Carbohydrate Polymers 82 (2010) 976–981

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Sodium periodate oxidized cotton yarn as carrier for immobilization of trypsin

Tanja Nikolic[a,b], Mirjana Kostic[a,*], Jovana Praskalo[a], Biljana Pejic[a], Zivomir Petronijevic[c], Petar Skundric[a]

[a] Faculty of Technology and Metallurgy, University of Belgrade, Karnegijeva 4, 11000 Belgrade, Serbia
[b] College of Textile Leskovac, Vilema Pusmana br. 17, 16000 Leskovac, Serbia
[c] Faculty of Technology, University of Nis, Bulevar oslobođenja 124 Leskovac, Serbia

ARTICLE INFO

Article history:
Received 9 May 2010
Received in revised form 1 June 2010
Accepted 14 June 2010
Available online 20 June 2010

Keywords:
Cotton yarn
Sodium periodate oxidation
Trypsin
Immobilization
Storage stability

ABSTRACT

Cotton yarn was first oxidized by sodium periodate to introduce aldehyde groups which were able to react with amino groups of trypsin to form Schiff's base, and result in cotton yarn immobilized trypsin. The effect of periodate oxidation on the chemical and physical properties of cotton yarn was evaluated by determining aldehyde group content, fineness and tensile strength of yarn. Measurements of protein load from Bradford assay and catalytic activity in hydrolysis of N-α-benzoyl-DL-arginine *p*-nitroanilide were made for the immobilized enzyme. The maximum amount of immobilized trypsin was 6.1 mg/g dried cotton yarn. Trypsin immobilized on oxidized cotton yarn retained 90% and 72% of the initial activity at 4 °C and 25 °C, respectively, over 60 days of storage in physiological solution.

1. Introduction

Cellulose is the most abundant natural, renewable and biodegradable polymer in the world. It has been used as a material for centuries in all kinds of practical applications. Social concerns for sustainable green products are encouraging the efficient exploitation of cellulose (Hon, 1994).

Cellulosic materials are generally strong, hydrophilic, insoluble in water, stable to chemicals, safe to living bodies, reproducible, recyclable and biodegradable. With these specific and advantageous characteristics of cellulose, modification techniques to reinforce these original properties or to add new functionalities to cellulose have been investigated. Chemical treatments have been positioned at the center of the field of cellulose modification (Hon, 1996; Isogai, 2001).

Chemical modification of cellulose using oxidizing agents is quite a frequent procedure in cellulose chemistry. During the oxidation of cellulose, aldehyde, ketone and carboxyl groups may be formed in the cellulose, depending on the nature of the oxidant and the conditions of oxidation. Most oxidations proceed with rather low selectivity. Oxidation of cellulose with periodates is a highly selective reaction (Calvini, Conio, Princi, Vicini, & Pedemonte, 2006; Kim, Kuga, Wada, Okano, & Kondo, 2000; Nevell, 1957; Potthast, Kostic, Schiehser, Kosma, & Rosenau, 2007; Potthast, Schiehser, Rosenau, & Kostic, 2009; Varma & Kulkarni, 2002). This reaction cleaves the C2–C3 bond of the glucopyranoside ring and leads to the introduction of two aldehyde groups at C-2 and C-3 positions; the resulting compound is the dialdehyde cellulose (DAC). One of the significant advantages of periodates over other oxidizing agents is that they minimize degradation and retain the mechanical and morphological properties of the starting material (Nevell, 1963). Obtained dialdehyde cellulose can be used to immobilize proteins (Carneiro-da-Cunha, Rocha, Garcia, & Gil, 1999; Varavinit, Chaokasem, & Shobsngob, 2001) or amino polysaccharides (Janjic et al., 2009; Liu et al., 2001) by reaction with their amino functions, as ion-exchange materials after further oxidation of the aldehydes to the corresponding carboxylic acids (Kim & Kuga, 2002), or as such for specific uses (Kim & Kuga, 2000; Kim, Wada, & Kuga, 2004).

Proteases are widely used in industrial and biomedical applications, among which trypsin was most extensively concerned. However, their applications are limited as regard to the problem of their instability and rapid losing of catalytic activity during the operational and storage periods resulting from autolysis, unfolding and aggregation of these enzymes (Villalonga, Villalonga, & Gómez, 2000). Enzyme immobilization can overcome these limitations; the immobilization stabilizes the structure and, hence, activity of enzymes. The key points in enzyme immobilization are a suitable carrier and immobilization method. Trypsin can be immobilized

* Corresponding author at: University of Belgrade, Faculty of Technology and Metallurgy, Department of Textile Engineering, Karnegijeva 4, Belgrade 11000. Tel.: +381 11 3303 628; fax: +381 11 3370 387.
E-mail address: kostic@tmf.bg.ac.rs (M. Kostic).

