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Protein Analysis by ^{31}P NMR Spectroscopy in Ionic Liquid: Quantitative Determination of Enzymatically Created Cross-Links

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ABSTRACT: Cross-linking of β-casein by *Trichoderma reesei* tyrosinase (TrTyr) and *Streptoverticillium mobaraense* transglutaminase (Tgase) was analyzed by ^{31}P nuclear magnetic resonance (NMR) spectroscopy in ionic liquid (IL). According to ^{31}P NMR, 91% of the tyrosine side chains were cross-linked by TrTyr at high dosages. When Tgase was used, no changes were observed because a different cross-linking mechanism was operational. However, this verified the success of the phosphitylation of phenolics within the protein matrix in the IL. Atomic force microscopy (AFM) in solid state showed that disk-shaped nanoparticles were formed in the reactions with average diameters of 80 and 20 nm for TrTyr and Tgase, respectively. These data further advance the current understanding of the action of tyrosinases on proteins on molecular and chemical bond levels. Quantitative ^{31}P NMR in IL was shown to be a simple and efficient method for the study of protein modification.

KEYWORDS: Tyrosinase, transglutaminase, β-casein, cross-linking, ^{31}P NMR, AFM

INTRODUCTION

Enzymatic modification of proteins via cross-linking is an attractive way to alter and improve the chemical and physicochemical properties of protein-based products such as yogurt and cheese in the food industry as well as of foams and emulsions in cosmetics and pharmaceuticals.[1] In food applications improved texture (mouthfeel), low fat content (energy content), and stable gel formation (sensation of satiety) are properties that consumers typically appreciate.[2,3] Milk proteins, such as caseins and whey proteins, are food components of particular interest in the dairy industry due to their importance in human nutrition and allergenicity.[4] Enzymatic cross-linking of caseins may be an attractive way to overcome milk-derived health problems in addition to improving the quality of products.[5] When enzymatic modifications are combined with chemical (organic solvents, salts, pH) and physical (heat, milling, pressure) treatments, the applicability of the technology may be further expanded.[6,7]

The effects of various cross-linking enzymes, for example, tyrosinases, laccases, peroxidases, and transglutaminases on proteins have been extensively reported in various publications.[2,8−10] In addition to direct cross-linking, laccases and peroxidases can modify protein material[9,11] using small phenolic molecules as mediators.[2] Cross-linking of proteins by enzymes is challenging to study at the chemical bond level, although several serious attempts have been performed recently using different analytical[2,8,9] and rheological methods.[2] The type, position, and extent of individual cross-links produced by various enzymes in proteins are still largely unknown due to the complexity of the cross-linked protein material and the lack of sensitive analytical techniques capable of detecting individual cross-links of different types. However, the chemical structure and the amount of individual cross-links are crucial criteria for understanding how cross-linking of proteins affects, for example, digestibility, allergenicity, processing, and other functional properties.

NMR spectroscopy is widely used to study protein structures,[12,13] folding,[14] and modifications[15,16] in solution. In addition to structures, noncovalent protein−protein interactions[13] have also been studied extensively by this technique. ^{31}P NMR is a widely used method for structural and chemical characterization of various biopolymers such as cellulose and lignin in wood.[17] In addition, the method has been exploited in food applications, for example, to analyze mono- and diglycerides in olive oils.[18] Typically, a phosphitylating reagent such as 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (PR[II]) has been employed in derivatization of the free hydroxyl groups, making them detectable by NMR as shown in Scheme 1.[19] An ether bond is formed in the case of phenolic and aliphatic hydroxyl groups, whereas in the case of carboxylic acid hydroxyl group, an ester bond is formed. This reagent allows excellent separation of aliphatic,

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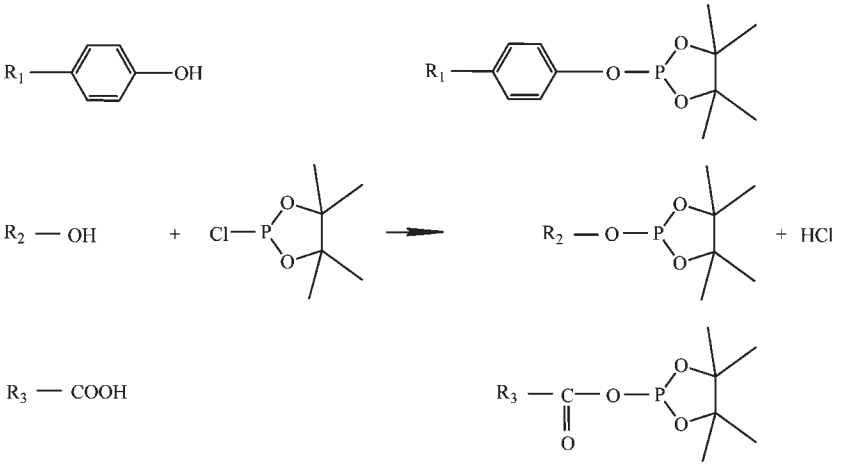
Scheme 1



phenolic, and carboxylic acid hydroxyl groups in the NMR spectrum.[18,20] However, until now this method has not been applied to protein material containing the corresponding functionalities. Thus, it is assumed that after the peak integration of phosphitylated hydroxyls of the various amino acid side chains in peptides and proteins, the amount of phopsphitylated hydroxyls could be quantitatively assessed against a stable internal standard such as *endo*-*N*-hydroxy-5-norbornene-2,3-dicarboximide (*e*-HNDI).[21] As the phenolic hydroxyls enzymatically cross-linked are not accessible to modification by PR[II], the amount of cross-links may be quantified from the decrease of the integrated signal.

Inorganic salts, buffers,[22] and organic solvents[23] have commonly been used to improve the solubility of proteins. During recent years a number of different ionic liquids (ILs) have also been tested for solubilization of proteins due to their attractive chemical and physical properties. They are thermally stable, nonvolatile, and nonflammable solvents,[24] which have replaced organic solvents, for example, in studies of recombinant spider silk proteins[25] and in analyses of wood components such as cellulose[17,26,27] and suberin.[28] Usually, 1-allyl-3-methylimidazolium chloride ([amim]Cl) has been used due to its low melting point and viscosity. In addition, it is easy to synthesize due to its high chloride content.[17] Clearly, ILs are attractive novel potential media for the characterization of enzymatically cross-linked proteins.

In this study the potential of the ^{31}P NMR method was evaluated for the quantitative analysis of free reactive functional groups in native and enzymatically cross-linked proteins dissolved in ILs. *Trichoderma reesei* tyrosinase (TrTyr) was used as a cross-linking enzyme forming covalent bonds via phenolic groups. Transglutaminase (Tgase), forming isopeptide bonds between lysine and glutamine side chains, was used as a reference enzyme to assess the quantitativity of the phosphitylation. In addition, atomic force microscopy (AFM) was used to study the morphology of the enzymatically cross-linked proteins.

