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Tyrosinase-catalysed coupling of functional molecules onto protein fibres

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ABSTRACT

Grafting, using oxidative enzymes shows a high potential for wool fibres functionalisation. In this work we attempt to graft on wool fibres with phenolic antioxidants in order to introduce and improve the properties of the fibre. The approach of tyrosinase to oxidize tyrosine residues in wool proteins to quinones, which can further react with free sulfhydryl (thiol), amino or phenolic groups of different substrates was exploited to couple different phenolic antioxidants (caffeic acid and chlorogenic acid) onto the wool fibre proteins. Tyrosinase-catalysed reactions were followed by different analytical methods like oxygen consumption, FT-NIR Raman and UV/vis spectroscopy. It was proved that phenolic compounds used are strongly cross-linked on the wool fibre resulting to an improved antioxidant activity.

1. Introduction

Wool is an extremely complex, highly cross-linked protein fibre. The use of enzymes for wool surface modification has gained considerable interest in recent years. The majority of this research has been directed towards shrink-resist and softening treatments where different proteolytic enzymes hydrolysing peptide linkages [1–7], alone or in combination with other enzymes (e.g. peroxidase, catalase or lipase) in pre-treatment stage [2,8], have been studied. Alternatively, protein cross-linking enzymes, e.g. transglutaminases [6] have shown potential to be used for this purpose. In addition a variety of new wool surface properties could be obtained by covalently linking of active functional compounds to the fibres using enzymes [7]. Oxidative enzymes (i.e. tyrosinases) could have also a potential to impart new properties to fibre surfaces via grafting reactions.

Chemical, physical, mechanical and biological properties of wool protein fibres afford a broad application field among which innovative biomaterials for medical devices, such as bioactive dressings, wound healing isolation materials, etc. [6,9–11] are became very promising. However, many natural fibre-based materials show limited UV-protection. Ultraviolet radiation (UVR)-induced skin damage includes acute reactions such as erythema and edema, as well as premature skin aging and carcinogenesis largely determined by chronic exposure. Reactive oxygen species cause injury by reacting with bio-molecules such as lipids, proteins, and nucleic acids, as well as depleting the human skin [12–14].

Antioxidants are used to preserve food and other biomaterials by retarding rancidity, discolouration or deteritoration due to auto-oxidation [15]. Antioxidants may intervene at different levels in the oxidative process (e.g. by scavenging of free radicals and lipid peroxyl radicals, removing oxidatively damaged bio-molecules) [12,13,16,17]. Chlorogenic acid (CHA) and caffeic acid (CA) are one of the most interesting non-flavonoid catecholic compounds, which are present in many plants possessing anti-inflammatory, antimutagenic and anticarcinogenic properties [12,13,16–18].

CA has proven medicinal properties, especially as an antioxidant agent. Despite this, few studies have been dedicated to the enzymatic oxidation mechanism of this substance. The enzymatic oxidation of CA in the presence of polyphenoloxidase has been studied [19]. CHA, an ester of CA and quinic acid, is a major phenolic compound in coffee while the daily intake was estimated to 0.5–1 g for coffee drinkers. CHA and CA are proven antioxidants *in vitro* and might therefore contribute to the prevention of cardiovascular disease [18]. Their protective effects are confined from the ability to inhibit lipid peroxidation, chelate redox-active metals and attenuate other processes involving reactive oxygen species. The antioxidant activity *in vitro* depends upon the spatial arrangement of functional groups in specific position at nuclear structure [20].

Cross-linking can lead to beneficial changes of the properties of various proteins. Unfortunately due to a limiting number of binding sites or steric inaccessibility, several proteins may not be cross-linked or modified due to enzymatic reaction. In the case of wool proteins, a covalent network can be generated by addition of reductive and denaturing agents, like dithioeritrithol, ascorbic acid

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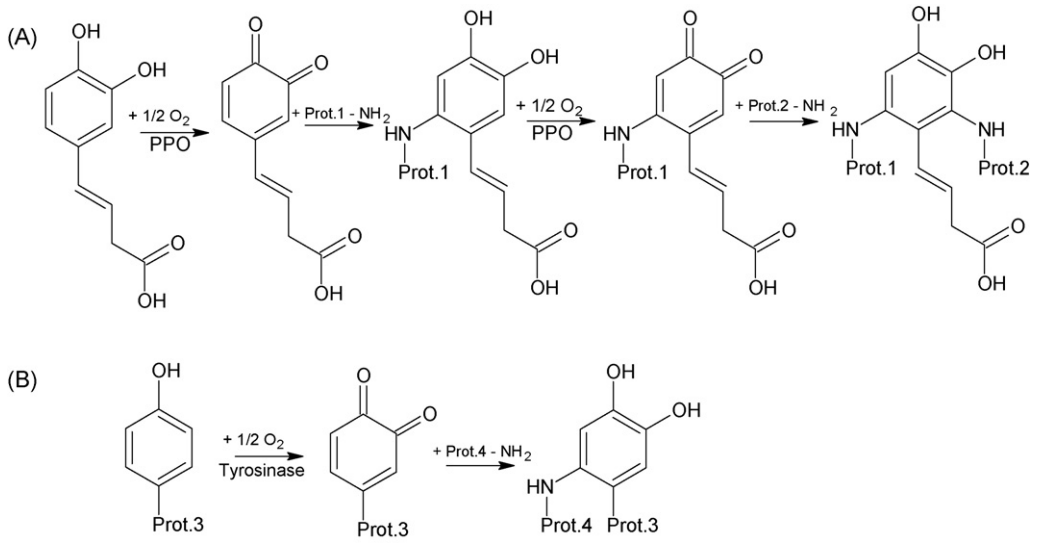

Fig. 1. Reaction principle of a polyphenol oxidase (PPO)-generated cross-linking of proteins in case of: (A) in the presence of CA as a low molecular weight phenolic reagent; (B) in the absence of a low molecular weight phenolic reagent. Prot.1–4—protein residues [21].

and others [12,21,22]. Alternatively, tyrosinases can be used for cross-linking via oxidation of tyrosine residues [23]. Oxidation of tyrosine residues in proteins leads to the corresponding quinones, which can further react with, e.g. free sulfhydryl (thiol) and/or amino groups resulting in formation of tyrosine–cysteine and tyrosine–lysine cross-links. Quinones have also been suggested to form tyrosine–tyrosine linkages by coupling together [11,21]. After step-wise oxidation of phenolic molecules to *o*-quinones the addition of a protein leads to a cross-link (Fig. 1a). In principle, it is also possible that tyrosine residues of the protein are oxidised via *o*-diphenols to *o*-quinone structures, resulting in direct protein–protein cross-link (Fig. 1b).

