

Thermostability of *endo*-1,4- β -xylanase II from *Trichoderma reesei* studied by electrospray ionization Fourier-transform ion cyclotron resonance MS, hydrogen/deuterium-exchange reactions and dynamic light scattering

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Endo-1,4- β -xylanase II (XYNII) from *Trichoderma reesei* is a 21 kDa enzyme that catalyses the hydrolysis of xylan, the major plant hemicellulose. It has various applications in the paper, food and feed industries. Previous thermostability studies have revealed a significant decrease in enzymic activity of the protein at elevated temperatures in citrate buffer [Tenkanen, Puls and Poutanen (1992) *Enzyme Microb. Technol.* **14**, 566–574]. Here, thermostability of XYNII was investigated using both conventional and nano-electrospray ionization Fourier-transform ion cyclotron resonance MS and hydrogen/deuterium (H/D)-exchange reactions. In addition, dynamic light scattering (DLS) was used as a comparative method to observe possible changes in both tertiary and quaternary structures of the protein. We observed a significant irreversible conformational change and dimerization when the protein was exposed to heat. H/D exchange revealed two distinct monomeric protein populations

in a narrow transition temperature region. The conformational change in both the water and buffered solutions occurred in the same temperature region where enzymic-activity loss had previously been observed. Approx. 10–30% of the protein was specifically dimerized when exposed to the heat treatment. However, adding methanol to the solution markedly lowered the transition temperature of conformational change as well as increased the dimerization up to 90%. DLS studies in water confirmed the change in conformation observed by electrospray ionization MS. We propose that the conformational change is responsible for the loss of enzymic activity at temperatures over 50 °C and that the functioning of the active site in the enzyme is unfeasible in a new, more labile solution conformation.

Key words: conformation, ESI-MS, H/D exchange, stability.

INTRODUCTION

Industrial processes require unique properties of enzymes with respect to pH optima, substrate specificity and thermostability. A detailed picture of the structure and function of such enzymes is needed to develop new species for a variety of different purposes. Thus, the relationships between dynamic structures of proteins and their biological or enzymological activities have long been of interest for protein chemists. A variety of analytical methods have been used in this research area. Recently, MS has provided useful information about biological molecules. The development of soft ionization techniques such as electrospray ionization (ESI) has shown MS to be useful in structural and functional studies of different proteins and their complexes [1,2]. The new low-flow-rate methods, nano- [3–7] and micro-electrospray [3,8] ionization, have led to various applications in protein MS due to their accuracy, sensitivity and low sample requirement. These ionization methods combine well with electrophoretic (e.g. capillary electrophoresis), chromatographic (e.g. HPLC) or hybrid (e.g. capillary electrochromatography) micro-separation techniques prior to MS [3].

ESI-MS and its applications have been used to investigate the thermostability of proteins. Increasing temperature can denature the native tertiary structure of a protein, leading to inactivity in catalysis or another functional feature. Different proteins have shown different behaviour in thermal denaturation, as confirmed by ESI-MS [9–12]. Certain proteins refold after heat treatment, whereas others lose their native structure irreversibly. These

conformational changes can be monitored with ESI-MS by observing changes in the charge-state distribution. In addition, hydrogen/deuterium exchange (H/D exchange) has made many advances in MS [13–15]. H/D exchange of proteins and peptides in the gas or liquid phase has been used to investigate protein dynamics [16], secondary structure [17], stability [18], conformational changes [19–22] and folding [23,24] as well as non-covalent interactions with metal ions [22,25] or molecular chaperones [26]. The effects of amino acid mutations on protein stability and conformation have also been studied using this method [21,27]. Since the H/D-exchange ratio of a protein in the liquid phase depends on its conformation present in solution, it can also be used to determine the thermostability and thermal denaturation of proteins [15,23,28] by exposing them to heat treatment under controlled conditions.

Another useful analytical method for studying protein tertiary or quaternary structure in solution is dynamic light scattering (DLS) [29–31]. DLS measures the translational diffusion coefficient (D_T), which can easily be converted to the hydrodynamic radius (R_H) of the particles of interest, e.g. protein molecules in solution. Size distribution (monomodal, bimodal, narrow, wide) of the molecules reflects the present conformation or/and the number of the species present in solution. Oligomer formation, aggregation or conformation of the protein molecules as well as the amount of impurities in the solution are critical parameters in successful crystallization. Thus, DLS is widely used prior to protein crystallography [32,33] to assess whether protein samples are able to form crystals. It is also a practical method to

Abbreviations used: DLS, dynamic light scattering; ESI, electrospray ionization; FTICR, Fourier-transform ion cyclotron resonance; R_H , hydrodynamic radius; XYNII, *endo*-1,4- β -xylanase II; H/D exchange, hydrogen/deuterium exchange.

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Figure 1 Three-dimensional structure of XYNII from *T. reesei*

The active site is located in a cleft formed in the middle of the overall β -sandwich structure of the enzyme.

investigate conformational changes [34,35], folding kinetics [36], oligomerization [37,38], thermostability [39,40] and the liquid-phase complex formation [41] of proteins. Together with MS, it can be a useful method to confirm the observed characteristics of the protein molecules [42]. DLS is a quick, non-destructive and sensitive method requiring relatively small amounts of purified material, typically less than 1 mg.

