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Monitoring of Deamidation and Lanthionine Formation in Recombinant Mugwort Allergen by CZE-UV and tCITP-CZE-ESI-TOF-MS

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ABSTRACT (250): The response to thermal stress is an important parameter relevant for characterizing the biological activity and long-term stability of recombinant proteins, which may show irreversible, pH-dependent structural changes under these conditions. We selected the recombinant pollen allergen of mugwort (*Artemisia vulgaris*) rArt v 3.0201 as a relevant model to study structural changes due to thermal and pH stress by means of CZE-UV and CZE-ESI-TOF-MS. Therefore, this recombinant protein was exposed to 95 °C under acidic (pH 3.4) and slightly alkaline (pH 7.3) conditions for up to 120 min. CZE-UV data showed a continuous degradation of the allergen accompanied by the gradual formation of several reaction products. Characterization of novel allergen variants occurring at longer migration times was done *via* CZE-ESI-TOF-MS using in-capillary transient CITP pre-concentration to facilitate the identification of minor variants. MS data revealed various modifications of rArt v 3.0201 in response to heating. Deamidations and sulfur-related modifications including both yield and loss of sulfur were identified at increased migration times. Desulfurization produced allergen variants with up to four lanthionines that replaced initial disulfide bonds. In addition, mass spectra revealed shifts in the charge state distribution which indicate concomitant conformational alterations. Moreover, several low-abundant oxidized variants were identified. With extended thermal stress the portfolio of variants increased and progressively shifted towards rArt v 3.0201 with high lanthionine content. The kinetics of conversion and the complexity of variant composition were pH-dependent and increased under alkaline conditions.

Conformation and structural stability of proteins are determined by secondary and tertiary structures that are both governed by the primary sequence and the formation of stabilizing disulfide bonds.^{1,2} The native protein conformation may be altered by mutations,³ post-translational modifications (PTMs),⁴ and changes in the protein ambience, such as the pH,^{5,6} the composition of the bulk solution,⁷ and the environmental temperature.⁸ The three-dimensional structure of proteins may be destroyed by denaturing reagents and/or the disruption of disulfide bonds by reducing agents. Heating of protein solutions also remarkably changes the protein structure - in some instances even irreversibly - without the need for chemical additives, and the response is protein specific.^{9,10}

Thermal stressing in combination with monitoring concomitant conformational responses constitutes a standard method in the physicochemical characterization of recombinant proteins and particularly of (recombinant) allergens. Thereby, the influence of temperature in combination with the bulk pH is investigated, addressing the melting temperature but also changes in secondary and tertiary structure as well as the reversibility of unfolding events when cycling back to the initial temperature.¹¹ Resilience against heat treatment and reversibility of conformational changes are protein specific and also depend on the selected pH, as shown for ovalbumin,¹² ovotransferrin,⁹ and the peach allergen Pru p 3.¹⁰ In the latter case, an irreversible unfolding at pH 7.5 was observed when heating the allergen to 95 °C. This was explained by a cleavage of disulfide bonds by β -elimination and a formation of novel

intermolecular disulfide bonds that induce aggregates, but no experimental proof for this assumption was provided.¹⁰ Thioethers formed between dehydroalanine (DHA) and a free cysteine, so-called lanthionines (LAN), provide an alternative reaction possibility.¹³⁻¹⁵ Both newly formed intermolecular disulfide bridges and LANs were found to contribute to the formation of protein networks.¹⁶

The formation of LAN has initially been described for wool after alkaline treatment with carbonate, targeting a favorable change of the textile structures.¹⁷ Additionally, DHA and LAN formation is associated with protein aging,¹⁸ for instance during cataract progression in the human eye.¹⁹ The propensity for LAN formation is protein specific and seems to depend on the vicinity between cysteine and basic amino acids.^{20,21} Although LAN formation is only marginally discussed in the literature, it does not represent an unusual modification, particularly in recombinant proteins.¹³ Remarkably, DHA variants of human proteins have also been observed *in vivo*, e.g. for albumin in plasma.²⁰ Thioether and LAN formation due to heat treatment with subsequent aggregation have been observed for various proteins, including wheat gluten,^{16,22,23} and casein.²⁴ Formation of multimers *via* thioether-based linkage has also been described for peptides.²⁵

Molecular modifications affecting disulfide bonds are most likely accompanied by changes of the three-dimensional protein structure.²⁰ Their characterization requires separation tools that allow for a resolution of (closely) related protein conformations which are subsequently identified by mass spectrometry.