In the present work, tyrosinase has been used to graft antioxidant phenolic substrates like CA and CHA onto the wool fibres. The efficiency of phenolic substrate enzyme grafting and the antioxidant properties of functionalised wool fibres are assessed.

2. Materials and methods

2.1. Wool preparation

Australian merino wool top (fineness of fibre 19.5 μm and length of fibre 70 mm) was cleaned by a sohxlet extraction using dichloromethane to remove fatty matters and possible impurities on the wool fibre. Thereafter the wool was washed with distilled water and air dried at about 40 °C.

Soluble wool proteins (Wh) were extracted from wool fibres by an extensive 12-h treatment with a 8 M Tris/urea buffer containing (50 mM) dithiotreitol as reduction agent at pH 9.3 and 25 °C. The reaction was stopped by using 20% iodacetamide. The extracted wool proteins were dialysed against distilled water for 4 days. The protein content of the enzymes and the wool hydrolysate were determined using the Lowry test method [24].

2.2. Enzymatic wool treatment

Mushroom tyrosinase was obtained from Sigma Chemical Co. Dried wool tops (0.5 g) were incubated with tyrosinase (2 kU/g) in 50 ml 0.1 M potassium phosphate buffer (pH 6.5) in a glass flask at 25 °C for 24 h. In addition, ascorbic acid solution (0.42 mg/ml) was added as a reducing agent. Stock solutions of CA and CHA were prepared by dissolving the chemicals in dimethylsulfoxide (DMSO) and further diluted with 0.1 M potassium phosphate buffer (pH 6.5) to obtain final concentrations of 0.5 and 5 mg/ml in treatment solution. Aliquots of the stock solutions were added to the above incubation mixtures. To terminate the reaction the enzyme was inactivated by increasing the temperature to 85 °C for 10 min. Thereafter, fibres were rinsed in distilled water and 1% (v/v) acetic acid solution for several times and air-dried. Tyrosinase activity was assayed according to Duckworth using L-tyrosine (Sigma) and L-dihydroxy phenylalanine (L-DOPA, Sigma) as substrates [25].

2.3. Measurement of oxygen consumption during enzyme treatment

Wool fibres were incubated with tyrosinase as described above. However, the buffer solution on ice was saturated with O_2 prior to the treatment for 30 min according to Niku-Paavola et al. [26]. Blank treatments were carried out similarly but without wool and L-tyrosine was used as an alternative substrate. The oxygen consumption was measured using the OXILAB V5 apparatus (WTW, Weilheim, Germany).

2.4. UV/vis spectral analysis of enzyme treatment

A Tecan Infinite 200 plate reader was used for the monitoring of the enzymatic oxidation of substrates and potential coupling reactions between phenol substrates and wool proteins. Tyrosine (1 mM) was used as control. The wool hydrolysate and the CA and CHA (Sigma) were measured from 200 to 700 nm. UV/vis microtiter plates using a 96-well plate (UV, flat bottom) (Nunc) with the volume of 350 μl were used and the temperature was adjusted to 24 ± 1 °C. Oxidation reactions was started by adding tyrosinase (1 kU/ml) into a reaction batch containing the phenolic substrates (0.5 mg/ml, dissolved in DMSO) and 1 mg/ml of hydrolysed wool protein in 50 mM phosphate buffer of pH 6.5. To prevent oxygen limitations, the reactions were performed in open vessels under continuous stirring. Control samples were incubated in the same conditions, with boiled tyrosinase.

2.5. Quantification of DOPA and DOPA quinone (DQ) formation during the tyrosinase treatment of Wh

1 kU/ml tyrosinase were added to 20-μl substrate solution (CA: 5 mg/ml; CHA: 5 mg/ml; wool hydrolysate: 1 mg/ml) after addition with 980 μl of distilled water, 70 μl of ethylendiamine, and 50 μl of 2 M ethylendiamine dihydrochloride (pH 11). The mixture was incubated at 50 °C for 2 h in the dark, and then the fluorescence intensity was measured using the Tecan Infinite 200 plate reader. The excitation and emission wavelengths were at 420 and 543 nm, respectively. The concentrations of the DOPA in the preparations were estimated using a standard fluorescence curve of DOPA and the concentrations of DOPA residues were calculated from published extinction coefficients [27].

For the quantification of DQ, MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate) was used. MBTH reacts with DQ to form a pink pigment with λ_{max} at 505 nm. The assay solution was prepared by mixing 480 μl of the enzyme/substrate reaction mixture, 980 μl of 4.3% (v/v) DMF (dimethyl formamide) in distilled water, and 580 μl of 20.7 mM MBTH. The total volume was 2 ml. The reaction mixture was incubated at 25 °C for 10 min before the absorbance measurement at 505 nm. The amount of DQ produced was calculated from ε_{505} = 28,800 for the MBTH–quinone adduct [27].