Endo-1,4- β -xylanase II (XYNII, EC 3.2.1.8) from *Trichoderma reesei* is a low-molecular-mass (21 kDa) hemicellulolytic enzyme with an alkaline isoelectric point (pI 9.0) and an activity optimum at pH 4–6. It belongs to the G/11 glycosidase protein family and has a β -sandwich structure (Figure 1) that is the shape of a right hand [43–45]. The protein has no stabilizing disulphide bridges. XYNII catalyses the hydrolysis of xylan, the most common hemicellulose in plant biomass, by cleaving specifically the *endo*-1,4- β -glycosidic bond between the subunits of the xylan polymer chain. Xylanases are used widely in the paper and pulp as well as the food and feed industries. Biobleaching of pulp material is one of the most beneficial applications of xylanases, reducing the use of hazardous chlorine-based chemicals; enzyme helps to remove the coloured lignin component of the xylan polymer chain without damaging the cellulose fibre itself. Moreover, xylanases are used to increase the nutritional value of pig and poultry feed and they can be used in baking processes to improve dough texture [43]. Previous activity measurements by biochemical methods have shown that the protein rapidly loses its enzymic activity when incubated at temperatures over 55 °C in aqueous citrate buffer solution at pH 4.5 [46]. However, residual activity of 50 % is maintained after 24 h of incubation at temperatures below 45 °C.

In this article, we describe our latest research in order to study the thermostability of XYNII by conventional and nanoelectrospray ionization Fourier-transform ion cyclotron resonance (FTICR) MS with liquid-phase H/D-exchange reactions and DLS. Our primary purpose has been to determine the structural characteristics causing the enzyme to lose its activity during heat treatment.

EXPERIMENTAL

Sample preparation

The purified, concentrated XYNII stock solution (43 mg/ml) was prepared as described elsewhere [43]. The solution was desalted further by ultrafiltration with a Millipore Ultrafree[®] 5 kDa cut-off centrifugal concentrator. The stock solution was first diluted to a total xylanase concentration of 20 mg/ml. An aliquot of 100 μ l was ultrafiltered in the concentrator at 10621 g and 4 °C for 5 min. The residual 10–12 μ l was then diluted back to 20 mg/ml with ultra-pure water. Ultrafiltration significantly reduced the sodium content in the sample, allowing better MS studies without highly sodium-adducted protein ions.

The thermostability of XYNII was investigated in three different solutions, methanol/water, water and ammonium acetate buffer. Liquid-phase H/D-exchange reactions were performed in totally deuterated solvents. Additional acetic acid was used in both the DLS and ESI-MS measurements. In DLS measurements, the use of buffer or acid is highly recommended for increasing the ionic strength of the solution, thus preventing the electrostatic interactions of protein molecules with each other. In pure water, these interactions can influence the diffusion coefficient and have an apparent effect on the calculated R_H value. In ESI-MS the additional acetic acid was used to adjust the solution pH as well as to assist the protonation of protein molecules. The xylanase concentration in ESI-MS measurements and precursory heat treatments varied from 0.1 to 0.5 mg/ml (5–25 pmol/ μ l), unless otherwise noted. In the DLS measurements a higher concentration (4.0 mg/ml) was used to obtain a sufficient photon count rate. It was noted that concentration values close to the threshold limit (0.7 mg/ml) gave R_H values that were too high, as discussed later in this article. In addition, an increase in solvent noise was obvious in lower concentrations, causing intense peaks under 0.1 nm. The pH values of the solutions were measured by a Orion 250A pH meter (Orion Research) assembled with a micro-electrode.

All solvents and reagents were the highest available quality. Deuterated solvents and reagents were purchased from Merck except methanol, which was purchased from Euriso-Top. Other solvents and reagents were purchased from Sigma-Aldrich.

Heat treatment

The purified enzyme was exposed to heat treatment by incubating samples in a thermostatically controlled Grant[®] heat-incubation block. Incubations were made in the final concentrations and in the solvents used in both the DLS and ESI-MS measurements, unless otherwise noted. The average incubation time in the heat treatments varied from 15 to 25 min, unless otherwise noted. Samples were allowed to cool at room temperature before measurements. All incubations in deuterated solvents were carried out in a constant time period from the start of the incubation to sample measurement to avoid the effect of time dependence on H/D exchange.

ESI-MS

All MS measurements were done by a Bruker BioAPEX II 47e FTICR mass spectrometer (Bruker Daltonics) using positive-mode ESI. The instrument is based on a 4.7 T passively shielded horizontal superconducting magnet (Magnex Scientific) and cylindrical Infinity[®] ICR-cell. The conventional dualspray ion source (Analytica of Branford) with an IRIS[®] radio frequency (rf)-only hexapole ion guide was used to measure all acetonitrile/water, methanol/water and some buffer solutions (Figure 2A). The buffer solutions were electrosprayed through the off-axis

