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Amyloid fibril formation in the presence of water structure-affecting solutes

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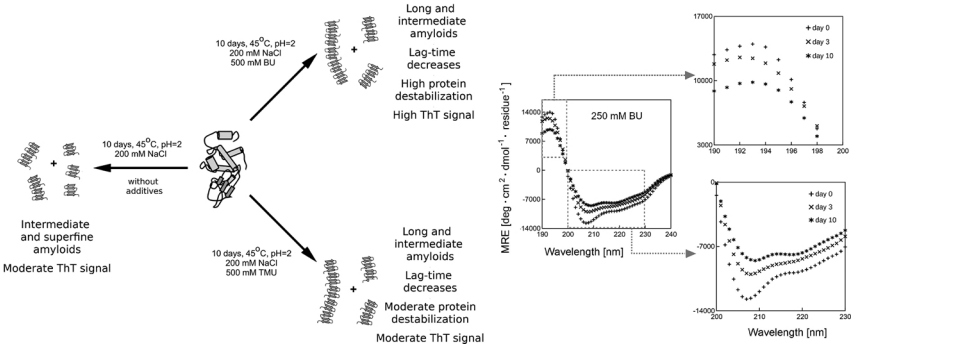
HIGHLIGHTS

- Influence of butylurea and tetramethylurea on lysozyme fibrillation was tested.
- Both isomers decrease thermal stability of lysozyme.
- Butylurea and tetramethylurea decrease lag time of amyloidogenesis.
- Only butylurea increases the efficiency of lysozyme fibrillation.

GRAPHICAL ABSTRACT



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ABSTRACT

The impact of the differently hydrated non-electrolytes (protein structure destabilizers) on the fibrillation of hen egg white lysozyme (HEWL) was investigated. Two isomeric urea derivatives i.e. butylurea (BU) and *N,N,N′,N′*-tetramethylurea (TMU) were chosen as a tested compounds. The obtained results show that butylurea exerts greater impact on HEWL and its fibrillation than tetramethylurea. Both substances decrease the time of induction of the fibrillation (lag time) but only BU increases the efficiency of amyloidogenesis. For the systems with equivalent reduction of the HEWL stability (250 mM BU and 500 mM TMU) the not-equivalent increase of the protein fibrillation was recorded (higher for BU). This fact suggests that specific interactions with protein, possibly water mediated, are responsible for the action of the tested substances.

1. Introduction

Amyloids are linear aggregates of proteins in which monomeric molecules are not covalently bonded to each other but only held together by physical interactions [1]. It is believed that the formation of amyloids is generic property of the polypeptide chain driven by universal mechanism. Amyloids are formed from misfolded proteins [2] and the first step of the amyloidogenesis is partial denaturation of the macromolecules [3]. The structure of the amyloids obtained from different proteins are similar. The secondary structure is rich in β-sheet content [4]. The fibrils are long, unbranched, composed of several twisted protofilaments [5] with the diameter ranging from around 2 to 12 nm [5,6].

Social and biological significance of amyloidogenesis is far-reaching. Around 40 human disorders have been associated with the

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formation of amyloids or amyloid-like intracellular inclusions [2]. The presence of the amyloids is regraded as a molecular hallmark of various diseases including Alzheimer13s and Parkinson13s diseases, amyotrophic lateral sclerosis, the spongiform encephalopathies and type II diabetes.

In this work we study the fibrillation of hen egg white lysozyme (HEWL). In humans, accumulation of amyloid deposits of lysozyme in liver and kidneys is responsible for the development of hereditary amyloidosis [7,8]. The homology of the both enzymes i.e. the human and the chicken is quite high [9,10], but in the present work we intend to use lysozyme in a wider context as a model protein. The structure and properties of the HEWL have been well described, it is well soluble and easy to handle protein. For these reasons its fibrillation has been studied by a number of research teams over the years [11] as well as by us [12,13].

Despite of the negative aspects of the amyloidogenesis, the large number of works is focused on the practical application of the protein fibrillation. The possible utilization of the amyloids includes fabrication of nanomaterials (metallic nanowires, silica or CdSe nanofibers), bone-like biomimetic composites, tissue scaffolds and many others [14]. Application of these materials ranges from medicine to bio-sensors and energy storage devices manufacturing [14].

Moreover, the food scientists strengthen their efforts to analyze the properties of the amyloids generated from edible proteins which, in natural form, are not fibrillar [15]. The studies cover the emulsifying activity of the fibrils, their foaming and gelation properties and the viscosity and texture modification abilities. The intentional modification of the food products leading to the amyloids generation appears to be controversial and more studies on the food safety are needed [16,17].

The aim of the present work is to examine the influence of differently hydrated non-electrolytes on the fibrillation of hen egg white lysozyme. As a tested compounds we selected two isomeric urea derivatives i.e. butylurea (BU) and *N*,*N*,*N*′,*N*′-tetramethylurea (TMU). Our previous reports [12,18,19] show that butylurea is hydrophobically hydrated while tetramethylurea manifests structure breaking properties. Additionally, our preliminary work proves that BU acts as a protein structure destabilizer [20]. As mentioned above, the protein fibrillation begins with partial unfolding of protein thus it could be expected that the changed HEWL stability will change the progress of amyloidogenesis.

According to the recently published work [21] the similarity of the hydration shells formed around non-electrolytes and the hydration shell of the protein is one of the decisive factors influencing the impact of the solutes on protein stability. In the light of this finding, it is appropriate to investigate how the properties of the solvent structure affect the protein fibrillation.

In the present work we investigate the hen egg white lysozyme fibrillation in the presence of butylurea and *N*,*N*,*N*′,*N*′-tetramethylurea at concentrations ranging from 50 mM to 500 mM. The samples were incubated in the acidic solutions (pH = 2, 200 mM NaCl) at 45 °C. We decided to perform the experiments at lower temperature than usual [1,22,23] to compensate for the decrease of the denaturation temperature of HEWL at the highest tested concentration of butylurea [20].

The obtained results reveal that BU and TMU decrease the time of induction of the amyloidogenesis but only BU increases the efficiency of the fibrillation. These effects cannot be attributed solely to the decrease of the denaturation temperature caused by the urea derivatives. The specific interactions or the solvation properties of the tested compounds are also of great importance.