MATERIALS AND METHODS

Chemicals. Serine and glutamic acid as well as 4-hydroxybenzoic acid and 2-hydroxyethanoic acid were obtained from Fluka (Taufkirchen, Germany). Tyrosine was purchased from Merck (Darmstadt, Germany). GYG peptide was obtained from Bachem (Bubendorf, Switzerland) and EGVYVHPV peptide from AnaSpec (Fremont, CA). Bovine β-casein (purity ≥ 85%) was obtained from Sigma-Aldrich (St. Louis, MO) and further purified as described under Results and Discussion. Urea was obtained from Merck, and imidazole and NaCl were obtained from Sigma-Aldrich (St. Louis, MO).

Characterization of Purified β-Casein. Fractions of chromatographically purified β-casein were collected and analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE; Bio-Rad Laboratories, Richmond, CA) to estimate the molecular mass (MM) of the main β-casein component after fractionation of 85% pure β-casein by Äkta purifier (Amersham Pharmacia Biotech, Piscataway, NJ). The gels used for the analysis were 10−20% Tris-HCl Criterion PreCast gels (Bio-Rad Laboratories, Richmond, CA). Coomassie Brilliant Blue staining (Pharmacia, Uppsala, Sweden) was used for visualization of the bands. The MM estimation was based on the method developed by Läemmli[29] using prestained MM standards (MM, 6.6−203 kDa) from Bio-Rad Laboratories (Hercules, CA).

The more accurate MM of the main β-casein fraction was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) using Autoflex II (Bruker Daltonics, Bremen, Germany) equipped with a UV/N_2-laser (337 nm/100 μJ). Sinapic acid (Bruker Daltonics) solubilized in Milli-Q water (Millipore, Molsheim, France) containing 50% acetonitrile and 0.1% TFA was used as matrix solution. The purified β-casein fraction was mixed with the matrix solution (1:1 v/v), and 1 μL of the mixture was applied on the stainless steel MALDI target plate. The sample spot was dried under a gentle air stream at ambient temperature. The mass spectrum (10−25 kDa) was measured in linear positive-ion mode. Protein standard solution I (206355, Bruker Daltonics) was used for the external molecular mass calibration.

Enzymes. Fungal tyrosinase from *Trichoderma reesei* was produced, purified, and characterized at VTT as described by Selinheimo et al.[30] Microbial transglutaminase from *Streptoverticillium mobaraense* was purchased from Ajinomoto Co. Inc. (Tokyo, Japan) and further purified, according to the method of Lantto et al.[31] TrTyr activity was measured spectrophotometrically using 15 mM L-3,4-dihydroxyphenylalanine (L-DOPA, Sigma-Aldrich) as substrate at pH 7.0 according to the method developed by Robb.[32] The activity of Tgase was measured using the *N*-carbobenzoxy (CBZ)-glutaminyl-glycine/hydroxylamine assay described by Folk.[33] The specific activities of the enzyme stock solutions were 380 and 420 nkat mg^{-1}, respectively.

Cross-Linking Reactions. For the cross-linking of β-casein by TrTyr and Tgase, purified β-casein was dissolved in Milli-Q (Millipore) water at 1 mg mL^{-1} concentration and incubated with the enzymes using three different enzyme dosages: 10, 100, and 1000 nkat g^{-1}. In the case of TrTyr a 24 h incubation time was used, whereas in the case of the

reference enzyme, Tgase, a 2 h incubation time was sufficient to produce β-casein with various degrees of cross-linking as described by Monogioudi et al.[8] The enzymatic reactions were performed at 40 °C[34] and pH 7.0. Constant stirring and the presence of ample oxygen, essential for the action of TrTyr, were ensured using open vessels during incubation of the samples. All enzymatic reactions were terminated by boiling for 10 min prior to characterization of the reaction products. Reference samples without enzyme addition were analyzed in parallel with the actual samples. All reactions were performed in duplicate.

^{31}P NMR Spectroscopy of Model Compounds and Protein-Based Materials. For the solubilization of selected model compounds and cross-linked proteins, two ILs, [amim]Cl and 1-butyl-3-methylimidazolium chloride ([bmim]Cl), were synthesized according to the methods of Granström et al.[26] and Dyson et al.,[35] respectively. Other chemicals such as chloroform, deuterated chloroform ($CDCl_3$), chromium acetylacetonate ($Cr(acac)_3$), *e*-HNDI, and PR[II] used in the phosphitylation were purchased from Sigma-Aldrich. Pyridine was obtained from Fluka.

For the analyses, the accurately weighed samples (ca. 40 mg) were stirred in [amim]Cl (0.5 mL, 500 mg) in a 5 mL screw-top glass sample bottle and gently warmed (~60 °C) until a clear homogeneous solution was obtained. Then pyridine (150 μL) was added in one portion, and the samples were vortexed for ca. 30 s until visibly homogeneous. After the samples had cooled to ambient temperature, PR[II] reagent (200 μL, 1.26 mmol) was added in one portion and vortexed for ca. 30 s until a visibly homogeneous, white to yellow, cream paste was formed. Then, a solution of $Cr(acac)_3/CDCl_3$ (1 mg mL^{-1}, 500 μL) was added in four portions (125 μL) with vortexing for ca. 30 s between each addition. Internal standard, that is, *e*-HNDI solution (121.5 mM in 3:2 pyridine/$CDCl_3$), was added (125 μL) in one portion,[36] and the solution was vortexed for ca. 30 s. Finally, 500 μL of $Cr(acac)_3/CDCl_3$ solution was added to further decrease the viscosity.

Quantitative ^{31}P NMR spectra were recorded from the samples in 5 mm o.d. tubes using a Varian Unity INOVA 600 MHz spectrometer equipped with a quadruple probe to detect ^{31}P nuclei in the purified and enzymatically cross-linked and phosphitylated β-casein. In the case of small model compounds a Bruker 300 MHz spectrometer (Newark, DE) was used to measure the ^{31}P NMR spectra. In all cases a total of 256 scans were acquired for each sample, with a relaxation delay time of 5.0 s and an acquisition time of 5.24 s, ensuring sufficient resolution and a good signal-to-noise ratio. All spectra were acquired at 25 °C. Typical 90° pulse lengths were 11−12 μs determined using lower molecular weight *e*-HNDI resonance recorded under standard conditions. The optimized long recycle times (5 s) in addition to an excess of $Cr(acac)_3$ as a relaxation agent ensured the quantitative measurements.[37] The chemical shifts of ^{31}P NMR spectra were calibrated according to the signal derived from the phosphitylated *e*-HNDI at 152.0 ppm. The phosphitylated hydroxyls of the various amino acid side chains in peptides and proteins as well as in single amino acids were quantified against the same internal standard after peak integration.

