# Laccase-catalysed protein–flavonoid conjugates for flax fibre modification

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**Abstract** The introduction of flavonoid compounds into proteins can improve the natural properties of proteins, being promising products which essentially require antioxidant property. The oxidative conjugation of protein–flavonoids was processed by laccase catalysis resulting in the synthesis of biologically functional polymers. The new reaction products were detected in terms of sodium dodecyl sulfate polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionisation-time of flight mass spectra, showing a greater molecular weight formation. Their characterisations were further carried out in terms of UV–Vis spectroscopy, photon correlation spectroscopy, differential scanning calorimetry and Fourier transform infrared (FT-IR) spectroscopy analysis. In addition, their application of protein–flavonoid conjugates onto flax fibres was exploited to supplement a suitable microorganism environment of protein-possessed fibres. The anchoring of conjugates onto cationised fibres was successfully performed by ionic interaction with negatively charged proteins. The level of anchoring efficiency was quantified in terms of measuring colour strength (*k/s*) and fluorescence microscopy analysis. The conjugates onto fibres presented acceptable durability in terms of washing resistance and the surface became hydrophilic when α-casein–catechin was applied (lower contact angle 48°). By the anchoring of protein–flavonoid conjugates onto flax fibres, the final products with new colour generation and antioxidant activity (>93%) were obtained.

**Keywords** Laccase · Protein–flavonoid conjugate · Surface modification · *O*-quinones · Antioxidant activity · Flax fibres

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## Introduction

Enzyme applications are broadly accepted in organic and polymer chemistry due to the environmentally friendly behaviour and resource-saving conditions. Compared with chemical catalysts, enzymes are highly selective and require milder conditions to process, e.g., room temperature, atmospheric pressure and neutral pH (Gübitz and Cavaco-Paulo 2003; Kobayashi and Higashimura 2003; Reihmann and Ritter 2006). Laccases (EC 1.10.3.2) are multicopper oxidases able to oxidise various aromatic and phenolic compounds with or without mediators (Chung et al. 2003; Claus 2004; Gianfreda et al. 1999). The phenolic substrates are oxidised, yielding resonance stabilised phenoxy radicals through one-electron transfer process. These radicals further play a role as coupling sites for other reactant phenoxy radicals (Mattinen et al. 2008; Mayer and Staples 2002). In addition, laccases are found to oxidise several amino acids such as tyrosine, tryptophan and cysteine, resulting in peptide polymerisation and in the cross-link of certain proteins as well as their fragmentation by radical generation reaction (Chung et al. 2003; Selinheimo et al. 2008; Steffensen et al. 2008). In the cross-link of the tyrosine-containing peptides, the proceeding of tyrosyl radicals was suggested to isodityrosine and small portion of dityrosine bonds (Mattinen et al. 2005). The oxidation of cysteines into cystine resulting in the formation of disulfide bonds is another example of cross-link of proteins by laccase catalysis (Labat et al. 2000). In those two amino acids oxidised by laccase catalysis, ferulic acid was generally used as a reaction accelerator. Comparatively, proteins showed poor reactivity with laccase due to the limited accessibility of reactive amino acid residues that are subjected to steric hindrance (Cura et al. 2009; Labat et al. 2000; Mattinen et al. 2005). For this reason, the presence of small molecules of mediator or auxiliary substances, easily oxidised by laccase

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and that react with the target substrates, is required (Cura et al. 2009).

Proteins such as collagen, gelatin, casein, zein, elastin and albumin are widely used in medical textiles for wound healing, tissue culture, tissue repair, etc. (Petrulyte 2008). The fabrics used for wound care can be divided into biodegradable and non-biodegradable, and natural cellulosic fabrics are preferred due to the shorter time degradation as opposed to synthetic fabrics. However, natural fibres provide a suitable environment for microorganism growth since they have a large surface area and potential to retain moisture that might be incremented in the presence of protein at the surface. Numerous chemicals have been subjected to an attempt to yield antimicrobial and antioxidant activity in spite of their toxicity and harmfulness to humans. With the effort of seeking non-toxic and non-harmful materials, flavonoids are getting much attention as potential chemical substitutes due to their high antioxidant property. Flavonoids are presented in the form of β-glucosides and are divided into four main groups: flavones, flavonols, flavonones and isoflavones based on their basic molecular structure (Heim et al. 2002; Kanaze et al. 2006; Kurisawa et al. 2003; Pietta 2000). With their specific properties, they are potentially useful for human health as protectors, e.g., anti-carcinogens, anti-inflammatory agents, as well as inhibitors of platelet aggregation in 'in vivo' and 'in vitro' aspects (Andersen and Markham 2006; Pietta 2000). Most flavonoid applications are related to their two fundamental properties, i.e., their antioxidant properties by electron and H-atom donation and their ability to interact with proteins (Andersen and Markham 2006; Arts et al. 2002). There are some studies focused on the interactions between flavonoids and proteins that resulted in the improvement of antioxidant properties of proteins, suggesting the possible applications in the medical, cosmetic and food industry (Kanakis et al. 2006). Through an antioxidant supply to proteins, it is possible to protect the oxidative damage involved in free radicals, which have a noxious effect on cells, thus causing the etiology of several diseases (Andersen and Markham 2006; Arts et al. 2002).

In this study, protein–flavonoid conjugates were produced by enzymatic radical generation and non-enzymatic covalent bonding, and their attachment was carried out onto both cationised and non-cationised flax fibres under mild conditions. Flax is a natural lignocellulosic fabric mainly consisting of cellulose compounds. Generally, phenolic polymers generated by laccase do not have affinity towards cellulose, resulting in non-covalent fixation (Hadzhiyska et al. 2006). Raw flax, however, contains the aromatic lignin compounds at the surface that are responsible for the natural colouration of the fabrics and can be removed by bleaching processes. In this research, the natural lignin compounds obtained after scouring were used for providing graft points for protein–flavonoid conjugate attachment. Laccase oxidises not only flavonoid phenolic compounds (catechin, quercetin) but also the phenolic lignin compounds of flax fabrics to reactive *o*-quinones (Mattinen et al. 2008). Therefore, the grafting points (reactive quinones) can further chemically react with the functional groups of amino acids in proteins or flavonoid *o*-quinones via enzymatic or non-enzymatic pathways.

A medium-sized random coil protein, α-casein and a large globular protein, bovine serum albumin, were studied as protein models. Catechin and quercetin were used as flavonoids. The molecular mass distribution of reaction products was analysed with matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Furthermore, the newly obtained protein–flavonoid conjugates were instrumentally analysed in terms of UV–Vis spectrophotometer, photon correlation spectroscopy (zeta-potential), differential scanning calorimetry (DSC) and fourier transform infrared spectroscopy (FT-IR) analysis. The level of conjugates binding onto flax fibres was estimated in terms of colour strength determination and fluorescence microscopic analysis. The surface properties like hydrophilicity and antioxidant behaviour were also quantified.

## Material and methods

### Enzyme, fabric and chemicals

Laccase (EC 1.10.3.2) from the ascomycete *Myceliophthora thermophila*, Novozym®51003 (340 $UmL^{-1}$ at RT), was obtained from Novozymes (Bagsvaerd, Denmark). The 100% raw woven flax fabric having 14/14 yarns (warp/weft)$cm^{-1}$ was kindly supplied by the "Institute of Natural Fibres" (Poland) and used for modification after alkaline scouring. Bovine serum albumin (BSA) and α-casein from bovine milk were commercially obtained from Sigma-Aldrich and used without further purification. Catechin, quercetin and other chemicals were purchased at high level of purity from Sigma-Aldrich. The anionic polyelectrolyte, poly(diallyldimethylammonium-chloride) (PDDA <10,000 MW) was obtained from Aldrich in liquid form.

### Laccase activity

Standard assays of laccase activity were performed spectrophotometrically by measuring the enzymatic oxidation of 0.5 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid (ABTS) at 420 nm. The molar extinction coefficient for the oxidation product was $3.6\times10^{4}$ $cm^{-1}$ $M^{-1}$. One unit of laccase (*U*) was defined as the amount of enzyme required to oxidise 1 μmol of ABTS per minute at room temperature (Wolfenden and Willson 1982). Laccase was diluted with 0.1 M acetate buffer at pH 5.

Cationic layer deposition onto flax fabric (cationisation)

The cationisation of flax fabric was performed using PDDA. The scoured flax fabrics were incubated in 2 $gL^{-1}$ of PDDA solution at 20:1 (liquor to fabric ratio) using Rotawash MK II apparatus (SDL International Ltd., Stockport, UK) for 30 min at 60°C. In order to validate the cationic layer deposition, the fabrics were further dyed with Coomassie brilliant blue dye G-250, which has affinity to positively charged groups.

Enzymatic conjugation of protein–flavonoid and their anchoring onto flax fibres

α-casein and BSA solutions were prepared in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution, pH 7.4 with protein concentration at 5 mg $mL^{-1}$ (obtained by Bradford method: Bio-Rad protein assay cat. No. 500-0006). The oxidation reaction was initiated by adding 2 $UmL^{-1}$ laccase in the presence and absence of flavonoid compounds. Flavonoid solutions were prepared with 10% ethanol in HEPES buffer (pH 7.4) (*v*/*v*). The incubation was carried out using a water bath at 120 rpm, for 24 h at 50°C. Control samples were also incubated under the same conditions in the absence of laccase. The anchoring of conjugates was carried out by incubating flax fabrics in previously prepared solutions containing 5 mg $mL^{-1}$ protein and 10 mM flavonoid in the presence and absence of laccase. Fabric samples were then washed in non-ionic surfactant solution (Lutensol AT 25, 1 $gL^{-1}$) at 20:1 (liquor to fabric ratio) for 10 min at 80°C using a Rota wash in order to remove the unlinked protein and flavonoids as well as any laccase still remaining at the fabric's surface.

