Peroxidases in food industry: crosslinking of proteins and polysaccharides to impart novel functional properties

C.G. BOERIU [a,b]
[a] *Wageningen University & Research Centre, AFSG, Division Biobased Products, The Netherlands*[a]; [b] *Universitatea "Aurel Vlaicu", Arad, Romania*[b]

[a]*Bornsesteeg 59, 6708 PD, Wageningen, The Netherlands;*[b] *Str. Elena Dragoi nr. 2, 310330 Arad, Romania*

Abstract

Covalent attachment of proteins to carbohydrates can significantly change the functional properties of the polymers, which may be useful industrially. To produce novel functional polymers for food applications, we explored the enzymatic crosslinking of proteins and polysaccharides. Heterologous conjugates of wheat arabinoxylan and α-lactalbumin were prepared by peroxidase-mediated cross-linking, using sequential addition of the arabinoxylan to the mixture of α-lactalbumin, peroxidase and hydrogen peroxide. Formation of reaction products was followed by size exclusion chromatography. The protein-arabinoxylan conjugates were separated by anion exchange chromatography and characterized by infrared spectroscopy. The viscosity and emulsifying properties of the novel polymers were measured.

Keywords: *peroxidase, α-lactalbumin, arabinoxylan, phenolic acids, crosslinking*

Introduction

Proteins and polysaccharides have found multiple applications in both food and non-food industry. Polysaccharides are structure-forming polymers that are mainly used as thickening agents and as stabilizers of foams and emulsions. Traditionally, proteins from animal origin have been used in conventional and fabricated foods. Although cheap and abundant, plant proteins have found less application mainly because of lack of desirable functional performance of these proteins in foods. For industrial application, it is necessary to modify the proteins to provide a particular property such as dispersibility, improved colour, foaming capacity, wettability, adhesive strength, viscosity, gel strength, colloid protective properties or a specific molecular size or shape.

The functional properties of proteins are fundamentally related to their physico-chemical and structural properties. Tailoring protein properties can be achieved by physical, chemical and enzymatic modifications. For food applications, however, the enzymatic route is preferred because of mild reaction conditions, increased specificity of the sites of modification, better control of the process and low toxicity of the initial reactants and products. Hydrolysis and cross-linking are the most important routes for enzymatic modification. Our work concerns cross-linking resulting in products with new functionalities. Usually protein molecules are cross-linked with each other (homo-crosslinking) by using transglutaminase [1], poly-phenoloxidases [2], lysyloxidase [3] and peroxidises [4]. Recently we have demonstrated the intermolecular cross-linking of β-casein, a protein with an extremely flexible structure, essentially random, with arabinoxylans containing ferulic acid residues (AX), in the presence of peroxidase and hydrogen peroxide, and we have characterized the hetero-adducts formed

under different reaction conditions [5]. We have showed that covalent attachment of arabinoxylan to β-casein modifies the hydrophilic-hydrophobic balance of the protein molecule. The properties of such protein-carbohydrate conjugates depend on the size, and the number of the attached carbohydrate fragments. In this study we report the synthesis of hetero-conjugates of arabinoxylan with α-lactalbumin via phenolic bridges, using the peroxidase - hydrogen peroxide catalytic system and applying the kinetic control method developed in model studies [5, 6, 7].

Materials and methods

Chemicals: Horseradish peroxidase (EC 1.11.1.7, Type VI, 250 U/mg) was obtained from Sigma-Aldrich (Zwijndrecht, Netherlands). α- Lactalbumin (EC 232-750-1, type III, calcium depleted, 85 % w/w) was obtained from Sigma-Aldrich (Zwijndrecht, Netherlands). Hydrogen peroxide (30 % solution, w/v) was from Merck (Darmstadt, Germany). Wheat arabinoxylan (arabinoxylan content: 97 % (w/w), containing approximately 0.2 % ferulic acid (w/w)) was from Megazyme (Bray, Ireland). All other reagents were of analytical grade.