AFM of Enzymatically Cross-Linked β-Casein. To further study the structure, that is, size and morphology, of the enzymatically cross-linked β-caseins, square-shaped mica substrates (area = 1 cm^2) were attached onto steel supports with double-sided tape. A NanoScope IIIa Multimode AFM (Digital Instruments/Veeco Instruments, Santa Barbara, CA) equipped with an "E" scanner was used for the measurements. Silicon cantilevers (NanoSensors GmbH, Wetzlar, Germany) with a nominal resonance frequency of ca. 300 kHz and a tip radius of ca. 10 nm were used in the analyses. For the actual experiments, 10 μL of cross-linked β-casein solution (1 mg mL^{-1}) was pipetted onto freshly cleaved mica surfaces and left to dry at ambient temperature. Imaging of the surfaces was performed in tapping mode with scan rates around 1 Hz. The ratio of set-point amplitude (A_{sp}) to free amplitude (A_f) was 0.7−0.85. For the image data analysis, a NanoScopeIII offline workstation and Scanning Probe Imaging Processor software (SPIP, Image Metrology, Hørsholm, Denmark) were used to visualize the data. The image processing step included flattening the baseline of the image, setting of a threshold level at 1 nm, and calculation of some specific parameters. Imaging was reproducible, as similar structures were visible at certain areas of all protein films prepared according to the method described above. Measurements were performed in duplicate.

RESULTS AND DISCUSSION

Selection Criteria of Model Compounds and Protein Materials. Enzymatic cross-linking of β-casein by TrTyr and Tgase, under the experimental conditions used in this study, has been evidenced in our previous studies.[8,37] However, in contrast to those studies, chromatographically purified β-casein was now used as a substrate to quantitatively study enzymatic cross-linking reactions. Thus, to facilitate interpretation and discussion of the data, the purified β-casein was modified by TrTyr and Tgase using the same reaction conditions as before. The use of 10 nkat g^{-1} enzyme dosage for cross-linking reactions whether by TrTyr or by Tgase did not have a significant effect on the polymerization of β-casein, whereas the use of a 10-fold higher enzyme dosage resulted in intermediately polymerized material. When the high enzyme dosage of 1000 nkat g^{-1} was used, β-casein was fully polymerized.[8]

Clearly, these two sets of cross-linked β-caseins provided a useful series of model polymers to be used as protein matrix, in addition to the individual organic model compounds, amino acids (tyrosine, serine, and glutamic acid), peptides (GYG and EGVYVHPV), and non-cross-linked β-casein, for the development of a ^{31}P NMR method for the analysis of protein modifications via various hydroxyl groups. To ensure the performance of the widely accepted ^{31}P NMR method in the selected reaction conditions for protein-based materials, 4-hydroxybenzoic acid and 2-hydroxyethanoic acid, containing all possible reactive hydroxyl groups for the PR[II] reagent, were used as single model compounds.

Purification of β-Casein for the Enzymatic Cross-Linking Reactions. For the quantitative determination of cross-links created by TrTyr, as well as to ensure that ^{31}P NMR in ILs is applicable for the analysis of modifications in the protein matrix in general, impure β-casein was fractionated according to a protocol of Kauf and Kensinger[38] and further developed and optimized in this study as described below. Prior to chromatographic fractionation by the Äkta purifier system equipped with 6 mL Resource Q anion-exchange column (GE Healthcare, Chalfont St. Giles, U.K.) and a UV detector operating at $\lambda \sim 280$ nm (Amersham Pharmacia Biotech), impure β-casein was dissolved in the eluent buffer (mixture of 1 M urea and 0.01 M imidazole, pH 6.75) at 100 mg mL^{-1} concentration. The optimized amount of injected protein was 10 mg. The optimized flow rate for the successful fractionation was 6 mL min^{-1} when using a stepwise gradient of NaCl in eluent buffer: 30 mL at 75 mM, 30 mL at 175 mM, and finally 60 mL at 500 mM NaCl concentration.

The chromatogram of the fractionated β-casein is shown in Figure 1A. Four well-resolved peaks were detected in addition to the main component (elution volume = 120 mL). After that, all fractions were analyzed by SDS-PAGE for the MM evaluation (data not shown). The SDS-PAGE of the purest β-casein fraction is shown in Figure 1B (lane 3). The bands of the unpurified β-casein are shown in lane 2. On the basis of the analysis, the main component of the fractionated β-casein is almost 100% pure β-casein. Comparison of the bands in lanes 2 and 3 to the

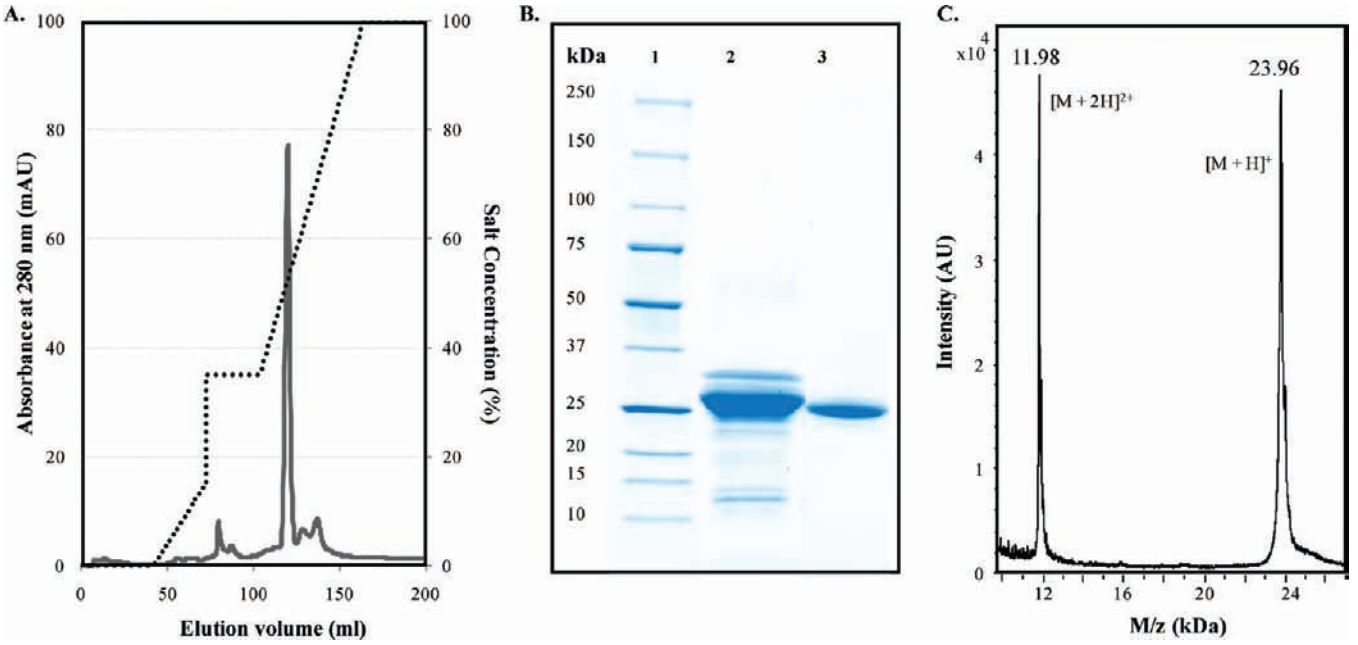


Figure 1. Purification and analysis of β-casein: (A) anion exchange chromatographic profile; (B) SDS-PAGE (lane 1, MM marker; lane 2, unpurified β-casein from Sigma-Aldrich; lane 3, purified β-casein fraction from anion exchange chromatography shown with a small arrow); (C) MALDI-TOF mass spectrum of the purified β-casein fraction (positive double- and single-charged ions of the protein are shown in the spectrum).

known MM marker (lane 1) confirmed this conclusion, as the theoretical MM of β-casein is 24 kDa. To further confirm the purity and MM of the purified fraction, it was analyzed by MALDI-TOF MS. As shown in Figure 1C, the correct mass, that is, 23.96 kDa for single-charged ion and 11.98 kDa for double-charged ion, was detected from the spectrum of purified β-casein. No additional peaks were detected from the spectrum, verifying the purity of the main β-casein fraction.