2.6. FT-NIR Raman analysis

Raman spectra were recorded on the PerkinElmer spectrophotometer with a FT-Raman module with Nd:YAG laser source. Spectra were accumulated from 64 scans at a resolution of 4 cm^{-1}. An optical bench alignment was performed before each Raman measurement to ensure that the spectrometer was fine-tuned and the detector signal maximized.

2.7. Physical–mechanical properties of enzyme-treated wool fibres

The efficiency of the oxidative enzyme treatment and the addition of antioxidants of the dried and conditioned (T = 20 °C, RH = 65% and t = 24 h) samples was evaluated by measuring the fineness-titer and the tenacity of enzyme-treated wool fibres, according to ISO 1973 and ISO 5079 test methods. The strength of fibre is defined as the force required for breaking load of fibre. The unit of tenacity determination is cN per Tex. The test for determination of the breaking force and elongation at break and fibre density of individual fibres were performed in conditioned state. The test is restricted to the use of constant-rate-of-extension testing apparatus using Vibrodyn and Vibroskop 400 (Lenzing, Germany).

2.8. Washing and light fastness of enzyme-treated and coated wool samples

The stability and antioxidant activity of enzymatic fibre functionalisation to washing (40 °C) and to light fastness were determined based loosely on the instrumental assessment of change in colour for the determination of grey scale rating by ISO 105-A05 and ISO 105-A04 and evaluation of the antioxidant activity after the washing procedures.

Light fastness was measured following the ISO 150-B04 standard. Treated specimens were stapled to the black side of test mask. The mask was placed in a XENOTEST 150S (No. 55007101, Heraeus) and exposed to a xenon light source at an irradiance of 1154 W/m^2 for 15 h. Colour changes in the specimens were assessed using the Blue Scale method and evaluation of the antioxidant activity after the exposure to the xenon light.

2.9. Colour measurements

The whiteness and the colour of the enzyme-treated samples were determined by reflectance measurements using a Spectraflash SF 600 PLUS spectrophotometer (Datacolor). CIE whiteness W was determined according to the ISO 105-J02:1999 test method and the colour was evaluated by CIELAB colour and CIELAB 1976 colour difference ΔE^* at D 65/10°. The K/S (colour depth) was calculated based on the absorption at minimum reflectance according to the Kubelka–Munk equation.

2.10. Antioxidant activity determination

The antioxidant activity of phenolic substrates was determined according to the ferric thiocyanate method with minor modifications. Each sample of treated wool (200 mg) was mixed with 2 ml distilled water and 5 ml linoleic acid emulsion (0.02 M, pH 7.0) and 5-ml phosphate buffer (0.2 M, pH 7.0). Linoleic acid emulsion was prepared by mixing 0.56 g of linoleic acid with 0.56 g of Tween 20 as emulsifier, and 100-ml phosphate buffer (0.2 M, pH 7.0), and then the mixture was homogenized. The reaction mixture was incubated at 37 °C. Aliquots of 0.1 ml were taken at different intervals during incubation. The degree of oxidation was measured by sequentially adding 4.7 ml ethanol (75%), 0.1 ml ammonium thiocyanate (30%), 0.1 ml sample solution and 0.1 ml ferrous chloride (0.02 mg, in 3.5% (v/v) HCl). The mixture was incubated for 3 min and then the peroxide value was determined at 500 nm [17]. During the linoleic acid oxidation, peroxides are formed leading to oxidation of Fe^{2+} to Fe^{3+}. The procedure was repeated every 3 h and later in 24 h intervals until the control reached its maximum absorbance value. High absorbance indicates high linoleic acid emulsion oxidation. The solutions without a treated sample were used as a control [26]. All data on total antioxidant activity are the average of triplicate experiments. The inhibition percentage of lipid peroxidation in linoleic acid emulsion was calculated by the following equation:

$$I_{\text{inhibition of lipid peroxidation}}(\%) = \left[1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right] \times 100$$

where A_{control} is the absorbance of control reaction and A_{sample} is the absorbance in the presence of CA, CHA or standard compounds [14,17,28,32].

3. Results and discussion

The objective of this work was to investigate enzymatic coupling of two antioxidants, CA and CHA, onto wool fibres in order to impart unique properties to both the structure and the function of the wool protein and consequently improve the fibre properties.

Oxidation of tyrosine residues in wool fibres was monitored via quantification of the O_2 consumption. The initial O_2 concentration was about 30 mg/l (enriched by pure oxygen); the results are presented in Fig. 2. There was a clear continuous decrease of the oxygen concentration indicating oxidation of tyrosine residues of wool fibres. To a second sample ascorbic acid was added as a reducing agent to increase the accessibility of tyrosine residues in wool proteins and enhance the conversion of tyrosine to DOPA [12,21]. In earlier work researcher used reducing agents like sodium bisulphite

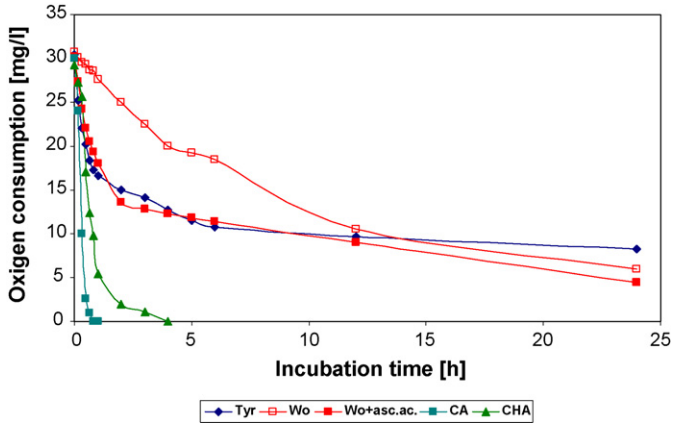

Fig. 2. Oxygen consumption during tyrosinase treatment of different substrates (Wo—wool fibres, Wo-asc.—acid–wool fibres with ascorbic acid supplement, CA—caffeic acid (0.5 mg/ml) and CHA—chlorogenic acid (0.5 mg/ml)).

for a mild reductive pre-treatment of wool for loosen the strongly cross-linked wool fibre cuticle [1,5,27]. Using ascorbic acid higher oxygen consumption was measured and the oxygen concentration dropped to 5 mg/l O_2 after 24 h of incubation. In the case of small monomer substrates like CA the oxygen concentration decreased much faster and reached the minimum of 1.2 mg/l already during the first hour of the enzyme treatment. CHA was oxidised slower than CA. The reason could be due to the sterical hindrance of the CHA in comparison to the smaller CA and also in redox potential differences of the substrates.

