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Horseradish Peroxidase-Catalyzed Cross-Linking of Feruloylated Arabinoxylans with β-Casein

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Heterologous conjugates of wheat arabinoxylan and β-casein were prepared via enzymatic cross-linking, using sequential addition of the arabinoxylan to a mixture of β-casein, peroxidase, and hydrogen peroxide. The maximal formation of adducts between the β-casein and the feruloylated arabinoxylan was reached at a protein-to-arabinoxylan ratio of 10:1, in combination with a molar ratio hydrogen peroxide to substrate of 2:1 and a molar protein-to-enzyme ratio between 10^2 and 10^4. The protein−arabinoxylan adducts were separated from the arabinoxylan homopolymers by size exclusion and anion exchange chromatography. The molar ratio protein:arabinoxylan in the purified conjugates varied between 0.1 and 5.6. This is the first report on the large-scale enzymatic preparation of heterologous protein−arabinoxylan conjugates.

KEYWORDS: Peroxidases; protein; arabinoxylan; cross-linking; heterocoupling; β-casein

INTRODUCTION

Proteins and polysaccharides have found multiple applications in both the food and the nonfood industry. Functional properties of proteins of importance for industrial application include viscosity, oil and water binding, gelling, foaming, emulsifying properties, adhesive strength, colloid protective properties, or a specific molecular size or shape (*1*). The protein properties are largely influenced by environmental conditions, such as solvent nature, pH, temperature, and ionic strength (*2*). Polysaccharides are structure-forming macromolecules that have been used as thickening, gelling, and suspending agents and as stabilizers in foams and emulsions.

Covalent coupling of proteins to polysaccharides, which maintains to a certain extent the structural integrity of the primary polymers, results in the formation of new macromolecular structures and may lead to improved or novel functional properties and increased resistance to heat, proteolytic attack, and organic solvents. The properties of such protein−carbohydrate conjugates will probably depend on the size, structure, and number of the attached protein and carbohydrate fragments.

Chemical coupling of proteins with polysaccharides has been reported. Usually, polysaccharides are coupled to proteins after random activation of functional groups along the internal part of the chain or after selective activation of their terminal reducing end group using a variety of activating agents (*3*). Recently, the potential of the Maillard reaction for preparation of protein−polysaccharide conjugates has been investigated. Kim and co-workers (*4*) prepared bovine serum albumin (BSA)−galactomannan conjugates containing 2.5−7 mol of galactomannan per mole of protein. These conjugates showed higher stability and emulsifying properties than BSA alone. Conjugation of gelatin to chitosan catalyzed by polyphenol oxidase has also been reported (*5*). The authors described the formation of gellike structures that differed mechanically from the gels that were obtained by cooling gelatin solutions and that could be broken down by chitosinase.

As a new approach to prepare protein−polysaccharide conjugates, we investigated the coupling of proteins to polysaccharides catalyzed by the peroxidase/hydrogen peroxide system. Water soluble wheat arabinoxylan (AX) containing ester-linked ferulic acid (FA, 3-methoxy-4-hydroxycinnamic acid) residues

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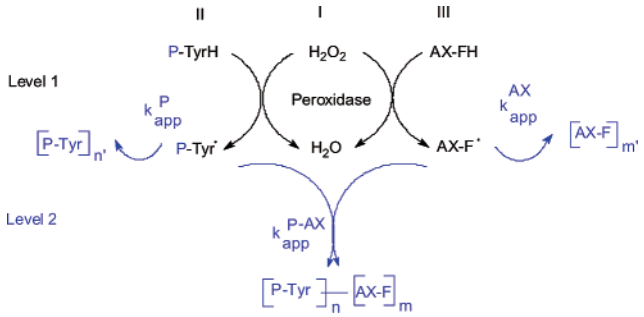

Figure 1. Schematic representation of the chemoenzymatic cross-linking of proteins with polysaccharides. P-TyrH, protein with reactive tyrosine; AX-FH, AX containing reactive FA residues; $(k^{P})_{app}$, apparent rate of enzymatic protein cross-linking; $(k^{AX})_{app}$, apparent rate of enzymatic AX cross-linking; $(k^{P-AX})_{app}$, apparent rate of enzymatic protein–AX cross-linking; *n*, *n*′, number of protein units in protein–AX and protein–protein adducts, respectively; *m*, *m*′, number of AX units in protein–AX and AX–AX adducts.