Characterisation of protein–flavonoid conjugates

*SDS-PAGE analysis*

The molecular weight distributions of the reaction products were detected by SDS-PAGE analysis according to the Laemmli method (Laemmli 1970). For the molecular mass estimations, 15% separating Tris–HCl gels (Bio-Rad, Richmond, CA) were prepared with wide range of molecular mass standard 7–206 kDa (Bio-Rad, Richmond, CA). The visualisation of proteins was performed with gel staining using Coomassie Brilliant Blue dye overnight.

*Mass spectrometry analysis*

The mass spectra of the reaction products of BSA and catechin incubated under enzymatic oxidation were analysed by MicroFlex MALDI-TOF mass spectrometry (Bruker Daltonics, German). Before collecting the samples for analysing, laccase was separated and eliminated from solutions by centrifugation with an Amicon ultra centrifugal filter (Ultra-15 MWCO 100 kDa). The samples were prepared with 2,5-dihydroxyacetophenone (DHAP). As matrix solution, a saturated solution of 0.1% (*v*/*v*) trifluoroacetic acid (TFA) in 50% acetonitrile was prepared. This matrix is mainly used for in-source-decay experiments on intact proteins.

*UV–Vis spectrophotometer analysis*

The molecular rearrangement of proteins by laccase catalysing oxidation was monitored with UV–Vis spectrophotometry using a diode-array J&M Tidas spectrophotometer (J&M Analytische Mess- und Regeltechnik GmbH, Esslingen, Germany). Spectra were collected at room temperature before and after adding laccase. After laccase introduction, the changes of spectra were followed at different time points to a maximum of 6 h.

*Zeta-potential determination*

The zeta-potential values were obtained using a Zetasizer Nano Series (Malvern Instruments Inc., Worcester, UK) by Dynamic Light Scattering (DLS) technique. The data were collected in terms of "Electrophoretic Mobility" degree. All the processes were carried out before and after enzymatic reaction, and the mean values were presented after triplicate measurements.

*Thermal analysis*

The oxidised solutions of protein–flavonoid conjugates were filtered using PD-10 columns packed with Sephadex™ G-25 to remove un-grafted flavonoids. The solutions were then lyophilised for complete removal of solvent. DSC (METTLER-TOLEDO, United States Co., Columbus, OH) measurements were carried out with a temperature range of 20 to 300°C with heating rate of 5°C/min and nitrogen gas passing at a flow rate of 60 mL/min. Lyophilised solid samples (2 mg) were taken, and their thermal behaviours were analysed. The weight of samples used for analysis was applied for the calculation of enthalpy changes ($\Delta H$).

*FT-IR spectroscopy analysis*

FT-IR spectra were taken with a Perkin Infrared Spectrophotometer. Before collecting, the background scanning was performed using KBr powder. The lyophilised samples were mixed with a small amount of KBr that was used as

matrix. At least 32 scans were obtained to achieve an adequate signal to noise ratio. The spectra were taken in the region of 450–4000 $cm^{-1}$ with a resolution of 8 $cm^{-1}$ at room temperature.

## Characterisation of protein–flavonoid conjugates anchoring to flax fabrics

### *k/s estimation*

The anchoring level of protein–flavonoid conjugates to flax surface was estimated by the measurement of the colour generation of the phenolic compounds oxidised by laccase catalysis (Kim et al. 2007; Kim et al. 2008). The level of attachment is directly proportional to the amount of flavonoid grafted onto the surface. *k/s* values were spectrophotometrically obtained by Datacolor apparatus at standard illuminant D65 and represented by the Kubelka–Munk's equation (Eq. 1). The data are the sum of all *k/s* values obtained at wavelength range (400~700 nm). The samples were measured by triplicate, and the values presented are the mean among them.

$$\frac{k}{s} = \frac{(1-R)^2}{2R} \tag{1}$$

Equation 1—Kubelka–Munk equation: where $k$ is the absorbance coefficient, $s$ is the scattering coefficient and $R$ is the reflectance ratio.

### *Fluorescence microscopy*

Flax samples treated with protein–flavonoid conjugates in the presence and absence of laccase were analysed using a fluorescence microscope LEICA DM 5000B. To visualise the proteins bound to fabrics under fluorescence microscopic, fluorescein isothiocyanate (FITC) conjugated to bovine albumin was introduced as protein substrate during samples incubation. The conditions for fabric incubation were applied as previously described. The samples were incubated overnight without light exposure. Scoured flax samples (cationised and non-cationised) were also analysed as references.

### *Contact angle*

The water contact angle was measured with a deionised water droplet on the treated fabric surfaces at room temperature. The data were determined by averaging values after measuring at three different points on each fabric sample. A dosing volume of water droplet was set as 15 μL using Hamilton 500-μL syringe type. The measurement conditions were selected as Ellipse fitting.

### *Antioxidant activity measurements*

The antioxidant activity of treated flax fabrics was evaluated according to ABTS radical cation decolourisation assay method (Re et al. 1999; Sousa et al. 2009). The $ABTS^{\bullet+}$ were produced by reaction with potassium persulphate in distilled water and stored for 12 h in the dark at room temperature. Fabric samples (30 mg each) were inserted in 2 ml of diluted $ABTS^{\bullet+}$ (absorbance 0.7± 0.02 at 734 nm) solution. After incubation of samples for 30 min, the absorbance of $ABTS^{\bullet+}$ solutions was measured at 734 nm. The $ABTS^{\bullet+}$ scavenging capacity of fabrics was obtained with Eq. 2.

$$\text{Antioxidant activity}(\%) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100 \tag{2}$$

Equation 2—evaluation of radical scavenging effect.

## Results

The molecular mass distributions of reaction products of protein and flavonoid by enzymatic catalysis were detected with SDS-PAGE analysis (Fig. 1). When laccase was applied in protein solutions without phenolic molecules, the fragmentation of α-casein was detected showing new bands below 29 kDa (line 4 in gel (a)). In the case of BSA, both fragmentation and cross-link were not detected by enzymatic oxidation (line 4 in gel (b)). This might be due to the different structure presented by α-casein and BSA proteins. When flavonoid molecules were included in the enzymatic process, band migration to higher molecular mass was detected in both α-casein and BSA (line 6 and 8 in gel (a) and (b)). The results demonstrate that both catechin and quercetin flavonoids acted as cross-linking agents, promoting the formation of new dimers or polymers. Laccase from ascomycete *Myceliophthora* with a molecular weight around 80~85 kDa was inserted in each gel (a) and (b) at line 3 (2 $U mL^{-1}$ of laccase in HEPES buffer (~0.006 mL $mL^{-1}$)). However, due to the low amount of enzyme used, no protein was detected.

MALDI-TOF mass spectra was also performed to analyse the reaction products (Fig. 2). In this study, BSA and catechin interaction in the presence of laccase was mainly carried out and the interesting peak was presented near molecular weight 100 kDa (Fig. 2a). BSA monomer has molecular weight near 67 kDa (presented in Fig. 2b). The peak of BSA–catechin conjugate observed in Fig. 2a indicates higher molecular weight than BSA monomer. As a result of oxidative conjugation, the difference of molecular weight (~33 kDa) between BSA monomer and BSA–

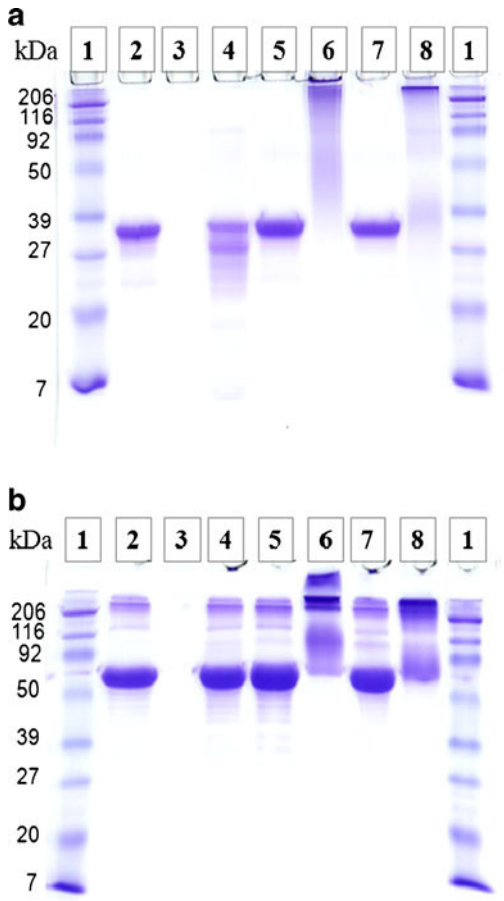


**Fig. 1** SDS-PAGE analysis of incubated proteins; α-casein: Gel (**a**) and BSA: Gel (**b**) proteins. *Line 1*: molecular weight standard 7–206 kDa; *line 2*: proteins; *line 3*: laccase (2 $UmL^{-1}$); *line 4*: protein incubated with 2 $UmL^{-1}$ of laccase; *line 5*: protein incubated with catechin; *line 6*: protein incubated with catechin and laccase (2 $UmL^{-1}$); *line 7*: protein incubated with quercetin; *line 8*: protein incubated with quercetin and laccase (2 $UmL^{-1}$) for 24 h

catechin conjugates might correspond to the conjugated catechin polymers into BSA monomers.