Synthesis of carbohydrate-protein conjugates: Oxidative coupling of protein with arabinoxylan with the peroxidase/hydrogen peroxide system was performed by adding 100 μl of horseradish peroxidase (1 mg/ml) and 100 μl of hydrogen peroxide 0.5 M to 2.8 ml of 0.1 M sodium phosphate, pH 7, containing 1% (w/v) protein. The mixture was allowed to equilibrate at 25° C and subsequently 3 ml of arabinoxylan solution (1 %) were gradually added. The reaction mixture was maintained at 25°C for 4 hours and then the reaction was stopped by inactivation of the enzyme (2 minutes/ 100°C). The samples were frozen in liquid nitrogen and kept at -20°C until further analysis. In the blanks (control experiments without protein or arabinoxylan), the protein and or arabinoxylan solutions, respectively, were substituted by 0.1 M sodium phosphate, pH 7.0.

Analytical size exclusion chromatography: SE-HPLC analyses were carried out using a Waters HPLC system consisting of a Waters 600E solvent delivery/control system with a Waters 717 automatic sampler injector and a Waters 2487 Dual Wavelength Absorbance Detector. The column used was TSK-Gel G-2000 SW_{XL} (300x7.8 mm), with a guard column (TSK-Gel SW, 7.5x7.5 mm). Both columns were from TosoHaas (Tokyo, Japan). Before analysis, the samples were diluted with the eluent buffer and filtered through 0.45 um Orange Scientific Gyrodisc-PES 13 filters, and 20 μl of the supernatant was injected into the column. The components were eluted with 0.1 M sodium phosphate buffer pH 7.0 containing 0.3 M NaCl, at a flow rate of 0.25 ml/min, and detected at 214 nm, 280 nm and 320 nm. The column was calibrated with standards of known molecular mass. The protein standards were α-lactalbumin (14.2 kDa), chymotrypsin (25.7 kDa), bovine serum albumin (66 kDa), aldolase (158 kDa) and catalase (232 kDa). The size exclusion limit of the column was ≈ 300 kDa. The void volume of the column was experimentally determined with blue dextran (2000 kDa) and corresponded to R_t = 27.8 min. The relative abundance of reagent protein and reaction products in the reaction mixture was expressed as the proportion (%) of the total peak area determined at 280 nm.

Anion exchange chromatography (AEC): Anion exchange chromatography was performed on a FPLC system AKTA explorer equipped with a UV-900 monitor and Frac-900 fraction collector (AmershamBiosciences, UK), using Q-Sepharose columns (0.7 x 2.5 cm for analytical runs and 2.6 x 40 cm for preparative runs, respectively). Samples were eluted with a buffer system consisting of 0.1M Tris-HCl buffer, pH=7.5 and Tris-HCl 0.1M, pH=7.5 with1M NaCl, with a linear salt gradient from 0 to 100%, at a flow rate of 1 ml/min. The

fractions collected were dialyzed (cut-off membrane 15 kDa) and lyophilized prior to further characterization.

Characterization of protein-arabinoxylan conjugates by FT-IR spectroscopy and chemometry: IR spectra of protein, arabinoxylan, mixtures thereof and products isolated from crosslinking reactions were obtained on a Bio-Rad Fourier transform infrared spectrometer (FTS-60A) equipped with a MTC detector and ATR accessory, with a ZnSe ATR crystal. MID-IR spectra were recorded between 700 and 4000 cm^{-1} at a resolution of 2 cm^{-1} in the ATR mode, on thin films. 64 Interferograms were co-added for a high signal to noise ratio. The spectra were baseline corrected prior to further analysis. Three replicate spectra were measured for every sample and the mean spectrum was used for data analysis. Partial least squares regression analysis (PLS) was used to correlate the FT-IR spectroscopic data with the chemical composition of arabinoxylan-protein conjugates in terms of (i) moles of protein bound per mole arabinoxylan and (ii) % of arabinoxylan in the sample (w/w), according to the method described by Boeriu et al. [5] Calibration set was built up from mixtures of arabinoxylan and α-lactalbumin for the concentration range 0-100% AX. PLS models were constructed using the 2^{nd} derivative of the spectra for the spectral region 750-1800. The PLS models were validated using the "full cross-validation" technique to ensure predictive validity, guarding against over-fitting. The statistical software Unscrambler 6.1 (Camo A/S, Norway) was used for chemometric calculation.
Viscosity measurements: The relative viscosity of arabinoxylan - protein solutions (1 %) before and after oxidative crosslinking were measured with an Ubbelohde capillary viscometer (Schott, mainz, Germany) submerged in a thermostatically controlled waterbath at 25°C. Capillary diameters ranged from 0,53 to 0.95 mm.