try (MS). Several reviews have surveyed the excellent applicability of capillary electrophoresis in distinguishing coexisting conformers.^{26,27} Analyte properties that govern the effective electrophoretic mobility (μ_{eff}) in capillary zone electrophoresis (CZE) comprise the hydrodynamic radius (r_{H}) and the protein net charge. As conformational changes can influence r_{H} or pI either individually or simultaneously, CZE is predestined in separating protein conformations particularly since - unlike in HPLC - the separation principle does not involve a mass transfer term.^{28,29} Kálmán *et al.* resolved proteins of identical net charge but differences in r_{H} below 0.50 Å by capillary electrophoresis.³⁰ Whereas CZE allows for an up-stream resolution of PTMs and (related) conformational variants, their identification requires subsequent mass spectrometric investigation. Indeed, variants related to disulfide bond cleavage and LAN species have successfully been characterized and identified by MS either in stand-alone operation^{21,31} or hyphenated to HPLC.^{20,32-34} Although CZE-ESI-MS is increasingly employed in the characterization of small analytes,³⁵ its application to the analysis of intact proteins is still discerning.³⁶ Remarkably, despite the stated advantages of CZE-ESI-MS this hyphenated method has not been applied in the analysis of variants resulting from disulfide bond decay so far.

The molecule chosen here for studying disulfide bond decay is recombinant Art v 3.0201 (rArt v 3.0201), a non-specific lipid transfer protein from pollen of mugwort (*Artemisia vulgaris*) representing an important allergen in the Mediterranean area.³⁷ Despite their high thermal stability, different recombinant variants of Art v 3, thereunder the isoform Art v 3.0201, showed a permanent change in the protein structure when heated to 95 °C.³⁸ Thus, this isoform was selected as a model analyte to elucidate the molecular reasons for irreversible structural changes in response to heat treatment. In order to investigate whether a decay of disulfide bonds and subsequent development of LANs is involved in this process, acidic and slightly alkaline solutions of rArt v 3.0201 were heated for different time intervals. Mass differences indicating alterations in the protein sulfur content shall reveal the changes in molecular structure initiated by the heat stress conditions. Postulated structural changes should be reflected by changed r_{H} and/or pI. Thus, CZE-ESI-MS will be established as an up-stream electrophoretic separation technique for folding variants followed by mass spectrometric characterization through comparing experimental masses of heated species with theoretical masses of predicted LAN and deamidation variants.

EXPERIMENTAL SECTION

Chemicals and materials. Ammonium formate ($\geq 99.995\%$, trace metals basis), sodium phosphate monobasic monohydrate, sodium phosphate dibasic dihydrate (p.a), methanol ($\geq 99.9\%$; Chromasolv[®] for HPLC) and formic acid (FA; 98-100%; puriss.) were purchased from Sigma Aldrich (Vienna, Austria). Isopropanol ($\geq 99.9\%$; LC-MS Chromasolv[®]) was obtained from Fluka Analytics (Steinheim, Germany). Orthophosphoric acid (85% w/v; for analysis) was purchased from Merck (Darmstadt, Germany). Sodium hydroxide standard volumetric solution (1.00 mol L⁻¹) and hydrochloric acid standard volumetric solution (1.00 mol L⁻¹) were purchased from AppliChem (Darmstadt, Germany). The ESI-L Low Concentration Tuning Mix was from Agilent Technologies (Waldbronn, Germany). Ultrapure water was produced with a resistivity of 18.2 MΩ.cm by a Milli-Q Integral 3 system from Merck Millipore Corporation (Darmstadt, Germany).

Methods. Measurement of pH was done with a Microprocessor pH Meter pH 3000 from WTW (Weilheim, Germany). Heat treatment under acidic conditions was performed with 10 mmol L⁻¹ NaH₂PO₄·H₂O buffer adjusted to pH 3.4 with orthophosphoric acid (85% w/v). Stressing under slightly alkaline conditions was performed with 10 mmol L⁻¹ Na₂HPO₄·2H₂O adjusted to pH 7.3 with ortho-phosphoric acid (85% w/v). The production and purification of rArt v 3.0201 is described in the supporting part. For thermal stressing, lyophilized rArt v 3.0201 was either reconstituted in 10 mmol L⁻¹ phosphate buffer, pH 7.3, or in 10 mmol L⁻¹ phosphate buffer, pH 3.4, providing a final protein concentration of 0.30 mg mL⁻¹, respectively. Aliquots of both preparations were heated to 95 °C in parallel applying a mixing frequency of 800 rpm on a Thermomixer comfort from Eppendorf AG (Hamburg, Germany). Aliquots were removed after 5, 15, 30, 45, 60, 90, and 120 min. A non-heated aliquot served as a control, respectively.