was used as the model polysaccharide. The milk protein β-casein, a protein with a flexible, essentially random structure, was used as the model protein. Horseradish peroxidase (EC 1.11.1.7; donor, hydrogen peroxide oxidoreductase) was selected as cross-linking biocatalyst since it is a versatile enzyme, which accepts a wide range of phenolic derivatives as hydrogen donors. In vitro peroxidase-mediated cross-linking via phenolic bridges has been reported both for proteins containing tyrosine residues (*6*−*8*) and for carbohydrate polymers carrying phenolic residues such as cereal AX (*9*−*12*) and beet pectin (*13*). The formation of hetero-cross-links between tyrosine moieties in a protein and phenolic residues in a polysaccharide by the action of peroxidase is therefore expected.

When a mixture of protein and AX is incubated with peroxidase and hydrogen peroxide, several competitive enzymatic and chemical (e.g., radical) reactions can take place, resulting in the formation of a range of protein−protein, AX−AX, and protein−AX conjugates (**Figure 1**). The relative contribution of each reaction is determined primarily by the difference in reactivity of the target groups with peroxidase. Also, the number and the accessibility of the reactive groups of each polymer control the reaction rate. Cross-linking of amino acids in a protein with functional moieties in a carbohydrate polymer requires exposed target groups and no or limited steric hindrance. Another factor that might affect cross-linking of the two macromolecules is their thermodynamic compatibility, which is related to the nature and intensity of the interaction as they approach each other (*23*, *24*).

Cross-linking of AX to proteins by oxidative enzymes has long been suggested (*14*, *15*) but never proven. Attempts of different research groups to cross-link proteins to AXs with either laccase or horseradish peroxidase (*16*) were unsuccessful.

Recently, we demonstrated the intermolecular cross-linking of a tyrosine-containing peptide (GYG) with FA by peroxidase-mediated dehydropolymerization in a kinetic-controlled reaction and characterized the heteroadducts formed under different reaction conditions (*17*, *18*). Moreover, we showed that cross-linking of both flexible and globular proteins by peroxidase-mediated oxidative dehydropolymerization in the presence of exogenous phenol is feasible (*19*). In this study, we report the synthesis of heteroconjugates of AX and β-casein, using peroxidase as the catalyst for the oxidative cross-linking reaction and applying the kinetic control concept developed in model studies (*16*, *17*).

Table 1. Parameters Studied on the Planned Factorial Design[a]

	level		
factor	−1	0	+1
molar ratio protein to AX (P/AX)	1	3	5
molar ratio substrate to enzyme (S/E)	10^2	5×10^3	10^4
hydrogen peroxide concentration (mM) (HP)	1	5	10
reaction time (h) (ζ)	1	2.5	4

[a] The following conditions were maintained constant: temperature, $T = 25$ °C; buffer molarity = 100 mM; and pH 7.0.

MATERIALS AND METHODS

Chemicals. Horseradish peroxidase (EC 1.11.1.7, type VI, 250 U/mg) was obtained from Sigma-Aldrich (Zwijndrecht, Netherlands). Bovine β-casein [90% β-casein based on mass, 95% β-casein based on nitrogen (w/w)] was obtained from Eurial (Rennes, France) and contained mainly the genetic variants A^1 and A^2. Hydrogen peroxide (30% solution, w/v) was from Merck (Darmstadt, Germany). Wheat AX [AX content, 97% (w/w), containing approximately 0.2% FA (w/w)] was from Megazyme (Bray, Ireland). All other reagents were of analytical grade.

Conjugation of AX to Proteins via Peroxidase/Hydrogen Peroxide Oxidative Polymerization. Oxidative cross-linking of protein with AX 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 7.0, containing 1% (w/v) protein. The mixture was allowed to equilibrate at 25 °C, and subsequently, 3 mL of 1% (w/v) AX solution was added in 10 aliquots of 300 μL each, at regular time intervals of 5 min, during 1 h. The reaction mixture was maintained at 25 °C for 4 h, and then, the reaction was stopped by inactivation of the enzyme (2 min/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 AX), the protein and/or AX solutions, respectively, were substituted by 0.1 M sodium phosphate, pH 7.0. All variations to the standard procedure are reported in the text.