2. Materials and methods

2.1. Solutions and fibrils preparation

Hen egg white lysozyme, HEWL (Fluka, Cat. No. 62971) was dialysed against pure water and lyophilized. The samples were prepared by dissolution of the protein in aqueous solution of HCl, pH = 2, containing 200 mM NaCl (VWR, purity 99.9%). The pH was adjusted by the addition of small volumes of HCl solution. The final concentration of the protein determined by weight was equal 25 mg/ml. The concentrations of urea derivatives i.e. butylurea, BU, (Fluka, purity 99%) and *N*,*N*,*N*′,*N*′-tetramethylurea, TMU, (Aldrich, purity 99%) were equal 0 mM, 50 mM, 250 mM and 500 mM. All samples were filtered by 0.1 μm syringe filters (Minisart Cat. No. 16553). Each of the solutions was divided into equal parts and placed in the separate, tightly closed test tubes. The samples were incubated in water bath (GFL 1086) at 45 °C with shaking (121–125 min^{-1}) for 10 days. After required time of incubation, the samples were removed from the thermostatic bath and diluted with water to the final concentration of the protein 3 mg/ml.

2.2. Thioflavin T fluorescence assay (ThT)

The Thioflavin T fluorescence assay was performed according to the known procedure [22,23]. The analysed solutions were prepared in the PBS buffer (10 mM phosphate buffer saline, 150 mM NaCl, pH = 7.0). The final concentration of ThT (Sigma-Aldrich Cat. No. T3516) in the sample was 9.5 μM and the concentration of the protein was 0.08 mg/ml. The fluorescence spectra were recorded on Jasco FP 8300 spectrofluorometer. The excitation wavelength was set to 440 nm and the emission spectra were collected from 460 nm to 550 nm. The scan speed was set to 200 nm/min with the data interval 0.5 nm. The excitation bandwidth and the emission bandwidth were set to 5 and 10 nm, respectively. The sensitivity (photomultiplier voltage) was 370 V. Five or six scans were averaged for every sample.

2.3. Atomic force microscopy (AFM)

Twenty microliters of the protein solution (0.015 mg/ml, diluted with water) was placed on the freshly cleaved surface of mica. After 5 min of incubation mica was flushed with two portions (50 μL) of filtered water (0.1 μm). The samples were dried for 3–4 days in desiccator over P_2O_5 and then stored over silica gel. The scans of the surface were performed using Nanosurf Easyscan 2 microscope in the contact mode. The applied force was equal 20 nN. The scans were collected with the resolution 512 × 512 px and the size of the scans were equal 9 × 9 μm. The data were analysed in Gwyddion 2.50.

2.4. Optical density measurements (OD)

The optical densities were measured at 350 nm using Thermo Evolution 300 spectrophotometer. The concentration of the protein in the samples was equal 3 mg/ml. The optical length was 1 cm and the samples were mixed using custom-made stirring accessory. Ten scans were averaged for each sample.

2.5. Circular dichroism spectroscopy (CD)

The samples of the protein were diluted with deionised and filtered water to the concentration of protein 0.15 mg/ml. The exact protein concentration was determined using UV-VIS spectroscopy (see below). The CD spectra were recorded using Jasco J-815 spectropolarimeter in the range from 190 to 260 nm. The signal in mdeg was recorded every 0.2 nm. All samples were blank subtracted using spectra measured for samples without protein. The scan speed was set to 50 nm/min and the optical length was 0.1 cm. For each sample 6 scans were averaged. The obtained data were analysed using CDSSTR algorithm with the help of the DichroWeb on-line service [24,25]. The dataset 7 was used as a reference [26].

2.6. Gel electrophoresis (SDS-PAGE)

The SDS-PAGE electrophoresis on gradient gels (10–16.5%) was performed under non-reducing conditions in Tris-tricine buffer

solution. The gels were stained by the Coomassie Brilliant Blue G250 dye. The marker was purchased from Thermo Fisher (Cat. No. 26628; the Spectra Multicolor Low Range Protein Ladder 1.7 to 40 kDa).

2.7. UV-VIS absorbance spectroscopy

The UV-VIS spectroscopy was used in order to determine the concentration of the protein in solutions analysed by circular dichroism spectroscopy. The spectra were collected using Thermo Evolution 300 spectrophotometer. The absorbance at 280 nm was measured and the extinction coefficient $\varepsilon = 2.65\ dm^3\ g^{-1}\ cm^{-1}$ was used in the calculations. Three scans were averaged for each sample.

2.8. Determination of temperature denaturation (nanoDSF measurements)

Thermal stability of the hen egg white lysozyme and the denaturation temperature T_m in the presence of the butylurea and tetramethylurea was determined using differential scanning fluorimetry (nanoDSF; apparatus Prometheus NT.48, NanoTemper). The concentration of urea derivatives increased from 0 mM to 500 mM with the interval of 100 mM. The concentration of the lysozyme was equal 25 mg/ml (determined by weight). The pH of the solutions was adjusted by HCl to pH = 2, the concentration of NaCl was 200 mM. The samples were excited at 280 nm and the intrinsic fluorescence of the protein was recorded at 330 and 350 nm. The temperature increased from 20 to 95 °C at rate of 2 °C min^{-1}.

2.9. Statistical analysis

The uncertainties of the measured values Δx were estimated using standard deviations $\Delta x = \sigma$ and were calculated from the equation:

$$\sigma = \sqrt{\frac{\Sigma(x_i - \bar{x})^2}{n-1}} \quad (1)$$

where: x_i and $\bar{x}$ are the observed values and the arithmetic mean, respectively. The n denotes the number of observations.

3. Results and discussion

The amyloid fibrils are often detected by the ThT fluorescence. In the current work we performed ThT binding assay to estimate the influence of the urea derivatives on the efficiency of the HEWL amyloidogenesis. The obtained ThT fluorescence spectra are shown in the supplementary materials (Figs. S1–S2). The results of this analysis are summarized in Fig. 1. For all tested samples the ThT fluorescence intensity increases with the incubation time. The magnitude of the increment depends on the solution composition, it proves that amyloids are formed but with different yield. The comparison of the results for 50 mM of BU and TMU with the sample without additives shows that the low concentration of urea derivatives has weak impact on the protein fibrillation. For the higher concentrations of BU and TMU (250 mM and 500 mM) the significant changes are observed.