0144-8617/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2010.06.028

by different methods, such as adsorption (Kotel'nikova, Mikhailova & Vlasova, 2007), entrapment (Monteiro et al., 2007) and covalent binding (Cavalcante, Carvalho, & Carneiro-da-Cunha (2006); Kumar & Gupta, 1998). Immobilization of enzymes by covalent coupling usually leads to very stable preparations compared with other immobilization procedures. Various carriers have been used for the immobilization of trypsin, e.g., chitosan-coated silica gel (Xi, Wu, Jia, & Lin, 2005), polyester fleece (Nouaimi, Möschel, & Bisswanger, 2001), chitosan and cellulose (Zelenetskii et al., 2003). Medical requirements considerably limit the number of carriers that can be used in the immobilization of enzymes for therapeutic uses. The carriers must be nontoxic, noncarcinogenic, biocompatible and in no way injurious in the biological environment. Dialdehyde cellulose has been recommended as a suitable matrix for the immobilization of drugs and hormones. Besides, it has been found that insoluble form of the dialdehyde cellulose itself inhibits the microbial growth, and this activity resists heat and washing (Hon, 1996).

In this paper, we report the oxidation of cotton fibers with sodium periodate solutions under different conditions in order to obtain a suitable carrier for subsequent immobilization of trypsin, an enzyme with anti-inflammatory properties. Cotton, as the purest natural form of cellulose, was chosen for periodate oxidation because of the following: it possesses excellent properties, such as regeneration, biodegradation, softness, affinity to skin and hygroscopic property, and is traditionally used as a bandaging material. Our data demonstrated that trypsin immobilized on selective oxidized cotton fibers was markedly stabilized for a long period of storage.

2. Experimental

2.1. Materials

Raw cotton ring yarn (fineness: 20.85 tex, CV = 3.4%; yarn twist: 755 t.p.m., CV = 5.5%), which is intended for gauze production, was obtained from Strumicanka (Strumica, FYR of Macedonia). Trypsin from bovine pancreas (EC 3.4.21.4) in a powder form and N-α-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (BAPNA) were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2. Oxidation of cotton yarn with sodium periodate

A sample of cotton yarn was immersed in solutions of sodium periodate in 0.1 M acetic buffer (ratio 1:50, w/v), pH 4.0 at concentrations of 2.0 mg/ml and 4.0 mg/ml, i.e. 0.2% and 0.4% (w/v). The mixture was then stirred in the absence of light, at room temperature, for 15, 30, 45, 60, 120, 180, 240, 300 and 360 min. After completion of the oxidation, the cotton yarn was washed with ice-cold distilled water several times to remove the oxidant. This oxidized cotton yarn was used for the immobilization of trypsin without drying.

The effects of reaction time and periodate concentration on the rate of oxidation of cotton fibers were studied. Consumption of one periodate molecule produces two aldehyde groups. Therefore, the rate of periodate consumption (i.e. the decrease in the periodate content of the solution, referred to the weight of fibers immersed in it, and expressed as molecules of periodate per 100 glucose units) may be identified with the rate of oxidation. Titrimetry was used to calculate the periodate consumption (Nevell, 1957).

The formation of soluble fragments, as a result of the cellulose destruction (i.e. cellulose chain scission caused by subsequent reaction, not by oxidation itself), was determined by measuring the weight loss of oxidized cotton yarn samples by applying the direct gravimetric method (Koblyakov, 1989).

2.3. Determination of aldehyde group content

The aldehyde content present in the oxidized cotton was measured according to the method described in literature (Kumar & Yang, 2002; Praskalo et al., 2009; Saito & Isogai, 2004). The aldehyde groups were selectively oxidized to carboxyl groups with sodium chlorite at pH 4–5, at room temperature for 48 h, and carboxyl group content was determined by calcium-acetate method modified by Praskalo et al. (2009). Before titrations all cotton samples were ion-exchanged into acid form by suspending in 0.01 M HCl for 1 h, followed by washing with distilled water. The aldehyde group content was calculated by subtracting the carboxyl content value determined in the starting cotton sample from that of chlorite oxidized samples.

2.4. Determination of fineness of oxidized cotton yarns

Fineness in tex was determined as per standard method (SRPS ISO 2060, 1994).

2.5. Tensile strength measurement

Cotton yarns were tested by using tensile tester Tex Test (Switzerland), with clamps spaced at 100 mm, and with strain rate (bottom clamp rate) of 150 mm/min, according to the usual procedure described elsewhere (Koblyakov, 1989). The tensile strength of the yarn was calculated as the mean value of 20 measurements.