Solubilization of the Model Compounds and Proteins in ILs. Although various organic solvents are commonly used to improve the solubilization of different polymers,[23] they are not applicable for the solubilization of large proteins. In our studies the singe amino acids and short peptides could be solubilized in adequate concentrations, that is, 30 mg mL^{-1}, but in the case of β-casein only ca. 10 mg could be solubilized in 1 mL of chloroform in order to perform phosphitylation and subsequent ^{31}P NMR measurements. However, it is well-known that ^{31}P NMR is not a sensitive method and that it requires larger amounts of sample, typically at least ca. 40 mg (depending on the content of reactive groups) in a 1 mL volume to be effective.[39] Thus, to overcome this problem, all model compounds as well as enzymatically polymerized β-caseins were dissolved in a solvent system based on chloride anion and imidazolium cation, that is, in [amim]Cl, which has successfully been used to dissolve wood and other plant-based materials.[17,21,28] Moreover, ILs have been reported to stabilize the three-dimensional structure of various proteins.[40] However, Swatloski et al.[27] have speculated that ILs with high chloride content may break the hydrogen bonds present in cellulose, and hence it is believed that the same phenomenon may occur in the case of proteins such as β-casein. If that is the case, in our studies it can be considered as an advantage, because the aim was to phosphitylate all nonreacted hydroxyl groups in cross-linked networked protein matrix. All amino acids, peptides, and proteins used in this study were easily dissolved in [amim]Cl in adequate, that is, 80 mg in 1 mL, concentration. However, [bmim]Cl, also tested for this purpose, was not suitable for this application due to its high melting point and high viscosity (data not shown).

Thus, it can be summarized that in the presence of imidazole-based ILs, high concentrations of protein material can be dissolved for further chemical modifications. This provides an attractive basis also for protein-based applications in addition to those described for wood components. In addition, ILs have high potential as green solvents due to their ability to dissolve a wide variety of biopolymers compared with conventional organic solvents.[41]

Performance of ^{31}P NMR Method for Analysis of Proteins in ILs. *Amino Acids and Small Organic Compounds.* 4-hydroxybenzoic acid, 2-hydroxyethanoic acid, serine, tyrosine, and glutamic acid were analyzed by ^{31}P NMR to evaluate the suitability of this method for the analysis of protein material. In general, analysis of single amino acids as well as small organic molecules by ^{31}P NMR in ILs resulted in well-resolved spectra enabling identification of various functional hydroxyl groups. ^{31}P NMR spectra measured from tyrosine, containing a phenolic ring and carboxylic acid group, and serine, containing an aliphatic hydroxyl and a carboxylic acid group, are shown in Figure 2, panels A and C, respectively. The phenolic hydroxyl signal of the tyrosine side chain (138.5−137.5 ppm) and the aliphatic hydroxyl signal of serine side chain (147.5−145.5 ppm) were duplets due to the dimerization of the amino acids in the IL solution, explaining also the lack of the signal of the hydroxyl group of carboxylic acid functionality (typically ~134 ppm). It is assumed that the dimerization occurs via intermolecular hydrogen bonds between amino and carboxylic acid groups of two different molecules.[42] The low pK_a values (2.2) of the carboxylic acid groups present in the amino acids tyrosine and serine explain their tendency to dimerization in [amim]Cl containing basic amine groups.[43] However, the accurate pH of the ILs used in the study could not be determined. In the case of dimeric serine (Figure 2C) the aliphatic hydroxyl group peaks are not of the