After 3 days of incubation, the ThT fluorescence for the samples of lysozyme incubated in the presence of butylurea (250 mM and 500 mM) is markedly higher than for the control sample. The similar effect is clearly observed for the highest concentration of tetramethylurea (500 mM). For samples containing BU, high fluorescence observed for third day of incubation is accompanied by the increase of the fluorescence for day 10. In the present study, the highest reported signal was detected for the samples incubated ten days with 250 mM and 500 mM BU, the fluorescence was much larger than for the samples without additives. In contrast, the initial increase of the signal for the samples heated for 3 days in the presence of 500 mM of TMU does not go together with increase of fluorescence for the samples incubated ten days. The signals recorded for the samples without additives and with 250 mM or 500 mM of TMU after ten days of incubation are comparable.

These results suggest that butylurea shortens the time of fibrillation initiation (so-called lag time) and raises the efficiency of the amyloidogenesis. The tetramethylurea also decreases the lag time of the amyloid fibril formation but not as effectively as BU. In the lower concentration of tetramethylurea (250 mM) ThT fluorescence signal recorded after 3 days of incubation is only slightly elevated in comparison with the control sample. Moreover, TMU does not increase the overall efficiency of HEWL fibrillation. This reported mutual independence of the time of the fibrillation initiation and the yield of amyloidogenesis has been reported previously by Uversky et al. for the systems containing α-synuclein and the electrolytes [27]. Similarly, the opposing effects were also detected for the fibrillation of lysozyme in the presence of different additives. The shorter lag time can result in either higher [28] or lower [29] efficiency of the amyloidogenesis.

To study the structural changes of the HEWL induced by the sample incubation at elevated temperature we used circular dichroism spectroscopy (CD) in the UV region. Although, the samples were diluted with pure water (see Materials and methods Section 2.5) the concentration of the highly absorbing compounds was too high for the CD measurements for the samples containing 500 mM of urea derivatives.

The observed differences in the CD spectra for the remaining samples were relatively small. The highest differences are present for the solutions containing 250 mM BU and 250 mM TMU (see Figs. 2 and 3). The circular dichroism spectra recorded for samples without additives and for the 50 mM solutions of BU and TMU can be found in the supplementary materials to the manuscript (Figs. S3, S4 and S5).

The changes, progressing with incubation time, are more pronounced for butylurea than for tetramethylurea solution. The influence of the TMU on the protein is rather small. These results are in agreement with the conclusions drawn based on ThT fluorescence.

The quantitative analysis of the measured data was made possible by implementation of deconvolution algorithms [24,25]. The estimated components of the secondary structure are shown in Table 1.

The initial structure of the HEWL determined in the present study for the non-heated samples at pH = 2 in the absence of the urea derivatives is consistent with the literature data [23,30,31]. In acidic conditions the calculated structural components of lysozyme are in the range reported for the protein in the neutral pH [32–34]. As can be seen in Table 1 low concentrations of the BU and TMU do not affect the structure of the protein significantly. However, in the solution containing 250 mM of BU the structure of the lysozyme is perturbed. In contrast to the system without additives the β-sheet content is reduced and the percentage of the unordered structure content increases by around 5%-points. The starting destabilization of the HEWL impacts the aggregation of lysozyme during the incubation of the protein at elevated temperature.

The secondary structure of the HEWL incubated without additives undergoes small but noticeable changes. The percentage of the α-helical component decreases with the time of heating while the unordered structure increases. Interestingly, the large increase of the β-sheet content was not detected in these samples. The presence of the amyloid fibrils was confirmed by ThT fluorescence and atomic force microscopy (see below) but due to the relatively short incubation time and low temperature of incubation the concentration of the fibrils was rather small. The analysis of the data for the samples with TMU shows similar pattern. The β-sheet content is changing in narrow limits, α-helix content decreases and protein structure becomes more unordered.

The high concentration of butylurea (250 mM) strongly influences the amyloidogenesis. During the sample incubation, the percentage of the α-helix decreases much more drastically than for the solutions without additives or in the presence of TMU (about 10%-point higher decrease). The increase of the unordered structures is also more pronounced. As mentioned above, the starting β-sheet content for the non-incubated samples containing 250 mM BU is reduced. However, during

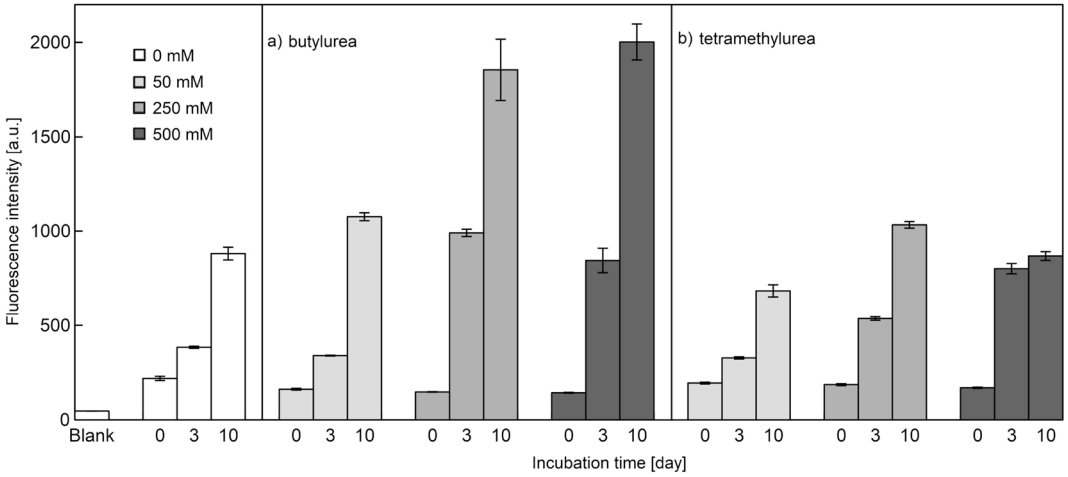

Fig. 1. Thioflavin T fluorescence intensity recorded at 485 nm for the samples containing butylurea and *N,N,N′,N′*-tetramethylurea (0 mM; 50 mM; 250 mM and 500 mM) incubated at 45 °C for 0; 3 and 10 days.

the course of the heating the β-sheet content reached 21%. It is the highest content of the β-sheet recorded in the present study.