2.6. Immobilization of trypsin on oxidized cotton yarn

A trypsin solution (0.8 mg/ml) was prepared by dissolving enzyme in 100 mM Tris–HCl buffer (pH 9.2) containing 10 mM $CaCl_2$. The above mentioned oxidized cotton yarn was incubated with the solution of trypsin (ratio 1:25, w/v) for 18 h at 4 °C. Subsequent to immobilization, the yarn was washed with physiological solution 9 times to remove unbound trypsin. The supernatants of each washing were collected for protein measurements. The resultant immobilized trypsin on cotton yarn was stored in physiological solution. All immobilizing tests were performed in duplicate.

2.7. Protein loading assay

The amount of trypsin immobilized on the cotton yarn was determined from a mass balance, i.e. difference between the content of protein in solution before and after immobilization, and in the combined washings, using following equation:

$$T_{iy} = \frac{T_{s0} - T_{si} - T_{sw}}{m}$$

where T_{iy} is amount of trypsin immobilized on the cotton yarn (mg/g of cotton); T_{s0} is content of trypsin in solution before and T_{si} after immobilization (mg), and T_{sw} is content of trypsin in combined washing solutions (mg) determined according to the method of Bradford (Kruger, 2002) and using bovine serum albumin as the standard, while m is the weight of absolute dry cotton yarn (g).

2.8. Trypsin activity assay

The trypsin activity in the solution and in the immobilized state was determined by a method based on the initial rate of hydrolysis of the substrate BAPNA, similar as described in the literature (Nouaimi et al., 2001; Ohta, Makinen, & Loesche, 1986; Xi et al., 2005). One unit of activity was expressed as the amount of

enzyme required to release 1 μmol of *p*-nitroaniline per min by use of the molar extinction coefficient of *p*-nitroaniline at 410 nm of 8800 M^{-1} cm^{-1}.

The reactions were carried out in a 0.1 M phosphate buffer solution, at pH 7.0 and 37 °C, approximately the physiological conditions. The reaction mixture consisted of 3 ml 0.1 M phosphate buffer (pH 7.0), 15 μl 0.1 M BAPNA prepared in DMSO, and suitable amount of trypsin, i.e. 50 μl of trypsin solution (0.8 mg/ml) and 5 mg dry wt. of modified cotton yarn, for the activity assay of soluble and immobilized trypsin, respectively. At fixed time intervals, the released *p*-nitroaniline was measured at 410 nm using UV–VIS spectrophotometer (SP6-550 Pye Unicam). Three repetitive values were determined and the mean value calculated.

2.9. Stability of free and immobilized trypsin during storage

The stability of free and immobilized trypsin was determined at 4 °C and 25 °C during 60 days of storage. Samples of cotton yarn with immobilized trypsin, stored in physiological solution, and trypsin solution (0.8 mg/ml) in 0.1 M phosphate buffer pH 7.0, were kept at 4 °C and 25 °C. During 60 days, their activities were periodically assayed, as described above. Storage stability of free or immobilized trypsin is given as relative activity, i.e. in the case of free enzyme activity of stored trypsin solution is related to the activity of freshly prepared trypsin solution (initial activity of free enzyme) and in the case of immobilized enzyme activity of stored cotton yarn with immobilized trypsin is related to the activity of the same sample immediately after immobilization (initial activity of immobilized enzyme).

2.10. Trypsin leakage from the carrier

In order to study the trypsin leakage from the carrier, three samples of cotton yarn with immobilized trypsin were stored in 5 ml of physiological solution. After 60 days, protein concentration in the solution was determined as described above.

3. Results and discussion

3.1. Obtaining of dialdehyde cotton yarns

Cotton yarn was first oxidized with sodium periodate to cleave the 2,3-vicinal diol of the cellulose glucose units and produce aldehyde groups, and then employed to immobilize trypsin. Several oxidations with different oxidation time and different concentration of periodate solution were carried out. The course of the reactions was followed by measuring oxidant consumption by cotton fibers from 0.2% and 0.4% $NaIO_4$ solutions, Fig. 1. The reaction course can be divided into three distinct phases, i.e. the rate of periodate consumption is relatively high at first 60 min, after that it diminishes and above 240 min becomes almost constant. Calvini et al. (2006) suggested three simultaneously occurring reactions during periodate oxidation: a fast initial attack of periodate in the amorphous region of cellulose, a second slow reaction attributed to the oxidation of the surface of crystallites, and a very slow third reaction due to the oxidation of the crystalline core. According to Nevell (1957) the initial fast reaction may be identified with the formation of a cyclic complex of periodate ion with vicinal hydroxyls. Curves for periodate consumption obtained in this study were similar to those reported by Janjic et al. (2009) for the oxidation of lyocell fibers with 0.2% and 0.4% KIO_4 solution.

