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# Stabilization of Collagen with biotechnological Approaches

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## 1. Introduction

Collagen is a popular material for a broad range of different applications. It is used for manufacturing technical products like leather and parchment as well as it is a raw material in the food and food packaging industry for the production of e. g. casings and gelatin (de Wolf, 2003). Furthermore, it is widely used for biomedical applications to produce drug delivery systems or scaffolds for cellular growth and tissue engineering (Lee *et al.*, 2001). In all these various applications, one important disadvantage is that collagen-based products are sensitive against the influence of water, humidity, temperature and enzymatic degradation. Therefore, they need to be stabilized by suitable chemical or physical processes.

One of the oldest stabilization methods known in collagen-industry is the tanning step during leather production where collagen from animal hides is turned into a heat resistant and stable material. Almost 90 % of the leather manufactured worldwide is tanned by using chromium because it is the most efficient and versatile tanning agent available, and it is relatively cheap (Covington 1997). However, it is not considered as environmentally friendly. Solid leather wastes as well as waste water from the tanning industry are still major issues for the global leather sector (Prentiss *et al.*, 2003).

Several alternatives for the stabilization of collagen materials have been explored using a variety of different reagents (Sreeram and Ramasami 2003). In general, these technologies include a) other mineral tanning agents such as Al(III), Ti(IV) or Zr(IV) (Covington 1988), b) aldehyde tanning (Fathima *et al.*, 2004), c) organic tanning with plant polyphenols or synthetic polymers (Covington *et al.*, 2005) and d) biomimetic approaches using natural substances like genipin (Ding *et al.*, 2006) or oleuropein (Antunes *et al.*, 2006). However, some of these processes involve chemicals that are considered to be cytotoxic or environmentally unfriendly (Nimni 2001), while other techniques do not confer sufficient hydrothermal stability to the collagen material (Collighan *et al.*, 2004). That is why novel collagen stabilization mechanisms that provide good thermal and mechanical properties are required.

Enzyme-mediated reactions represent one possible approach to find efficient and non-toxic methods for stabilizing collagen-based materials. Beside microbial transglutaminase which has gained much attention in this respect (Lastowka *et al.*, 2005; Stachel *et al.*, 2010), other enzymes such as tyrosinases or laccases are known to cross-link proteins. Tyrosinases (EC 1.14.18.1) are widely distributed in higher plants, animals, and microorganisms and are binuclear copper containing enzymes that catalyze the formation of reactive quinone precursors on the side chain of tyrosine residues in proteins (Sanchez-Ferrer *et al.*, 1995). These quinones may then either further react with nucleophiles such as adjacent amino and sulphhydryl residues or condense with each other to undergo direct cross-linking (Freddi *et al.*, 2006). Laccases are polyphenol oxidases (EC 1.10.3.2) that oxidize a wide range of mono- and poly-phenolic

substrates to form free radicals. These reactive radicals can react further and lead to polymerisation, hydration and disproportionation (Thurston, 1994). Laccases have a wide substrate specificity and, in addition to mono- and diphenols, they have been found to be capable of oxidizing various aromatic compounds such as substituted phenols, diamines, aromatic amines or thiols. In proteins, the enzymes directly oxidize tyrosine as well as cysteine residues leading to the formation of cross-links (Lantto *et al.*, 2005). The substrate specificity of both, tyrosinases and laccases, can be broadened by the addition of small molecular weight compounds called mediators. Although both enzymes have been used for the functionalisation of biopolymers (Jus *et al.*, 2008) as well as for the cross-linking of gelatin (De Jong 2005), there is only very little information about the action of laccases and tyrosinases towards collagen as the substrate.

Another possibility to stabilize collagen-based materials is to use natural sources such as various plant extracts similar to the process of vegetable tanning. In this regard, the iridoid structures genipin as well as oleuropein have been extensively investigated as novel cross-linking agents and it was shown that incubation with these substances results in hide materials with thermal stabilities of up to 80 °C (Ding *et al.*, 2006; Zotzel *et al.*, 2008). However, a side effect of both reactions is a characteristic staining of the tanned material which appears dark blue in the case of genipin or green after treatment with oleuropein. This might confine potential applications.