Results and Discussion

Wheat arabinoxylan used in the experiments consisted of a mixture of low and high molecular mass polymers. The main component, representing more than 90 % of the mixture, consists of high molecular weight polymers with an average molecular weight of 350 kDa, as determined from SEC-HPLC (not shown). The minor component consisted of low molecular weight oligosaccharides with a mass ranging from 1 to 10 kDa. Both the high and low molecular weigth arabinoxylan fractions contained ferulic acid residues, as determined from ester hydrolysis and quantification of liberated ferulic acid (not shown). The ferulic acid moieties in the polysaccharide chain can be involved in cross-linking reactions mediated by peroxidase. When arabinoxylan (AX) was incubated with α-lactalbumin, peroxidase and hydrogen peroxide, a complex mixture of products was obtained. Figure 1 shows the anion exchange chromatography of incubations of α-lactalbumin with arabinoxylan. Arabinoxylan, which is a neutral polymer not containing any charged residue, will elute at the void volume of the anion exchange column. However, it is not possible to detect the arabinoxylan polymers in the chromatographic trace with UV detection, since at the concentrations used in the experiments; the UV response of this polysaccharide is below the detection limit. α-Lactalbumin elutes at a slat gradient of 31 % (insert of Figure 1).
A first major fraction (F-1 and F-2) eluted at the void volume as a broad, overlapping peak, and accounted for about 30 % of the product mixture (Figure 1). This peak was assigned to protein-arabinoxylan adducts, based on the absorption at 280 nm, characteristic for the aromatic residues in polymers, and the absorption at 320 nm, which is specific for C-C covalent aromatic linkages. The C-C aromatic bonds might have been generated by cross-

linking of feruloyl residues in arabinoxylan with the tyrosine residues of the α-lactalbumine. Modelling showed that α-lactalbumine, a globular protein, has solvent exposed tyrosines that can be targeted by peroxidase. Both fractions F-1 and F-2 consisted of a population of arabinoxylan-protein with relatively high protein content, as determined from infrared analysis (Table 1). However, the presence of homo-conjugates of arabinoxylan might not be excluded.

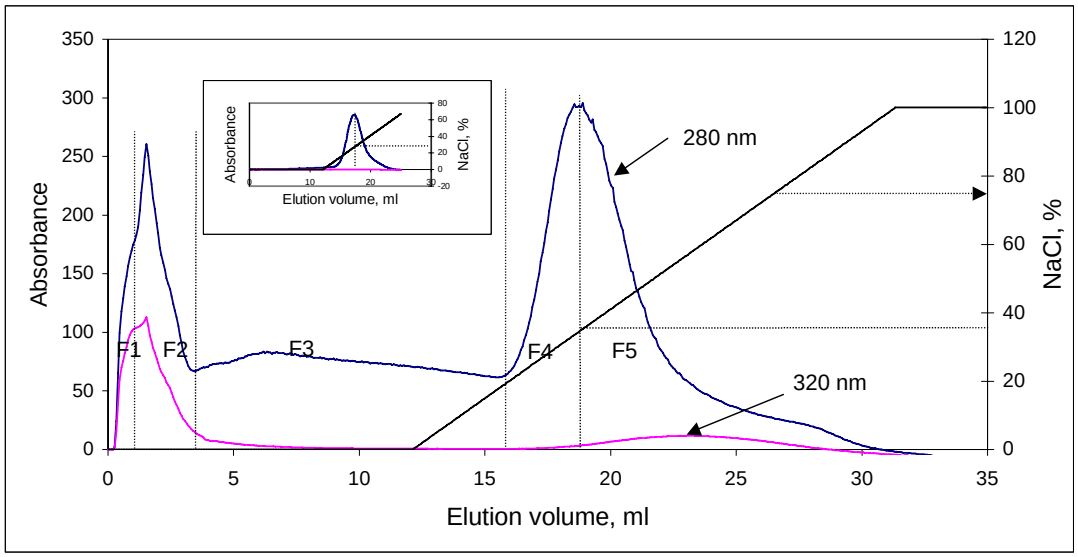

Figure 1. Anion exchange chromatogram of α-lactalbumin – arabinoxylan reaction at pH 7, 25 °C, 500nM HRP and 4 h; Blue line: detection at 280 nm, on left Y-axis; purple line: detection at 320 nm, on the right Y-axis. The insert shows the anion exchange elution pattern of a 1 % α-lactalbumin.