The effect of periodate oxidation on cotton fibers was then assessed by determining the aldehyde group content. As shows Fig. 2, no increase in aldehyde group content during the first 30 min of oxidation; after that, with increasing the oxidation time, the

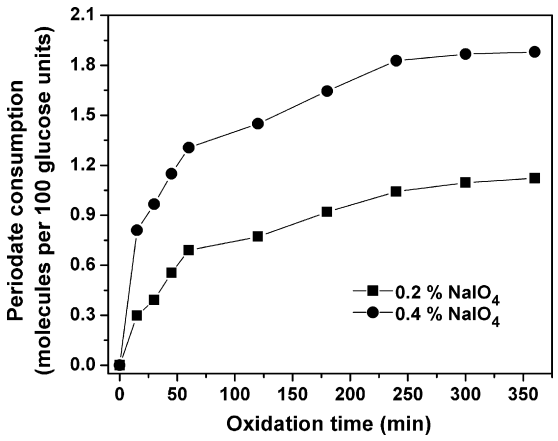

Fig. 1. Relationship between the periodate consumption and the oxidation time and the concentration of $NaIO_4$ used for the oxidation.

number of aldehyde groups rises for both periodate concentrations used. The cotton fibers oxidized with 0.4% $NaIO_4$ had higher increase in aldehyde group content (up to 282%) compared to the fibers oxidized with 0.2% $NaIO_4$ (increase up to 210%). Also, by comparison aldehyde group content against periodate consumption (Figs. 1 and 2) it is evident that during the first 30 min of treatment periodate was consumed probably by non-cellulosic material, since raw unbleached cotton yarn was used in this study. The non-cellulosic material at the cotton fiber surface was determined to be a complex mixture of fatty acids, alcohols, alkanes, esters and glycerides (Mitchell, Carr, Parfitt, Vickerman, & Jones, 2005).

The formation of soluble fragments, as a result of the cellulose destruction, was examined by measuring the weight loss of oxidized cotton yarn. Increasing reaction time and periodate concentration had no significant influence on weight loss. All samples showed the loss in weight in the range of 2.03–2.56%, which is considerably less compared to the weight loss values (up to 21%) determined for lyocell fibers oxidized under similar conditions with KIO_4 (Janjic et al., 2009). Similar results have been reported by Calvini et al. (2006) who obtained negligible weight loss of Whatman paper samples oxidized with periodate.

The mechanical properties of oxidized and native yarn were determined to establish the relationship between chemical modification and the resulting physical properties. The effect of oxidation conditions on the tensile strength of the cotton yarn is shown in Fig. 3. The tensile strength of the oxidized cotton yarn did not change remarkably for the oxidation time in the range of

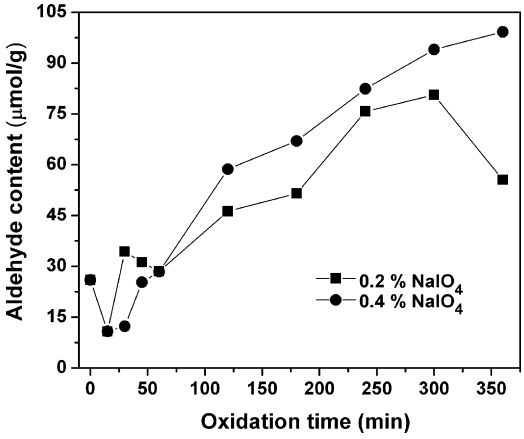

Fig. 2. Relationship between the aldehyde group content and the oxidation time and the concentration of $NaIO_4$ used for the oxidation.

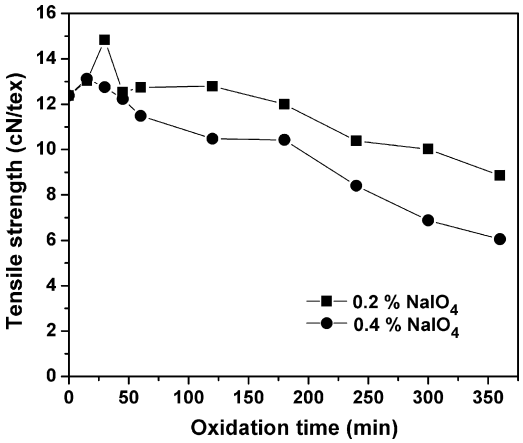

Fig. 3. The effect of oxidation time on the tensile strength of the oxidized cotton yarn.