The laccase-catalysed reactions of reactive *o*-quinone of catechin and the following coupling reactions with the reactive amino acid groups in proteins were spectrophotometrically studied using a UV–Vis spectrophotometer. The spectra were collected before and after laccase introduction in each protein and catechin reaction solutions (Fig. 3). The laccase-catalysed protein–catechin solutions turned to dark brown, and a new band emerged at around 450 nm resulting from enzymatic oxidation. Moreover, an increase of the band intensity in the visible area was detected in both α-casein and BSA incubated with catechin and laccase (Fig. 3a, b). In the UV area, the intensity of the two significant bands in both proteins (i.e., approximately at 238 and 274 nm) increased with the oxidation time.

As controls, α-casein–catechin and BSA–catechin solutions were also spectrophotometrically analysed for 6 h without laccase addition in the reaction medium (Fig. 3c). Different from Fig. 3a, b, there were no changes of peaks' shapes and intensity in both UV and visible area showing a maintenance of peaks shape from 0 time to final time of incubation.

The surface electrical properties of proteins were analysed in terms of zeta-potential measurement in aqueous reaction medium and presented in Table 1. In this experiment, the effects of enzymatic oxidation and of the binding of flavonoids to protein structure on the electrical property of proteins were studied. During measurements, the samples were diluted ten times using HEPES buffer and the experiments were carried out at room temperature.

α-casein and BSA protein values in a HEPES buffer solution at pH 7.4 presented zeta-potential (ζ) values at around −31 and −29 mV, respectively. Before introduction of laccase in the reaction medium, the ζ absolute values of both α-casein and BSA decreased temporarily in the presence of phenolic molecules, e.g., catechin and quercetin. However, after an enzymatic incubation at 50°C for 24 h, the ζ absolute values of proteins were differently modified. In the case of α-casein, a high increase of ζ absolute values was detected in the case of incubation with flavonoids in the presence of enzymatic oxidation.

In this work, the thermal behaviour of proteins conjugated with flavonoids was studied by laccase catalysis with DSC analysis (Fig. 4) in order to characterise their stability and binding properties.

Control samples of α-casein and BSA proteins without flavonoid or laccase were also prepared by lyophilisation after incubation in HEPES buffer solution pH at 7.4, presenting an intense peak near 82.9°C and 84.0°C, respectively. These peaks are attributed to the denaturation of proteins caused by heating. The oxidised protein–flavonoid conjugate samples reveal a shift of the peak to slightly higher temperatures. In the case of α-casein, the two endo peaks in both catechin and quercetin conjugated were detected.

FT-IR microscopy was used for the analysis of the chemical bonds formed by laccase-catalysed reaction protein–flavonoid conjugate (Fig. 5). Both α-casein and BSA presented typical protein bands at 1,650 and 1,540–1,530 $cm^{-1}$ attributed to amide I and amide II, respectively. Two absorption bands were observed near 1,180 and 1,040 $cm^{-1}$ corresponding to the C–N stretching or C–O stretching bonds of proteins. The intensity of these characteristic bands decreased after enzymatic incubation with laccase, meaning that new products were formed by enzymatic oxidation. Also, other characteristic protein bands at 2,830 and 3,000 $cm^{-1}$ attributed to an aliphatic and aromatic C–H stretching bond and broad O–H stretching bond were detected, and no modification after enzyme addition was observed. In addition to the above-

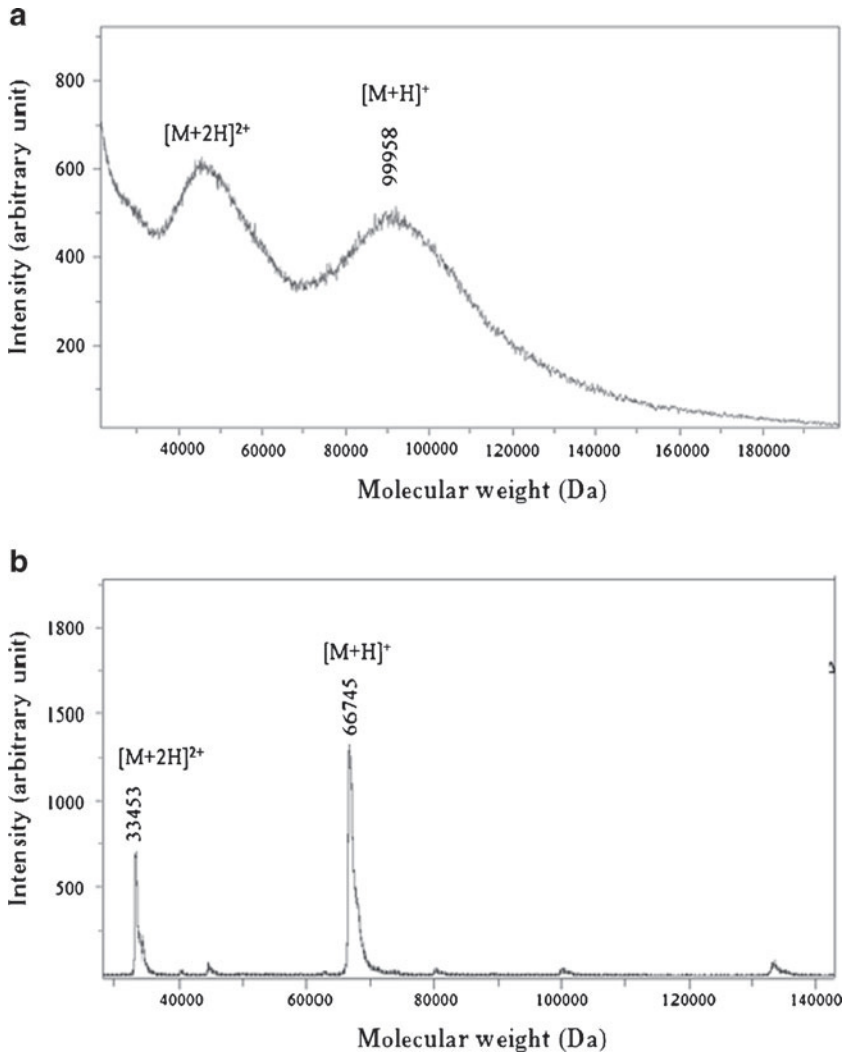


**Fig. 2** MALDI-TOF mass spectra measured from BSA–catechin conjugates obtained by laccase catalysis of BSA–catechin conjugates (**a**) and BSA monomers (**b**). [*M*] represents a neutral BSA–catechin conjugate molecule in (**a**) and a neutral BSA monomer molecule in (**b**)

described spectral properties, a weak carbonyl band of ester bond near 1,770 cm $^{-1}$ attributed to C=O stretching vibration bond was detected.

In this study, the anchoring of new polymers resulting from laccase-catalysed oxidation was carried out onto flax fabrics. The anchoring process was performed onto flax fabrics previously cationised with PDDA and non-cationised.

Firstly, the cationisation was carried out in order to positively increase the fabric surface charge, improving the affinity of the protein–flavonoid conjugates. The cationic layer deposited onto flax fabrics was evaluated using Coomassie blue dye colouration. This dye is commonly used for staining and detecting proteins due to its affinity with amino groups (Georgiou et al. 1996). Coomassie dye is negatively charged and stable in anionic form in solution; therefore, it can have a broad affinity with any type of protonate amino groups. Thus, positively charged flax fabrics can react strongly with Coomassie blue dye through ionic interaction. The colour strength of flax fabrics after colouration with Coomassie dye was measured in terms of *k*/*s*. The *k*/s values are related to the amount of dye absorbed by the surface of the fabrics. Compared with the non-cationised fabrics, cationised ones presented remarkable colour strength values taken at maximum Coomassie dye absorption (*k*/*s* at 600 nm: cationised fabric 12.22: non-cationised fabric formed 1.05). After incubation of flax fabrics with protein–flavonoid solutions, colour generation resulting from catechin or quercetin oxidation by laccase was evaluated by means of *k*/*s* determination. The values are presented as checksum of *k*/*s*, obtained in the visible region from 400 to 700 nm (Fig. 6). Colour generation is proportional to the amount of conjugate attached to the surface of the fabrics and can be presented as a measurement of the anchoring efficiency.

The *k*/*s* results also present the effect of reaction factors, e.g., the sort of proteins and flavonoids, enzymatic reaction, and the charge property of flax fabric.

Comparatively to polyquercetin, polycatechin presented higher *k*/*s* values indicating higher polymerisation level and