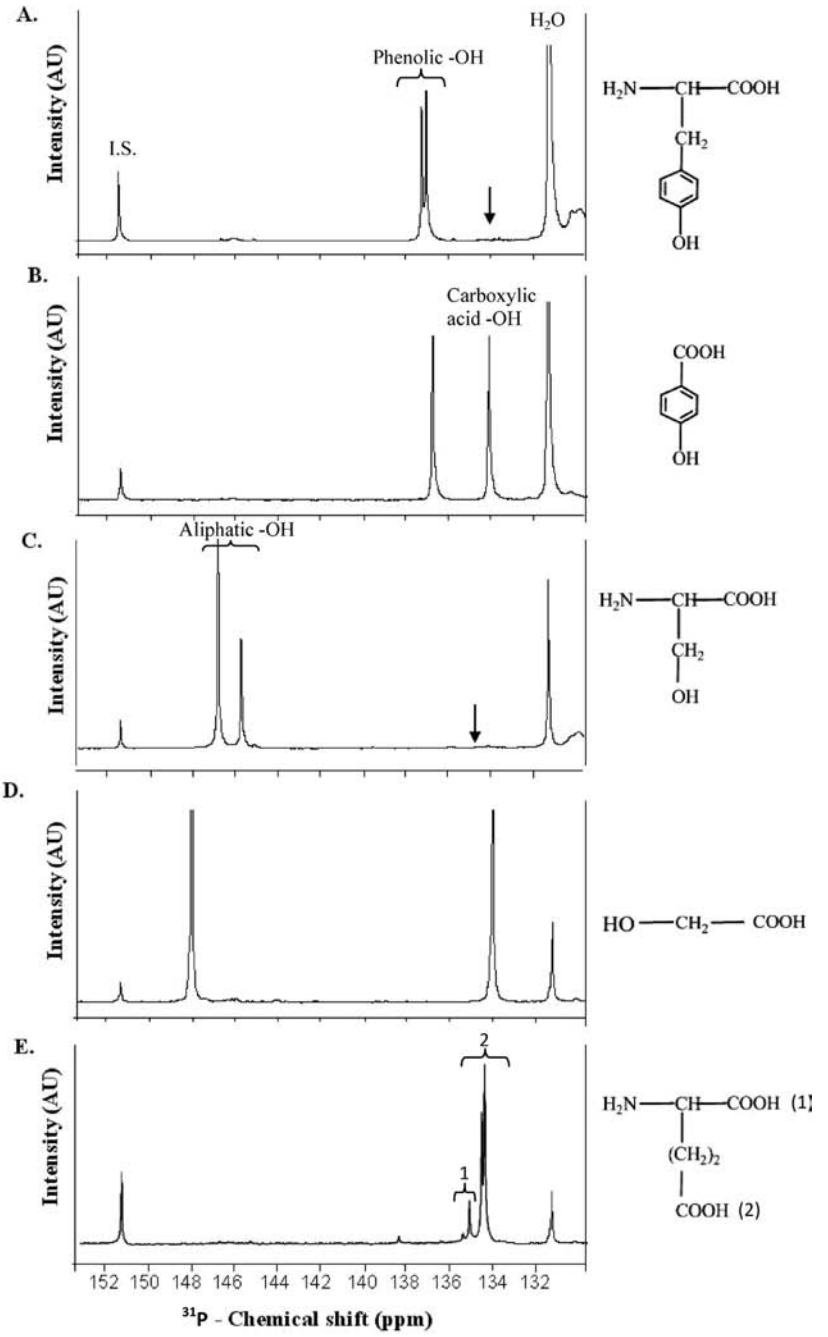


Figure 2. ^{31}P NMR spectra measured from (A) tyrosine, (B) 4-hydroxybenzoic acid, (C) serine, (D) 2-hydroxyethanoic acid, and (E) glutamic acid solubilized in ionic liquid. In addition to the chemical structures of the small model compounds, the assignments of the various phosphitylated functional hydroxyl groups (aliphatic, phenolic, carboxylic acid, water, and internal standard, I.S.) are shown above the corresponding peaks. The positions of the carboxylic acid hydroxyls that are not present due to dimerization are marked with an arrow. In the case of glutamic acid (E), the positions of the peaks of the carboxylic acid hydroxyl groups are marked by 1 and 2.

same intensity. This difference could be attributed to the presence of serine monomer having an internal hydrogen bond between amino and carboxylic acid groups. The peak of the monomeric aliphatic hydroxyl group is overlapping with the peaks that have resulted from the dimer. The error for the quantitative results of the phenolic group of tyrosine and the aliphatic group of serine side chains was <5%. The same phenomenon of dimerization was observed also in the case of glutamic acid (Figure 2E) containing two carboxylic acid groups having different pK_a values, that is, 4.3 for the group in the amino acid side chain and 2.2 for the group located next to the amino group. Also in this case, the signal resulting from the side-chain carboxylic acid group was a duplet (135−133 ppm) yielding quantitative results and having an average error of <5%. As in this case the dimerization via other carboxylic acid group was not stable and complete. Hence, a somewhat smaller duplet signal (~135 ppm) appeared next to the side-chain peak (Figure 3A, mixture of different amino acids).

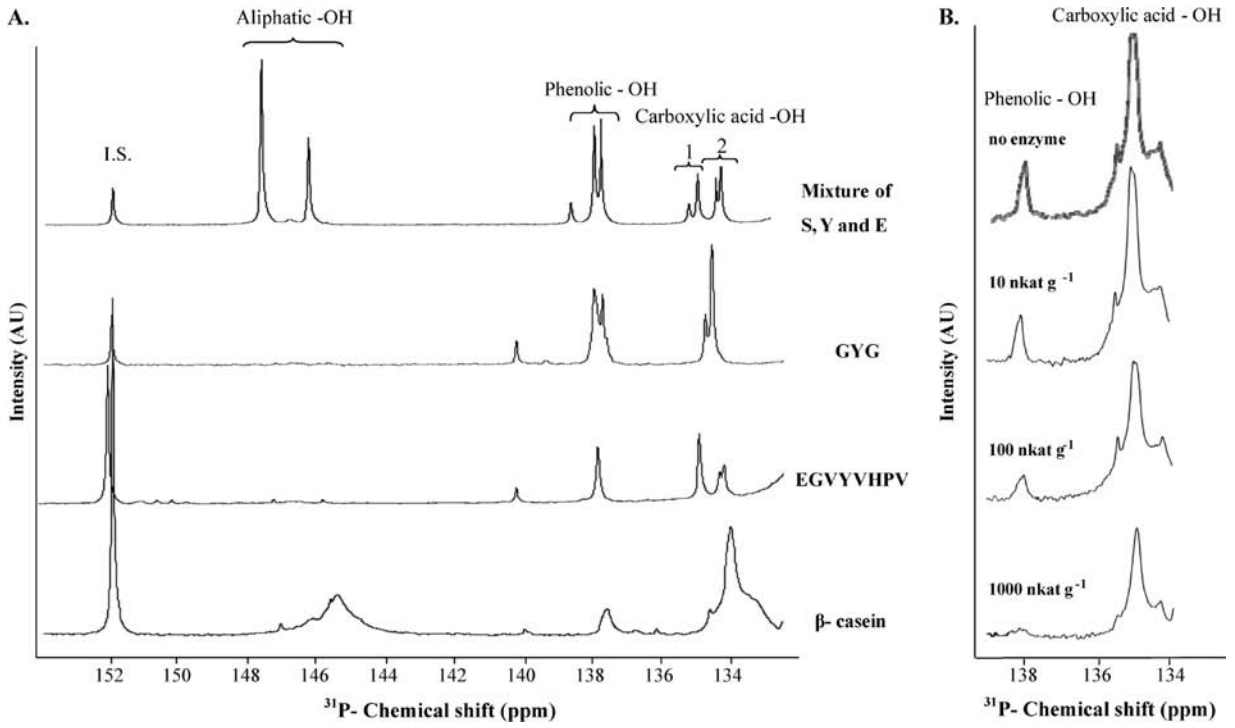


Figure 3. ^{31}P NMR spectra measured from various size model compounds: (A) mixture of single serine, tyrosine, and glutamic acid amino acids and short GYG and EGVYVHPV peptides as well as purified β-casein solubilized in ionic liquid prior to derivatization and acquisition (the positions of the peaks of the carboxylic acid hydroxyl groups of glutamic acid are marked by 1 and 2 as in Figure 2); (B) selected area of the ^{31}P NMR spectra measured from purified β-casein cross-linked by TrTyr using different enzyme dosages and 24 h incubation time. In both panels, the assignments of the various phosphitylated functional hydroxyl groups (aliphatic, phenolic, carboxylic acid, water, and I.S.) are shown above the corresponding peaks.

To verify the somewhat unexpected results described above, two small organic molecules, that is, 4-hydroxybenzoic acid and 2-hydroxyethanoic acid, were chosen as reference compounds for comparison to tyrosine and serine. In the case of 4-hydroxybenzoic acid acid both reactive groups, that is, the phenolic hydroxyl and the carboxylic acid hydroxyl group, could be successfully derivatized by PR[II] reagent in the selected solvent system as shown in Figure 2B. A singlet peak was detected for each functional hydroxyl group. The intensities of the peaks were almost identical, indicating that both functional groups reacted to an equal extent with the PR[II] reagent. No splitting was observed, due to the lack of an amino group in the molecule. In the case of 2-hydroxyethanoic acid both of the reactive functional hydroxyl groups became visible in the spectrum as singlets at equal intensities, indicating no preference between the hydroxyl groups as shown in Figure 2D. The quantification of the various hydroxyl peaks revealed excellent results with an error of <1%. In contrast to tyrosine, serine, and glutamic acid, the carboxylic acid groups of 4-hydroxybenzoic acid and 2-hydroxyethanoic acid have much higher pK_a values (4.48 and 3.83, respectively) than carboxylic acid groups next to amino groups in selected amino acids. Thus, no dimerization occurred and the hydroxyls of the carboxylic groups could be successfully derivatized by PR[II] reagent and detected as singlets in the spectra. In all spectra (Figures 2A−D), signals deriving from the phosphitylated residual water and internal standard were detected.