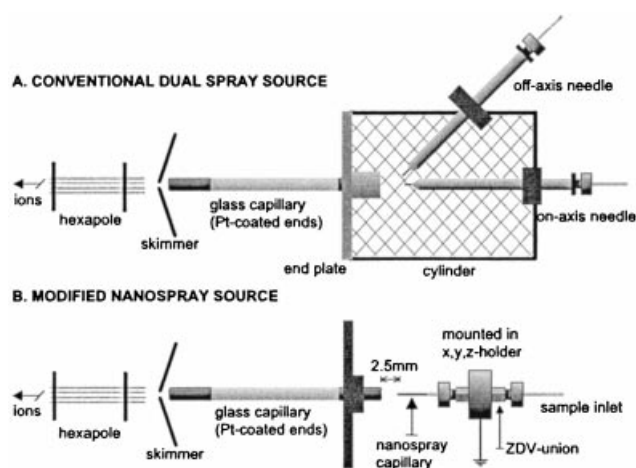


Figure 2 Schematic diagram of two different ion sources used in the ESI-MS experiments

ZDV, zero dead volume.

needle. A modified continuous-flow nanoelectrospray source was used in all other ESI-MS measurements (Figure 2B). The modification of the ion source was carried out in our laboratory. A gold-coated, 50 cm-long, 360 μm outer diameter and 50 μm inner diameter TaperTip[®] fused-silica capillary (New Objective) was cut to 3 cm and sleeved into a ZDV (zero dead volume)-union with microtight ferrules and sleeves, purchased from Upchurch Scientific. The normal ESI source chamber, containing a metal capillary and cylinder electrode, was removed and the end-plate electrode was replaced by a plastic end plate (Bruker Daltonics). The grounded (zero potential, 0 V) nanospray needle was adjusted in front of the ESI-source desolvation glass capillary by an *x-y-z* positioning holder. The distance of the nanospray needle was approx. 2.5 mm, nearly on-axis, from the front end of the glass capillary. The ionization voltage in the front end of the glass capillary was adjusted from -2100 V to -3200 V depending on the solvent used. Usually the water or buffer solutions needed a higher negative voltage for sufficient ion current and desolvation. Samples were introduced into the ion sources by a glass syringe (Hamilton) of 25 or 250 μl total sample volume, with a syringe-infusion pump (Cole-Parmer). The sample rates varied from only 5 to 300 $\mu\text{l}/\text{h}$ (0.08–5.0 $\mu\text{l}/\text{min}$), depending on the ion source used. Heated (250–300 $^{\circ}\text{C}$) ultra-pure nitrogen (AGA) was used as a drying gas with the conventional ESI-source. Additional needle gas (20–40 p.s.i., 138–276 kPa) was used when buffer solutions were applied via the off-axis needle. No drying gas was needed with nanospray assembly. Other instrumental parameters of the modified nanospray source were the same as the parameters used with the conventional ESI. Although two different ionization sources were employed, the results from samples measured by both conventional and nanospray methods were identical, and therefore results were not dependent on the ionization method used.

The mass spectra were calibrated against an acetonitrile-based ES Tuning Mix (Hewlett Packard) in the m/z range 200–2700. Data acquisition and processing were performed by Bruker XMASS[®] 5.0.6 software. Ions were accumulated for 500 ms using broad-band detection mode. The average time-domain size of the measurements was 128 000 and 32–64 scans were added depending on the signal intensity. Final spectra were zero-filled up to 256 000 bandwidth. Molecular masses of the samples were

calculated from obtained charge-state distributions using an XMASS[®] macro.

DLS

The light-scattering measurements were performed with a Dyna-PRO99 instrument (Protein Solutions) equipped with an MSTC (micro sampler, temperature controlled) sample holder and temperature-control box, 831.7 nm laser diode, Flex-30 single-channel correlator and 12 μl quartz sample cuvettes. Sample preparation was carried out with a Protein Solutions Micro Filter system with 0.1 μm Anodisc 13[™] membrane filters (Whatman) in order to remove any residual gas bubbles or solid impurities. Dynamics[™] 5.19.06 software was used in data collection and processing. The average number of acquisition scans was 20 and total acquisition time was set to 5.0 s. An aqueous buffer solution with a refractive index of 1.333, viscosity of 1.019×10^{-3} Ns/m² and temperature coefficient of 2024 was chosen from the calibration menu. The threshold (S/N) of the measurements was set at 2.0 with full detector sensitivity and scattered light was collected from an angle of 90 $^{\circ}$.

RESULTS AND DISCUSSION

ESI-MS measurements

The ESI spectra of XYNII measured in the water and methanol/water solutions greatly resembled each other at room temperature (Figures 3A and 3B), giving the strongest intensity on the peak of the charge state 9+. A small shift in the charge-state distribution into the lower charge states was observed when protein was measured in pure water without any acid (Figure 3C). In the ammonium acetate buffer solutions a similar shift was noticed but in these cases the peak of charge state 8+ was the most abundant in the spectra (Figure 3D). The shift from 9+ to 8+ in the most intense charge state may be due to different solution conditions, with buffer having apparent stabilizing effect on lower charge states. All spectra showed a narrow charge-state distribution from 7+ to 10+, which is in good agreement with