Optimization of Reaction Conditions. To study the influence of reaction parameters on the cross-linking reaction, a fractional factorial design accommodating four variables, each one at two levels (−1/+1), was made. At the medium point (0), independent replicates were run to estimate the standard deviation. The variation between the central replicates reflects the variability of all design. After the runs, the response obtained [in this case, the yield in high molecular weight (HMW) adducts] was submitted to the algorithm of Yates to calculate the effects of each parameter (*20*). The response was used to calculate the coefficients of a first-order equation that shows the dependence of the product yield on all effects (parameters):

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_4 \quad (1)$$

where y is the theoretical response (the value predicted by the model), x_i (i from 1 to 4) represents the factors, and b_i represents the coefficients determined by matrix calculation.

The reaction parameters whose influence on the product yield was studied are molar ratio protein to AX (P/AX), molar ratio total substrate (protein and AX) to enzyme (S/E), H_2O_2 concentration (HP), and reaction time (ζ). **Table 1** gives the corresponding values at each level.

Analytical Size Exclusion (SE) Chromatography. SE-high-performance liquid chromatography (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, a TSK-Gel G-2000 SW_{XL} (300 mm × 7.8 mm), was fitted with a matching guard column (TSK-Gel SW, 7.5 mm × 7.5 mm) and was maintained at 25 °C. Both columns were from TosoHaas (Tokyo, Japan). Before analysis, the samples were diluted with the eluent buffer also containing 1% (w/v) sodium dodecyl sulfate (SDS) to minimize casein self-aggregation and were 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 280 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), BSA (66 kDa), aldolase (158 kDa), and catalase (232 kDa). The SE 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 proportion (%) of the total peak area determined at 280 nm.

Anion Exchange Chromatography. Anion exchange chromatography was performed on a FPLC system AKTA explorer equipped with a UV-900 monitor and Frac-900 fraction collector (Amersham-Biosciences, United Kingdom), using Q-Sepharose columns (0.7 cm × 2.5 cm for analytical runs and 2.6 cm × 40 cm for preparative runs, respectively). Samples were eluted with a buffer system consisting of 0.1M Tris-HCl buffer, pH 7.5, and 0.1 M Tris-HCl, pH 7.5, with 1 M NaCl, with a linear salt gradient from 0 to 100%, at a flow rate of 1 mL/min. The fractions collected were dialyzed (cutoff membrane, 15 kDa) and lyophilized prior to further characterization.

Preparative SE Chromatography. Preparative SE experiments were performed using a FPLC system AKTA explorer equipped with a monitor UV-900 and Frac-900 fraction collector on a column of Sephacryl S-300 HR (2.6 cm × 60 cm). Samples were eluted with 0.1 M phosphate buffer, pH 7, at a flow rate of 1 mL/min. For samples containing β-casein, an eluent consisting of 0.1 M sodium phosphate, pH 7, containing 1% (w/v) SDS and 0.15 M NaCl, was used to minimize protein self-association. The column was calibrated with standards of known molecular mass. The protein standards were α-lactalbumin (14.2 kDa), chymotrypsin (25.7 kDa), BSA (66 kDa), aldolase (158 kDa), and catalase (232 kDa). The fractionation range of the column was between 10 and 1500 kDa for globular proteins and 2 and 400 kDa for polysaccharides, respectively. Peak elution was monitored at 280 and 320 nm. The void volume of the column was determined with blue dextran (2000 kDa) and corresponds to 38 mL.

Characterization of Protein−AX Conjugates by Fourier Transform Infrared (FT-IR) Spectroscopy and Chemometry. IR spectra of protein, AX, and mixtures thereof and of products isolated from cross-linking reactions were obtained on a Bio-Rad FT-IR 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. Sixty-four interferograms were coadded 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 (PLS) regression analysis was applied to correlate the FT-IR spectroscopic data with the chemical composition of AX−protein conjugates in terms of (i) moles of protein bound per mole AX and (ii) % of AX in the sample (w/w). A calibration set was built up from mixtures of AX and casein covering the concentration range 0−100% AX and a molar ratio [P]/[AX] between 0 and 20. The molar ratio protein to AX was calculated using M_w = 24 kDa for β-casein and an average molecular mass of 300 kDa for the wheat AX. The average molecular mass of AX was determined from SEC-HPLC with RI detection (not shown). PLS models were constructed using the 2nd derivative of the spectra for the following spectral regions: 750−1800 cm^{-1} for [P]/[AX], 1250−1800 cm^{-1} for % protein, and 750−1100 cm-1 for %AX, respectively. The PLS models were validated using the "full cross-validation" technique to ensure predictive validity and guarding against over-fitting. The predictive ability of the calibration models is described by the root mean squares error of prediction (*20*). The PLS calibration models were used to determine the composition of each fraction isolated from the incubations of AX with β-casein. The statistical software Unscrambler 6.1 (Camo A/S, Norway) was used for chemometric calculation. **Figure 2** shows the calibration lines of the PLS models developed for the molar ratio protein to AX {[P]/[AX] and AX concentration (%, w/w), respectively}.