^{31}P NMR was also used for analyzing a mixture of three selected amino acids (in equal concentrations) representing different functionalities in the amino acid side chain. Thus, tyrosine, serine, and glutamic acid were dissolved efficiently in the IL and analyzed by ^{31}P NMR, as shown in Figure 3A. The signals in the spectrum measured from the mixture of amino acids were identical to those measured for the individual amino acids. The variation between the theoretical and experimental values for the amount of reactive groups was again found to be small, that is, <5% for amino acid side chains. In general, the chemical shifts of the functional groups derivatized by PR[II] in protein material are in good agreement with the studies conducted by Wroblewski on the ^{31}P NMR analysis of organic compounds containing phenols, alcohols, and acids.[44]

Overall, with an average error of <5%, the quantitative ^{31}P NMR of the amino acids and small molecules presents strong evidence for the potential of the IL solvent system for quantification of phenolic structures as well as other hydroxyl groups in large molecules such as peptides and proteins as described below.

Peptides. To further evaluate the possibility of using the IL solvent system, that is, [amim]Cl, for the quantitative analysis of reactive hydroxyl groups present in larger molecules, two peptides, GYG and EGVYVHPV, were used as model compounds. The tripeptide GYG and the octapeptide EGVYVHPV were successfully analyzed by ^{31}P NMR after derivatization by PR[II] as shown in Figure 3A. In the case of GYG small doublets appeared in the reactive phenolic (~137.7 ppm) group signal, whereas in the case of the longer peptide the reactive groups appeared as singlets at 137.7 ppm without any splitting. The dimerization of the protein-based molecules appears to diminish as a function of the size of the molecules. Furthermore, the pK_a value of the carboxylic acid group at the C-terminus, ca. 3.1, is higher as in the case of individual amino acids having a minor tendency to dimerization.[45] In the case of peptides the error of the method was found to be approximately 10%.

Proteins. Finally, to evaluate the performance of the quantitative ^{31}P NMR method at protein level, purified β-casein was

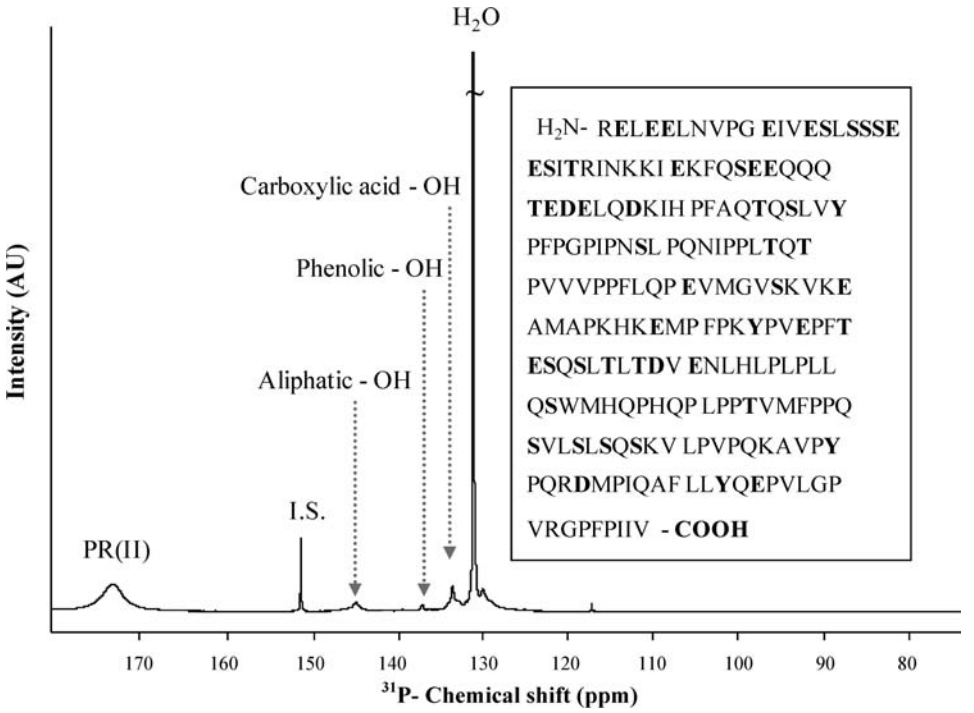


Figure 4. Complete ^{31}P NMR spectrum of purified β-casein measured in ionic liquid. The assignments of the various phosphitylated functional hydroxyl groups (aliphatic, phenolic, carboxylic acid, water, and I.S.) are shown above the corresponding peaks. The amino acid sequence of β-casein is shown in the inset. The amino acids containing reactive groups are in boldface in addition to the C-terminus (S = 16, T = 9, Y = 4, D = 4, E = 19).

analyzed by this technique as shown in Figure 3A. Three broad peaks resulting from the aliphatic (145.5 ppm), phenolic (137.7 ppm), and carboxylic acid hydroxyl (134.2 ppm) groups could be detected in the spectra as well as one sharp peak resulting from the addition of the internal standard for quantification. The quantitative determination of the various reactive groups showed an average error of approximately 12%. The largest errors were observed for the carboxylic acid hydroxyl groups due to the partial overlapping of the signal with the signal derived from the residual moisture from IL. Although the protein samples were freeze-dried prior to the NMR analyses, it is possible that small quantities of absorbed water remained in the protein material. The water signal could also originate from atmospheric humidity and/or organic solvents used in the experiments. To ensure that no extra signals originated from the IL itself or other derived functional groups, a wider spectrum from the spectral region of 180−70 ppm was measured for the purified β-casein as shown in Figure 4. Only one additional unknown peak, most likely a decomposition product of PR[II] water adduct, was detected below the water signal at 130 ppm.[21]

^{31}P NMR Study of Enzymatically Cross-Linked β-Casein. After it had been established that different kinds of protein material can be solubilized in [amim]Cl and that various hydroxyl groups can be quantitatively derivatized by PR[II] reagent, enzymatically cross-linked β-caseins were analyzed using the ^{31}P NMR method optimized for protein material. The cross-linking of chromatographically purified β-casein by TrTyr and Tgase to different degrees was achieved by using different enzyme dosages as described in our previous work.[8] The hydroxyl groups present in cross-linked β-casein (phenolic, aliphatic, and carboxylic acid) were found to be fully phosphitylated when Tgase was employed for the cross-linking. Only small changes in the intensities of the peaks were detected in the spectra, independent of the enzyme dosage used for the cross-linking reaction (data not shown). This result was expected, as cross-linking occurs via isopeptide bonds between lysine and glutamine side chains and not via hydroxyl groups. This confirms the applicability of the ^{31}P NMR method in IL for the analysis of modifications via hydroxyl groups in high molecular mass networked protein material.[8]