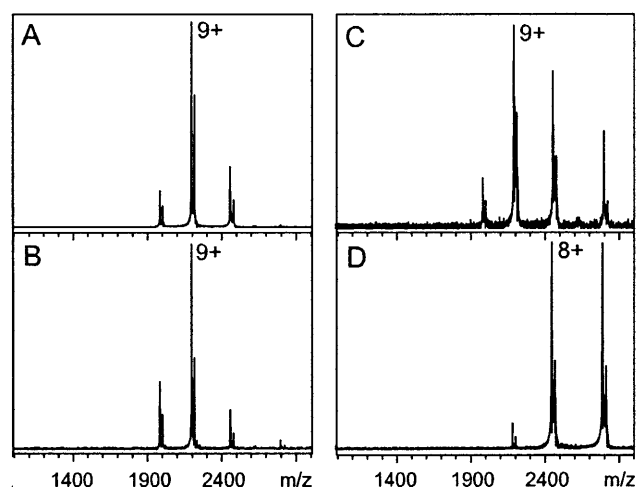


Figure 3 ESI mass spectra of XYNII in different solutions

(A) Water + 0.1% acetic acid (pH 3.8); (B) methanol/water (50:50, v/v) + 1% acetic acid (pH 3.2); (C) 100% water (pH 6.5); (D) 10 mM ammonium acetate (pH 3.8, adjusted by acetic acid). All spectra were measured by nanospray (sample rate, 7 $\mu\text{l}/\text{h}$; total xylanase concentration, 0.2 mg/ml). Peak notation: numbers ($n+$) correspond to ions, $[\text{M} + n\text{H}]^{n+}$, of the most abundant charge state.

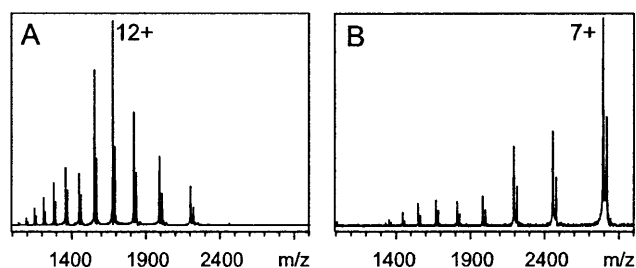


Figure 4 ESI mass spectra of XYNII in acetonitrile solutions

(A) Acetonitrile/water (50:50, v/v) + 0.5% acetic acid (pH 3.2); (B) acetonitrile/5 mM ammonium acetate (50:50, v/v, pH 4.9, adjusted by acetic acid). Spectra were measured by conventional ESI [sample rates, 250 $\mu\text{l/h}$ (A) and 40 $\mu\text{l/h}$ (B)]; total xylanase concentration, 0.5 mg/ml.

the native structure of XYNII, with 8–10 basic amino acid residues on the surface (three Lys, five Arg and two His, depending on pH). The accessibility (hydrophobicity) of each residue was calculated from the X-ray structure of XYNII using the NACCESS program ([47], for information see <http://wolf.bms.umist.ac.uk/naccess>). The native structure was furthermore confirmed by DLS, as described below. The pH dependence of the structure was also investigated in different solutions. Samples with an additional 0.2–2.0% acid in both the water and buffer solutions caused only slight changes in the charge-state distribution. The decrease in pH to 2.2 in ammonium acetate solution shifted the charge-state distribution slightly towards the one obtained in water. XYNII retains full activity for 24 h in the pH range 3.0–8.5 at room temperature, as shown by previous activity and stability measurements [46]. However, ESI experiments performed in solutions containing 50% acetonitrile (Figure 4A) revealed a strong denaturing effect on the protein structure. A broad distribution of charge states up to 20+ was noted in both the acetonitrile/water and acetonitrile/ammonium acetate/water solutions. The increase of pH to nearly 5 in the acetonitrile/ammonium acetate/water solution changed the spectra (Figure 4B) to resemble those obtained in ammonium acetate buffer solutions, again indicating the stabilizing effect of the buffer.

The measured molecular mass of the enzyme was 20825 ± 1 Da, averaged over the charge-state distribution. A mass decrease of 17 Da (equivalent to an NH_3 group) compared with the protein sequence (theoretical mass, 20842 Da) was due to the N-terminal glutamine residue blocked in its pyrrolidinecarboxylic acid form, which is seen clearly in the crystal structure of the enzyme [43]. All spectra were dominated by two adjacent peaks in every charge state due to another post-translational modification. About 30% of the protein has a 203 Da increase in molecular mass, indicating covalently bound *N*-acetylglucosamine (referred to here as A). The relative amount of A was calculated using absolute intensities of $[\text{M} + \text{H}]^{n+}$ (indicating *n*-protonated protein) and $[\text{M} + \text{A} + n\text{H}]^{n+}$. N-glycosylation of the enzyme is conceivable, according to the homology consensus Asn-Xaa-Ser/Thr-Z, where Z is not Pro and Xaa is any amino acid residue [48]. There are three such putative N-glycosylation sites in the sequence of XYNII. The behaviour of N-glycosylated species (M + A) was identical with that of the free enzyme (M).