Complete oxidation of 1 mol of tyrosine to DOPA quinone consumes 1.5 mol of O_2. Wool contains about 0.35 mmol tyrosine residues per gram of fibres most likely located at the cuticle (0.258 mmol/g) [10,12]. Limited accessibility of tyrosine residues for tyrosinase could be one reason for the slower O_2-consumption when compared to tyrosine. The surface of untreated wool fibre is heavily cross-linked by disulphide bridges, thus hindering penetration of enzymes into the fibre. However, due to the smaller size of the phenolic substrates CA and CHA, used in this study, this limitation should be less pronounced.

Tyrosinase activity on different substrates (Tyr, CA, CHA and wool hydrolysate) was determined spectrophotometrically. The tyrosine, CA and CHA solution treated with active tyrosinase developed a pale yellow brown colour very quickly.

Fig. 3a–c presents the oxidation reaction of the control substrate tyrosine, Wh—wool hydrolysate and CA during the incubation period. Based on a decrease of the peak at 285 nm in the case of tyrosine and in the case of wool proteins, the absorption peak at 265 nm gradually decrease. This spectral change is typical for the conversion of tyrosine to DOPA residues [27]. The conversion of phenol in the CA structure into quinone was seen at 420 nm (brown orange colour) and the formation of reactive *o*-quinones was indicated with the shift of the peak at 310 nm. The tyrosinase-catalysed oxidation reaction of tyrosine residues in wool proteins was observed as a decrease of the peak at 265 nm (Fig. 3b) until the accessible tyrosine content was exhausted and a shift of the peak were observed at 285 nm. These could be the consequence of the formation of cysteinyl–DOPA and the presence of proteins [28,29]. The spectrophotometric examination of the reaction showed that the initial absorption maximum of DOPA at 280 nm was gradually replaced during the incubation by an absorption maximum at 292 nm corresponding to 5-*S*-cysteinil–DOPA [28]. The high absorption level after tyrosine oxidation at 265 nm of the protein content might belong to other aromatic amino acids like tryptophan and phenilalanin of wool proteins which cannot be oxidised by tyrosinase.

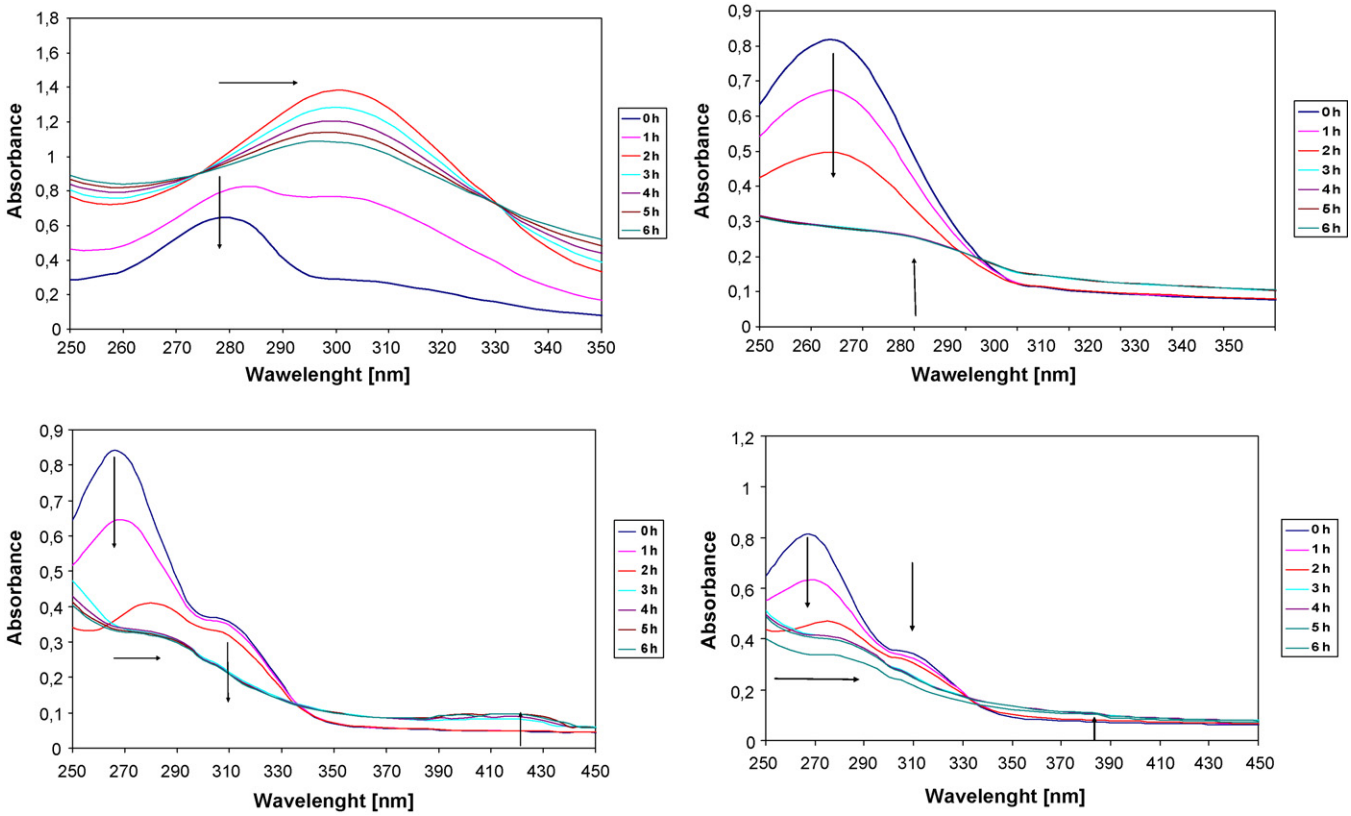

Fig. 3. Specrophotometric course of tyrosinase action on different substrates ((a)Tyr—tyrosine, (b) Wh—wool hydrolysate, (c) CA and (d) CA + Wh) at pH 6.5 and 25 °C during 6 h of incubation.

