethyl-ester (BAEE). To evaluate its enzymatic activity, coupon SS-SC-SIL-GA-TBP and SS-SC-PEI-GA-TBP were immersed in a suspension of N_α -benzoyl – L – arginine-ethyl-ester-hydrochloride and its turbidity was monitored spectrophotometrically during 6 h. Similar experiments were performed to assess the efficiency of the immobilization procedure. The SS-SC, SS-SC-SIL, SS-SC-PEI coupons were dipped overnight in a solution of TBP. The enzymatic activity of the resulting and samples was assayed on the N_α -benzoyl – L – arginine-ethyl-ester-hydrochloride suspension accordingly. The enzymatic activity calculated for the various samples (see equation 1) is reported in figure 6. In parallel, for

correction of nonspecific effects, the turbidity of a substrate suspension alone or in the presence of stainless steel coupon was monitored.

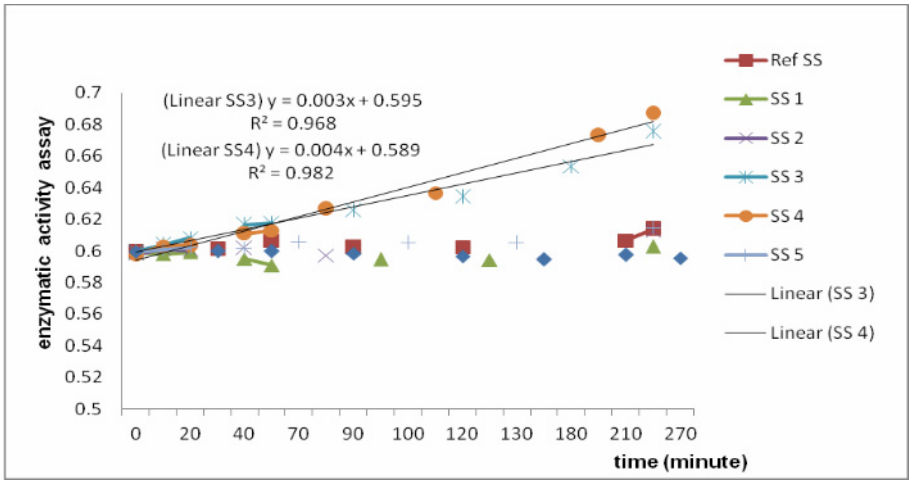

Figure 6. *First enzymatic activity of several trypsin-treated stainless steel coupons on BAEE solution: SS 1 – SS-SC-SIL-TBP; SS 2 – SS-SC-PEI-TBP; SS 3 – SS-SC-SIL-GA-TBP; SS 4 – SS-SC-PEI-GA-TBP; SS 5 – SS –SC-PEI-GA-TBP (reduction Schiff base with $NaCHBH_3$)*

In order to compare the activity of the covalently immobilized trypsin with the *free* trypsin that is physical adsorbed, the enzymatic activities of different situations were measured after incubation in 0.1 M phosphate buffer at pH 8.0 refrigerate at 4-5 °C for different intervals of time. After each given time interval (between 1– 30 days), the sample was quickly rinsed by pure water to room temperature and the enzyme activity was assayed immediately. After one day there were selected the following samples: SS-SC-SIL-GA-TBP and SS-SC-PEI-GA-TBP. Figure 7 shows approximate 40% loss of enzymatic activity for these samples that it was observed after storage for one week at 4^0C and 70% loss after 3 weeks, respectively. The last result was maintained for 2 weeks more.

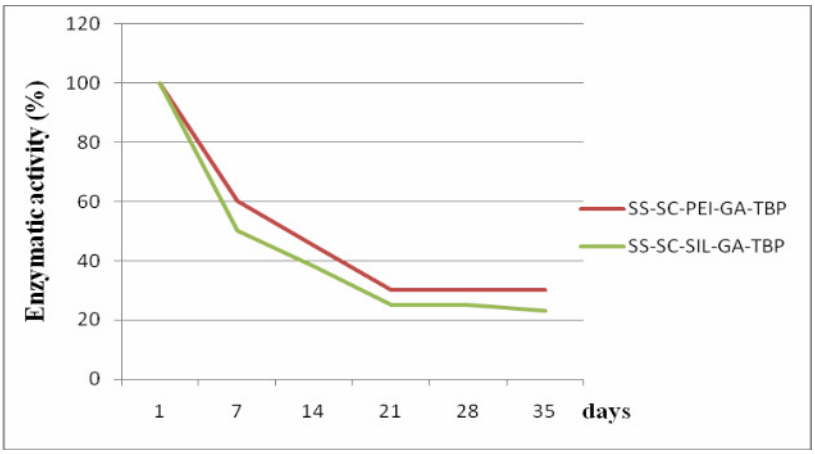

Figure 7. *Variation of enzymatic activity of trypsin - treated stainless steel coupons for 1 month*

CONCLUSIONS

Trypsin was immobilized by all stainless steel samples used in this study. From the experimental data, the best result was obtained in the following procedure: pretreatment of stainless steel coupons by sulfochromic acid, activation of stainless steel surface by adsorption of 1% PEI solution or, alternatively, aminosilanization by 0.12 % solution of APTES in a 3:1 EtOH/H_2O mixture and fixation of glutaraldehyde just to be sure the trypsin is immobilized by covalent binding. The efficiency of the covalently immobilized trypsin was measured for one month. The enzymatic activity of trypsin following this procedure was better than those for immobilizing lysozyme on stainless steel with glutaraldehyde as the cross linker molecule [17]. Immobilized trypsin onto SS was inhibited in all situations by reduction of the formed Schiff base after fixation of glutaraldehyde. Those physico-chemical properties of immobilized trypsin of practical importance were improved after immobilization. This suggests it has significant potential applications. Forthcoming studies will target the immobilization of a bigger amount of active trypsin by better controlling of all steps of described procedure or by using another immobilization strategy.

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