Altogether, the results obtained from circular dichroism spectroscopy are in excellent agreement with ThT fluorescence. The presence of BU promotes the protein fibrillation, additionally this compound facilitates partial unfolding of the HEWL. The impact of TMU on amyloid fibrils formation is rather weak.

The changes of the turbidity of the samples were studied by optical density measurements at 350 nm. The results are shown in Fig. 4. The turbidity of the samples increases, with the time of incubation, for all of the analysed solutions. This increase is higher for the samples containing BU than TMU at the same concentration of urea derivative.

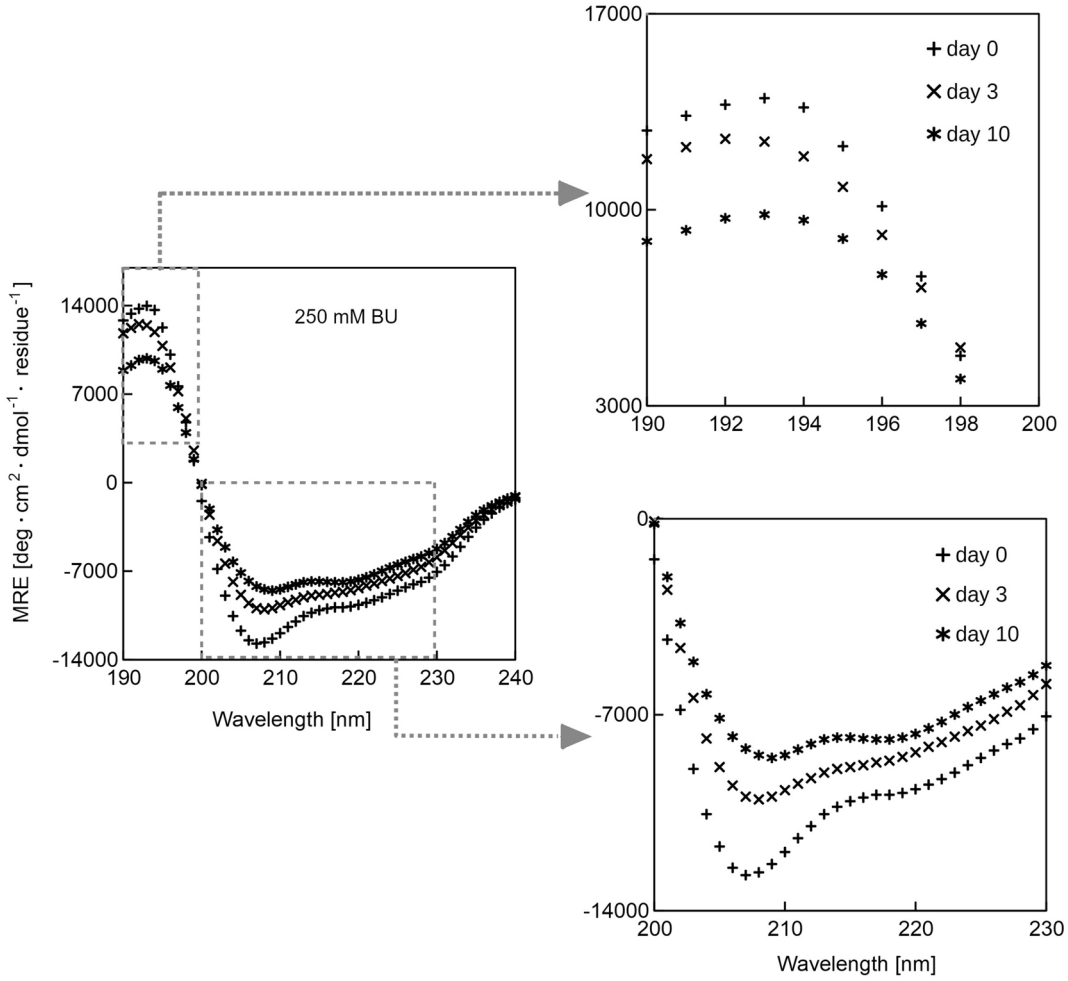

Fig. 2. The UV circular dichroism spectra (MRE mean residue ellipticity) measured for the solutions of HEWL incubated in the presence of 250 mM butylurea at 45 °C for 0; 3 and 10 days.