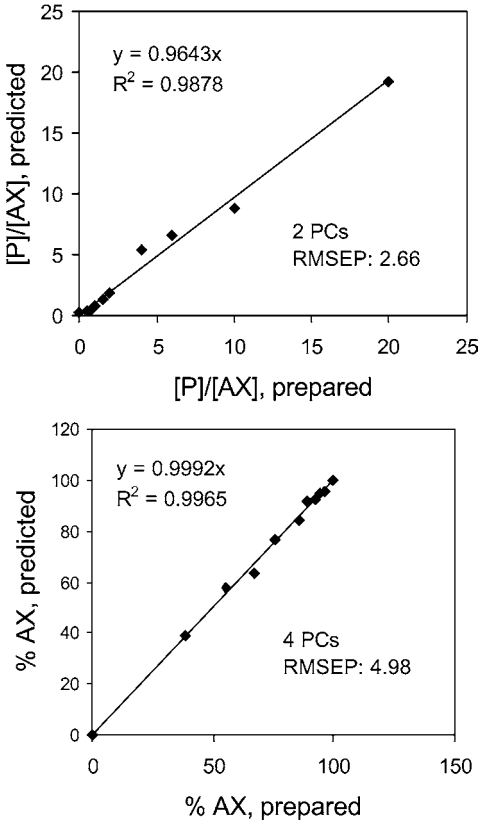

Figure 2. FT-IR-based calibration models.

Determination of the Amount of Arabinose and Xylose. Pure AX, protein, and the products isolated from the cross-linking reaction were hydrolyzed by stirring in 1 M H_2SO_4 for 2 h at 100 °C. The hydrolysates were filtered through a Whatman GF/C glass fiber filter and neutralized with $BaCO_3$. Samples (10 μL) of the neutralized hydrolysates were analyzed for arabinose and xylose by using a HPLC system (Pharmacia LKB low-pressure mixer, HPLC pump 2248 and autosampler 2157) equipped with a Carbopack PA 1 column (250 mm × 4 mm, Dionex). The eluents consisted of Milli-Q water and 150 mM NaOH. Compounds were detected with a Dionex pulsed amperometric detector equipped with a gold working electrode. The applied potentials were set at E_1 = 0.1 V, E_2 = 0.6 V, and E_3 = −0.6 V against an Ag/AgCl reference electrode. Pulse durations for the applied potentials were 500, 100, and 50 ms, respectively. Mannitol, added after hydrolysis of the samples, was used as the internal standard. The sum of arabinose and xylose was used to estimate the amount of AX in the isolated reaction products and the starting materials.

RESULTS AND DISCUSSION

Cross-Linking of β-Casein with AX; Optimization of Reaction Conditions. The SE chromatogram of a concentrated solution of AX (1%) monitored at 280 nm showed that the AX consists of a mixture of low and high molecular mass species (insert, **Figure 3**). The main fraction has HMW, $M_w \cong$ 350 kDa, and elutes between 28 and 32 min. The minor fraction has a broad molecular mass distribution, ranging from 1 to 10 kDa, and elutes between 45 and 65 min. Both the high and the low molecular mass AX fractions contain FA residues, as seen from the absorbance at 280 nm, and consequently can be involved in cross-linking reactions.