The aim of this study was to assess novel approaches for the stabilization of collagen materials by incubating insoluble collagen dispersion with different enzymes (tyrosinase and laccases). In order to characterise the cross-linking effect, the resulting modified collagen materials were analysed with respect to their thermal stability, their mechanical properties and their behaviour towards enzymatic degradation.

## 2. Materials and Methods

### 2.1 Collagen Materials

During this study, insoluble collagen dispersion was used for the cross-linking reactions with different enzymes. For its preparation, porcine skin obtained from a local abattoir was extensively washed and soaked in a solution of sodium hydroxide at pH 12.5 overnight. The swollen skin was split two times on a conventional splitting machine followed by soaking in ammonium sulphate solution to adjust the material to a pH of 7. After another washing step and treatment with 1 % $H_2O_2$, the split was minced using a meat chopper, acidified to pH 4 with hydrochloric acid and further treated in a colloid mill to obtain a homogenous collagen dispersion. For the experiments, the dry matter content of this dispersion was either adjusted by centrifugation or freeze drying and resolubilization (Meyer *et al.*, 2010).

### 2.2 Enzymes

One tyrosinase as well as two laccases were supplied from the Institute of Environmental Biotechnology at the University of Technology in Graz, Austria. Individual properties of these enzymes are indicated in Table 1.

### 2.3 Cross-linking Reaction

Insoluble collagen dispersion was treated with the enzymes by suspending 0.5 % (w/w) in a neutral (pH 6) Soerensen-buffer that had been modified by the addition of tyrosinase or laccases. The samples were then incubated at 30 °C for 24 h. To study the influence of small phenolic molecules, 150 µl from a stock solution of catechin (CAT) were added to some of the

samples after 20 h of enzyme incubation and the reaction was continued for further 4 h. After incubation, the collagen was centrifuged to stop the reaction and the samples were washed 5 times with acetic acid at pH 3.7 to remove the enzymes. The enzymatically modified collagen pellets were dried prior to further analysis. All experiments were performed in duplicates. Control samples were incubated at the same conditions but without addition of the enzyme or plant material.

Table 1: Properties of the enzyme preparations used for collagen stabilization

| Enzyme | Specific Activity [U/mg] | Protein concentration [mg/ml] | Molecular weight [kDa] |
|---|---|---|---|
| Tyrosinase (E3) | 5528,3 | 1,4 | 53,9 |
| Laccase 1 (THL) | 10,8 | 0,6 | 59,9 |
| Laccase 2 (TVL) | 22,5 | 0,2 | 51,1 |

2.4 Sample Characterization

Thermal stability was determined using a Mettler Toledo DSC821$^e$ differential scanning calorimeter. Collagen samples were incubated with 10 µl PBS-buffer in 40 µl aluminium crucibles (Mettler, ME-26763) for at least 2 h at room temperature before heating from 20 to 90 °C with a constant heating rate of 10 K/min.

Mechanical properties of the collagen samples were analysed using a Texture Analyser XTPlus (Stable Microsystems, UK). Dried collagen strips were soaked in PBS buffer for at least 2 h at room temperature and either elongated until rupture with a speed of 0.5 mm/s or compressed to 50 % of their initial height using a cylindrical piston with a diameter of 10 mm and a speed of 1.0 mm/s. To evaluate tensile strength, the measured force values were normalized to the cross-section areas of the collagen strips.

To determine the resistance of the modified collagen towards enzymatic degradation, samples were incubated using collagenase (*Clostridium histolyticum* type H, Sigma) in ammonium hydrocarbonate buffer pH 8 at 37 °C. Prior to analysis, the samples were soaked in the same buffer for at least one hour to allow wetting and swelling of the specimens. The incubations were carried out over a time period of 24 h. To stop the reaction, samples were centrifuged and the supernatants were removed. After vacuum-drying, the collagen was reweighed and the soluble fraction was calculated from the determined weight loss.