CZE-UV separations. CZE-UV analyses were performed with 100 mmol L⁻¹ NaH₂PO₄·H₂O buffer adjusted to pH 2.50 with 85% (w/v) ortho-phosphoric acid. Separations were run on an Agilent 7100 Capillary Electrophoresis system equipped with a diode array detector (190-600 nm) operating under 3D-CE ChemStation Rev.B.04.03 (16). Data treatment was done on OpenLAB CDS ChemStation Edition for CE & CE/MS Systems Rev. C.01.07 (27). Peak areas are generally reported as migration-time-normalized and background corrected values. A bare fused-silica capillary [50 µm i.d., 360 µm o.d., a total length (L_{T}) of 49.2 cm and an effective length (L_{D}) of 41.3 cm] from Polymicro Technologies (Phoenix, AZ, USA) was used for CZE separations. The capillary was conditioned by rinsing with 1.00 mol L⁻¹ NaOH (10.0 min), ultrapure water (15.0 min), 0.10 mol L⁻¹ HCl (10.0 min), and background electrolyte (BGE) (20.0 min) (all with 93.0 kPa at 35.0 °C).³⁹ Finally, 15.0 kV (0.17 min ramp) were applied for 20.0 min with the capillary filled with BGE. Prior to each run the capillary was treated as follows: 0.10 mol L⁻¹ NaOH (3.0 min), ultrapure water (2.0 min), 0.10 mol L⁻¹ HCl (2.0 min), ultrapure water (2.0 min), and BGE (3.0 min) (all with 93 kPa, 25.0 °C). Injection was performed hydrodynamically with 3.5 kPa for 10.0 s. A separation voltage of 15.0 kV (normal polarity, with 0.17 min ramp time) was applied at 25 °C, which resulted in ~57 µA.

CZE-ESI-TOF-MS analyses. For CZE-ESI-TOF-MS measurements 1.00 mol L⁻¹ FA was prepared by dilution of 98-100 % FA with ultrapure water which resulted in a final pH of 1.80. In CZE-ESI-TOF-MS, transient capillary isotachopheresis (tCITP) was applied for in-capillary pre-concentration.⁴⁰ Therefore, 100 mL of a 500 mmol L⁻¹ ammonium formate stock solution, pH 4.00, was prepared which was applied as leading electrolyte (LE). An appropriate volume of the 500 mmol L⁻¹ ammonium formate stock solution (pH 4.00) was added to the sample to provide a final concentration of 125 mmol L⁻¹ ammonium formate. Under tCITP conditions the injection duration was increased to 50 s (maintaining 3.45 kPa). CZE preceding TOF-MS detection was done on a ProteomeLabTM PA 800 Capillary Electrophoresis system from Beckman Coulter (Fullerton, CA, USA) operating under 32 Karat version 8.0. The Beckman Coulter ProteomeLabTM PA 800 Capillary Electrophoresis system was hyphenated to a micrOTOF mass spectrometer from Bruker Daltonics (Bremen, Germany) by a coaxial MS-sprayer from Agilent Technologies (Waldbronn, Germany). A bare fused-silica capillary was installed in the CE system and aligned in the electrospray interface. The capillary conditioning protocol