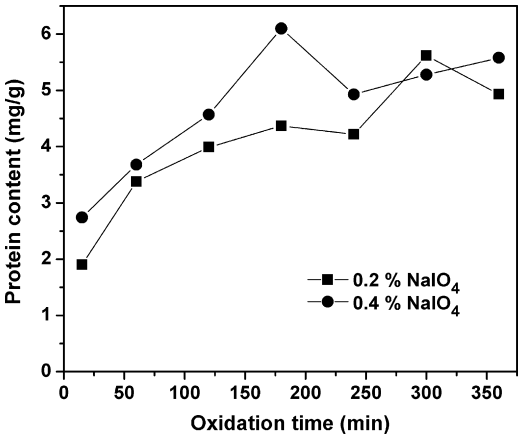

Fig. 4. Relationship between the amount of trypsin immobilized on cotton fibers and the oxidation time and the concentration of $NaIO_4$ used for the oxidation.

0–180 min. Cotton yarn oxidized by 0.2% and 0.4% $NaIO_4$ during 180 min retained 97% and 84% of its initial tensile strength, respectively. However, tensile strength strongly decreased when the time of oxidation was over 180 min. The oxidation by sodium periodate breaks to some extent the crystalline structure of cellulose in the native cotton yarn, and long oxidation time weaken the mechanical properties of the oxidized cotton yarn.

The mechanical properties of the oxidized cotton fibers are considerably less degraded compared to those determined for oxidized man-made cellulose fibers (lyocell) by Janjic et al. (2009), who reported that tensile strength of oxidized lyocell fibers was reduced of more than 80% when the oxidation time was above 60 min. This difference can be attributed to the different structure of natural and chemical cellulose fibers, mainly in degree of polymerization and crystallinity, fibrillar arrangement, etc.

The above mentioned weight loss of the oxidized cotton yarns, with no observed yarn contraction during the modification, is responsible for changes in the oxidized yarn fineness. Oxidized cotton samples became finer, i.e. fineness value decreased up to 5.28%, namely from 20.85 tex for starting yarn to 19.75 tex for oxidized yarn (data not shown).

3.2. Obtaining of cotton yarn with immobilized trypsin

The cotton fiber, previously activated by periodate treatment, was used for immobilization of trypsin through the formation of Schiff base product. In this process, the N-terminal amino group, as well as the amino groups from lysines, react with the aldehyde groups on the oxidized cellulose fiber. Trypsin contains 14 lysine residues (Mikes, Holeysovsky, Tomasek, &Sorm, 1966), which means a sufficient amount of amino groups for multipoint attachment between the enzyme and the oxycellulose (Villalonga et al., 2000; Xi et al., 2005).

The amount of immobilized trypsin was estimated from a mass balance, as difference between the amount of protein in solution before immobilization and sum of protein in solution after immobilization and in the combined washings. Fig. 4 shows the relationship between the reaction times of periodate oxidation of the cellulose fiber and the amount of trypsin covalently immobilized on this modified fiber. The amount of immobilized trypsin increased with the oxidation time during the first 180 min, when reached the maximum value 6.1 mg/gram of dried cotton yarn, which is characteristic of carriers that have low specific surface area (Seabra & Gil, 2007). The protein content in the modified cotton fiber was higher for fiber oxidized by 0.4% $NaIO_4$ than fiber oxidized by 0.2% $NaIO_4$. When the oxidation time was over 180 min, there was no further increase in the protein content. This can be attributed to the difference in the reaction site of the oxidation and Schiff's base formation in the cellulose (Liu et al., 2001). The small periodate ion is able to enter the cellulose fiber interior and the glucose unit both inside and on the surface of the fiber can be oxidized. Trypsin is a huge molecule that cannot access aldehyde groups formed inside the fiber and the attachment occurred on the surface of the cellulose fiber. Furthermore, the trypsin molecules in large amounts cover the dialdehyde cellulose chain and form a shield over a significant portion of the aldehyde groups.

After the immobilization process, the obtained cellulose fibers with different amounts of bound enzyme were assayed for activity. Relative activity of trypsin immobilized on cotton fibers oxidized in different conditions is presented in Fig. 5. The activity of immobilized trypsin increased with the oxidation time during the first 180 min, whereas it became nearly constant with further increase of oxidation time. Immobilized activity was higher for fiber oxidized by 0.4% $NaIO_4$ than fiber oxidized by 0.2% $NaIO_4$. These results are in accordance with the protein content data. The maximum activity of the immobilized trypsin was 1.22 U/gram of dried cotton yarn, which is 14% of the total initial activity of trypsin in solution before immobilization. Finally, it should be mentioned that the incubation of trypsin with unoxidized cotton fiber yielded a fiber with very low immobilized activity. According to these results and values obtained for the mechanical properties, cotton yarn oxidized by 0.4% $NaIO_4$ during the 180 min was chosen for further storage stability characterisations.