## 3. Results and Discussion

Cross-linking or tanning of collagen materials usually results in enhanced hydrothermal stability which is characterized by an increase in denaturation or shrinkage temperature. The latter can be easily obtained during DSC-analysis and is often used as a measure of successful collagen cross-linking. Figure 1 shows the denaturation temperatures determined for insoluble collagen dispersion after incubation with tyrosinase and laccases. As can be seen, no significant differences between the starting material and the samples treated with the enzymes alone were observed. However, an increase of ~ 2 K was detected after addition of catechin (CAT) to the enzymatic reaction. To allow dissociation of intermolecular aldimine-type cross-links and to remove residual phenolic molecules, the samples were dialysed against acetic acid at pH 3.7 (Trelstad 1982). After this treatment, the denaturation temperatures of collagen materials that had been incubated with or without the enzymes in the presence of CAT did not change significantly indicating that CAT was integrated in the collagen material in a stable way (Figure 1). In contrast, the denaturation temperatures of the samples not treated with CAT

decreased because of the cleavage of acid-labile cross-links. The latter finding was not as pronounced for the E3-treated collagen and excluded the TVL-cross-linked material where acidic-dialysis did not significantly decrease denaturation temperature. In summary, enzymatic cross-linking with laccases or tyrosinase did only slightly increase the thermal stability of scaffold materials manufactured from insoluble collagen dispersion.

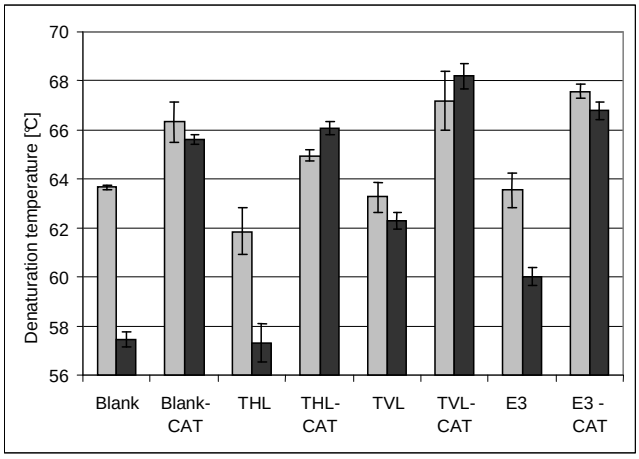


Figure 1: Denaturation temperature of enzymatically cross-linked insoluble collagen dispersion before (grey) and after acidic dialysis at pH 3.7 (black)

In addition to the hydrothermal stability, the mechanical strength of enzymatically modified collagen was measured. After treatment with laccases or tyrosinase, the tensile strength of the samples decreased slightly (Figure 2). However, enzymatic incubation in the presence of CAT led to a substantial increase in tensile strength indicating stronger interaction of the fibres. In comparison, addition of CAT alone did not have a significant effect on the tensile strength of the collagen material.

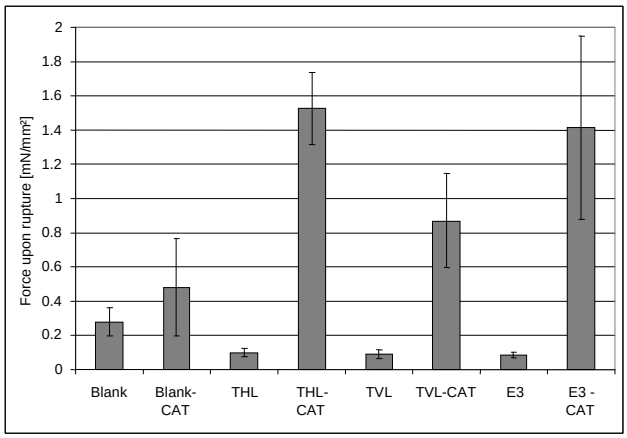


Figure 2: Tensile strength of insoluble collagen dispersion after cross-linking with laccases or tyrosinase in the presence or absence of catechin (CAT)

Unlike tensile strength, compressive forces measured after enzymatic treatment with laccases or tyrosinase did not differ significantly from the value determined for the untreated control (Figure 3). Even after addition of catechin to the enzymatic incubation, no increase in compressive strength could be observed. In part, this might be due to the high porosity of the collagen scaffolds which were fully soaked with PBS buffer prior to mechanical testing. Hence, the compressive forces were mainly determined by squeezing out the buffer. Anyway, from these results it can be concluded, that enzymatic treatment of insoluble collagen dispersion with

either laccases or tyrosinase led to an increase in tensile strength whereas the resistance to compression was not influenced.