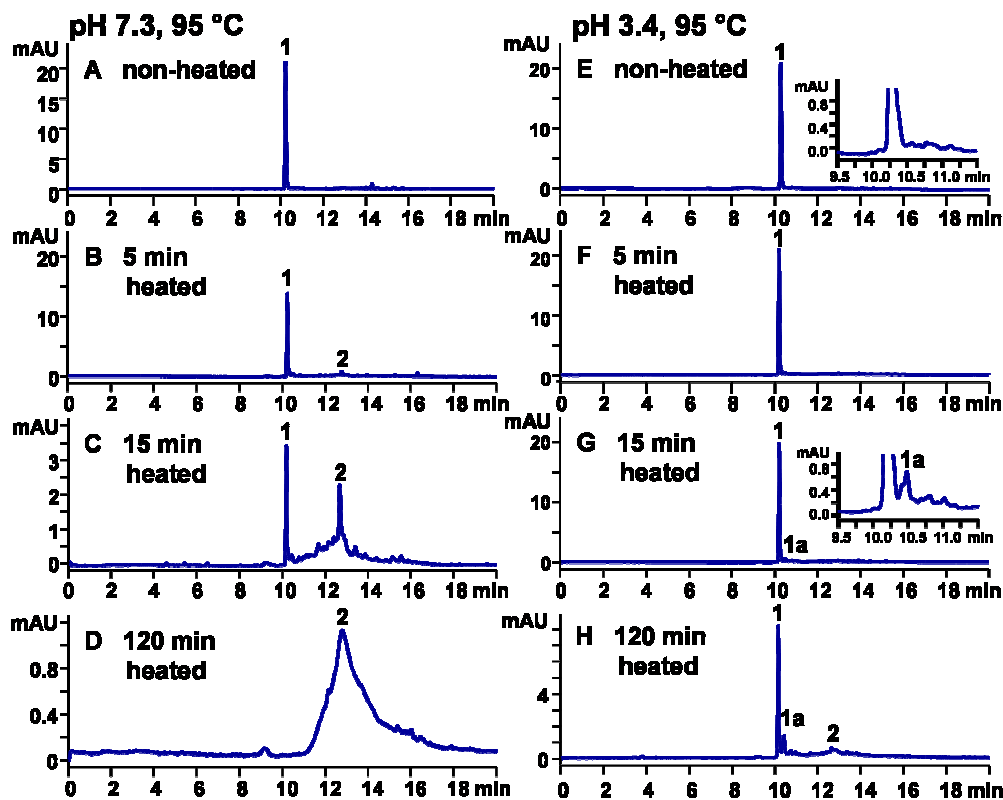


Figure 1. CZE-UV profiles of rArt v 3.0201 after heat treatment at 95 °C and pH 7.3 (A-D) and 3.4 (E-H) for different durations. Sample: 0.15 mg mL⁻¹ rArt v 3.0201 at pH 7.3 or pH 3.4. Capillary: bare fused-silica L_D 41.3 cm; L_T 49.2 cm. BGE: 100.0 mmol L⁻¹ NaH₂PO₄, pH 2.50. Injection: 3.5 kPa, 10.0 s. CZE separation: 15.0 kV (cathode at the detection side, 0.17 min ramp), 25.0 °C. Detection: 210.0 nm, 4.0 nm bandwidth (bw); reference wavelength 360.0 nm with 100.0 nm bw. Peak annotations: 1: non-modified rArt v 3.0201; 1a, 2: heat induced variants of rArt v 3.0210.

was slightly adapted and comprised the following steps: 1.00 mol L⁻¹ NaOH (10 min), ultrapure water (15.0 min), 0.10 mol L⁻¹ HCl (10 min), BGE (10 min) (all with 200.0 kPa at 35.0 °C).

After conditioning, the protective polyimide layer was thermally removed with a Weller[®]Pyropen[®] Jr. from Weller Tools GmbH (Besigheim, Germany) at the capillary outlet. The cleaned capillary outside was then aligned in the coaxial electrosprayer. For the final equilibration the capillary was rinsed with BGE (10 min, 200 kPa) followed by application of 25.0 kV (10 min with 1.0 min ramp time), all at 35.0 °C.

Prior to each sample injection the capillary was rinsed with BGE (2.0 min, with 100 kPa, 25.0 °C). Hydrodynamic sample injection was done with 3.45 kPa for 10 s (for CZE) or for 50 s (in case tCITP preceding CZE was applied for in-capillary pre-concentration). Subsequent CZE separations were performed at 25.0 kV (1.0 min ramp time; normal polarity), all at 25.0 °C. The UV detector was bypassed in CZE-ESI-MS setup.

Sheath liquid (SL) was prepared by mixing 75 mL methanol with 25 mL ultrapure water followed by the addition of 100 µL FA.⁴¹ The SL was delivered by a MicroProEldex syringe pump (Napa, CA, USA) at a flow rate of 6.0 µL min⁻¹.

The MS was tuned manually in positive ion mode *via* direct infusion of the rArt v 3.0201 pH 3.4 control sample (0.30 mg mL⁻¹) through the Beckman Coulter ProteomeLab[™] PA 800 system at 20.0 kPa. The nebulizer gas was set to 0.4 bar. For the dry gas a temperature of 190 °C and a flow rate of 4.0 L min⁻¹ were applied. The endplate voltage was set to -500 V and the capillary voltage to 4500 V. The transfer time was 73.0 µs and pre-pulse storage time was 20 µs. A scan range of

500 – 2000 *m/z* was selected. The *m/z* scales depicted in subsequent figures diminish the displayed range omitting sections without signals for an improved visualization of spectra. Calibration was performed by direct infusion of the undiluted ESI tuning mix from Agilent Technologies via the Beckman Coulter ProteomeLab[™] PA 800 system at 20 kPa.

Bruker Daltonics DataAnalysis, vers. 3.3, and BioTools software, vers. 2.2, both from Bruker Daltonics (Bremen, Germany), were utilized for evaluation of mass spectrometric data. Protein mass spectra were deconvoluted using the Maximum Entropy Algorithm implemented in BioTools.

Statistical treatment. Statistical evaluation of data was done by means of IBM[®] SPSS[®] Statistical Software Package version 23. Results of performed tests were considered significant at *p* < 0.05.