Figure 3 shows the analytical SE chromatograms of incubations of β-casein with the peroxidase/hydrogen peroxide system with (1) and without (2) AX. When β-casein was incubated with peroxidase and hydrogen peroxide, without AX (trace 2), only the formation of a low molecular mass casein adduct was

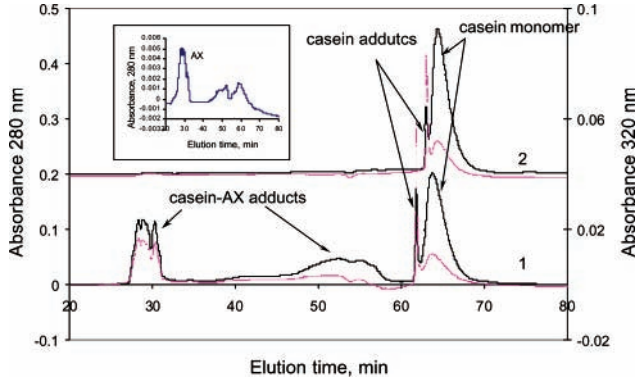

Figure 3. SE-HPLC of β-casein–AX reaction at pH 7, 25 °C, 500 nM HRP, and 4 h. Trace 1: 0.4 mM β-casein, 80 μM AX, 10 mM hydrogen peroxide, and AX sequentially added. Trace 2: 0.1 mM β-casein and 10 mM hydrogen peroxide. Black line: detection at 280 nm, on left *Y*-axis. Purple line: detection at 320 nm, on the right *Y*-axis. The insert shows the SE-HPLC elution pattern of a 1% AX solution.

observed. Sequential addition of feruloylated AX to a solution containing β-casein, peroxidase, and hydrogen peroxide and incubation at pH 7.0 resulted in the formation of higher molecular mass protein-containing conjugates (trace 1). Two groups of new products were formed, one of relatively low molecular mass (LMW-AXP), eluting between 48 and 60 min, representing about 28% of the mixture, and a fraction of high molecular mass (HMW-AXP). The high molecular mass products eluted with the void volume (elution between 28 and 32 min) and accounted for about 38% of the mixture. These protein-containing adducts, absorbing at both 280 (characteristic for polymers containing aromatic residues) and 320 nm (specific for C−C aromatic linkages), are unambiguously identified as AX−β-casein conjugates, since both AX and AX−AX conjugates do not show any significant absorption at these wavelengths for the concentration used in the experiments.

Fractionation of the reaction mixture was achieved by sequential preparative SE and ion exchange chromatography. **Figure 4A** shows the elution pattern of the reaction mixture on a Sephacryl-300 HR column. Four fractions (F1−F4) of different molecular mass were collected, as indicated on the chromatogram and further fractionated on a Q-Sepharose column (**Figure 4B**,**C**).

Figure 4B shows the anion exchange chromatogram of the high molecular mass fraction F-2 obtained from gel filtration. A first small fraction (F2-1) eluted at the void volume and consisted essentially of (cross-linked) AX, as seen from the infrared spectrum (**Figure 5**, lane 1).

The first major fraction (F2-2), representing about 90% of fraction F2, eluted with the running buffer and consisted of a population of AX−casein conjugates with a variable but relatively high content of protein, as determined from infrared analysis (**Table 2**). The last fraction (F2-3), eluted at 30% salt gradient. Infrared spectral analysis revealed that this fraction contained an even higher amount of protein. **Figure 4C** shows the anion exchange chromatogram of fraction F-4 isolated from SE chromatography. The main product of this fraction eluted with the salt gradient (Ev = 26 mL, 50% NaCl).