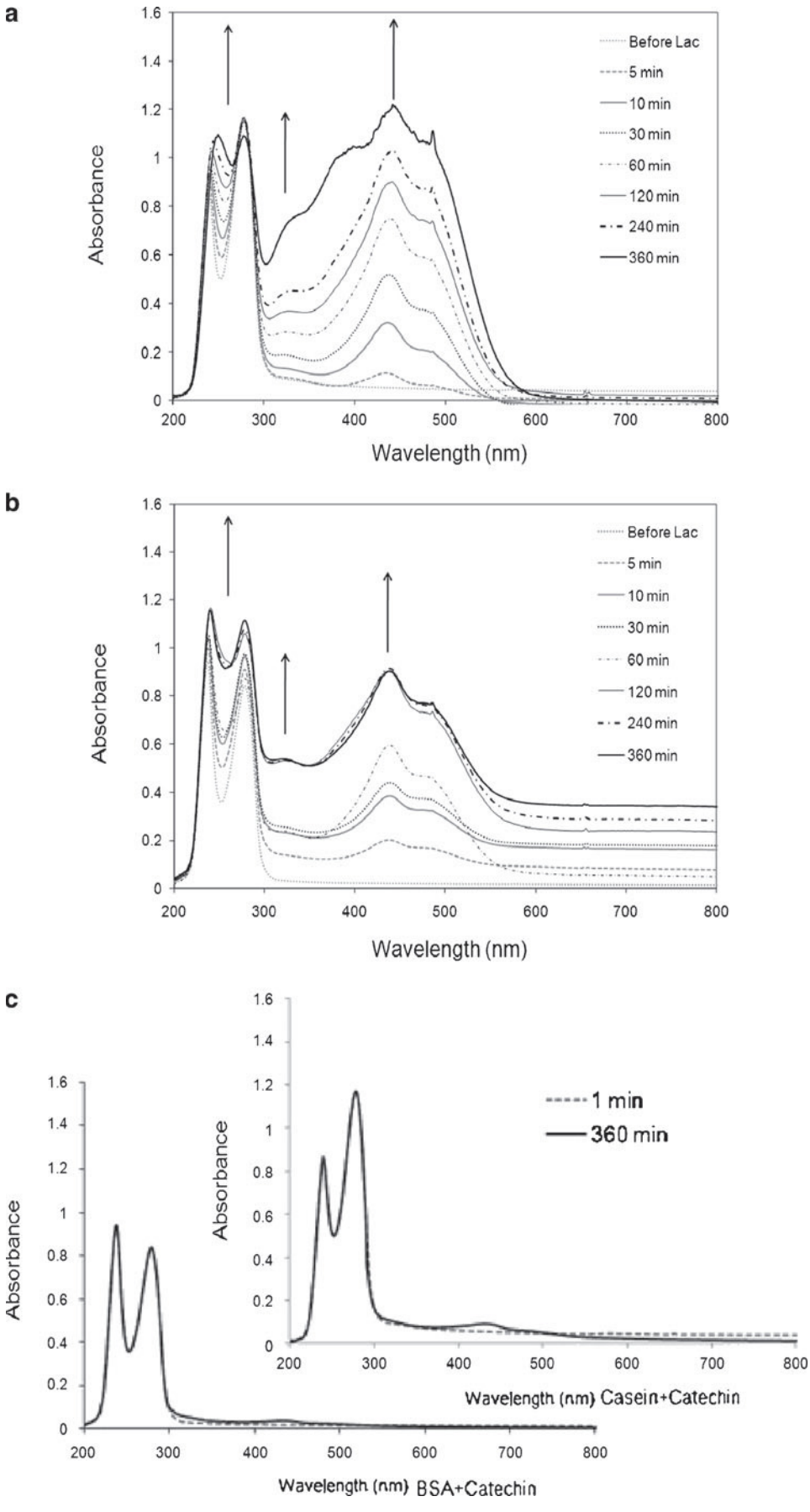


**Fig. 3** UV/Vis spectrophotometric analysis of the oxidation reaction of α-casein incubated with catechin and laccase (**a**), BSA incubated with catechin and laccase (**b**). Spectra were collected at different time points till 6 hours. The three positions where the intensity of band increases or new band emerges with oxidation time are pointed by *arrows*. Controls were presented on graph (**c**); α-casein + catechin and BSA + catechin incubated without laccase

**Table 1** Zeta potential (ζ) values of proteins obtained before and after enzymatic oxidation

| | Casein | Casein+catechin | Casein+quercetin |
|---|---|---|---|
| Before IC | −30.9±0.2 | −27.8±3.5 | −29.1±0.7 |
| After IC | −29.3±0.3 | −37.5±3.8 | −35.2±3.2 |
| | BSA | BSA+catechin | BSA+quercetin |
| Before IC | −29.1±0.6 | −24.2±0.7 | −26.0±2.8 |
| After IC | −27.4±5.6 | −23.9±2.5 | −26.7±5.2 |

*IC* incubation with laccase (2 $UmL^{-1}$) for 24 h at 50°C

affinity to flax fabrics. The polyphenolic compounds also presented higher affinity to fabrics in the absence of protein in the conjugate. In the case of the protein–flavonoid conjugate, some grafting points are occupied by the functional groups of the protein (BSA, α-casein). Therefore, the amount of possible sites for polymer attachment decreased. The type of protein used, α-casein or BSA, was a determinant factor for the difference obtained for catechin conjugates. The conjugate BSA–catechin and polycatechin presented higher *k*/*s* difference (30%) than the α-casein–catechin conjugates and polycatechin (14%) on the surface of cationised flax fabrics. As previously stated, a higher level of oxidation was achieved when α-casein is present in the conjugate, influencing therefore the amount of product that attaches onto flax fabrics. In the case of quercetin, the *k*/*s* results showed very similar behaviour in both α-casein and BSA presenting 30% of colour strength difference between protein–quercetin conjugates and polyquercetin (Fig. 6a, b). A remarkable increase of *k*/*s* values (2~4 times higher) was exhibited by the cationised samples in comparison to the non-cationised samples. The results also show low levels of protein–flavonoid conjugate attachment when the grafting points obtained by laccase catalysis of lignin were only available (non-cationised).

The measurement of *k*/*s* of samples was efficient to prove the attachment of phenolic compounds onto flax fabrics. However, a more accurate technique needed to be applied to detect protein attachment. A fluorescence microscopic analysis was performed to detect proteins bound onto flax fabrics using FITC conjugated with BSA (Fig. 7). The incubation process was carried out under the same conditions as previously described, without light exposure. The scoured and cationised flax samples were also analysed using fluorescence microscopic as references. The samples coated with proteins in the absence of laccase were also analysed.

From the observation of Fig. 7, it can be verified that high fluorescence is obtained for the samples that were previously cationised and incubated under enzymatic oxidation ((A)-d through (B)-d). The cationic layer deposited is the determinant factor for protein attachment where the negatively charged proteins can react by ionic interaction.

The resistance of conjugates binding onto fabrics was evaluated in this work in terms of washing fastness. The standard test method for colour fastness used for domestic and commercial laundering (ISO 105-C06 2010) was followed, and the results were presented in Table 2 as the percentage values of colour vanishing after washing and air drying.

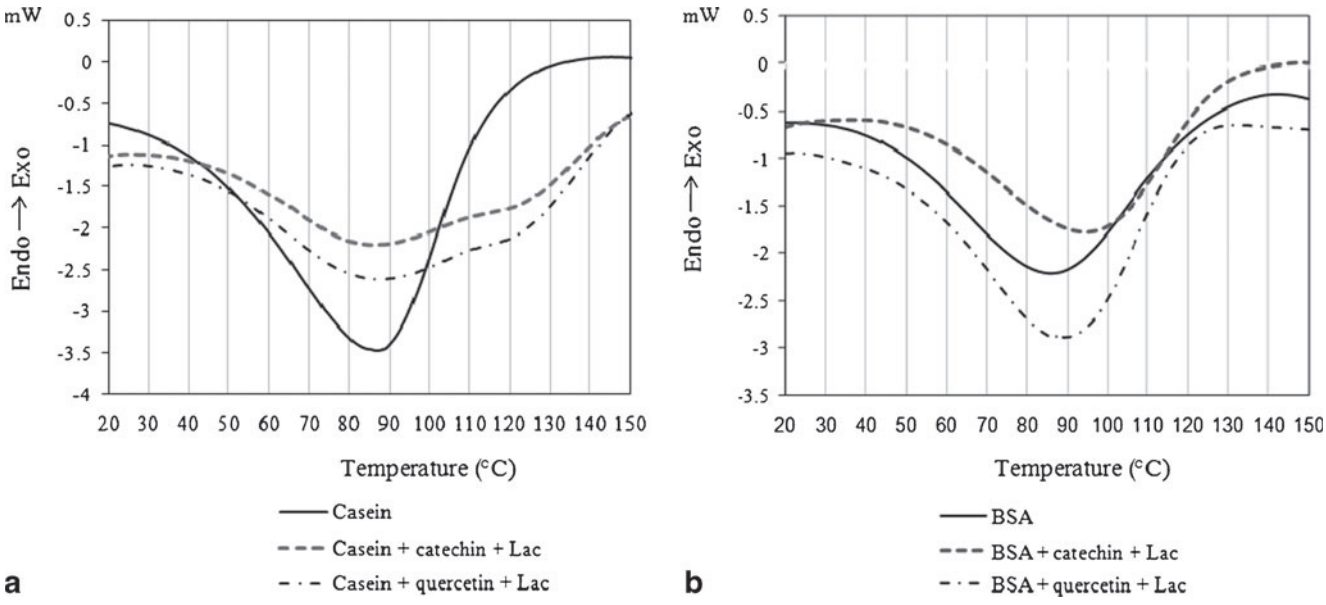


**Fig. 4** Thermal behaviour of proteins analysed by DSC. Exo-thermal peaks of α-casein and α-casein+catechin with and without laccase addition (**a**), and BSA and BSA+catechin with and without laccase addition (**b**) in the reaction medium are presented