In the case of β-casein cross-linked by TrTyr, it was evident that whereas the integrals of the peaks corresponding to the carboxylic hydroxyl acid and aliphatic hydroxyl groups remained nearly unchanged, the signal for the phenolic hydroxyl group decreased as a function of enzyme dosage as shown in Figure 3B. The decreases in phenolic hydroxyl group signals were found to be 15, 53, and 91% when using enzyme dosages of 10, 100, and 1000 nkat g^{-1}, respectively. The decrease of phenolic hydroxyls and thus the amount of covalent bonds created in the cross-linking by TrTyr was surprisingly high when the highest enzyme dosage of 1000 nkat g^{-1} was used. However, as shown in a previous study,[8] β-casein could be fully polymerized up to 1500 kg mol^{-1} when these reaction conditions were employed. This verifies that cross-linking reactions by TrTyr on β-casein occur covalently via tyrosine side chains present in the proteins and not via aggregation. According to current knowledge, various amino acid side chains such as −SH and −NH$_2$[46] can react with diquinone, the reactive intermediate produced in TrTyr-catalyzed reactions,[46] in addition to coupling of two diquinones. It is therefore clear that the variation in the chemical structure and environment of the individual cross-links produced in the TrTyr-catalyzed reactions is vast in addition to low quantity, making them impossible to detect by NMR spectroscopy. However, decrease of the phenolic hydroxyl signal as a function of enzyme dosage could be detected by this method showing covalent cross-linking of β-casein by TrTyr not evidenced before at chemical bond level.

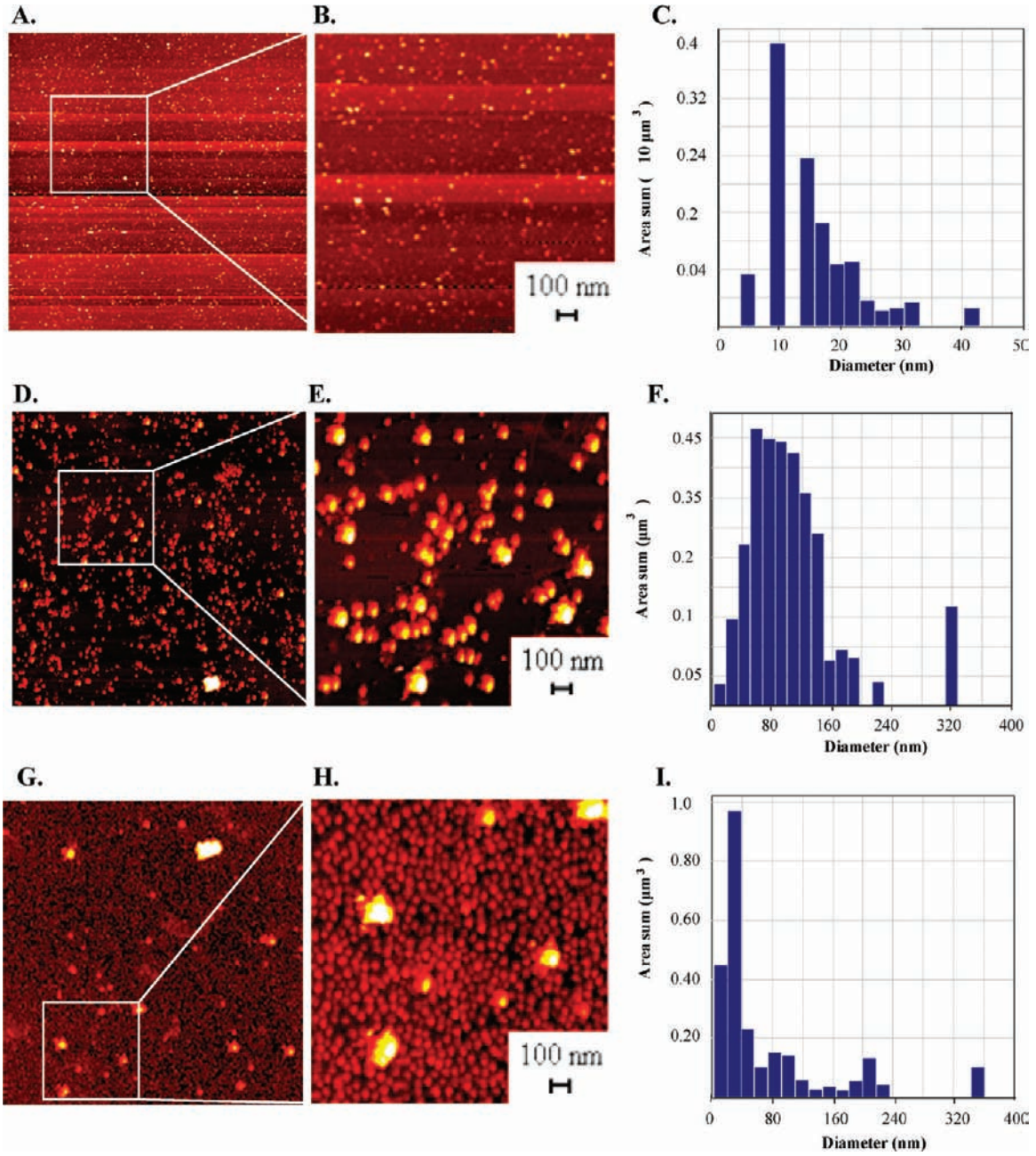


Figure 5. Morphology of enzymatically cross-linked purified β-casein as analyzed by AFM: (A) reference (purified β-casein); (B) selected, magnified area of the surface of the reference sample; (C) area-weighted histogram of the reference sample; (D) β-casein cross-linked by TrTyr (enzyme dosage, 1000 nkat g^{-1}; incubation time, 24 h); (E) selected, magnified area of the surface of β-casein cross-linked by TrTyr; (F) area-weighted histogram of the TrTyr sample; (G) β-casein cross-linked by Tgase (enzyme dosage, 1000 nkat g^{-1}; reaction time, 2 h); (H) selected, magnified area of the surface of β-casein cross-linked by Tgase; (I) area-weighted histogram of the Tgase sample.

The ^{31}P NMR spectra of nonpurified β-casein cross-linked by TrTyr were also analyzed (data not shown). As expected, the overall pattern of the spectra was identical to that of the purified β-casein cross-linked by TrTyr. However, the calculated cross-linking efficiencies were different, as the commercial β-casein contains some impurities such as whey proteins that cannot be cross-linked by this enzyme.[8] Specifically, the data for the phenolic groups in impure β-casein cross-linked using TrTyr dosages of 10, 100, and 1000 nkat g^{-1} were 12, 23, and 68%, respectively. In the case of the lowest enzyme dosage the results with the nonpurified and purified β-casein were almost identical. However, when using the highest TrTyr dosage and nonpurified β-casein, the phenolic signal was only 75% of that obtained using chromatographically purified protein as substrate. This is most probably due to the presence of the above-mentioned whey proteins that could not be cross-linked by TrTyr.[8] Overall, the results obtained by ^{31}P NMR method are compatible with our previous findings.[8]

Morphology of Enzymatically Cross-Linked β-Casein. An AFM image of chromatographically purified β-casein is presented in Figure 5A (magnification in panel B), showing formation of disk-shaped particles via aggregation. The area-weighted average diameter of β-casein was found to be ca. 10 nm, although some

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larger particles with maximum diameter ca. 42 nm were also detected as shown in Figure 5C. The rms roughness for nontreated purified β-casein was 0.2 nm, indicating the formation of a smooth protein surface on the mica.