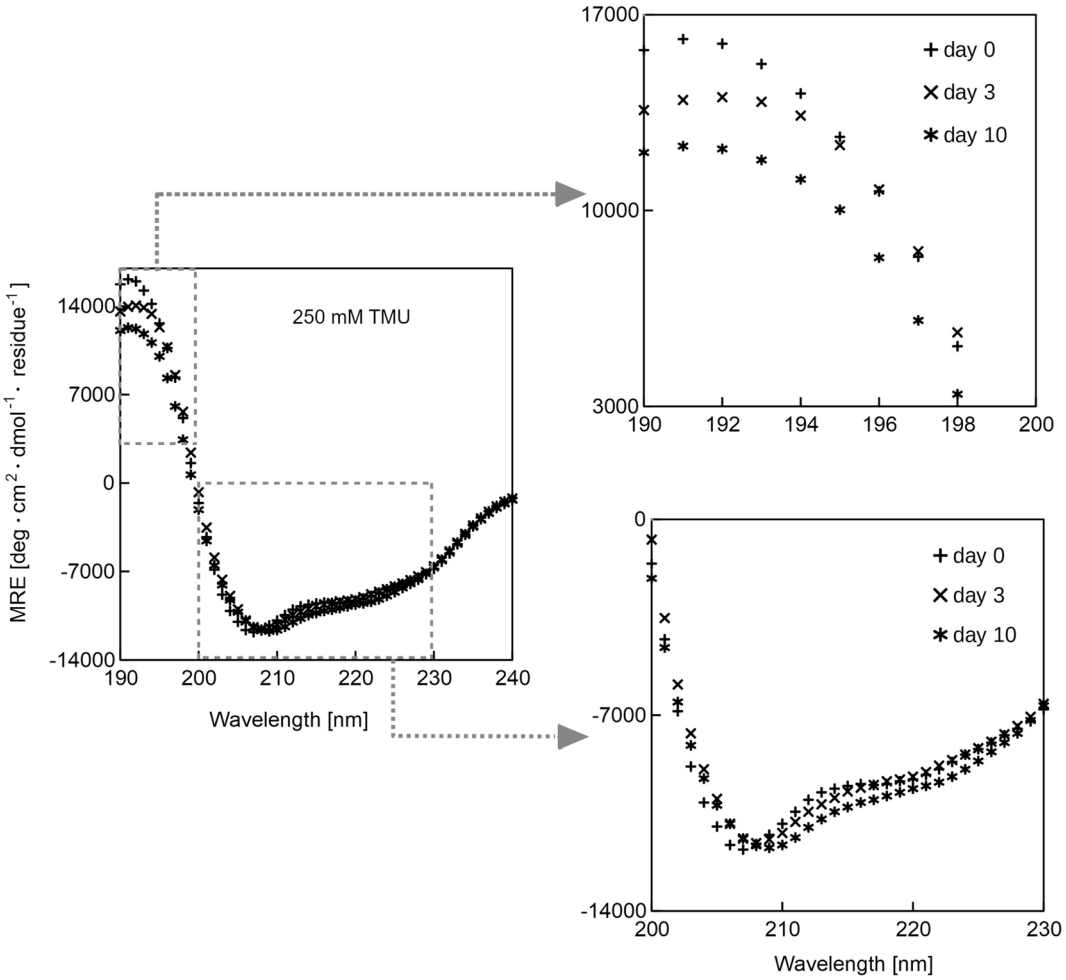

Fig. 3. The UV circular dichroism spectra (MRE mean residue ellipticity) measured for the solutions of HEWL incubated in the presence of 250 mM *N,N,N′,N′*-tetramethylurea at 45 °C for 0; 3 and 10 days.

Table 1
The components of the secondary structure for the samples of HEWL incubated at 45 °C in the presence of butylurea or *N,N,N′,N′*-tetramethylurea and without additives obtained based on circular dichroism measurements.

Sample		*α*-helix [%]	*β*-sheet [%]	Turns + unordered [%]
Samples incubated without additives				
Day 0		36	15	48
Day 3		35	17	47
Day 10		33	13	53
Samples incubated in the presence of butylurea				
Day 0	50 mM BU	36	13	50
Day 3	50 mM BU	36	14	50
Day 10	50 mM BU	32	15	53
Day 0	250 mM BU	37	10	53
Day 3	250 mM BU	29	18	51
Day 10	250 mM BU	22	21	55
Samples incubated in the presence of N,N,N′,N′-tetramethylurea				
Day 0	50 mM TMU	36	14	48
Day 3	50 mM TMU	35	16	48
Day 10	50 mM TMU	33	14	52
Day 0	250 mM TMU	36	14	49
Day 3	250 mM TMU	34	15	50
Day 10	250 mM TMU	32	14	54

Butylurea effects the lysozyme aggregation to a larger extent than tetramethylurea and, in this sense, these results are in agreement with the ThT and CD measurements. Surprisingly, the recorded turbidity was the highest for the 250 mM BU solution of HEWL incubated for 10 days.

The morphology of the amyloids was analysed using atomic force microscopy (AFM). The resulting height images are presented in Figs. 5 and 6 . The AFM confirms the presence of amyloids. There are no drastic changes in the fibrils morphology formed in the samples with different concentrations of additives. The large changes in macroscopic structure of fibrils have been observed by us in our previous work [23] for the samples incubated under different ionic strength and in the presence of different ions.

In the our samples at least 3 different populations of fibrils were present: superfine (around 0.2 μm), intermediate (around 0.8 μm) and long (2–3 μm, for some samples even longer).

As expected, in the solution without additives the number of fibrils increases with the heating time. It appears that the increasing concentration of BU causes the increase of the length of fibrils. The number of the superfine fibrils is the highest for the samples containing 50 and 250 mM of BU after 10 days of the incubation. To our surprise, it seems that the shortest fibrils are not present in the samples with the highest concentration of BU.

In the samples containing TMU all three types of fibrils were also detected. The longer incubation time increased the amount of amyloids and the formed fibrils were longer. Similarly to BU, in the 250 mM solution of TMU the number of superfine fibrils was the highest. For the samples containing 500 mM of TMU the superfine fibrils seem to be absent.

The results of gel electrophoresis are shown in Fig. 7. The lines were slightly overload to detect small amounts of products formed in the solutions. Apart from the large fraction of the lysozyme, the incubated

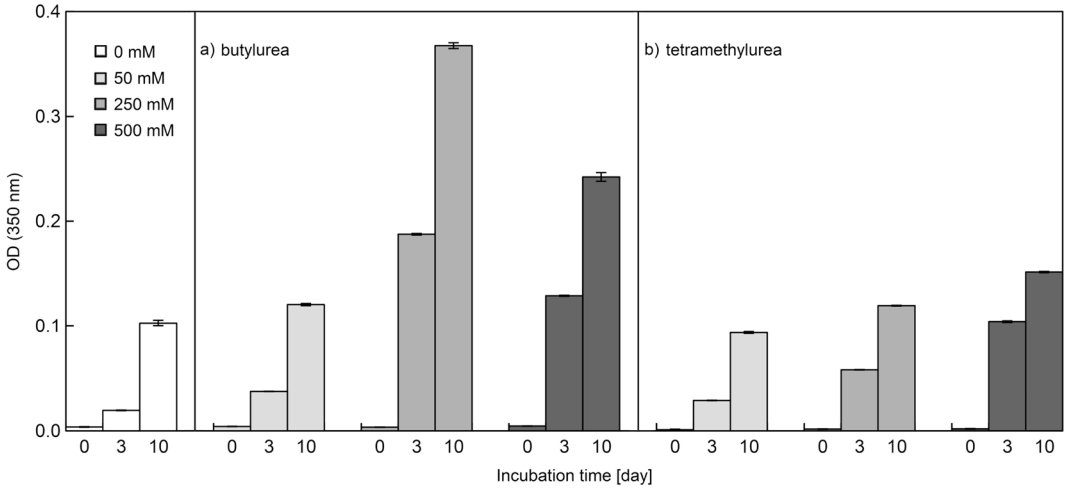

Fig. 4. The optical densities (OD) measured at 350 nm for the samples containing butylurea and *N,N,N′,N′* -tetramethylurea (0 mM; 50 mM; 250 mM and 500 mM) incubated at 45 °C for 0; 3 and 10 days.

samples contain hydrolytic fragments of the protein and covalently bonded HEWL dimers. For all samples the protein hydrolysis and dimerisation increase with the time of incubation. Two fractions of protein fragments were detected, one slightly smaller than native protein (band just below native HEWL) and the fragment smaller than 10 kDa.