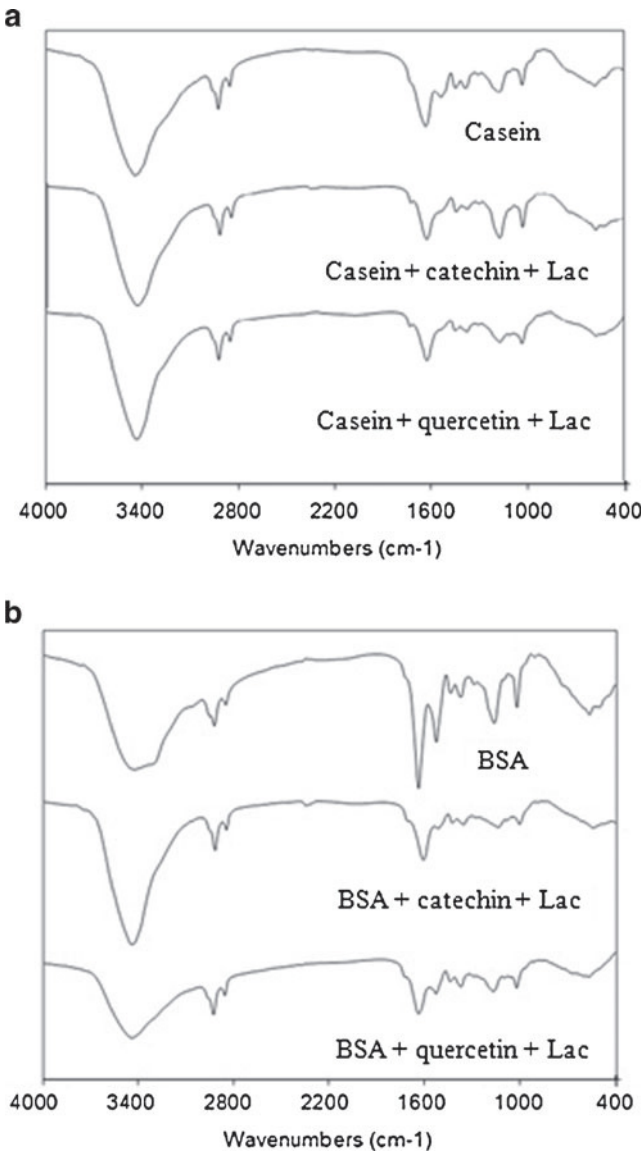


**Fig. 5** FT-IR spectra of α-casein–flavonoid (**a**) and BSA–flavonoid (**b**) conjugates obtained by laccase oxidation. *Lac*—laccase

Following the results obtained for the washing fastness test, it was possible to affirm that modifications on the levels of water absorption could be obtained after protein–flavonoid conjugate deposition.

In accordance with the results of the washing fastness test, the conjugates produced by laccase catalysis are quite resistant to the washing process due to their low solubility (Hadzhiyska et al. 2006). Moreover, the polyelectrolyte assembled at the surface can modify not only the properties of conducing dyes, polymers or biomaterials but also the hydrophilic/hydrophobic property of the surface (Polowinsky 2005). The values of water absorption were obtained by contact angle measurement of coated samples, and the scoured and cationised flax samples were taken as references.

After attachment of protein–flavonoid conjugates onto flax fabrics, the samples presented variations in terms of their hydrophilicity. The water contact values obtained show that the cationisation process increases the hydrophobicity of the samples (contact angle 120±1°) (Fig. 8). The scoured flax fabrics incubated in protein–flavonoid solutions in the presence and absence of laccase showed no changes of hydrophilic property, and water contact angles were very low (~25±3°, both right (R) and left (L): Fully wetting).

Flavonoids have the property of inhibiting autoxidation and scavenging of free radicals. For this reason, the antioxidant ability was expected to be present in protein–flavonoid conjugates attached onto flax fabrics and evaluated by following the $ABTS^{\bullet+}$ cation decolourisation assay method and presented as the $ABTS^{\bullet+}$ cation scavenging capability (Table 3) (Re et al. 1999; Sousa et al. 2009). Scoured or cationised samples, with non-attachment of conjugates presenting 0% of antioxidant activity, were considered as controls.

Superior capacity level of the $ABTS^{\bullet+}$ cation scavenging was detected on previously cationised fabrics as opposed to non-cationised ones. The polyquercetin developed much higher antioxidant activity on flax fabrics than polycatechin using both α-casein and BSA proteins (>93%).

## Discussion

Laccases have been reported to cross-link or polymerise specific milk and cereal proteins. These reactions are highly enhanced in the presence of small phenolic molecules, which play an important role as a binding agent between protein molecules (Mattinen et al. 2005; Selinheimo et al. 2008; Steffensen et al. 2008). In this work, the flavonoids were newly subjected to an attempt to develop molecular modification of proteins with recourse to the cross-link method giving rise to higher molecular weight products compared to former monomers.

The hydroxyl group of B-ring in flavonoids, possessing electron-donating properties and being a radical target, formed phenolic radicals by laccase catalysis. The phenolic radicals may react with a second radical to form *o*-quinones, which are highly reactive electrophilic molecules and spontaneously polymerise in a non-enzymatic pathway (Kurisawa et al. 2003; Shin et al. 2001). Besides the reaction with themselves, the *o*-quinones are also covalently cross-linked with functional groups in proteins, e.g., sulfhydryl, amine, amide, indole and imadazine substitutes by Schiff bases or Michael addition reactions (Bittner 2006). The expected schematic coupling reaction between reactive *o*-quinones enzymatically produced from flavonoids and reactive amino acid groups in protein structures is shown in Fig. 9.

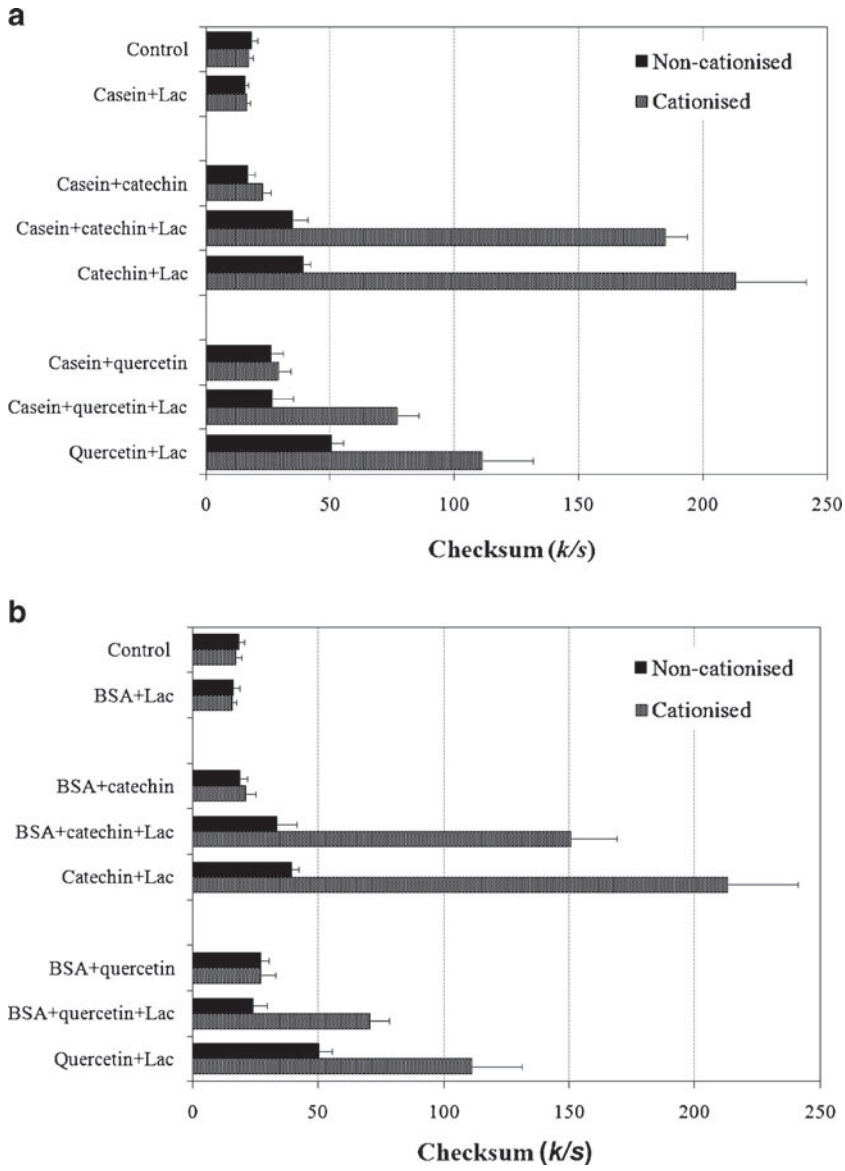


**Fig. 6** Estimation of conjugate binding onto flax fabrics measured in terms of colour strength (*k/s* values were obtained and summed in the visible range from 400 to 700 nm): (**a**) α-casein–flavonoid conjugate; (**b**) BSA–flavonoid conjugate

In this work, the reactive *o*-quinone of catechin and the following coupling reactions with the reactive amino acid groups in both proteins by laccase-catalysed oxidation was detected by UV–Vis spectrophotometry analysis showing a new band emerging near 350 nm (Fig. 3). The intensity of the band consistently increased with the level of cross-linking between amino acid groups and *o*-quinones by Michael addition reaction (Anghileri et al. 2007; Sousa et al. 2009). Changes of band shape and new bands emerging in ultraviolet and visible areas by laccase catalysis were commonly detected in both α-casein and BSA. However, the level of intensity increase was greater in the case of α-casein–catechin oxidation by laccase than BSA–catechin. An explanation for this has to do with the protein structure that is less accessible to oxidation than α-casein. Therefore, low amounts of amino acids are available to react with the *o*-quinone of flavonoid. As a result of the Michael addition reaction, the molecular weight increase was detected by SDS-PAGE analysis (Fig. 1) and MALDI-TOF mass spectra (Fig. 2).