When β-casein was cross-linked by TrTyr using the lowest enzyme dosage (10 nkat g^{-1}), disk-shaped particles resembling the control sample were formed, and no linear polymerized structures as in the case of small phenolic compounds[47] were detected from the AFM surface (data not shown). However, the average diameter of these particles was approximately 2-fold higher, that is, 19 nm, than that of the control sample (non-cross-linked β-casein), showing activity of the enzyme. Unexpectedly, a 10-fold increase in the enzyme dosage (100 nkat g^{-1}) resulted in slightly smaller particles having an average diameter of around 15 nm (data not shown), suggesting nonlinear proceeding of the enzymatic reaction. When β-casein was cross-linked with the highest enzyme dosage (1000 nkat g^{-1}), large particles were formed as shown in Figure 5D. The formation of disk-shaped cross-linked particles is evident in the magnified Figure 5E. In this case the average diameter of the dried cross-linked β-casein particles was 80 nm. However, larger particles were also formed with a diameter of approximately 340 nm (Figure 5F). The roughnesses rms values were 0.2, 1.3, and 2.0 nm, respectively, when TrTyr dosages of 10, 100, and 1000 nkat g^{-1} were used for the cross-linking of β-casein. The rms roughness of β-casein cross-linked by 10 nkat g^{-1} of TrTyr was equal to that of untreated β-casein. This correlates well with our previous results, showing that only some polymerization occurred when using the lowest enzyme dosage.[8]

When β-casein was cross-linked by the reference enzyme, Tgase, the lowest enzyme dosage of 10 nkat g^{-1} also resulted in disk-shaped particles of 22 nm average diameter (data not shown). Use of the intermediate enzyme dosage (100 nkat g^{-1}) resulted in somewhat larger particles with an average diameter of 39 nm (data not shown). The analysis of β-casein cross-linked by Tgase using an enzyme dosage of 1000 nkat g^{-1} resulted in an average particle diameter of 20 nm as shown in Figure 5G (magnification in Figure 5H). In addition to these uniform and rather homogeneous nanoparticles, as also reported in our previous studies,[8] larger aggregates of approximately 360 nm were formed in the presence of the highest Tgase dosage. The rms roughness of the sample surfaces appeared to increase along with increasing enzyme dosage, that is, 0.3, 0.9, and 1.5 nm. In the case of 10 nkat g^{-1} of Tgase, the rms roughness is slightly higher than that of untreated β-casein, correlating with our previous data according to which β-casein cross-linked by Tgase (10 nkat g^{-1} for 2 h) was only slightly polymerized.[8] In general, the cross-linking of β-casein by Tgase resulted in small particles with an average diameter of 20 nm regardless of the enzyme dosage used for the cross-linking reaction. Tight cross-linking of β-casein into spherical nanoparticles by Tgase can easily be understood, as β-casein contains many reactive sites for transglutaminase, that is, 11 lysines and 20 glutamines, enabling extensive cross-linking as evidenced earlier.[8] The radius of gyration obtained for the cross-linked protein particles was rather small for such a large cross-linked polymer. The elution order of cross-linked β-casein in size exclusion chromatography (SEC) obtained using 1000 nkat g^{-1} enzyme dosage and 2, 6, and 24 h incubation times also supported the above conclusions, as the cross-linked material produced in the extreme reaction conditions eluted last. Thus, it can be postulated that the protein network formed after Tgase treatment is very tight, also indicating the formation of a large number of intramolecular cross-links in addition to intermolecular cross-links responsible for the polymerization reactions.

In general, the apparent radii of gyration (R_g) were smaller for all enzymatically cross-linked samples when analyzed by AFM in comparison to the values obtained in our previous studies by SEC-UV/vis-MALLS.[8] In SEC-MALLS the hydrodynamic diameter of the polymer is determined by dynamic light scattering in the solution. By contrast, in AFM the polymer samples are dried on the mica surface, and enzymatically cross-linked protein particles may shrink during the drying. A similar phenomenon was reported by Martin et al. for casein micelles analyzed by SEM.[48]

It has also been shown that cross-linking of β-casein by Tgase using a dosage of 1000 nkat g^{-1} forms products which are slowly digested by the pepsin, in comparison to β-casein cross-linked by TrTyr or native β-casein.[37] These observations support the above AFM results as the protein particles formed in the cross-linking by Tgase had much smaller diameter and tighter morphology when compared to the particles formed by TrTyr. Thus, it is suggested that the cross-linking by Tgase largely occurs via intramolecular bonding. By contrast, the cross-linking of β-casein by TrTyr produced particles with larger diameter, thus indicating the predominant role of intermolecular bonding as β-casein contains only four reactive sites, that is, tyrosine residues for cross-linking by TrTyr.

It has been shown that various protein materials can be dissolved in chloride-based IL for further analysis of enzymatic (as well as chemical) modifications by ^{31}P NMR spectroscopy. In addition, it was verified that the method can be applied for quantitative determination of aliphatic, phenolic, and carboxylic acid hydroxyls present in native and cross-linked proteins. The extent of cross-linked phenolics in β-casein, that is, 91% produced by TrTyr, was surprisingly high, showing on a chemical bond level that nearly all tyrosine residues are involved in the cross-linking reactions. AFM results indicated on a molecular level that the cross-linking of β-casein by TrTyr largely occurs via intermolecular bonding, whereas Tgase was found to form largely also via intramolecular cross-links. The data are important for exploitation of tyrosinases in different applications as complete cross-linking of random coil protein was confirmed.

■ ABBREVIATIONS USED

AFM, atomic force microscopy; [amim]Cl, 1-allyl-3-methylimidazolium chloride; [bmim]Cl, 1-butyl-3-methylimidazolium chloride; ^{31}P NMR, phosphorus nuclear magnetic resonance; $Cr(acac)_3$, chromium(III) acetylacetonate; PR[II], 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane; IL, ionic liquid; K, lysine; Q, glutamine; S, serine; E, glutamic acid; Y, tyrosine; D, aspartate; MALDI-TOF MS, matrix-assisted laser desorption/ionization−time-of-flight mass spectroscopy; MM, molecular mass; L-DOPA, L-3,4-dihydroxyphenylalanine; R_g, radius of gyration; rms, root-mean-square; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; SEM; scanning electron microscopy; VTT, Technical Research Center of Finland.

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