RESULTS AND DISCUSSION

Response of rArt v 3.0201 to thermal stress. The different responses of rArt v 3.0201 to heat treatment under acidic and slightly alkaline conditions are illustrated in Figure 1. While unheated control samples yielded identical profiles at pH 7.3 or pH 3.4 (Figure 1A and 1E), CZE-UV revealed progressively changing electrophoretic profiles of rArt v 3.0201 with increasing duration of the heat treatment. Over the selected heating period of 120 min, novel peaks with longer migration times occurred at the expense of original allergen peak intensity. The extent of the profile alteration is pH related (Figure 1B-D and 1F-H). At pH 7.3, the pronounced changes in the electrophoretic profiles indicate considerable thermal lability of rArt v 3.0201. Heating for 5 min already induced a pronounced diminution of the height of the original peak in comparison to the control run (Figure 1A and 1B). After 15 min

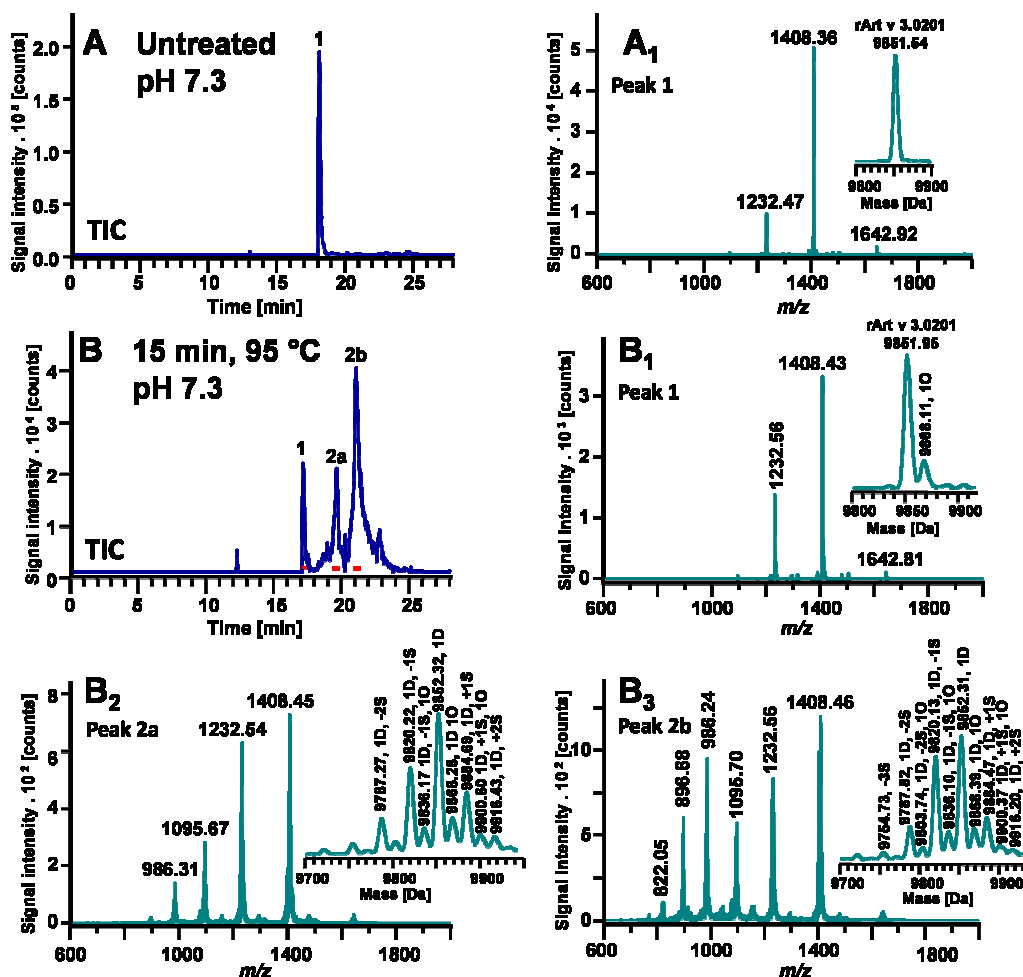


Figure 2. tCITP-CZE-ESI-TOF-MS monitoring of the development of modifications to rArt v 3.0201 at pH 7.3 unheated (A, A₁) and heated to 95 °C for 15 min (B, B₁-B₃). (A, B) TIC electropherograms, (A₁, B₁-B₃) extracted raw mass spectra and deconvoluted mass spectra depicted as inserts. Sample: 0.23 mg mL⁻¹ rArt v 3.0201 at pH 7.3 either unheated (control) or heated to 95 °C for 15 min with addition of 125 mmol L⁻¹ ammonium formate, respectively. Injection: 3.45 kPa, 50.0 s. CZE separation: 25.0 kV (1.00 min ramp time, anode at the inlet side), 25.0 °C. Sheath liquid: methanol:water:FA 75%:24.9%:0.1% (v/v/v). Nebulizer gas 0.4 bar. Drying gas: 4.0 L min⁻¹, 190 °C. End plate offset: -500 V. MS transfer capillary voltage: 4.5 kV (positive ion mode). Abbreviations in peak annotations represent: D=deamidation; +S=gain of sulfur; -S= loss of sulfur (LAN formation); O=oxidation.