The charge generation on proteins is mainly determined depending on the amino acids' locations at the surface of their structure. However, other ions from the solution environment can be responsible for the surface charge modification at the moment of the binding process (Mukai et al. 1997). The ζ absolute values allow to estimate the level of enzymatic oxidation of proteins and the amount of *o*-quinones bound into protein molecules (Table 1). The *o*-quinones of catechin and quercetin produced by enzymatic radical generation are highly reactive electrophilic compounds (Cavalieri et al. 2002). Furthermore, they can covalently link to reactive amino acid residues in a non-enzymatic pathway. For this reason, the *o*-quinones linked to protein molecules might affect the electrophoretic mobility of proteins. Differently from BSA, a greater

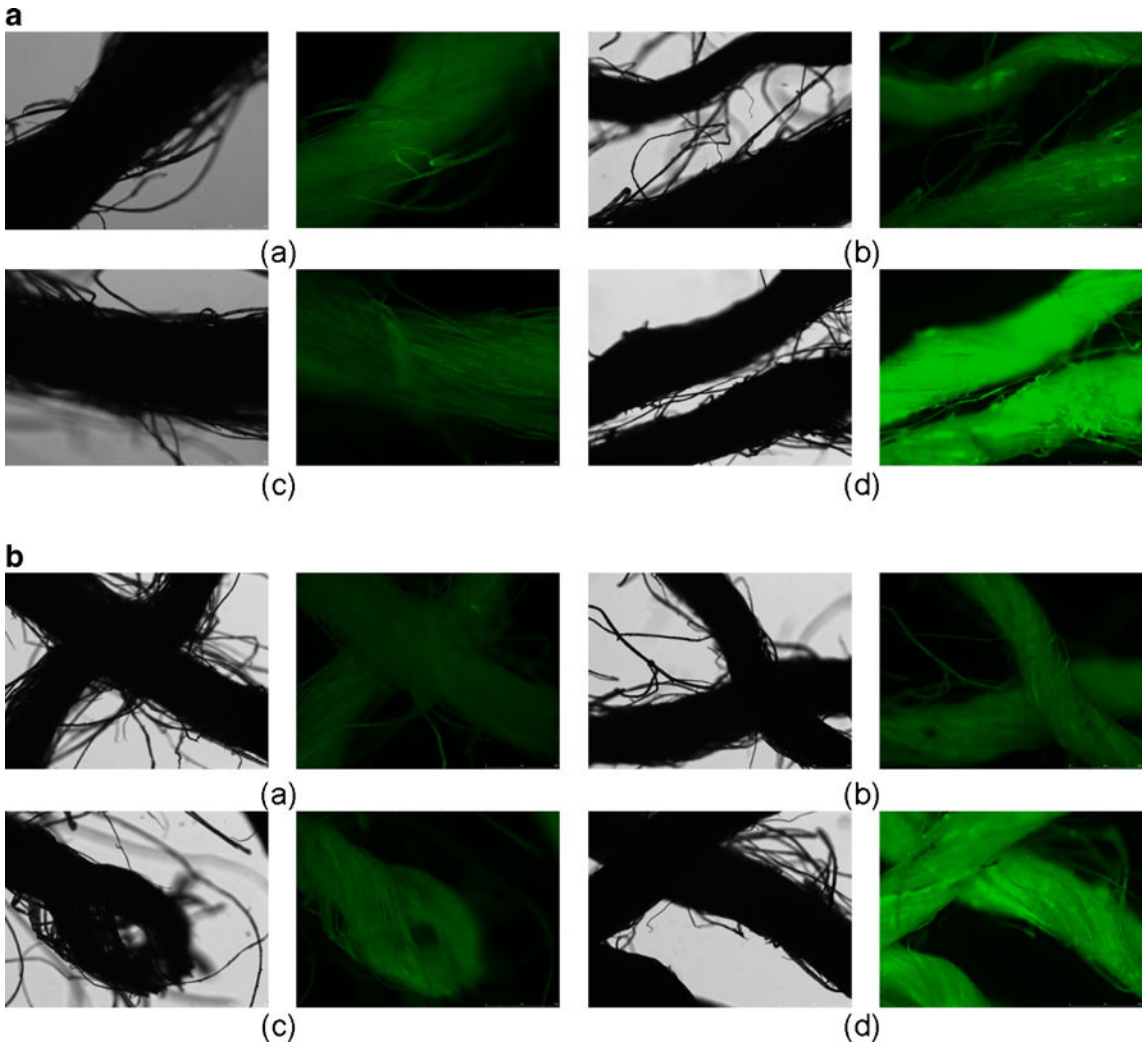


**Fig. 7** **a** Fluorescence microscopic images of cationised and non-cationised flax fibres after treatments with casein FITC-BSA+catechin in the presence and absence of laccase catalysis for overnight anchored to flax fibres: (*a*) BSA+catechin; (*b*) BSA+catechin+accase; (*c*) C-BSA+catechin; (*d*) C-BSA+catechin+laccase. *C*—cationised scoured flax fabrics. **b** Fluorescence microscopic images of cationised and non-cationised flax fibres after treatments with FITC-BSA+quercetin in the presence and absence of laccase for overnight BSA anchored to flax fibres: (*a*) BSA+quercetin; (*b*) BSA+quercetin+laccase; (*c*) C-BSA+quercetin; (*d*) C-BSA+quercetin+laccase. *C*—cationised scoured flax fabrics

increase of ζ absolute values was detected in α-casein after an enzymatic incubation in the presence of phenolic molecules. This might be caused by the covalent binding of the reactive *o*-quinones to molecules of α-casein. In the case of BSA, the charge of the particles was maintained after laccase introduction. Moreover, as previously reported, the oxidation level obtained for BSA was lower than the one obtained for α-casein.

DSC is a particularly suitable technique to study the thermodynamics controlling conformational transitions in proteins as well as the binding interactions between proteins and small molecules, drugs and other proteins (Bruylants et al. 2005). This can be detected by changes in the denaturation temperature of proteins which are related with the stability of protein structure. When the bound molecules stabilise the proteins or higher molecular weight

**Table 2** Colour degradation (percent) of flax samples incubated with protein–flavonoid conjugate in the presence of laccase

| | Casein+catechin+laccase | BSA+catechin+laccase | Casein+quercetin+laccase | BSA+quercetin+laccase |
|---|---|---|---|---|
| Non-cationised | 55.1 | 56.2 | 34.2 | 45.8 |
| Cationised | 34.8 | 28.3 | 37.3 | 40.8 |

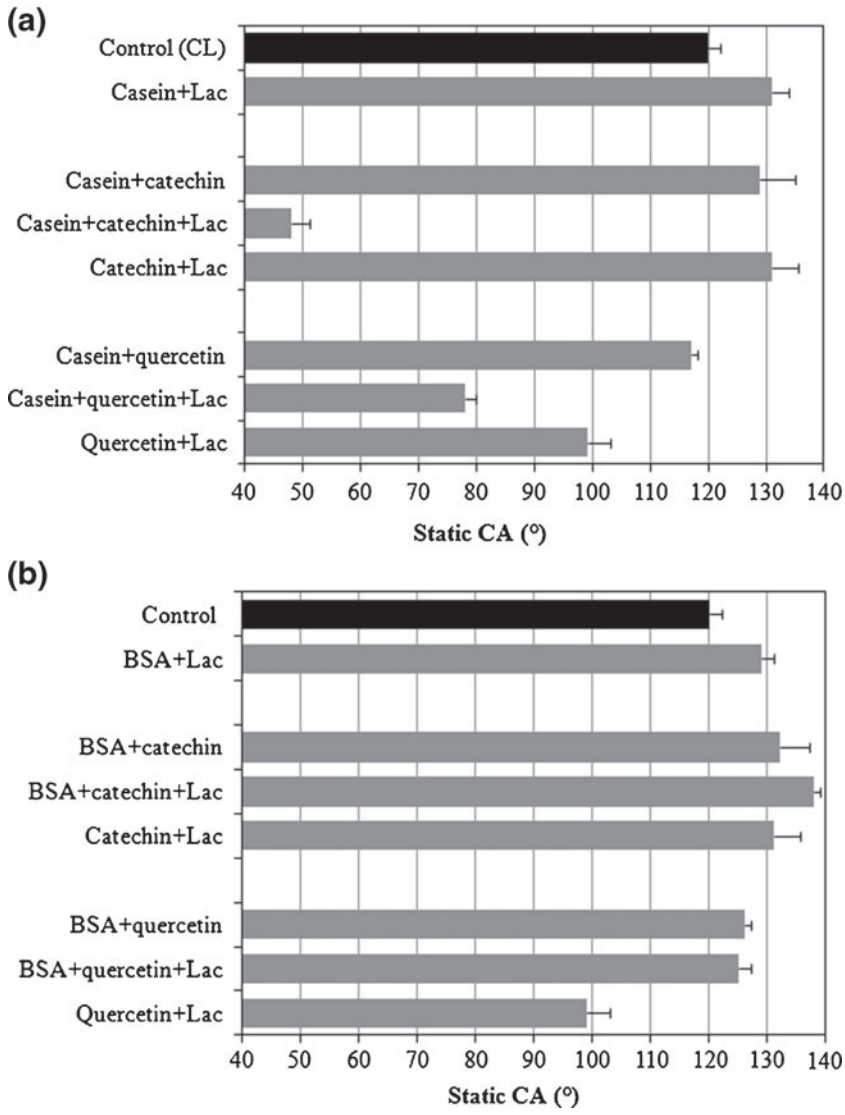


**Fig. 8** Hydrophilicity/hydrophobicity was evaluated on evaluation of cationised flax samples after treatments at different conditions by measurements of water contact angle: α-casein (**a**), BSA (**b**). The static values were presented as mean values obtained after triple measurements at different points of flax fibre samples. *CL*—cationised scoured flax fabrics, *CA*—contact angle

forms, it results in an increase of the denaturation temperature. In the case of protein–flavonoid conjugates, the increase in molecular weight of the protein–flavonoid conjugate is the main factor for the increase observed in the denaturation temperature (Mukai et al. 1997). These results corroborate those obtained by SDS-PAGE analysis (Fig. 1) and MALDI-TOF mass spectra (Fig. 2).