The presence of the band just below 14 kDa has been reported previously for the samples incubated at 60 °C [23]. In the current work, better separation conditions and lower concentration of protein improved the detection. As can be seen, the longer incubation increases the intensity of this band. However, neither the ionic strength [23] nor the presence of BU and TMU influences it significantly.

The HEWL fragment with the mass below 10 kDa was also detected in our earlier experiments conducted at 60 °C. This time, however, single band is clearly visible, not two as for 60 °C. Its lower intensity might be caused by mild incubation conditions (45 °C) leading to less effective hydrolysis, the presence of relatively high concentration of salt (200 mM NaCl) and the smaller overload of the gel lines with protein.

As far as the HEWL dimers are concern, their concentration increases with incubation time and concentration of the urea derivatives. The higher degree aggregates, larger than dimers, were detected in the samples incubated for 10 days. The observed disturbances for the TMU samples at the longer incubation time were probably caused by the presence of the large amorphous aggregates.

The influence of the butylurea and tetramethylurea on the protein was assessed by nanoDSF technique [35–37]. Denaturation temperature T_m determined in these experiments is a measure of destabilizing effects of the urea derivatives on the HEWL structure. The concentration of BU and TMU ranged from 0 mM to 500 mM with the interval of 100 mM. The intrinsic fluorescence of the HEWL was measured at 350 and 330 nm and the ratio of the fluorescence *F350/F330* was plotted against temperature. For the precise determination of the denaturation temperature, the temperature derivative $\partial(F350/F330)/\partial T$ was calculated and the maxima of the obtained functions were estimated. Resulting plots are shown in Figs. 8 and 9 . The denaturation temperatures are collected in the Table 2.

In our earlier paper we investigated influence of the butylurea on the lysozyme dissolved in the pure water [20]. The current study presents the results of more detailed experiments in the acidic solutions (pH = 2) containing 200 mM of NaCl. Preliminary experiments conducted in water [20] showed that the BU decreases the T_m of the HEWL by about 12 °C. The BU exerts greater effect on the stability of HEWL in the acidic solution, the T_m is lowered by around 18 °C. Moreover, the T_m for lysozyme itself is lower at pH = 2 (52.8 °C; Table 2) than in pure water (74.7 °C) [20].

At the high concentration of BU (400 and 500 mM) the denaturation is two-step process. The second step involves smaller fluorescence changes which means that the structure alternations around fluorophores are smaller than in the first step.

Tetramethylurea also decreases the T_m but this effect is weaker than for BU and the protein unfolding is one-step process in the whole concentration range of this additive. For both of the urea derivatives, the T_m depends linearly on the concentration of the additives. Consequently, the slope of this relations is less steeper for the TMU than BU. The temperature of the second step of denaturation recorded for the BU also decreases as the concentration of the additive increases (Table 2).

As can be seen, low concentrations of BU and TMU have weak impact on the lysozyme unfolding. For 100 mM BU solution the T_m decrease is around 3 °C and for 100 mM TMU solution T_m decreases by around 1.5 °C. Assuming the linear dependency of the denaturation temperature on urea derivative concentration, these decreases for 50 mM solutions would be equal 1.5 °C and 0.8 °C for BU and TMU, respectively. This fact explains poor influence of the low concentration of the additives on protein fibrillation and aggregation and is in agreement with the other data obtained in this work.

For the highest concentration of additives (500 mM) the impact of the urea derivatives on the protein stability is very large. Butylurea affects the T_m to greater extent than TMU, this explains the stronger influence of BU on lysozyme amyloidogenesis. The fact that increased efficiency of the protein fibrillation correlates with the presence of the more effective destabilizer is not unexpected. It has been suggested before that partial unfolding of the protein is the first step of amyloidogenesis [3]. Commonly, the partial destabilization of the protein is achieved by performing the fibrillation at temperature close to denaturing conditions. Our work proves that the addition of the protein destabilizers can fulfill a similar role. The circular dichroism measurements for the samples containing 250 mM of BU showed that this compound destabilizes the lysozyme even in the unheated samples. Consequently, the denaturation temperature of HEWL is lowered. This destabilization makes the structure of the protein more labile. The hydrophobic residues of the amino acids and the backbone groups which in native protein are buried inside the folded molecule becomes exposed [38]. The polypeptide chain in this state is prone to rearrangement and the amyloids can be formed. By catalyzing the first step of amyloidogenesis i.e. the partial unfolding, the protein fibrillation could be accelerated. However, it is necessary to maintain the balance. The high concentration of the protein fibrillation accelerating agents can lead to the amorphous aggregation [23].