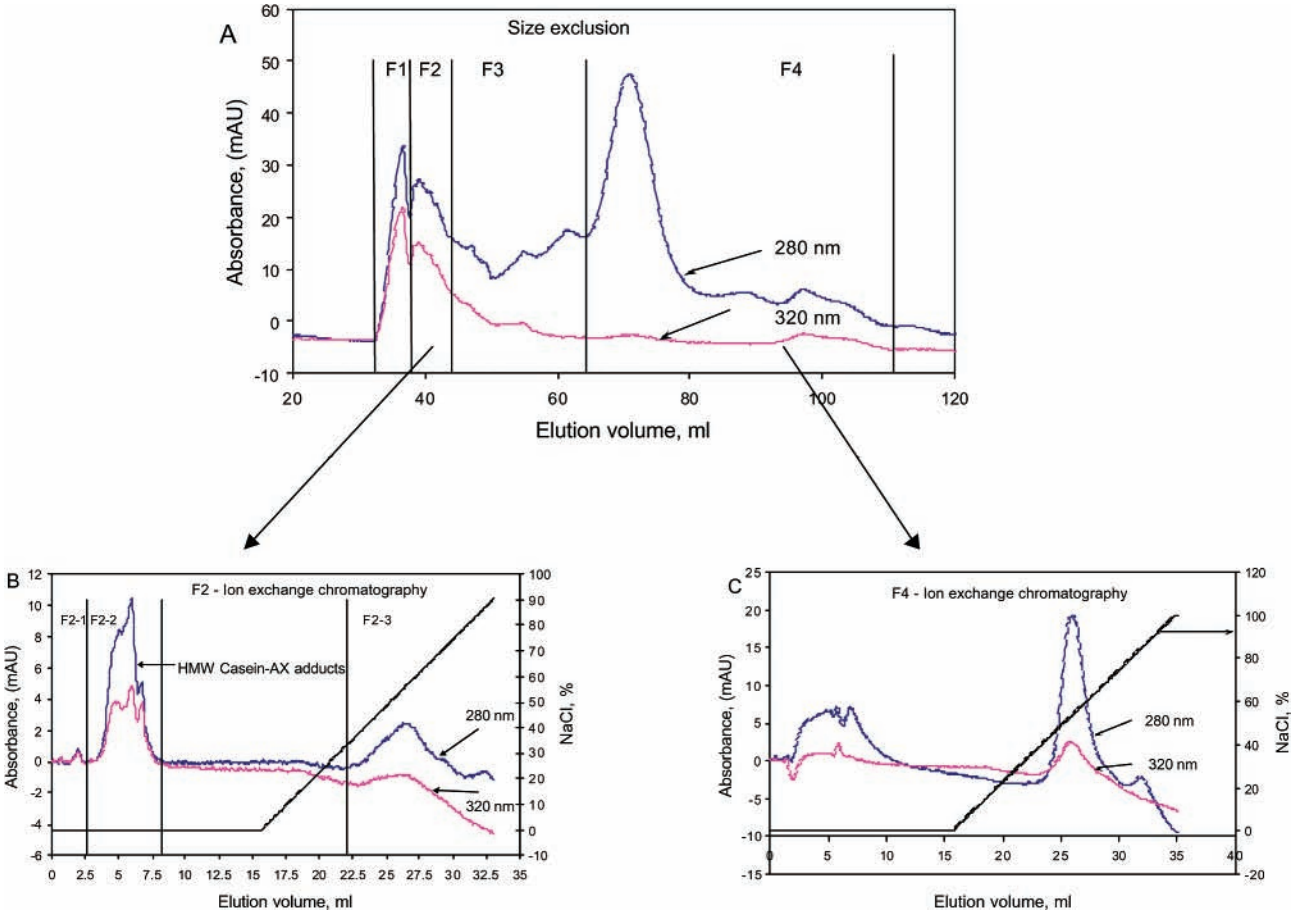

Figure 4. Fractionation of cross-linking products by consecutive gel filtration and anion exchange chromatography. Cross-linking conditions: 80 μM AX, [P]/[AX] = 10, [S]/[E] = 10^3, [H_2O_2]/[S] = 2, pH 7, T = 25 °C, and 24 h. Blue trace, absorbance at 280 nm; purple, absorbance at 320 nm; and black, NaCl gradient.

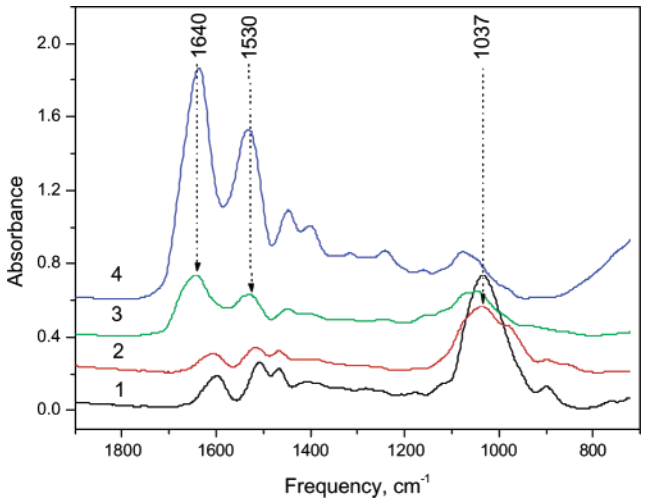

Figure 5. FT-IR spectra of products isolated from anion exchange chromatography. Lane 1, F2-1; lane 2, F2-2; lane 3, F2-3; and lane 4, F4.