**Table 3** Antioxidant activity (percent) of protein–flavonoid conjugates anchored onto flax fabrics; control: scoured or cationised samples, without conjugate attachment (0% antioxidant activity)

| | Non-cationised | Cationised |
|---|---|---|
| Casein | −18.0 | 6.04 |
| Casein+catechin+laccase | 3.47 | 48.58 |
| Casein+quercetin+laccase | 15.85 | 96.23 |
| BSA | −9.72 | −2.64 |
| BSA+catechin+laccase | 3.40 | 32.17 |
| BSA+quercetin+laccase | 15.66 | 93.40 |

The α-casein–flavonoid conjugate samples presented similar exothermal behaviour, showing the formation of two distinct peaks corresponding to the denaturation of conjugates with different molecular weights (Fig. 4a). BSA, however, presented a different behaviour: in this case, the oxidation level achieved is lower than the one obtained for α-casein (Fig. 4b). Therefore, the shift of the denaturation peak can be attributed only to the small amount of protein dimers. These results indicate that both catechin and quercetin are very promising molecules for protein modification by means of an intermolecular binding. Nonetheless, α-casein and BSA proteins showed different reaction behaviours demonstrating divergent analytic results. In the case of BSA, the tyrosine amino acid residues, which are supposed to act as dominant role for enzymatic cross-link,

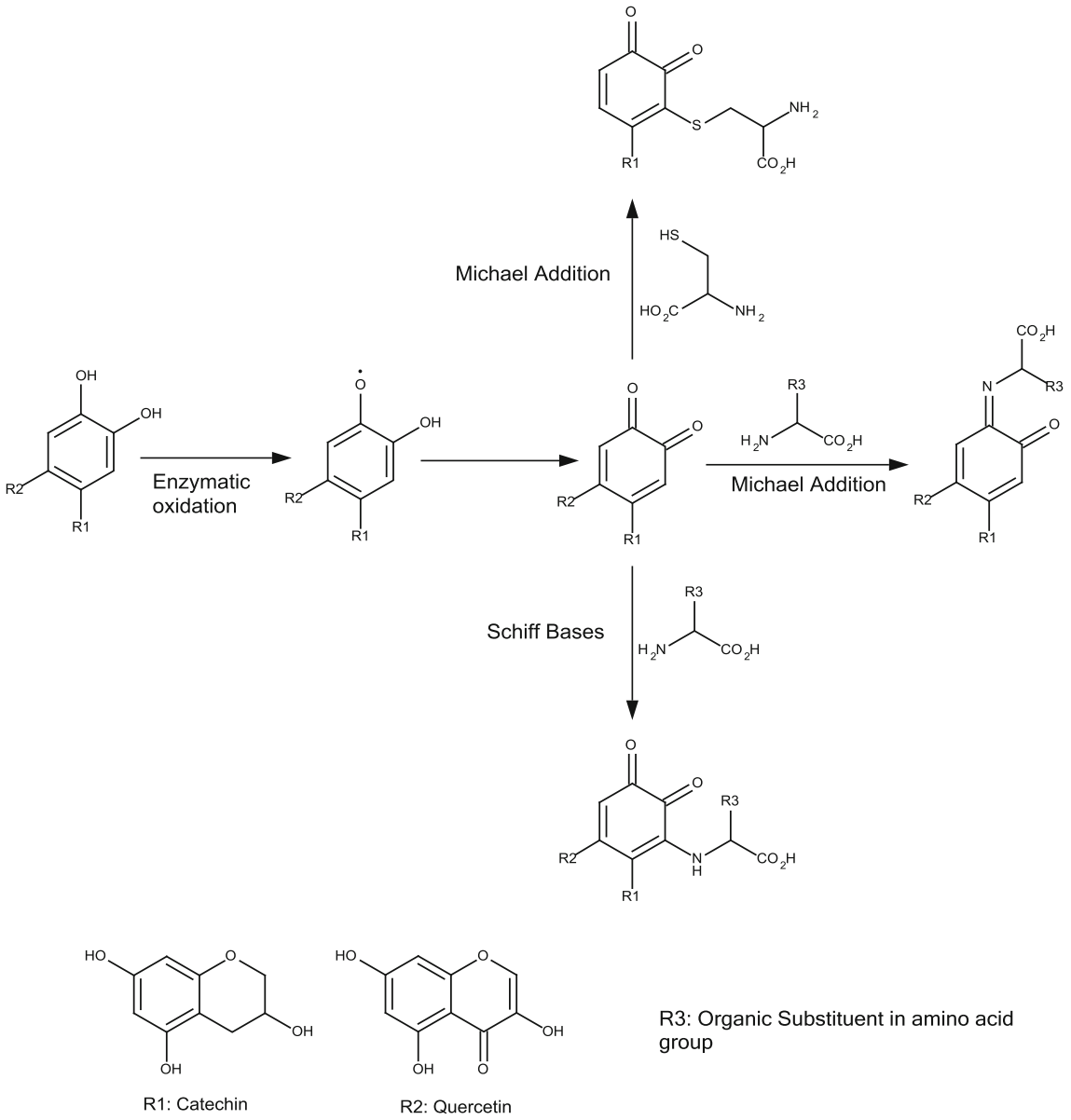


**Fig. 9** Schematic representation of enzymatic and non-enzymatic conjugation of flavonoids and reactive amino acids

are present at the surface of the globular structure. However, their 3-D structure is very compact, decreasing laccase accessibility to tyrosine residues, which consequently reduces enzymatic modification (Mattinen et al. 2006).

To verify the chemical bonds caused by laccase catalysis of protein and flavonoids, FT-IR analysis was carried out. The characteristic bands of proteins were clearly detected, and a weak carbonyl band of ester bond near 1,770 $cm^{-1}$ attributed to C=O stretching vibration bond was also detected from the data analysed in Fig. 5. This weak band can be generated by the polymerisation of phenolic monomers or by covalent bonding between flavonoids and proteins (Mattinen et al. 2005).

The biomimetic process using oxidative enzymes is interested in the "in situ" production of biopolymers and further application onto fabrics, satisfying an environmentally friendly concept by replacing chemical processes (Hadzhiyska et al. 2006; Kim et al. 2007; Kim et al. 2008). The ability of cationised flax fabrics to anchor new products like flavonoid polymers and proteins was further studied by comparing it with the non-cationised substrates. Protein–flavonoid conjugates can link to non-cationised flax fabrics in two different ways: the polymer part can link via lignin constituent and the protein can link to fabrics by coupling to *o*-quinones from lignin part. Additionally, protein and phenolic polymers can interact with the cationised flax fabrics via ionic interactions.

The anchoring efficiency of the conjugated protein–flavonoid onto flax fabrics was performed by following two different methods, i.e., colour strength measurement and fluorescence microscopic analysis. Colour generation is commonly detected in enzymatic polymerisation since the subsequent coupling reactions result in some biosynthetic pathways such as melanin and tannin formation. For this reason, the flax fabrics incubated in the protein–flavonoid solutions in the presence of laccase presented colour generation on the surface due to the anchoring of protein–flavonoid conjugates.

The cationisation of fibres was the most determinant factor for an improvement of the affinity of enzymatically produced conjugates towards fabric surface. In this research, the charge of proteins might be responsible for the interactions established with the cationised flax fabrics. α-casein and BSA proteins have the isoelectric point at pH 4.5 to 5. As their charge behaviours depend on pH, they get negative charge at pH 7.4, where the reactions were carried out. The negatively charged proteins can react with the cationised fabric surface through ionic interactions. Both *k*/*s* and fluorescence microscopic analysis results are in accordance, and a higher colour deposition was observed from samples coated with protein–flavonoid conjugates onto previously cationised surfaces (Figs. 6 and 7). The high level of polymerisation of flavonoic phenolic compounds promoted by laccase can also be responsible for a greater level of conjugated attachment. A higher molecular weight polyphenol attached to the protein seems to have a greater ability to link to flax surfaces especially if the surface has been previously cationised. The detection of the parallel increase of phenolic polymers as well as amino acids in fabrics is a potential evidence of conjugate formation between proteins and flavonoids by laccase and their grafting onto fabrics.

Surface modification of textile fabrics is mostly accomplished by the introduction of functional polymers compromising the diverse properties such as dye-uptake increase, antibacterial activity, UV protection, self cleaning, and wrinkle free as well as mechanical strength improvement (Mondal and Hu 2007). However, the utility of those modified fabrics is highly dependent on the resistance of polymer binding onto fabrics and the linking strength between fabric and polymers. The polymers generated by enzyme oxidation are broadly studied for colouration of several materials such as cotton, hair, wool as well as flax fabrics (Kim et al. 2007; Kim et al. 2008; Hadzhiyska et al. 2006; Shin et al. 2001). These colourising polymers are less soluble than the mother compounds; therefore, their release to water is prevented during wet processes (Hadzhiyska et al. 2006). However, colour resistance results show that the scoured fabrics coated with the conjugates have poor fastness resistance, showing a high percentage of colour degradation (34~56%) after washing (Table 2). The cationisation of fibres seems to be a positive factor to increase the linking strength between conjugates and fabrics involving less colour release when the samples are in contact with water (28~40%) (Table 2). As mentioned before, the charged functional layers formed by polyelectrolyte deposition are electronically drawing the oppositely charged materials by ionic interaction. Therefore, the protein–flavonoid conjugates were not easily released to water. Compared to protein–quercetin conjugates, protein–catechin conjugates presented much less colour degradation.

The hydrophilic and hydrophobic properties are some of the major factors to determine the utility of the fabrics. As surface becomes either more oxidised or more ionised, hydrogen bonding with water is easily formed, resulting in a high speed of the water droplet spread and lower contact angle (Mondal and Hu 2007).