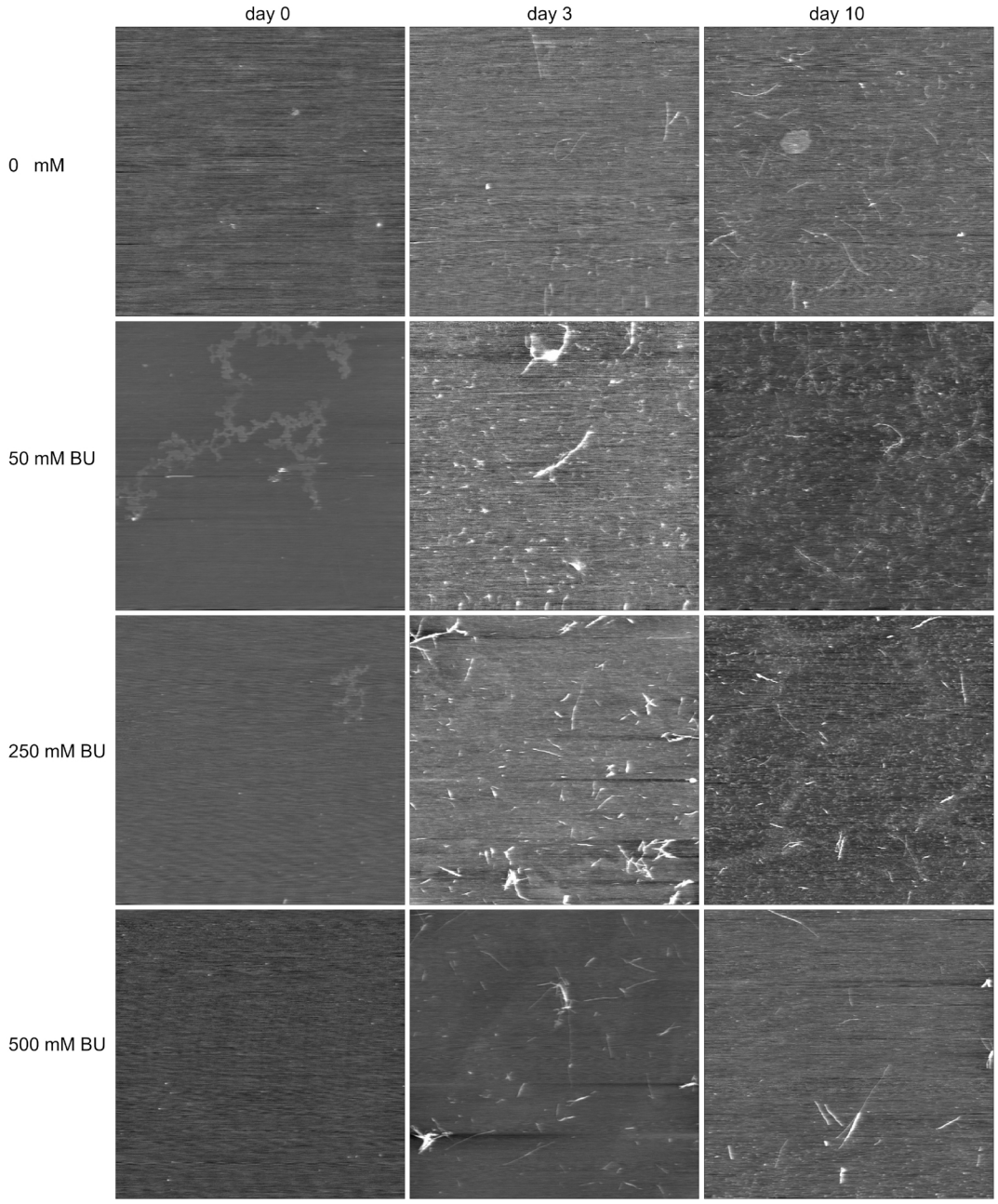

Fig. 5. Atomic force microscopy height images for the samples containing butylurea (0 mM; 50 mM; 250 mM and 500 mM) incubated at 45 °C for 0; 3 and 10 days. Size of the scans 9 × 9 μm.

As explained above, there is certain correlation between lower protein stability, lower denaturation temperature and higher tendency of the HEWL towards fibrillation. However, the extent to which BU enhances amyloidogenesis is somewhat unexpected. For the highest tested concentration of BU the destabilizing effect is tremendous (T_m is decreased by around 18 °C) but it translates into rather moderate increase of the protein fibril formation. One might expect that the impact of BU on amyloidogenesis would be greater. Understanding the causes of such action requires further studies.

To obtain the information whether the increased fibrillation is caused by lower stability of the protein or by specific interaction it is worth to compare the results for high concentration of urea derivatives. It appears that, the influence of the 250 mM solution of BU on T_m is comparable with the impact of 500 mM of TMU. Recalculation of data presented in Table 2, based on linear interpolation, shows decrease of T_m for 250 mM BU by 9 °C and for 500 mM TMU by 8 °C but the measured ThT fluorescence is much higher for the sample containing 250 mM BU than for 500 mM of TMU. It means that the specific interactions or the properties of the solvation sphere formed around BU may play substantial role in the promotion of the fibrillation. The simple destabilizing actions of the urea derivatives are not solely responsible for enhanced amyloidogenesis. Two investigated compounds have comparable impact on the denaturation temperature but have different influence on the amyloid fibril formation. This finding may be potentially useful in protein engineering and selective control of the various processes involving nano-materials production.

The obtained results could be applied for the scaffold manufacturing produced from HEWL. The addition of BU would increase the efficiency of the amyloidogenesis and the process can be conducted at relatively low temperature. It seems that in the presented way it is possible to alternate the length of fibrils. This feature can be useful for the modification of the properties of the nano-composites. The question remains