The products separated by anion exchange chromatography were characterized by infrared spectroscopy, and their chemical composition was determined by the PLS calibration method, as described in the Materials and Methods section. The infrared spectra of the isolated products (**Figure 5**) show the specific vibrations for the amide I (1640 cm^{-1}) and amide II (1530 cm^{-1}) of the protein as well as for the COC (900−1220 cm^{-1}) in polysaccharides. **Table 2** gives the composition of the products isolated from anion exchange column as determined by using the PLS calibration models developed.

Within the experimental error of the method, we can conclude that F2-1 consists mainly of AX and AX homopolymers. This sample was uncharged and could be well-separated from the protein-containing adducts. The products isolated from fractions F2-2 and F2-3 contained about 3.8 and 14.8 mol of protein per mole of AX, indicative for different AX−β-casein conjugates. These heteroconjugates were well-separated from the homo-AX species and from each other due to the difference in their charge density. The product isolated from fraction F4 of the preparative gel filtration column consisted mainly of β-casein.

These results clearly show the formation of AX−β-casein heteroconjugates by peroxidase-mediated cross-linking. The feruloyl radicals generated by the peroxidase iron-oxo species react with the tyrosyl radicals generated on β-casein to form AX−protein adducts. Because the protein substrate is in excess, the hetero-cross-linking reaction might be favored against homopolymerization of the AX.

Optimization of Reaction Conditions. Further experiments focused on determination of optimal reaction parameters that allow tuning of the reaction toward the formation of protein−AX heteroadducts. **Table 3** shows the yield in high molecular mass heteroadducts (HMW-AXP) for each experimental condition varied according to the experimental design. The relative abundance of different products in the mixture was determined from SE chromatograms and expressed as proportion (%) of the total peak area determined at 280 nm.

The effects of the variables on the yield of high molecular mass AX−casein adducts are given in eq 2.

$$\%\ \text{HMW-AXP} = 0.48(\text{P/AX}) + 5.4 \times 10^{-4}(\text{S/E}) - 1.54(\text{HP}) + 2.37\,\zeta + 17.62;\ R^2 = 0.81 \quad (2)$$

This equation suggests that maximal formation of β-casein−AX adducts will be reached at a high protein-to-AX ratio, in combination with a low hydrogen peroxide concentration, long reaction time, and a molar ratio protein to enzyme between 10^2 and 10^4.

Table 2. Composition of the Protein–AX Cross-Linking Products Isolated by Sequential SE and Anion Exchange Chromatography

product	[P]/[AX]	% β-casein	% AX
F2-1	0.1	0.8	99.6
F2-2	3.8	17.8	89.2
F2-3	14.8	56.4	40.4
F4	40	95	0

Table 3. Product Yields (%) of Levels –1/+1 and Center Replicates[a]

sample	[P]/[AX]	[S]/[E]	H_2O_2 (mM)	time (h)	HMW-AXP (%)	LMW-AXP (%)	unreacted casein (%)
S-1	1	100	1	1	18.2	42.5	39.3
S-2	5	100	1	4	38.0	28.0	30.3
S-3	1	10000	1	4	37.4	27.0	35.4
S-4	5	10000	1	1	27.0	18.0	33.1
S-5	1	100	10	4	13.1	44.6	42.3
S-6	5	100	10	1	12.5	23.0	43.7
S-7	1	10000	10	1	10.6	32.2	54.4
S-8	5	10000	10	4	8.1	20.0	44.9
S-9	2.5	5000	5	2.5	15.7	24.5	54.4
S-10	2.5	5000	5	2.5	17.8	35.8	40.8

[a] Reaction conditions: 80 μM AX; pH 7.0; and T = 25 °C.