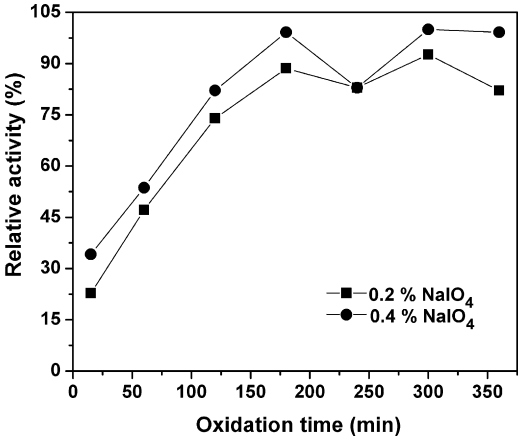

Fig. 5. Activity of trypsin immobilized on cotton fibers oxidized in different conditions. Activities are related to maximal immobilized activity.

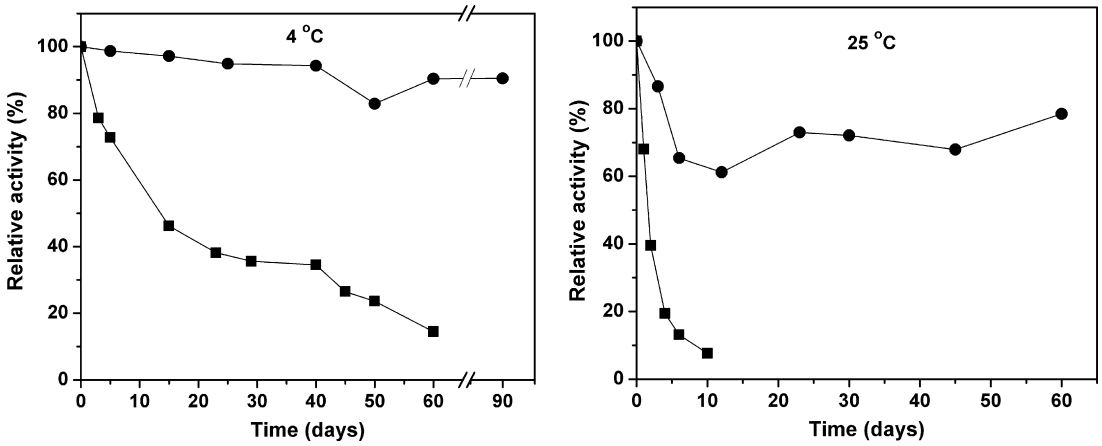

Fig. 6. Stability of free and immobilized trypsin at 4 °C and 25 °C for long storage. Activities are related to that at the first day (100%).

3.3. Storage stability of free and immobilized trypsin

The stability of free and immobilized trypsin was evaluated over the 60 days of storage at 4 °C and 25 °C. As it could be seen from Fig. 6, the free enzyme lost 85.5% of its initial activity after storage for 60 days at 4 °C, whereas at room temperature a 92.4% loss of activity was observed after 10 days. This severe decrease in activity of the free trypsin might be due to its autolysis (Kumar & Gupta, 1998; Villalonga et al., 2000; Xi et al., 2005). Trypsin immobilized on cotton yarn retained about 90% and 72% of its activity at 4 °C and 25 °C, respectively, over 60 days. Furthermore, the trypsin-cotton yarn sample stored at 4 °C showed the same activity retention after additional 30 days of storage. This improved stability is probably a result of the prevention of autolysis and unfolding of trypsin molecules by immobilization. Similar results have been reported by Cavalcante et al. (2006) and Seabra and Gil (2007) who evaluated the stability of a covalently immobilized trypsin at 4 °C. These authors found activity retention of 86.5% after 54 days and 85% over 50 days, respectively. For the immobilized trypsin stability at room temperature, activity retention of 65–85% depending on the coupling method (Xi et al., 2005) and 30% (Seabra & Gil, 2007) over 30 days have been reported.

The results presented above indicate that the storage stability of the immobilized trypsin was significantly improved compared with the free enzyme, and it was more efficient at 4 °C, which had to do with less amount of unfolding that occurred in the immobilized trypsin at a lower temperature.