heating, the height of the original allergen peak was reduced to less than 20% of the initial signal intensity and a novel peak cluster occurred at longer migration times (Figure 1C). Finally, after 120 min treatment at 95 °C the initial allergen peak had entirely disappeared and a broad novel peak representing several new protein species occurred at ~13 min (Figure 1D). Compared to pH 7.3, the thermally induced changes in the peak patterns of rArt v 3.0201 were considerably less pronounced at pH 3.4. No essential changes were observed after 5 min heating (Figure 1F). Stressing for 15 min caused only minute changes in the peak profile whereby the height of the original main peak was only slightly decreased and a minor novel peak became visible at 10.5 min migration time (Figure 1G). Even after a heating duration of 120 min the initial rArt v 3.0201 signal still represented the most prominent peak with only a cluster of minor peaks emerging at the migration time that corresponds to the novel peak occurring after 120 min thermal stress at pH 7.3 (Figure 1D and 1H). At pH 3.4, 50% degradation of the intact rArt v 3.0201 was observed after 90 min as compared to only 7.5 min required at pH 7.3 (see supplemental Figure S-1A).

Kinetics of rArt v 3.0201 degradation. In order to derive a mass balance, the migration time-normalized and background-

corrected peak areas were calculated and summed for all observed species in the CZE-UV electropherograms. The time course of normalized peak areas is outlined in supporting Figure S-1, which clearly illustrates the different kinetics of rArt v 3.0201 degradation at pH 7.3 and 3.4. Semilogarithmic plots of $\ln A_t/A_0$ versus incubation time were linear, suggesting that degradation follows a first-order-kinetics⁴² with kinetic constants of $1.3 \cdot 10^{-4}$ and $2.1 \cdot 10^{-3} \text{ s}^{-1}$ at pH 3.4 and 7.3, respectively (see supporting Figure S-1B and S-1C). Time-normalized peak areas measured by CZE-UV and summed over all variants (native and heat induced) were statistically evaluated over all 8 individual time points (three replicate injections each) for pH 7.3 and 3.4, respectively. Normal distribution and homogeneity of data were proven for pH 3.4 and 7.3 by Shapiro Wilk test ($p \geq 0.117$) and Levene's test ($p \geq 0.508$). Therefore, one-way ANOVA was applied and revealed no significant difference ($p \geq 0.764$) between total peak areas of the control and thermally stressed samples over the duration of thermal treatment with pH 3.4 and 7.3, respectively. Consistently, progressive heating did not induce allergen precipitation irrespective of the tested pH.

The long-term stability of the allergen at room temperature was tested in a separate control experiment. Thus, rArt v

Despite the fact that the portfolio of single deamidated species and oxidized siblings was identical in both peaks (peaks 2a, 2b in [Figure 2B](#)), their migration times differed. Within the primary sequence of rAart v 3.0201 six Asn residues representing possible deamidation sites are found at positions 9, 36, 50, 59, 77 and 89 (numbering includes N-terminal methionine; for sequence see supporting material).³⁸ The deamidation propensity for individual Asn residues is related to the primary sequence, the protein structure and properties of the bulk solution. The amino acid on the carboxylic side of Asn influences the deamidation propensity as outlined elsewhere.^{45,46} Further details are given in supporting [Figure S-3](#). The position of deamidation might thus differ between both peaks resulting in different μ_{eff} either due to changes in the pI and/or r_H . Higher

The TIC electropherogram and related deconvoluted mass spectra of protein species generated after 120 min stressing at 95 °C and pH 7.3 are depicted in [Figure 3](#). Since mass spectrometric data extractions were essentially equivalent to the data shown in [Figure 2](#), raw mass spectra can be found in the supporting information ([Figure S-4](#)). Spectra shown in [Figure 3A₁](#) and [3A₂](#) were extracted from two clearly separable peaks (2a, 2b), while [Figure 3A₃](#) represents the tailing shoulder (peak 2c) of the relatively broad second peak. It can be seen that spectral complexity was growing with increasing migration times, which is a clear indicator that the acquired protein modifications lead to a significant change either in the total charge, the hydrodynamic radius, or both. These are indicators of structural disintegration of the protein. Within experimental error, mass assignments for the peaks were congruent between

the three spectra. Therefore, we annotated the mass signals in Figure 3A₃ only with the corresponding protein modifications, based on the measured mass differences with respect to intact rArt v 3.0201. It can be seen that deamidation, oxidation, and LAN formation represent the most abundant modifications occurring under thermal stress.

The lowest mass detected in Figure 3A₂, 9690.21 Da, corresponds to a mass shift of -33.86 Da with respect to the adjacent species that has already lost four sulfurs and carries one deamidation (9724.07 Da). This mass shift is indicative of a loss of H₂S from a LAN containing species leading to the formation of two DHAs.