When wool proteins and CA were simultaneously incubated with tyrosinase (Fig. 3d) a strong peak decrease at 265 nm and the shift to the higher wavelengths was seen this is due to the formation of reactive cysteinyl–DOPA at 285 nm (wool proteins) and *o*-quinones at 310 nm resulting from CA oxidation. The absorbance increase in the 350 nm region could be consistent with a reaction between the quinone and amino groups [30]. The subsequent decrease of these peaks confirms the non-enzymatic reaction with the primary amino groups of wool proteins and the possible doublephenolic coupling reaction between the substrates. Thus, booth tyrosine residues in wool and CA are oxidised in the simultaneous treatment. A similar behaviour was observed for enzyme treatment of CHA and wool proteins (spectra not shown).

The tyrosinase oxidizes the tyrosine in proteins to yield L-β-dihydroxyphenyl-α-alanine (DOPA), followed by converting the DOPA into DQ residues. DOPA and DQ are significantly responsible for both intermolecular cross-linking and the interfacial adsorption properties of many proteins. For a better understanding of the oxidation mechanism during the enzymatic treatment and to quantify the DOPA transformation, we employed the fluorescent technique which was originally reported by Yagi and Nagatsu and then optimized for the analysis on a DOPA-containing protein [24]. As reported by Kuboe et al., DOPA and DQ are significantly responsible for both the intermolecular cross-linking and the interfacial adsorption properties of the proteins [27].

In the case of tyrosine (Tyr) as substrate 8.0 μg/ml DOPA were detected after 1 h of incubation. However, the amount of DOPA decreased thereafter due to further oxidation to DQ (Figs. 4 and 5). In contrast a smaller amount of DOPA (residues) was measured when wool protein Wh was used as a substrate.

The simultaneous enzyme treatment of Wh with CA or CHA resulted in two reaction steps. In a first step there was fast increase of DOPA concentration as a consequence of oxidation of wool tyrosine residues followed by a gradual decrease. The decrease of the DOPA concentration after longer incubation time was the consequence of the further oxidation of DOPA to DQ (Fig. 4).

The conversion of DOPA into DQ is shown in Fig. 5. In the case of the Wh substrate mixture with CA or CHA the conversion is increasing during the first 2 h of enzyme treatment and reached the maximum after 5 h of incubation in the case of the Wh/CA mixture. The increase was lower in the case of the Wh/CHA mixture. We noticed also a slight increase in DQ formation after 5 h of enzyme incubation in the case when only Wh was used as substrate, which is the possible result of the small content of tyrosine residues. After 5 h of incubation, the conversion slightly decreased in all cases. The reason for this fact could be found in the supplementary auto-

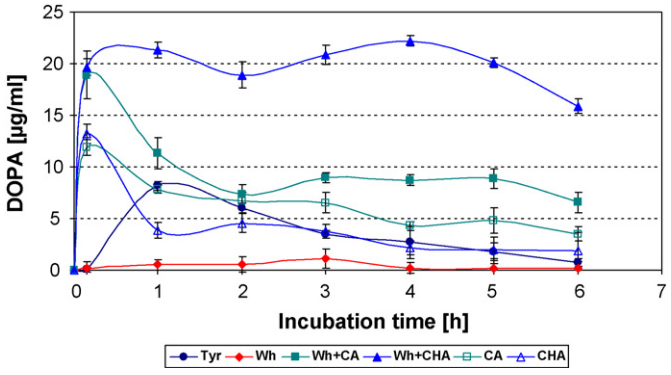

Fig. 4. Conversion of tyrosine residues of different substrates and substrate mixtures (Tyr—tyrosine, Wh—wool protein solution, CA—caffeic acid, CHA—chlorogenic acid) to DOPA.

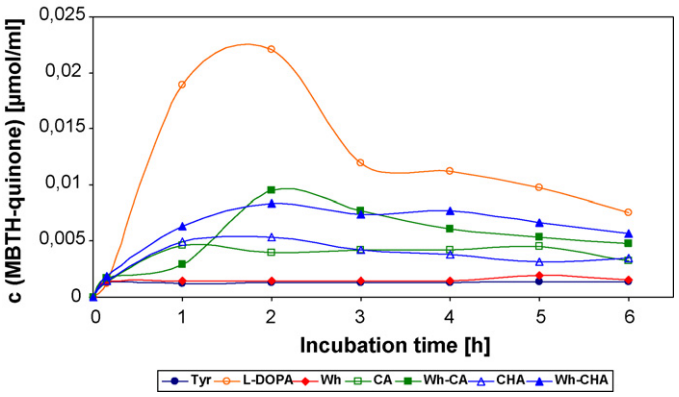

Fig. 5. Enzymatic conversion of DOPA into DQ in different substrates (Tyr—tyrosine, Wh—wool hydrolisate, CA—caffeic acid and CHA—chlorogenic acid) depending on the incubation time.

oxidation of DOPA. Thus, the conversion of DOPA to DQ involves the enzymatic and nonenzymatic mechanism [24].