.

Table 1. The composition of protein-polysaccaharide conjugates containing fractions isolated by AEC from incubation of α-lactalbumin with arabinoxylan and peroxidase.

Sample	[P]/[AX], mol/mol	% Protein	% AX
F-1	4.2	18.4	82.6
F-2	11.4	48.7	51.3
F-3	n.d.	100.0	0
F-4	n.d.	99.2	0
F-5	n.d.	97.2	2.8

The second major peak in the chromatographic trace, eluting at a salt gradient of 38 %, can be assigned to a mixture of unreacted α-lactalbumin (fraction F4) and a population of high-protein crosslinked conjugates (fraction F5). This fraction, containing about 98 % protein, might consist of homo-protein conjugates and heteroconjugates of α-lactalbumin with the law-molecular weight arabinoxylan oligosaccharides. A third minor fraction (F3) elutes with the wash and consis mainly of protein.

The products isolated by fractionation of the reaction mixture obtained from incubations of α-lactalbumin and arabinoxylan with peroxidase/hydrogen peroxide were characterized by FT-IR. The infrared spectra of the isolated products (Figure 2) show the specific vibrations for the amide I (1640 cm^{-1}) and amide II (1530 cm^{-1}) of the protein as well as the vibrations arising from the C-O-C bonding (900-1220 cm^{-1}) in polysaccharides. A shift towards lower frequency of the amide I and amide II vibrations in the FT-IR spectra of F-1, F-2 and F-5 was observed, that can be associated with a change of the protein conformation upon binding.

Table 1 shows the composition of the fractions isolated from AEC as determined by infrared spectroscopy and chemometric analysis.

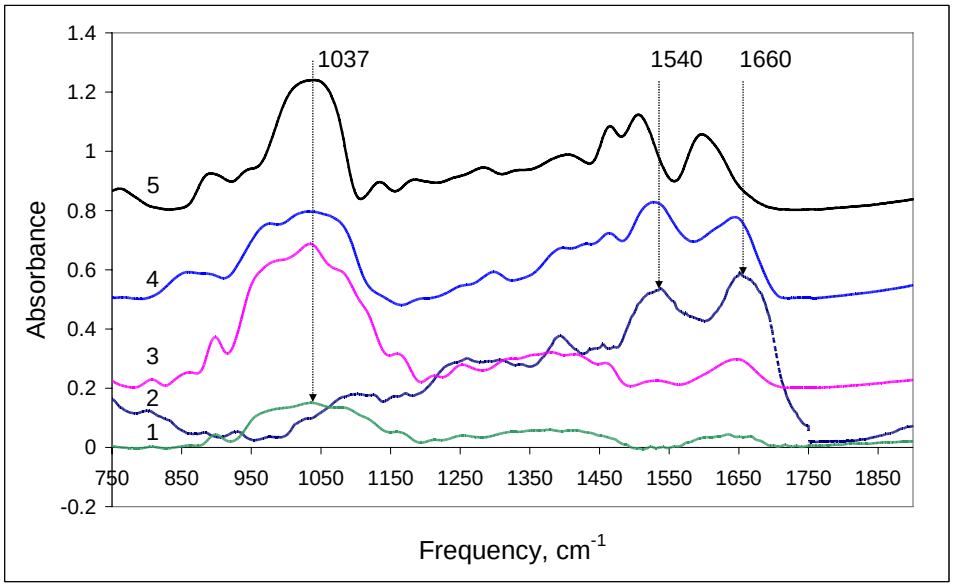

Figure 2. FT-IR spectra of isolated products. Lane 1: Arabinoxylan; Lane 2: α-lactalbumin; Lane 3: Fraction F-1; Lane 4: Fraction F-2; Lane 5: Fraction F-5

Functional properties of the fractions isolated by AEC were measured and compared with the properties of the starting materials. Fractions F-1 and F-2, consisting of mixtures of hetero-polymers containing 4.2 and 11.2 moles of protein bound per mole of arabinoxylan, respectively, showed higher viscosity as compared with the starting proteins and a physical mixture of α-lactalbumin and arabinoxylan (Figure 3).