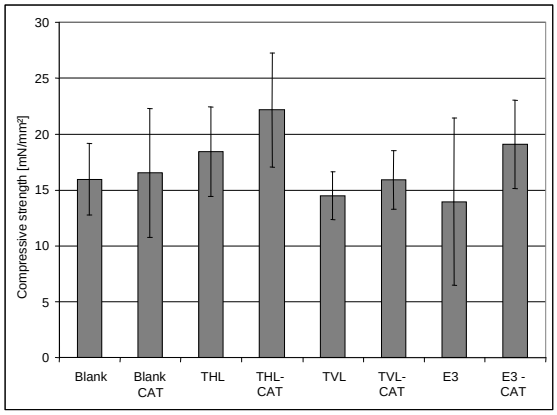


Figure 3: Compressive strength of insoluble collagen dispersion modified by treatment with laccases or tyrosinases with or without the addition of catechin

As a third parameter to characterise enzymatically treated collagen materials, their resistance to enzymatic degradation by collagenase was determined. This property is especially important for *in vivo*-applications of collagen products, e. g. as implant materials. Samples that had been incubated with laccasses or tyrosinase could be enzymatically disintegrated into soluble fragments by more than 90 % after 24 h (Figure 4). However, in the presence of CAT only 60 % of the collagen material was soluble after degradation by collagenase. This resistance against enzymatic hydrolysis increased even more when collagen was treated with both, enzyme and catechin. After incubation with CAT and the laccase TVL, e. g., only about 40 % of collagen were degraded to soluble fragments under the tested conditions. Thus, laccase and tyrosinase cross-linking in the presence of phenolic molecules such as CAT allowed to significantly enhance the resistance of collagen materials to enzymatic cleavage.

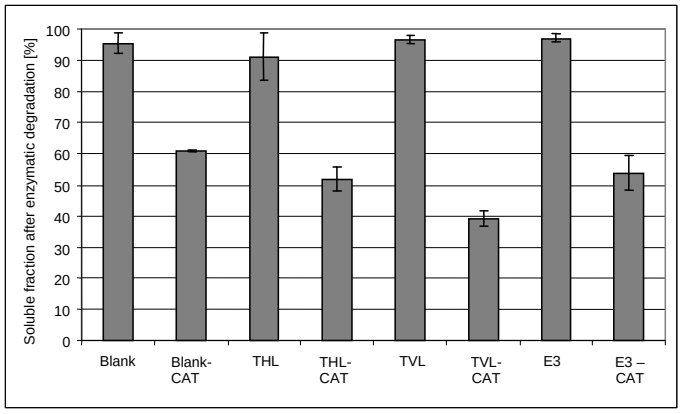


Figure 4: Soluble fractions of insoluble collagen dispersion modified with laccases or tyrosinases in the presence or absence of catechin (CAT) after degradation by collagenase

## 4. Conclusion

In this study, the potential use of specific enzymes for the stabilization of collagen was investigated. As a frequently used parameter for structural stability, the shrinkage temperatures of the modified samples were determined by using DSC. The results show, that the thermal stability of scaffold materials manufactured from insoluble collagen dispersion increased only slightly after enzymatic cross-linking with laccases or tyrosinase. Nevertheless, this

demonstrates the ability of the tested enzymes to cross-link and, hence, stabilize the collagen network.
In addititon to improved thermal stability, the mechanical strength of collagen significantly increased after enzymatic treatment. The highest values were obtained by combining enzymatic incubation with the addition of small phenolic compounds. Furthermore, the resistance of collagen materials against enzymatic degradation using collagenase could be reduced by treatment with laccases or tyrosinase.
In conclusion, these novel and biotechnological approaches for collagen stabilization provide effective and ecological alternatives to the chemical treatments used in collagen processing today.

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## 6. Acknowledgements

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