Thermostability

Clear changes were observed in the ESI spectra when the protein was exposed to heat treatment under different solution conditions

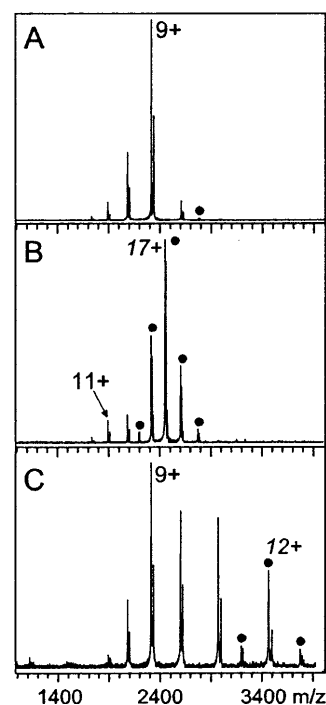


Figure 5 ESI mass spectra of XYNII after heat treatment in different solutions

(A) Water + 0.5% acetic acid (pH 3.4) at 60 °C; incubation time, 25 min; sample rate, 7 $\mu\text{l/h}$ (nanospray). (B) Methanol/water (50:50, v/v) + 0.1% acetic acid (pH 3.8), at 50 °C for 20 min; sample rate, 40 $\mu\text{l/h}$. (C) Ammonium acetate (10 mM; pH 3.8, adjusted by acetic acid), at 60 °C for 20 min; sample rate, 250 $\mu\text{l/h}$; total xylanase concentration, 0.2 mg/ml. Dimer peaks are denoted by ● (the most abundant charge states are shown in italics).

(Figure 5); both tertiary-structure changes and specific oligomerization of the enzyme were found. In the water and buffer solutions, the heat treatment produced notable changes in charge-state distributions at temperatures over 50 °C. In water, the shift was mainly to higher charge states (Figure 5A). In the ammonium acetate buffer solution there was also a shift to lower charge states (Figure 5C), due to the stabilizing effect discussed earlier. These observations indicate some changes in the structure of the enzyme. More conformational heterogeneity must be due to the heat treatment according to the charge-state distributions. Also, protein dimerization was observed. About 10–30% of the enzyme was specifically dimerized at temperatures over 50 °C in the water and buffer solutions. Because the protein sample consists of M (68%) and M + A (32%) forms, therefore 2M (50%), 2M + A (39%) and 2M + 2A (11%) forms were observed due to the dimerization process. These amounts correlate perfectly with the theoretical ones, indicating that glycosylation has no effect on the dimerization process. The lowest amount of dimer was formed in the water solution and the reason is unknown.

Interestingly, in methanol/water much higher amounts of dimer were formed due to heat treatment (Figure 5B) compared with the water and buffer solutions. In temperatures over 50 °C almost 90% of the protein was dimerized. If the acetic acid content of solution was increased from 1 to 5%, about 100% of the protein was dimerized during a 48 min incubation at 50 °C. However, decomposition of the dimer was not observed, even at low pH values, indicating a strong electrostatic character of the dimeric protein form. Conformational change was also noted at the same temperatures at which dimerization took place; the

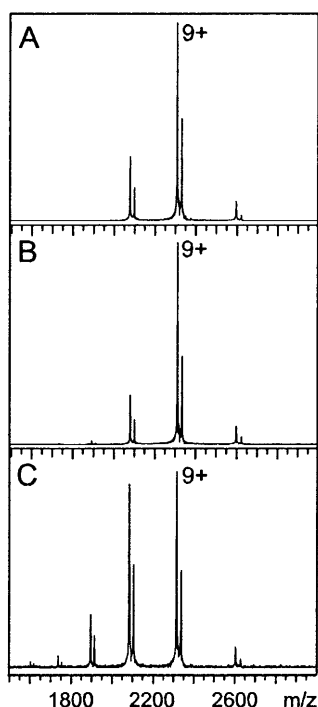


Figure 6 Heat treatment of a high concentration of XYNII (4.0 mg/ml) prior to ESI-MS

Incubation was performed at (A) 30 °C, (B) 50 °C and (C) 70 °C prior to ESI-MS in water + 1% acetic acid. Samples were re-diluted to a total xylanase concentration of 0.2 mg/ml after incubation (incubation time, 15 min; sample rate, 10 μ l/h).

monomer still present in solution was slightly opened up according to the charge-state distribution. The same conformational change was observed in the monomer of the dimeric protein that was formed by increasing the capillary-skimmer-region voltage, known as capillary-skimmer dissociation. The dimer was easily decomposed back to two monomers by high capillary voltages. The methanol content in the solution clearly lowered the transition-temperature region of the conformational change by 15 °C compared with the water and buffer solutions and furthermore increased the amount of dimer formed.

No dependency on the incubation time was noticed in any case when heat treatment was carried out for 15–48 min. Decomposition of the monomeric protein structure was not observed in any solvent conditions, even at temperatures over 80 °C, although the incubation time was extended up to 24 h. This observation indicates strong heat resistance of the primary structure of the protein. The conformational change was irreversible under all solution conditions because no refolding was perceived after extended cooling times. Despite the heat-induced conformational change, the overall structure was still maintained in solutions according to the charge-state distribution; random denaturation as observed in acetonitrile-based solutions was not noted under any circumstances.

To investigate the effect of protein concentration, heat treatment in water was also carried out with a higher protein concentration (4 mg/ml), which was used in DLS measurements. After incubation, samples were re-diluted to a protein concentration of 0.2 mg/ml and measured as in previous experiments. The results (Figure 6) were identical with those observed in lower concentrations (0.1–0.2 mg/ml). The protein showed a similar change in conformation and no dependency on concentration was noted at any temperature.