Finally, the study of the trypsin leakage from the carrier fiber showed that after 60 days of keeping cotton yarn with immobilized trypsin in physiological solution, no protein was found in the storage solution. This result meant that trypsin remained firmly attached to the cotton yarn, which is characteristic of covalent immobilization of enzymes.

4. Conclusion

Cotton yarn with covalently bound trypsin was obtained by the reaction between oxidized cotton fiber and the trypsin solution. Cotton yarn was first oxidized with sodium periodate to produce aldehyde groups, and then employed to immobilize trypsin. Different oxidation times and periodate concentrations were used to increase aldehyde content in cotton fibers. The periodate oxidation during the first 180 min did not have a significant effect on the mechanical properties of yarn.

The resulting aldehyde group on the cellulose fiber was able to react with an amino group of trypsin to form the corresponding Schiff base. The amount of immobilized trypsin, as well as its activity, increased with the oxidation time during the first 180 min, and than became nearly constant. In order to ensure enough amount of bound trypsin on the cotton yarn and preserve the tensile strength of the oxidized cotton yarn, the optimal concentration of the periodate is 0.4% and the optimal oxidation time is 180 min.

Trypsin immobilized on oxidized cotton yarn exhibited excellent storage stability. Trypsin possesses wound-healing properties, while cotton is traditionally used as a bandaging material, therefore the resulting composition of trypsin with cellulose has potential for medical applications.

Acknowledgment

This study has been supported by Ministry of Science and Technological Development of the Republic Serbia (Project TR 19009).

References

Calvini, P., Conio, G., Princi, E., Vicini, S., & Pedemonte, E. (2006). Viscometric determination of dialdehyde content in periodate oxycellulose Part II. Topochemistry of oxidation. *Cellulose*, *13*, 571–579.

Carneiro-da-Cunha, M. G., Rocha, J. M. S., Garcia, F. A. P., & Gil, M. H. (1999). Lipase immobilisation on to polymeric membranes. *Biotechnology Techniques*, *13*, 403–409.

Cavalcante, A. H. M., Carvalho, L. B., Jr, & Carneiro-da-Cunha, M. G. (2006). Cellulosic exopolysaccharide produced by *Zoogloea* sp. as a film support for trypsin immobilisation. *Biochemical Engineering Journal*, *29*, 258–261.

Hon, D. N.-S. (1994). Cellulose: A random walk along its historical path. *Cellulose*, *1*, 1–25.

Hon, D. N.-S. (1996). Cellulose and its derivatives: Structures, reactions, and medical uses. In S. Dumitriu (Ed.), *Polysaccharides in medical application* (pp. 87–105). NewYork, USA: Marcel Dekker.

Isogai, A. (2001). Chemical modification of cellulose. In D. N.-S. Hon, & N. Shiraishi (Eds.), *Wood and cellulosic chemistry* (pp. 599–626). NewYork, USA: Marcel Dekker.

Janjic, S., Kostic, M., Vucinic, V., Dimitrijevic, V., Popovic, S., Ristic, K., et al. (2009). Biologically active fibers based on chitosan-coated lyocell fibers. *Carbohydrate Polymers*, *78*, 240–246.

Kim, U.-J., & Kuga, S. (2000). Reactive interaction of aromatic amines with dialdehyde cellulose gel. *Cellulose*, *7*, 287–297.

Kim, U.-J., & Kuga, S. (2002). Ion-exchange separation of proteins by polyallyllamine-grafted cellulose gel. *Journal of Chromatography A*, *955*, 191–196.

Kim, U.-J., Kuga, S., Wada, M., Okano, T., & Kondo, T. (2000). Periodate oxidation of crystalline cellulose. *Biomacromolecules*, *1*, 488–492.

Kim, U.-J., Wada, M., & Kuga, S. (2004). Solubilization of dialdehyde cellulose by hot water. *Carbohydrate Polymers*, *56*, 7–10.

Koblyakov, A. (1989). *Laboratory practice in the study of textile materials*. Moscow: Mir Publishers. [p. 141–144, 192–200].

Kotel'nikova, N. E., Mikhailova, S. A., & Vlasova, E. N. (2007). Immobilization of proteolytic enzymes trypsin and α-chymotrypsin to cellulose matrix. *Russian Journal of Applied Chemistry*, *80*, 322–329.

Kruger, N. J. (2002). The Bradford method for protein quantitation. In J. M. Walker (Ed.), *The protein protocols handbook* (pp. 15–21). Totowa, NJ: Humana Press.

Kumar, A., & Gupta, M. N. (1998). Immobilization of trypsin on an enteric polymer Eudragit S-100 for the biocatalysis of macromolecular substrate. *Journal of Molecular Catalysis B: Enzymatic*, *5*, 289–294.