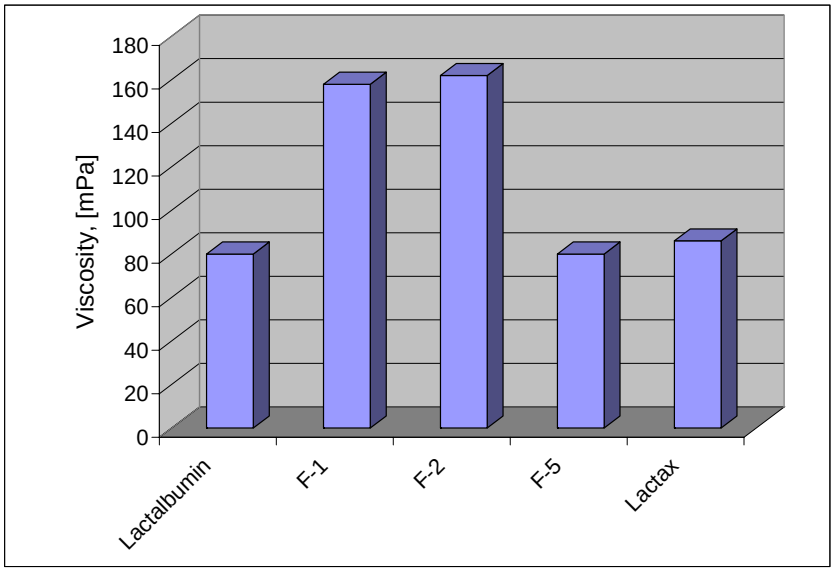

Figure 3. Dynamic viscosity of α-lactalbumin and isolated α-lactalbumin – arabinoxylan conjugates. Lactalbumin: a solution of 1 % α-lactalbumin; Lactax – the product from a control incubation of mixture of α-lactalbumin and arabinoxylan, wihout peroxidase and hydrogen peroxide.

Conclusions

This study shows that peroxidase can be used to cross-link feruloylated arabinoxylan and α-lactalbumin. A range of arabinoxylan - α-lactalbumin conjugates with different protein concentration were obtained by chromatographic fractionation.
The novel protein-polysaccharide conjugates can be potentially used in food products as thikening agents.

References

TANIMOTO S.Y., KINSELLA J.E., *Enzymatic Modification of Proteins: Effects of Transglutaminase Cross-Linking on Some Physical Properties of β-Lactoglobulin,* J. Agric. Food Chem. **36**, 281-285**,** 1988.
MATHEIS G., WHITAKER J.R., *A review: enzymatic cross-linking of proteins applicable to foods,* J. Food Biochem. **11**, 309-327, 1987.
KIM M.H., *The Effect of Ascorbic Acid on the Enzyme Reaction in Pyridinoline Formation during Soluble Collagen Maturation,* J. Korean Soc. Food Sci. Nutrition. **27**, 305-312, 1998.
MATHEIS G., WHITAKER J.R., *Modification of proteins by polyphenol oxidase and peroxidase and their products* , J. Food Biochem. **8**, 137-162, 1984.
BOERIU C.G., OUDGENOEG G., SPEKKING W., VANCON L., BOUMANS H., GRUPPEN H., VAN BERKEL W.H.J., LAANE C., VORAGEN A.G.J., *Horseradish Peroxidase-Catalyzed Cross-Linking of Feruloylated Arabinoxylans with β–Casein,* J. Agric Food. Chem, **52(21)**, 6633-6639, 2004.
OUDGENOEG G., HILHORST R., PIERSMA S.R., BOERIU C.G., GRUPPEN H., HESSING M., VORAGEN A.G.J., LAANE C., *Peroxidase mediated cross-linking of a tyrosine. containing peptide with ferulic acid,* J. Agric. Food. Chem. **49**, 2503-2510, 2001.
OUDGENOEG G., DIRKSEN E., INGEMANN S., HILHORST R., GRUPPEN H., BOERIU C.G., PIERSMA R.S., VAN BERKEL W.H.J., LAANE C., VORAGEN A.G.J., *Horseradish peroxidase-catalyzed oligomerization of ferulic acid on a template of a tyrosine-containing tripeptide*, J. Biol. Chem. **277**, 21332-21340, 2002.