Samples coated with the polyelectrolyte presented higher levels of conjugate attachment that confer a hydrophobic character to the fabric surface. A different behaviour was observed for the α-casein–catechin and α-casein–quercetin conjugates, showing a low water contact angle, i.e., 48° and 79°, respectively, indicating comparably hydrophilic properties (Fig. 8). This value corroborates the assumption that α-casein has a greater ability to be oxidised by laccase, being in higher percentage in the referred conjugate. The increase of hydrophilicity can be due to the presence of the protein at the surface of the fabric, overlapping the polymer's ability to promote hydrophobic behaviour. The difference obtained for both proteins is related to the fact that casein has the ability to form micelles where the hydrophobic is the inner part and the hydrophilic is the outer part. Therefore, when applied onto flax surfaces, there is a probability that the hydrophilic part of the protein is more exposed, and water absorption increases with the number of hydrophilic groups at the surface (Fig. 8a).

Finally, the expected antioxidant property on the surface of flax by anchoring protein–flavonoid conjugates was studied in terms of measuring antioxidant activity following $ABTS^{\bullet+}$ cation decolourisation assay method. From the results of *k*/*s* measurements and fluorescence microscopic analysis of modified fabrics, protein–catechin conjugates have a greater affinity onto flax fabrics, and a greater antioxidant activity was expected from the anchored fabrics than protein–quercetin conjugates anchored fabrics. Protein–quercetin conjugates, however, presented much higher antioxidant activity compared to protein–catechin conjugates (2~3 times higher). This can be explained by the structural differences between the catechin and quercetin flavonoids used in these experiments. Generally, it is considered that a higher number of hydroxyl group substituents in flavonoid structure results in a superior antioxidant activity (Amić et al. 2003; Heijnen et al. 2001;

Pekkarinen et al. 1999). Although both catechin and quercetin possess the same number of substituted hydroxyl groups, quercetin possesses a 4-oxo functional group and insaturation in the C-ring unlike catechin, which can play a role in the enhancement of the radical scavenging activity (Amić et al. 2003; Heijnen et al. 2001; Pekkarinen et al. 1999).

This study highlights the ability of laccase to catalyse the oxidation of proteins (α-casein and BSA) and flavonoids, promoting the formation of conjugates with antioxidant properties. The level of enzymatic oxidation and further conjugation were measured and α-casein seems to be the most promising protein for this purpose. Protein–flavonoid conjugates were successfully attached onto previously cationised flax fabrics leading to hydrophobic surfaces in all cases except in the case of α-casein–catechin conjugate, which gave rise to a higher fabric hydrophilic behaviour. The more exposed hydrophilic part of the α-casein protein could be responsible for this.

Protein–flavonoid conjugates can improve the natural properties of proteins, being promising products to be used in medical, food and polymer fields where antioxidant ability is an essential feature.

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## References

Amić D, Davidović-Amić D, Bešlo D, Trinajstić N (2003) Structure-radical scavenging activity relationships of flavonoids. Croat Chem Acta 76:51–61

Andersen OM, Markham KR (2006) Flavonoids: chemistry, biochemistry and applications. CRC Publishing Ltd., New York

Anghileri A, Lantto R, Kruus K, Arosio C, Freddi G (2007) Tyrosinase-catalyzed grafting of sericin peptides onto chitosan and production of protein–polysaccharide bioconjugates. J Biotechnol 127:508–519

Arts M, Haenen G, Wilms LC, Beetstra S, Heijnen C, Voss H, Bsat A (2002) Interaction between flavonoids and proteins: effect on the total antioxidant capacity. J Agric Food Chem 50:1184–1187

Bittner S (2006) When quinones meet amino acids: chemical, physical and biological consequences: review. Amino Acids 30:205–224

Bruylants G, Wouters J, Michaux C (2005) Differential scanning calorimetry in life science: thermodynamics, stability, molecular recognition and application in drug design. Curr Med Chem 12:2011–2020

Cavalieri E, Li KM, Balu N, Saeed M, Devanesan P, Higginbotham S, Zhao A, Gloss ML, Rogan EG (2002) Catechol ortho-quinones: the electrophilic compounds that form depurinating DNA adducts and could initiate cancer and other diseases. Carcinogenesis 23:1061–1077

Chung JE, Kurisawa M, Uyama H, Kobayashi S (2003) Enzymatic synthesis and antioxidant property of gelatine–catechin conjugates. Biotechnol Lett 25:1993–1997

Claus H (2004) Laccases: structure, reactions, distribution. Micron 35:93–96

Cura DE, Lantto R, Lille M, Andberg M, Kruus K, Buchert J (2009) Laccase-aided protein modification: effects on the structural properties of acidified sodium caseinate gels. Int Diary J 19:737–745

Georgiou CD, Grintzalis K, Zervoudakis G, Papapostolot I (1996) Mechanism of Coomassie brilliant blue G-250 binding to proteins: a hydrophobic assay for nanogram quantities of proteins. Analyt Bioanalyt Chem 391:391–403

Gianfreda L, Xu F, Bollag J (1999) Laccases: a useful group of oxidoreductive enzymes. Biorem J 3:1–25

Gübitz G, Cavaco-Paulo A (2003) New substrates for reliable enzymes: enzymatic modification of polymers. Curr Opin Biotech 14:577–582

Hadzhiyska H, Calafell M, Gibert JM, Dagà JM, Tzanov T (2006) Laccase-assisted dyeing of cotton. Biotech Lett 28:755–759

Heijnen C, Haene G, van Acker F, van der Vijgh F, Bast A (2001) Flavonoids as peroxynitrite scavengers: the role of the hydroxyl group. Toxicol In Vitro 15:3–6

Heim K, Tagliaferro A, Bobilya D (2002) Flavonoids antioxidants: chemistry, metabolism and structure–activity relationships. J Nutr Biochem 13:572–584

ISO 105-C06 (2010) Textiles—tests for colour fastness—part C06: colour fastness to domestic and commercial laundering. International Organization for Standardization, Geneva, Switzerland

Kanakis CD, Tarantilis P, Polissio M, Diamantoglo S, Tajmir-Riahi H (2006) Antioxidant flavonoids bind human serum albumin. J Mol Struct 798:69–74

Kanaze F, Kokkalou E, Niopas I, Georgarakis M, Stergiou A, Bikiaris D (2006) Thermal analysis study of flavonoid solid dispersions having enhanced solubility. J Therm Anal Calorim 2:283–290

Kim SY, Moldes D, Cavaco-Paulo A (2007) Laccase for enzymatic coloration of unbleached cotton. Enzyme Microb Tech 40:1788–1793

Kim SY, Lopez C, Gübitz G, Cavaco-Paulo A (2008) Biological coloration of flax fabrics with flavonoids using laccase from *Trametes hirsuta*. Eng Life Sci 8:324–330

Kobayashi S, Higashimura H (2003) Oxidative polymerization of phenols revisited. Prog Polym Sci 28:1015–1048

Kurisawa M, Chung JE, Uyama H, Kobayashi S (2003) Laccase-catalyzed synthesis and antioxidant property of poly(catechin). Macromol Biosci 3:758–764

Labat E, Morel MH, Rouau X (2000) Effects of laccase and ferulic acid on wheat flour doughs. Cereal Chem 77:823–828

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Mattinen ML, Kruus K, Buchert J, Nielsen JH, Andersen HJ, Steffensen C (2005) Laccase catalysed polymerization of tyrosine containing peptides. FEBS J 272:3640–3650

Mattinen ML, Hellman M, Permi P, Autio K, Kalkkinen N, Buchert J (2006) Effect of protein structure on laccase-catalyzed protein oligomerization. J Agric Food Chem 53:8883–8890

Mattinen ML, Suortti T, Grosselink R, Argyropoulos DS, Evtuguin D, Suurnakki A, de Jong E, Tamminen T (2008) Polymerisation of different lignins by laccase. BioRresources 3:549–565

Mayer AM, Staples RC (2002) Laccase: new functions for an old enzyme. Phytochemistry 60:551–565

Mondal S, Hu JL (2007) A novel approach to excellent UV protecting cotton fabric with functionalized MWNT containing water vapour permeable PU coating. J Appl Poly 103:3370–3376

Mukai Y, Iritani E, Muras T (1997) Effects of protein charge on cake properties in dead-end ultrafiltration of protein solutions. J Membrane Sci 137:271–275

Pekkarinen S, Heinonen M, Hopia A (1999) Flavonoids quercetin, myricetin, kaemferol and (+)-catechin as antioxidants in methyl linoleate. J Sci Food Agric 79:499–506

Petrulyte S (2008) Advanced textile materials and biopolymers in wound management. Dan Med Bull 55:72–77

Pietta PJ (2000) Flavonoids as antioxidants. Nat Prod 63:1035–1042

Polowinsky S (2005) Polyelectrolyte layer-by-layer processed coated textiles. Fibres Text East Eur 13:50–52

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C (1999) Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic Biol Med 26:1231–1237

Reihmann M, Ritter H (2006) Synthesis of phenol polymers using peroxidases. Adv Polym Sci 194:1–49

Selinheimo E, Lampila P, Mattinen ML, Buchert J (2008) Formation of protein–oligosaccharide conjugates by laccase and tyrosinase. J Agric Food Chem 56:3118–3218

Shin H, Gübitz G, Cavaco-Paulo A (2001) "In situ" enzymatically prepared polymers for wool coloration. Macromol Mater Eng 286:691–694

Sousa F, Gübitz G, Kokol V (2009) Antimicrobial and antioxidant properties of chitosan enzymatically functionalized with flavonoids. Process Biochem 44:749–756

Steffensen C, Andersen ML, Degn PE, Nielsen J (2008) Cross-linking proteins by laccase-catalyzed oxidation: importance relative to other modifications. J Agric Food Chem 56:12002–12010

Wolfenden BS, Willson RL (1982) Radical-cations as reference chromogens in kinetic studies of one-electron transfer reactions: pulse radiolysis studies of 2,2′-azinobis-(3-ethylbenzthiazoline-6-sulphonate). J Chem Soc Perkin Trans 2:805–812