The enzymatic surface modification of wool fibre was studied using FT-NIR-Raman spectroscopy. Broad and non-symmetrical amide I band with maximum at approximately 1650 cm^{-1} can be utilised as the criterion for the determination protein conformation [33–35]. Compared to untreated wool, changes in this region (1654 cm^{-1}) can be observed for both, the enzyme-treated and phenolics-grafted samples (Figs. 6 and 7). The decrease of the shoulder at 1615 cm^{-1} representing tyrosine and tryptophan of the wool [31] and the decrease of weak bands at 1209 and 1180 cm^{-1} arising from the C_6H_5–C stretching of phenylalanine and Tyr of the wool which are the consequence of enzymatic tyrosine oxidation. Simultaneously, an increase of tyrosine doublets at 852 and 835 cm^{-1} (ring vibration of *para*-disubstituted benzenes) and the appearance of the peak at 650 cm^{-1} (ring deformation) are the evidence of phenolic coupling between the wool tyrosine residue and CA (Fig. 6). These phenomena were also observed when CHA is used (Fig. 7). The presence of a benzene ring is verified by absorption bands at 1460 and 1520 cm^{-1} of aromatic C–C stretching vibrations [35,36]. Accordingly, changes in this region could be due to the presence of additional phenolic residues that confirm the enzyme catalysed coupling reaction of phenolic substrates with tyrosine, primary amine and thiolic groups of the wool protein.

After extreme washing (30 °C and 2 h), the intensity of these peaks is reduced (Fig. 8) and the tyrosine band at 640 cm^{-1} is become more intense.

Functional properties of enzimatically grafted wool fibres by CA or CHA were investigated.

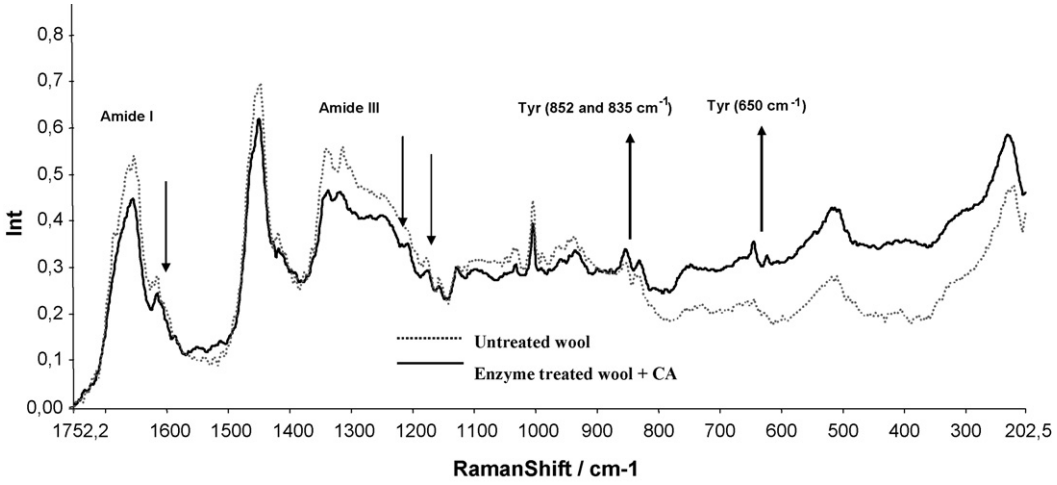

Fig. 6. NIR-Raman spectra of untreated wool (···) and enzymatic treated wool using soluble phenolic substrate CA—caffeic acid (5 mg/ml) (—).

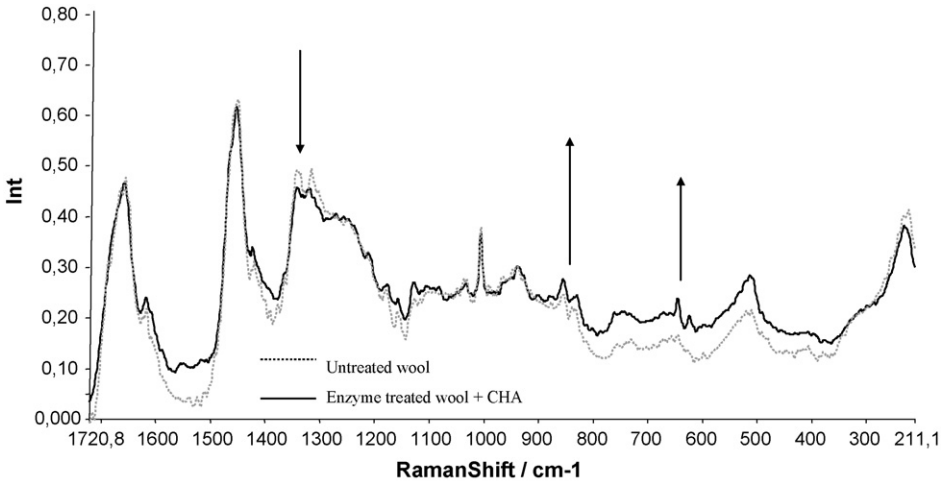

Fig. 7. Raman spectra of buffer treated wool (···) in comparison with tyrosinase treated wool with CHA—chlorogenic acid (5 mg/ml) (—).