The average mass deviation in 52 mass determinations was 24 ppm which corresponds to a delta mass of 0.24 Da for a 9800 Da molecule (with mass deviations very rarely exceeding 50 ppm, mostly for very low abundant signals). Therefore, even deamidation with a mass shift of -0.984 Da is clearly detectable. In total, 25 different protein species, containing up to two deamidations, four LAN formations, two DHA formations, and one oxidation were distinguishable in our tCITP-CZE-ESI-TOF-MS analysis approach (see supporting Table S-2). The four most abundant species were consistently deamidated and LAN-containing species.

In order to corroborate the PTMs induced by the stressing, peptide mapping and peptide mass fingerprinting of the heat-stressed rArt v 3.0201 preparations was performed (see supporting information). Indeed, PTMs, such as deamidation, oxidation, and DHA formation, found at the whole-protein level were also readily confirmed by peptide mass fingerprinting in abundances ranging from a few to more than 45% (see supporting Figure S-3, Table S-3). PTMs were also confirmed and localized by HPLC-ESI-MS/MS (see supporting Figure S-5 and S-6; oxidation is exemplarily shown later for the heat treatment at pH 3.4 but data at pH 7.4 are identical). LAN formation, on the other hand, is not easily detectable by conventional peptide mapping, as the thioether link combines two peptides that are not cleaved under reducing conditions. This leads to the failure of conventional database searching routines. Nevertheless, upon manual calculation of the masses of the linked peptides (for details, see supporting information), we were able to confirm LAN formation between the cysteines C14-C28, C29-C74, C49-C88, and C14-C88 (see supporting Figures S-7-10 and Table S-1).

The evolution of protein patterns during thermal stressing at pH 3.4 clearly corroborated the profiles already observed with CZE-UV analysis: products were formed much slower, but resulting species were similar to pH 7.3. Thus, the degradation products observed after 120 min treatment at pH 3.4 were also found after 15 min at pH 7.3 (supporting Figure S-11 and Table S-2). Oxidation of the N-terminal Met residue was confirmed by HPLC-ESI-MS/MS (supporting Figure S-12) as in all other oxidized variants at pH 3.4 and 7.3 (data not shown). The intact rArt v 3.0201 molecule displayed a relatively compact structure as evidenced by a low number of only three charge states observable in the spectrum (Figure 2B₁ and supporting Figure S-11A₁). Oxidized, deamidated, and/or LAN containing species, on the other hand can be assumed to possess a looser structure, as can be deduced from longer migration times and broader charge state distributions (supporting Figure S-11C, C₁, C₂).

Mechanistic interpretation of detected reaction products. Deamidation represents one of the most frequent reactions of proteins under stress conditions, especially at elevated pH, when the elimination of ammonia is effected upon hydrolysis of a succinimide intermediate, while deamidation at acidic pH

is believed to follow a typical hydrolytic mechanism (see supporting Figure S-13A and B).⁴² rArt v 3.0201 contains six asparagines in total, which are potential sites of deamidation. At the whole protein level, we were able to detect species with two deamidations at positions that are not revealed by the mass measurement of the whole protein. However, they could be assigned to positions 36, 50, and 59 upon peptide mapping. Indeed, Asn59 showed the highest propensity for deamidation (see supporting Figure S-3). The observation of faster deamidation at slightly alkaline pH is also in-line with previous reports on lysozyme deamidation.⁴⁷ Although deamidation replaces an amido-group with a carboxylic group, we can assume that the total charge of rArt v 3.0201 remains practically unchanged, since the carboxylic acid (pK_a~3.9) is almost fully protonated at the pH of 1.0 mol L⁻¹ FA (pH=1.80). Therefore, the observed increase in migration time most probably is due to an increase in r_H of the deamidated protein species (see supporting Figure S-4 and S-11).

Mass shifts of 32 Da or multiples thereof, indicative of sulfur gain or -loss, are more prominent at pH 7.3 and the abundances of the corresponding variants increase with the duration of thermal treatment. Reaction mechanisms involved include β -elimination^{2,15,48} with subsequent sulfur release and formation of DHA, which can finally react with cystine *via* Michael addition to form LAN (see supporting Figure S-13C). Moreover, stepwise loss of 32 Da and corresponding mass shifts up to -128 Da can be observed for heat treatment at pH 7.3, indicating that all four disulfide bonds in rArt v 3.0201 can be converted to LANs.

After 120 min heating at pH 7.3 a variant of 9690.21 Da was detected corresponding to a loss of -33.86 Da relative to the adjacent species that had already lost four sulfurs and carries one deamidation (9724.07 Da, Figure 3A₂, 3A₃). This mass can be explained by elimination of H₂S (33.99 Da) from LAN, resulting in the formation of DHA (supporting Figure S-13D). Indeed, this has been confirmed by HPLC-ESI-MS/MS by a conversion of LAN formed between C14-C88 (supporting Figure S-10) resulting in two DHA residues at the same positions, respectively (supporting Figure S-6).