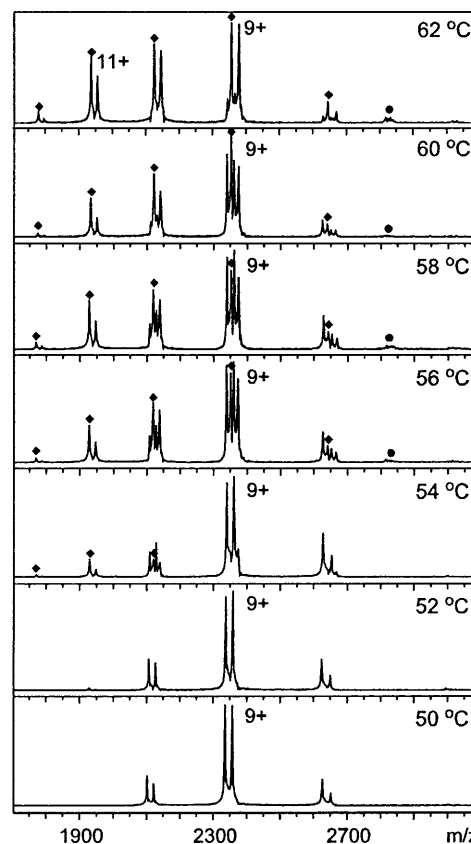


Figure 7 ESI spectra of XYNII after H/D exchange in $^2\text{H}_2\text{O}$ + 0.5% [^2H]acetic acid (pH 3.4) at different temperatures

Peak notation: \blacklozenge , M' monomer; \bullet , dimer. Incubation time, 20 min.

H/D exchange

H/D exchange showed interesting behaviour in the conformational change induced by heat in both the water and methanol/water solutions. Two different monomeric protein populations were found in certain transition-temperature regions, one (M) representing native-like conformation and the other (M') having a more open conformation. These two populations were observed through their different exchange rates, producing two new adjacent peaks in the spectra (Figures 7 and 8). M' exchanged over 100 deuteriums more than the original monomer M. The deuterium content in M' converged on the theoretical amount of exchangeable hydrogens in XYNII. We have calculated that there are 362 labile, exchangeable hydrogens in the structure of XYNII. A slight shift in the charge-state distribution was also noted between M and M' forms. Peaks of the strongest intensity in the charge-state distributions of M and M' were 9+ and 11+, respectively. The transition-temperature regions, where both monomers were present, were 50–62 °C for water and 37–42 °C for methanol/water solutions. In the methanol/water solution, both of the monomeric forms M and M' showed a nearly linear increase in H/D exchange against temperature (Figure 9A). In the water and buffer solutions the exchange behaviours were quite similar (Figures 9B and 9C). However, the deuterium content in the M form indicated a slight opening of the structure at temperatures over 50 °C, but the M' form showed constant deuterium incorporation. Thus it is obvious that the conformational change in all solutions follows a two-state co-operative transition mechanism [28,49], where only two

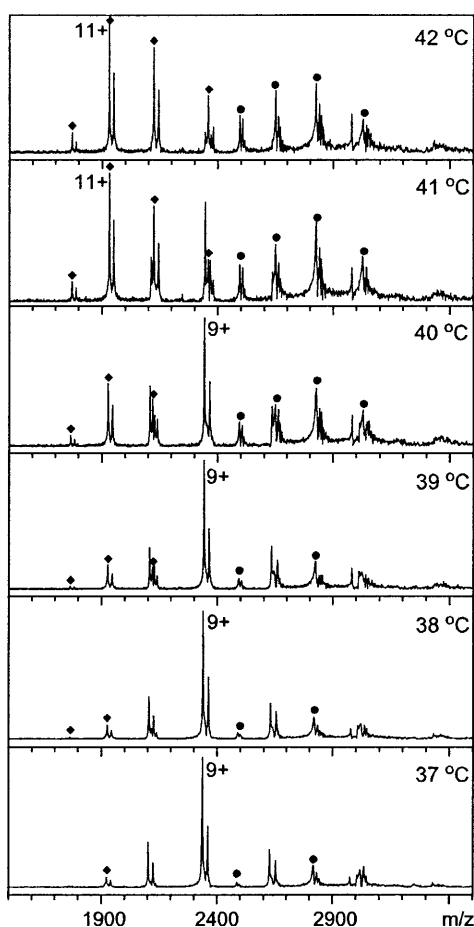
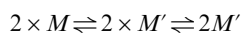


Figure 8 ESI spectra of XYNII after H/D exchange in ^{2}H methanol/ $^{2}\text{H}_2\text{O}$ (50:50, v/v) + 1% ^{2}H acetic acid (pH 3.2) at different temperatures

Peak notation: ◆, M' monomer; ●, dimer. Incubation time, 15 min.

distinct monomeric protein populations are present. The essential difference between these solutions was the temperature at which the conformational change between M and M' occurred (Figure 10A). The relative abundance between M and M' occurred (Figure 10A). The relative abundance of the dimer increased in the same temperature region, where the conformational change from M to M' was strongest (Figure 10B). In the methanol/water solution, 31% of the protein was dimerized at 40 °C. Dimerization of protein must have taken place through M' having a more open conformation than native-like M. This was verified by decomposition of the dimer by capillary-skimmer dissociation (Figure 10C). The monomer of the dimeric protein form exchanged almost the same amount of protons as the calculated amount of the two M' monomers together (Figure 9B). Therefore dimerization can be presented by the following:



In water and buffer solutions, where dimerization took place only to a minor extent, the dimer formed had exactly the same amount of deuterium as in M' monomer existing in solution.