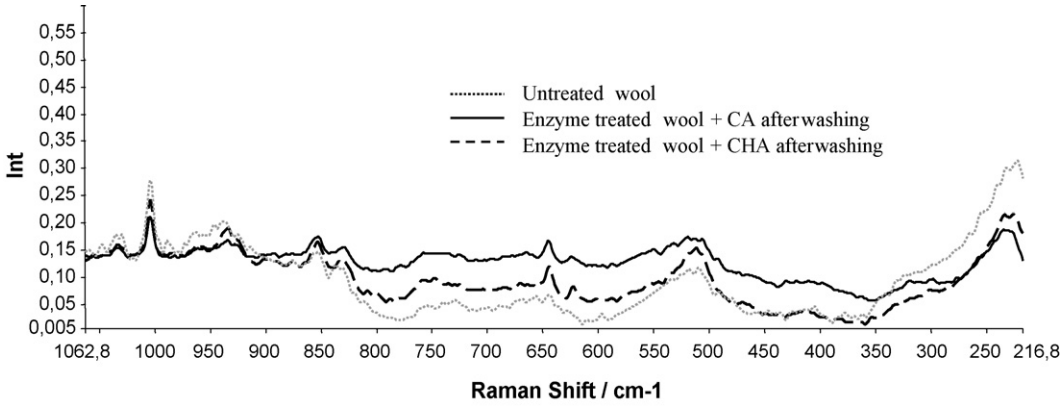

Fig. 8. NIR-Raman spectra of untreated wool and enzymatic treated wool using soluble phenolic substrates (a) CA—caffeic acid (5 mg/ml) and (b) CHA—chlorogenic acid (5 mg/ml) of samples after washing.

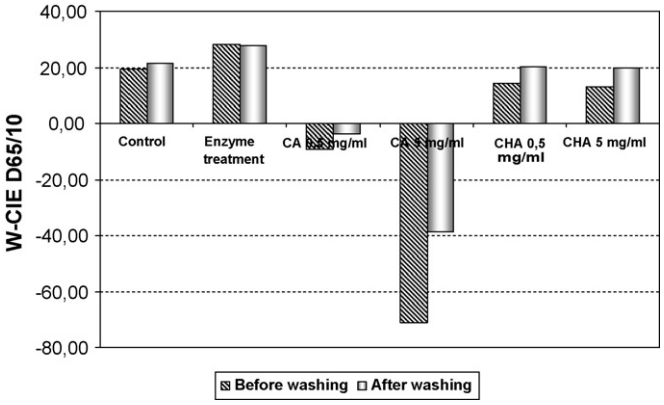

Fig. 9. CIE whiteness of untreated wool (control) and enzymatic treated wool using soluble phenolic substrates CA—caffeic acid (0.5 and 5 mg/ml) and CHA—chlorogenic acid (0.5 and 5 mg/ml) before and after washing procedure.

It is obvious from Fig. 9 that only the treatment of wool using tyrosinase alone significantly increased the whiteness in comparison with the untreated wool (19.3) for almost 46% (28.2). The reason could be found in the elimination of protein-based impurities and pigments from the fibres caused by simultaneously addition of ascorbic acid as a weak reduction agent for wool [11,21]. In the case of simultaneous treatment with CHA, the whiteness was increasing in comparison to the untreated control but it reached a lower level than in the absence of these molecules. The strong decrease of whiteness in the case of treatment with CA (−71.0) was a consequence brown orange colour appearance that increased with higher substrate concentration.

The results summarised in Table 1 shows the colour differences physico-mechanical properties of the fibres after the enzyme treatment and grafting with soluble phenolic substrates CA and CHA.

The enzymatic treatment of the wool fibres and the addition of ascorbic acid as a reducing agent have a considerable effect on the whiteness of the fibre and also on the colour difference ΔE. The highest values of colour change were observed in the case of enzyme-treated sample ($\Delta E_{CA5}{}^{*} = 17.494$), the reason for this phenomena was the intensive orange brown colour appearing during oxidation of CA and the enzymatic catalysed polymerisation of the phenolic substrates among themselves during the incubation. In the case of CHA the differences were also noticeable but to a lower degree. The used concentration of the phenolic substrate has also a slight impact on the level of fibre grafting. On the one hand of the amount of tyrosine residues in wool fibre is limited and on the other they also contain lysine and cysteine residues capable of covalently cross-linking with the phenolic substrates.

Table 1
Physico-mechanical properties of wool and the CIELAB colour difference (ΔE^{*}) of enzyme-treated wool samples using different protein substrates

Samples	ΔE^{*}	K/S	Titer (dtex)	Tenacity (cN/tex)
Native wool	–	0.4866	5.1 ± 1.2	12.5 ± 1.4
Enzyme-treated Wo	4.242	0.2988	4.56 ± 1,67	13.1 ± 1.9
CA (0.5 mg/ml)	4.047	0.5827	4.81 ± 1.54	13.1 ± 1.7
CA (5 mg/ml)	17.494	1.086	4.34 ± 1.55	12.6 ± 2.0
CHA (0.5 mg/ml)	3.980	0.3788	4.49 ± 1.43	12.4 ± 1.6
CHA (5 mg/ml)	1.713	0.266	4.56 ± 1.28	12.5 ± 1.6

The enzyme treatment had a slight impact on the finess and the tenacity of the enzyme-treated wool fibres. The strength and density of the wool fibres described as tenacity and titer of the enzyme-treated wool fibres was also changed. While the titer decreased slightly in all cases, a small increase of the fibre tenacity after the enzyme treatment was observed as the possible consequence of newly generated cross-links between primary amino and thiolic groups of the wool protein [8]. Based on the results of the physical and mechanical properties of the enzyme-treated wool fibre it can be concluded that the grafting with CA and CHA had no significant negative effect on the fibre properties.

The total antioxidant activity of wool proteins grafted with CA and CHA was determined by the ferric thiocyanate method in the linoleic acid system. The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation of Fe^{2+} to Fe^{3+} which is the primary product of oxidation [15,25,32]. The results of activity assays are summarised in Fig. 10 and they devote the inhibition (%) for the oxidation of the linoleic acid media. Untreated wool substrate was used as control. A standard test of antioxidant ability is the action of a substance in inhibiting peroxidation of lipid membranes such as erythrocytes, tissue homogenates, liposomes or microsomes [14]. In order to determine the durability and the effectiveness of enzymatic coupled substrates on the wool fibres, the wool was heavily washed under extreme conditions (2 h and 30 °C). The results of antioxidant activity assays of the samples before (CA and CHA) and after washing and exposure to the xenon light (W-CA, W-CHA, L-CA and L-CHA), were given during the incubation period of 72 h with linoleic acid emulsion. The results are summarized as inhibition (%) data and presented in Fig. 10.