Kumar, V., & Yang, T. (2002). HNO_3/H_3PO_4-$NaNO_2$ mediated oxidation of cellulose-preparation and characterization of bioabsorbable oxidized celluloses in high yields and with different levels of oxidation. *Carbohydrate Polymers*, *48*, 403–412.

Liu, X. D., Nishi, N., Tokura, S., & Sakairi, N. (2001). Chitosan coated cotton fiber: Preparation and physical properties. *Carbohydrate Polymers*, *44*, 233–238.

Mikes, O., Holeysovsky, V., Tomasek, V., & Sorm, F. (1966). Covalent structure of bovine trypsinogen. The position of the remaining amides. *Biochemical and Biophysical Research Communications*, *24*, 346–352.

Mitchell, R., Carr, C. M., Parfitt, M., Vickerman, J. C., & Jones, C. (2005). Surface chemical analysis of raw cotton fibres and associated materials. *Cellulose*, *12*, 629–639.

Monteiro, F. M. F., Silva, G. M. M., Silva, J. B. R., Porto, J. B. R., Carvalho, C. S., Jr, Lima-FilhoF L. B., et al. (2007). Immobilization of trypsin on polysaccharide film from *Anacardium occidentale* L. and its application as cutaneous dressing. *Process Biochemistry*, *42*, 884–888.

Nevell, T. P. (1957). The mechanism of the oxidation of cellulose by periodate. *Journal of the Textile Institute*, *48*, T484–T494.

Nevell, T. P. (1963). Oxidation. In R. L. Whistler (Ed.), *Methods in carbohydrate chemistry* (pp. 164–189). New York, USA: Academic Press.

Nouaimi, M., Möschel, K., & Bisswanger, H. (2001). Immobilization of trypsin on polyester fleece via different spacers. *Enzyme and Microbial Technology*, *29*, 567–574.

Ohta, K., Makinen, K. K., & Loesche, W. J. (1986). Purification and characterization of an enzyme produced by *Treponema denticola* capable of hydrolyzing synthetic trypsin substrates. *Infection and Immunity*, *53*, 213–220.

Potthast, A., Kostic, M., Schiehser, S., Kosma, P., & Rosenau, T. (2007). Studies on oxidative modifications of cellulose in the periodate system: Molecular weight distribution and carbonyl group profiles. *Holzforschung*, *61*, 662–667.

Potthast, A., Schiehser, S., Rosenau, T., & Kostic, M. (2009). Oxidative modifications of cellulose in the periodate system–Reduction and beta-elimination reactions. *Holzforschung*, *63*, 12–17.

Praskalo, J., Kostic, M., Potthast, A., Popov, G., Pejic, B., & Skundric, P. (2009). Sorption properties of TEMPO-oxidized natural and man-made cellulose fibers. *Carbohydrate Polymers*, *77*, 791–798.

Saito, T., & Isogai, A. (2004). TEMPO-mediated oxidation of native cellulose. The effect of oxidation conditions on chemical and crystal structures of the water-insoluble fractions. *Biomacromolecules*, *5*, 1983–1989.

Seabra, I. J., & Gil, M. H. (2007). Cotton gauze bandage: a support for protease immobilization for use in biomedical applications. *Brazilian Journal of Pharmaceutical Sciences*, *43*, 535–542.

SRPS ISO 2060. (1994). *Yarn from packages—Determination of linear density (mass per unit length)—Skein method*.

Varavinit, S., Chaokasem, N., & Shobsngob, S. (2001). Covalent immobilization of a glucoamylase to bagasse dialdehyde cellulose. *World Journal of Microbiology & Biotechnology*, *17*, 721–725.

Varma, A. J., & Kulkarni, M. P. (2002). Oxidation of cellulose under controlled conditions. *Polymer Degradation and Stability*, *77*, 25–27.

Villalonga, R., Villalonga, M. L., & Gómez, L. (2000). Preparation and functional properties of trypsin modified by carboxymethylcellulose. *Journal of Molecular Catalysis B: Enzymatic*, *10*, 483–490.

Xi, F., Wu, J., Jia, Z., & Lin, X. (2005). Preparation and characterization of trypsin immobilized on silica gel supported macroporous chitosan bead. *Process Biochemistry*, *40*, 2833–2840.

Zelenetskii, A. N., Akopova, T. A., Kildeeva, N. R., Vikhoreva, G. A., Obolonkova, E. S., et al. (2003). Immobilization of trypsin on polysaccharides upon intense mechanical treatment. *Russian Chemical Bulletin*, *52*, 2073–2077.