DLS measurements

DLS was performed to investigate possible changes in the overall structure of the enzyme during heat treatment. Experiments were carried out with the standard parametrical conditions recom-

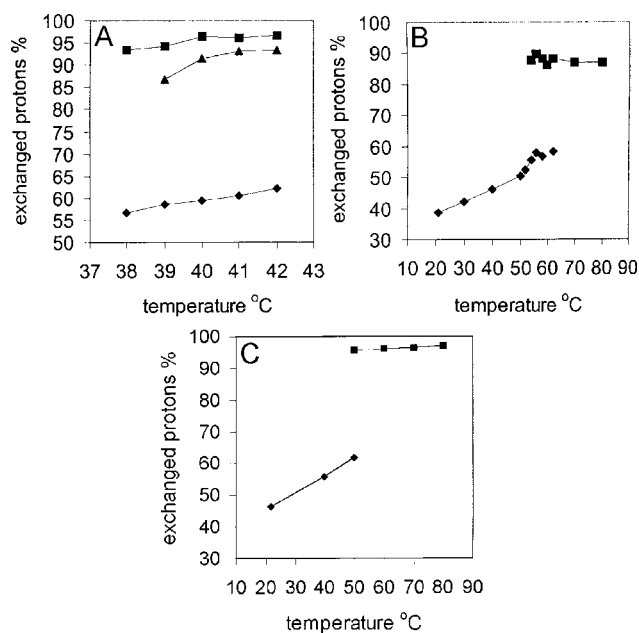


Figure 9 Temperature dependences on exchanged protons of M (◆), M' (■) and 2M (▲) in (A) $^{2}\text{H}_2\text{O}$ + 1% ^{2}H acetic acid, (B) ^{2}H methanol/ $^{2}\text{H}_2\text{O}$ (50:50, v/v) + 1% ^{2}H acetic acid, and (C) 10 mM ^{2}H ammonium acetate/ $^{2}\text{H}_2\text{O}$ (pH 3.8, adjusted by ^{2}H acetic acid)

Percentages of exchanged protons were calculated using the relation: $362 \text{ } ^2\text{H} = 100\%$.

mended by the instrument supplier. Pure water with 1% acetic acid was used as a solvent in all measurements. At temperatures of 20–40 °C, the enzyme showed a quite narrow monomodal size distribution with an average R_H value of 1.9 nm (Figure 11), which correlates well with the native structure of the enzyme obtained from the crystallographic data [43]. XYNII is folded into one domain structure with approximate dimensions of $3.4 \times 3.4 \times 3.6$ nm, which leads to an approximate R_H value of 1.73 nm. The relative difference of 12% between these two R_H values may be due to the somewhat different conditions of the molecules; some were calculated from a static crystal structure while others were calculated from the labile solution structure. We therefore assume that the DLS data represent the native solution structure under these conditions. If the protein concentration was decreased to close to the threshold level of the instrument (0.7 mg/ml), R_H values that were too high (2.3–2.4 nm) were obtained, which is why all data were collected with a total xylanase concentration of 4.0 mg/ml.

A clear difference was noted in the size distribution as well as in the R_H values at elevated temperatures. In the temperature region of 50–70 °C, R_H increased considerably up to 3.4–3.6 nm. The polydispersity of the size distribution also became broader, although maintaining its monomodal character. Other peaks, representing high-order aggregates or decomposition of primary structure, were not observed after heat incubation at any temperature. This indicates a clear heat-induced conformational opening of the structure, which is in good accord with MS studies. The ESI-MS and DLS methods give nearly the same transition-temperature region of the conformational change in water. The greater polydispersity of the size distribution may be due to the more labile solution structure of the protein, which also explains the increase in H/D exchange at temperatures over 50 °C. It is obvious that the overall structure is still maintained in the solution, denoted by a mono-modal size distribution and