The untreated wool control sample shows an insignificant inhibition (%) effect of 3.2% after 3 h of incubation and it is decreasing during the whole incubation period. The possible explanation for the negative values of decreasing peroxidation inhibition is the fact that wool fibres that we used as control contain fatty matters

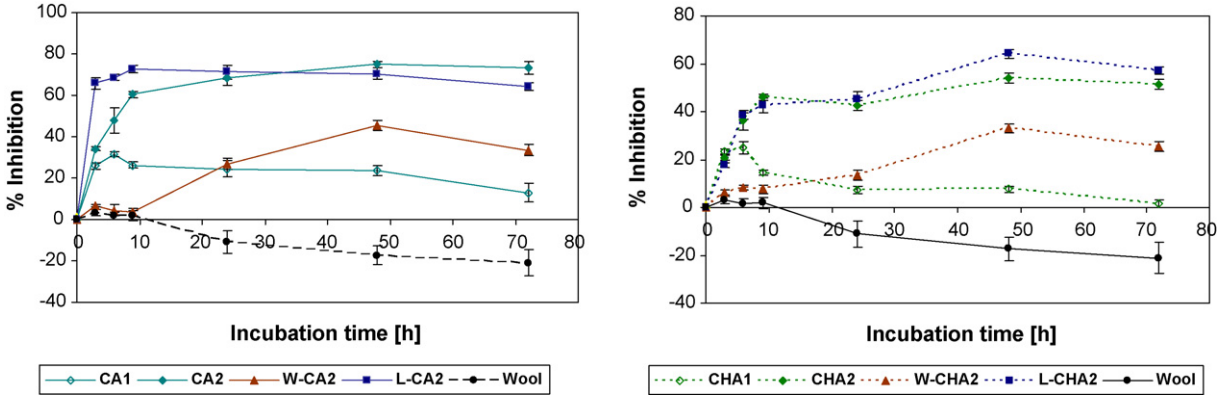

Fig. 10. Total antioxidant activity (AA) as inhibition (%) of the enzyme grafted wool samples with phenolic compounds: (1) 0.5 mg/ml and (2) 5 mg/ml before (CA1, CA2, CHA1 and CHA2) and after washing procedures (W-CA2 and W-CHA2) and the exposure to light (L-CA2 and L-CHA2).

of lanolin (wool grease) and other internal lipids that are peroxidised during incubation. However, the oxidation of linoleic acid was decreased by the enzyme-grafted antioxidants even after a strong washing treatment (W-CA2 and W-CHA2) and the long-lasting exposure to xenon light. This effect was higher when higher concentrations of the antioxidants were used in the grafting. The highest AA showed CA (5 mg/ml) and it was increasing during the whole incubation period and reached the maxima after 48 h (75.2%). After the strong washing procedure the AA of this sample decreased and reached the maximum after 48 h of incubation 45.5% (WCA-5 mg/ml). When the light fastness were assessed the AA of the exposure samples show an increase of antioxidant activity already during the starting incubation period (L-CA2 and L-CHA2). In the case of using CA (5 mg/ml) it reached the maxima after 12 h (72.9%) and in the case of using CHA (5 mg/ml) after 48 h (64.1%). From this point of view we can conclude that the exposure to the xenon light has a synergistic effect of the antioxidant activity and the values increased. One possible explanation for these phenomena is the UV/VIS irradiated decomposition of the surface grafted phenolic residues to more stable quinones as earlier reported by Ilisz and Dombi [36,37].

The lower concentration of used phenolic substrates shows a minor effect of the antioxidant activity, the maxima were reached by the use of CA after 24 h (24.4%) and CHA (7.5%) and also after washing procedure the AA is decreasing (data not shown). The caffeic acid and chlorogenic acid treated wool samples exhibited potent antioxidant activities with 75.2 and 51.4% inhibition of linoleic acid peroxidation, respectively.

4. Conclusion

In the present work, tyrosinase was successfully used to graft the antioxidant phenolic molecules CA and CHA onto wool. The antioxidant activity of grafted wool fibres was indeed enhanced and yielded a modified textile fibre with new properties and characteristic. Various analytic procedures were used to confirm tyrosinase-catalysed oxidation of tyrosine residues in wool and wool hydrolisates.

The NIR-Raman spectra showed changes in the characteristic amid I, and III regions as the consequence of the coupling of phenolics with the wool tyrosine residues and the remnant of the added phenolics (CA and CHA). The decreasing shoulder at 1615 cm^{-1}, assigned to tyrosine and trypthophan amino acids of the wool protein structure, and tyrosine peaks at 1209 and 1180 cm^{-1} are the consequence of enzymatic tyrosine oxidation. In addition, the intensive increase of the tyrosine doublets at 852 and 835 cm^{-1} and the appearance of the peak at 650 cm^{-1} are evident for phenolic coupling between tyrosine/cysteine/lysine residues of the wool protein and CA or CHA. Weak bands at 1208 and 1180 cm^{-1} arising from the C_6H_5–C stretching of phenylalanine and the Tyr mode of the wool substrate are additional absorption bands confirming the enzyme catalysed coupling reaction of phenolic substrates with tyrosine, primary amine and thiolic groups of the wool.

Higher antioxidant activity (75.2%) was obtained by CA when compared to CHA (51.4%). The antioxidant activity of the enzyme-treated wool considerable increase after the exposure to light. The novel approach of wool functionalisation could have a potential in the development of a protective device against degenerative skin disease.

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