During the heating also mass shifts of +32 Da and/or +64 Da were found at pH 3.4 and pH 7.4 (Figure 2B₂, 2B₃, supporting Figure S-11C₂, and supporting Table S-2). These mass shifts cannot be explained by multiple oxidations, since the observed masses disappeared after extended exposure to 95 °C (Figure 3). Alternatively, such mass shifts can be explained by polysulfide-formation, as already described for human growth hormone,³¹ recombinant allergens,⁴⁹ and monoclonal antibodies.^{31,33,50} Similarly, trisulfides have been described for salmon calcitonin after heating to 60 °C at pH 5-6.⁵¹ Several authors proposed reaction schemes,^{31,50,52} involving heterolytic cleavage that results in DHA and thiocysteine (supporting Figure S-13E). The latter decomposes *via* a loss of sulfur released either as H₂S or HS⁻ depending on the bulk pH.^{2,53} Nielsen *et al.*⁵⁰ proposed the formation of a trisulfide species by the nucleophilic attack of HS⁻ on a disulfide bond. This resulted in an intermediate which carries a perthiol (-SSH) and a thiol (-SH) group. Even under favorable environmental conditions, polysulfides are not persistent⁵⁴ which corroborates the temporary appearance of species with +32 and +64 Da (Figure 2 and 3). HPLC-ESI-MS/MS data confirmed two trisulfide structures involving C28-C29 and C49-51 (supporting Figure S-14A, 14B). Under the current conditions, a transient formation of two trisulfide bridges in rArt v 3.0201 was thus detectable.

Finally, oxidation was observed under practically all stressing conditions. The amino acid most prone to oxidation is

methionine,^{55,56} which is incorporated as starting codon in the recombinant protein. Since only one methionine is present in the sequence of rArt v 3.0201 and the oxidation of other amino acids such as tryptophan or histidine requires relatively harsh conditions for oxidation, predominantly monoxidation was detectable (supporting Figure S-12).

CONCLUSIONS

Thermal treatment of a recombinant protein containing several asparagines and disulfide bridges initiates the formation of a number of different protein species that can be readily monitored by CZE-ESI-MS. New species are formed at pH 7.3 with a first-order reaction constant in the order of $2 \cdot 10^{-3} \text{ s}^{-1}$, which is almost one order of magnitude faster than at pH 3.4. Except (methionine-) oxidized protein, none of the species formed at 95 °C can be observed even after prolonged storage at room temperature, which underlines the importance of elevated temperature for the processes involved.

The assignment of protein species is possible upon considering mass shifts that are due to conversion of amides into carboxy groups and/or addition as well as loss of sulfur. Whereas modifications of single amino acids can be readily corroborated by tandem MS analysis and database searching of proteolytic peptides, the detection of LAN-linked peptides is feasible upon manual calculation of the corresponding peptide masses and extracting appropriate ion current chromatograms.

For the first time, CZE-ESI-MS is shown to be a practical method to detect and identify LAN as well as trisulfide variants in a recombinant protein. The observed shifts in electrophoretic mobility also clearly correlate with corresponding changes in the three-dimensional structure of the protein. Finally, the revealed LAN formation explains irreversible conformational changes that have been observed several times in heat treated allergens. However, until now they have been postulated to originate from disulfide shuffling with subsequent allergen aggregation.

ASSOCIATED CONTENT

Supporting Information

Supporting Information available: protein expression and characterization methods, kinetic data, additional electropherograms and mass spectra, detailed schemes of degradation reactions, Table of molecular mass data, HPLC-ESI-MS(MS) data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

L. G. S., S. W., G. G., and H. S. conceived the study. L. G. S., S. W., C. G. H., C. R., and H. S. performed the experimental sections, data analysis, and interpretation. L. G. S., S. W., G. G., C. G. H., and H. S. drafted the manuscript. All authors reviewed the manuscript.

Notes

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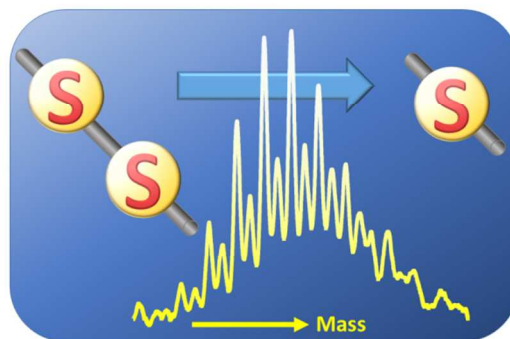
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