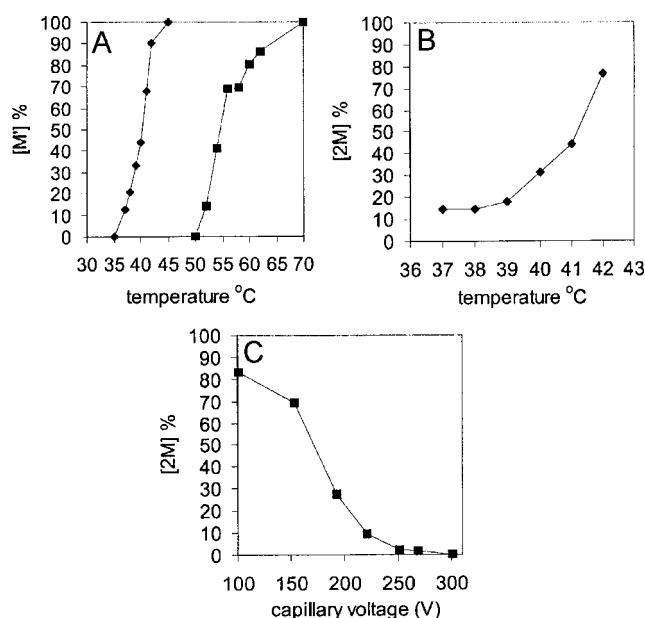


Figure 10 Temperature dependences

(A) Relative amount of M' in water (■) and methanol/water (◆) with 1% acetic acid. (B) Relative amount of 2M dimer in methanol/water (50:50, v/v) + 1% acetic acid. (C) Relative amount of 2M as a function of capillary voltage. Relative amounts of M' and 2M were calculated using equations: $[M'] = \text{absolute intensity } (M^{10+}) / \text{absolute intensity } (M^{10+} + M^{10+}) \times 100\%$ and $[2M] = \text{absolute intensity } (2M^{15+}) / \text{absolute intensity } (M^{9+} + 2M^{15+}) \times 100\%$.

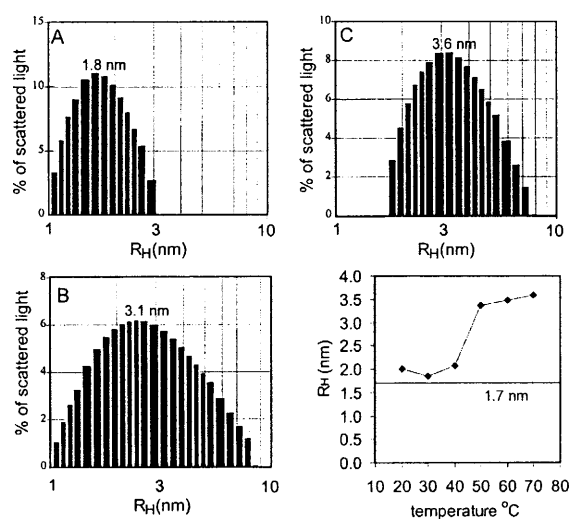


Figure 11 DLS data

Size distributions after heat treatment at (A) 30 °C, (B) 50 °C and (C) 70 °C and (D) mean R_H values at 20–70 °C of XYNII in water + 1% acetic acid (pH 3.2; total xylanase concentration, 4.0 mg/ml). The R_H value of 1.7 nm represents the radius of native protein molecules observed by X-ray crystallography.

moderate increase in R_H value, although a strict conformational change has occurred.

Conclusions

The thermostability of XYNII from *T. reesei* was investigated by two different methods, MS and DLS. The results of these two

methods greatly resembled each other, showing a clear irreversible conformational change and dimerization of the enzyme induced by heat. In addition, H/D-exchange reactions revealed two distinct monomeric protein populations in certain transition-temperature regions, depending on the solvent used. According to the relative amount of exchanged protons, the change in conformation is remarkable. However, the charge-state distribution seemed to be relatively insensitive to conformational change, leading to only a moderate shift towards the higher and lower charge states. This verified that the protein still possessed its overall structure after heat treatment. The change in conformation is assumed to break down some part of the hydrogen-bonding network in the native structure of the enzyme, leading to a more labile solution structure. Under these circumstances the active site of the enzyme is also considered to be prevented from performing catalysis. The functional conformational changes and open-closed movement of the active-site environment in XYNII have previously been studied by X-ray crystallography and molecular-dynamics simulations [45]. This conformational feature of the enzyme has been thought to play an important role in the function of XYNII. It could be suggested that this dynamic open-closed behaviour of the active site is made irreversible through heat treatment, since the hydrogen-bonding network has been broken. Although an irreversible opening in conformation was observed, the decomposition was not noted at any temperature, indicating the strong stability of the protein primary structure with regard to heat. In the methanol/water solution the dimerization was considerably higher than in the water and buffer solutions. In addition, the methanol content in the solution lowered the transition-temperature region of conformational change. Methanol is known as a denaturant affecting the conformation and folding of proteins [50]. However, random denaturation was not observed and the nearly native structure was still maintained, according to the charge-state distribution. Results also show that the dimerization was invariably accompanied by conformational change. H/D exchange showed that the dimer consisted of two monomers having a more open conformation, rather than two native ones. In addition, no dimer was formed until the conformational change between the two monomeric forms occurred. DLS studies in the water solution showed a considerable increase in the R_H values of protein molecules at temperatures over 50 °C, verifying the conformational change observed by ESI-MS. The conformational change in water, observed by both ESI-MS and DLS, occurs in the same temperature region that, according to previous activity measurements [46], enzymic activity disappears completely. Thus it is strongly suspected that the conformational change induced by heat is responsible for the previously found activity loss of the enzyme.

Our results show how nanoelectrospray ionization MS and DLS can be used as combined methods to study the thermostability of proteins. Nanoelectrospray is a very sensitive ionization method, allowing very low sample rates and the use of solvents with relatively low volatility, such as pure water. Additional studies by means of MS are still needed to learn more about the details of the conformational changes in XYNII on heat treatment. It would also be interesting to obtain crystallographic data from protein molecules that have undergone conformational change in order to specify the structural characteristics leading to inactivity.

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