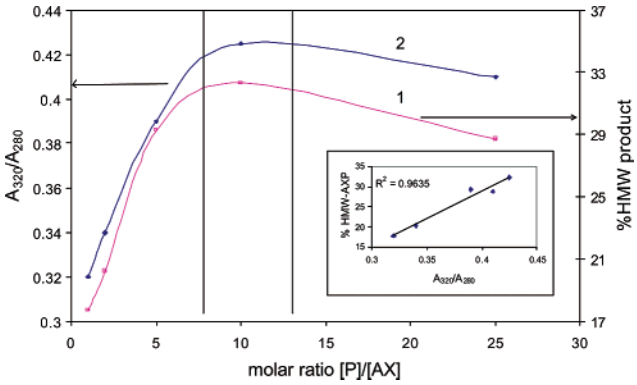

Figure 6. Determination of the optimal molar ratio protein/AX from SE-HPLC data. Reaction conditions: 80 μM AX, [S]/[E] = 10^3, [H_2O_2]/[S] = 2, pH 7, T = 25 °C, and 4 h. Trace 1, A_{320}/A_{280}; trace 2, % HMW-HRP.

In the range of values studied, the response surface suggests that an increase in the molar ratio protein to AX enhances the yield in heteroadducts (no further data shown). **Figure 6** shows the variation of the HMW-AXP product yield for an extended molar ratio, ranging from 1 to 25. High yields in HMW heteroadduct (HMW-AXP product) are obtained for a molar ratio protein to AX ranging between 5 and 12.5, with an optimum at [P]/[AX] = 10. There is a good linear correlation (R^2 = 0.96) between the product yield and the ratio of the intensity of the peak absorbance at 320 nm (characteristic for phenolic cross-links) to that at 280 nm (specific for protein). This implies that the absorbance ratio (A_{320}/A_{280}) can be used for monitoring the cross-linking reaction and the quantification of polymeric adducts coupled through phenolic linkages.

Preparative Cross-Linking Reactions and Characterization of Products. Preparative (gram) scale cross-linking reactions were performed under the predicted optimal reaction conditions previously determined, to synthesize casein−AX and

Table 4. Influence of Reaction Time on the Extent of Cross-Linking[a]

fraction	elution volume (mL)	A_{320}/A_{280} 4 h	A_{320}/A_{280} 24 h
F-1	30–38	0.53	0.64
F-2	38–42	0.42	0.52
F-3	42–70	0.04	0.25
F-4	70–80	0.08	0.08
F-5	>80	0.2	0.1

[a] Reaction conditions: 21.2 μM AX, [P]/[AX] = 10, [S]/[E] = 10^3, [H_2O_2]/[S] = 2, pH 7, and T = 25 °C; total reaction volume, 150 mL.

Table 5. Protein and Carbohydrate Composition of Protein–Polysaccharide Conjugates Isolated from Incubation of AX with β-Casein[a]

	FT-IR method		HPLC method			
sample	[P]/[AX]	% P	arabinose (mg/mg)	xylose (mg/mg)	Ara/Xyl	% AX
AX	0	0	0.28	0.64	0.44	91.7
AE-1	2.8	20.0	0.21	0.47	0.43	68.4
AE-2	5.6	22.6	0.16	0.37	0.43	53.4
AE-3	43.5	97.2	4.8×10^{-2}	5.7×10^{-2}	0.84	1.2

[a] Reaction conditions: 21.2 μM AX, [P]/[AX] = 10, [S]/[E] = 10^3, [H_2O_2]/[S] = 2, pH 7, T = 25 °C, and 24 h; total reaction volume, 150 mL.

AX conjugates. For this purpose, the reaction time was extended to 24 h. This resulted in a significant increase of cross-linking, as shown by the ratio A_{320}/A_{280} determined from SE chromatograms (**Table 4**). Fractionation of the reaction products of the large scale synthesis by SE and ion exchange chromatography resulted in three major products: AE-1, eluting with the void volume of the anion exchanger; AE-2, eluting by the running buffer; and AE-3, eluting with the salt gradient. The products were characterized by FT-IR, and the amount of AX was determined also by HPLC, after hydrolysis of the samples. Products AE-1 and AE-2 were identified as AX−β-casein adducts, containing 68.4 and 53.4% AX, respectively (**Table 5**). AE-3 mainly consisted of protein and contained only 1.2% AX. The AX contained in this product had a higher ratio arabinose to xylose than the standard wheat AX used in the reaction.

In conclusion, the results of this study show that feruloylated arbinoxylan and β-casein are compatible polymers that have the ability to form heterologous conjugates in a peroxidase-mediated cross-linking reaction. Fractionation of the reaction mixture by sequential SE and anion exchange chromatography resulted in a range of AX−β-casein heteroadducts with different protein contents.

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