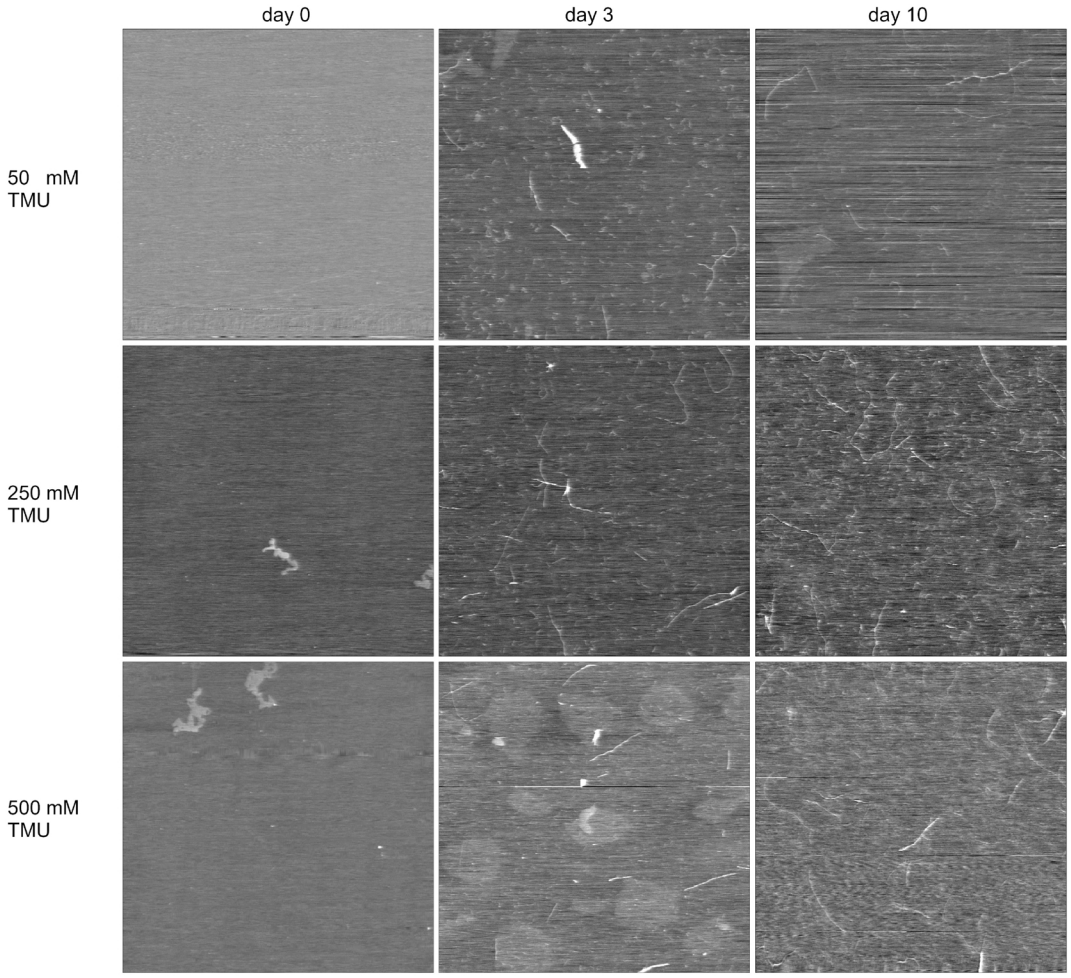

Fig. 6. Atomic force microscopy height images for the samples containing *N,N,N′,N′* -tetramethylurea (50 mM; 250 mM and 500 mM) incubated at 45 °C for 0; 3 and 10 days. Size of the scans 9 × 9 μm.

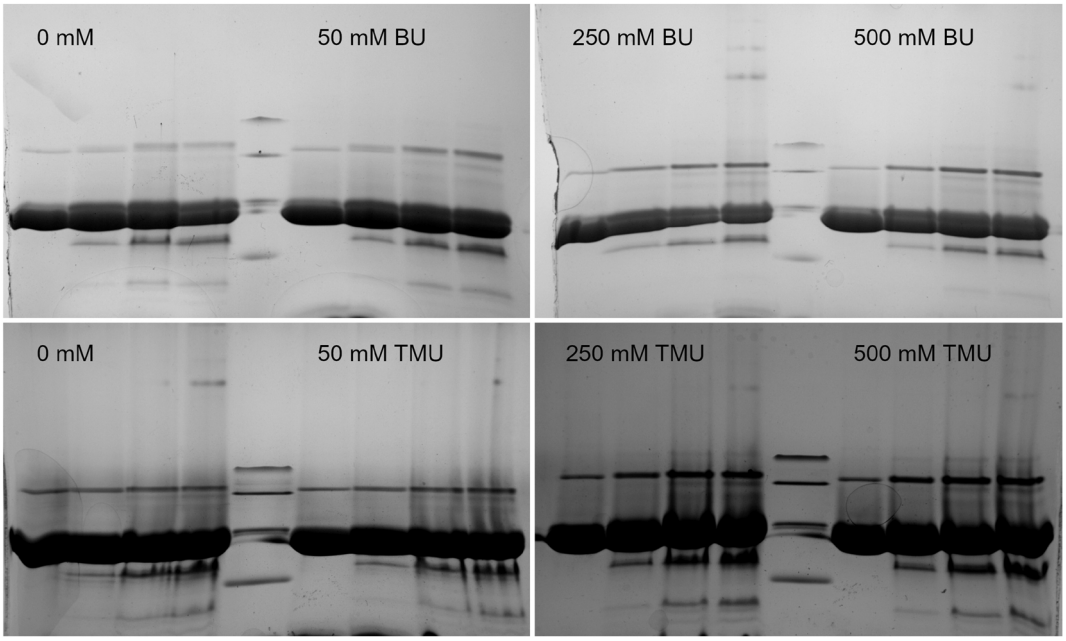

Fig. 7. The SDS-PAGE gels for HEWL incubated in the presence of butylurea (BU) and *N,N,N′,N′*-tetramethylurea (TMU) at 45 °C. The lines in each section correspond to the 0, 7, 3 and 10 days of incubation (from the left). Marker size 40, 25 15 and 10 kDa (middle line).

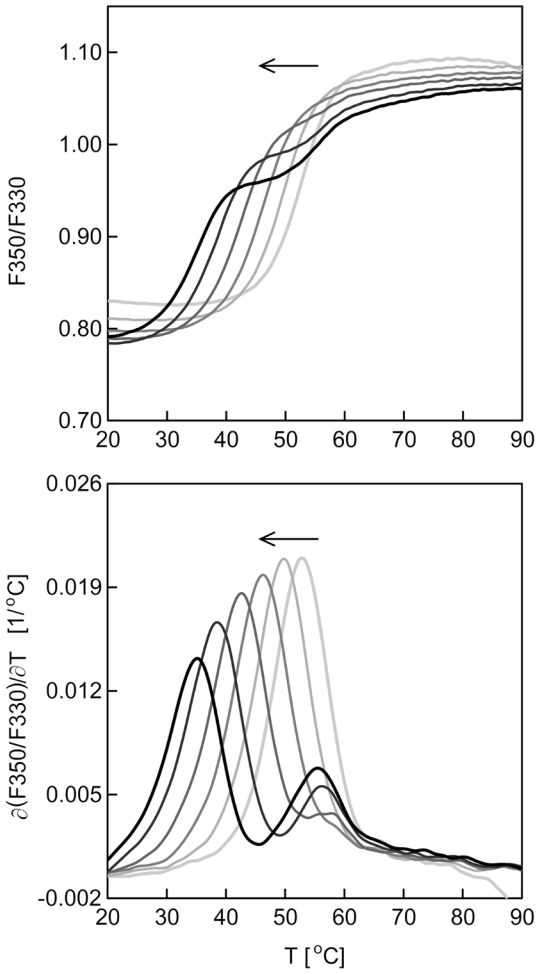

Fig. 8. The ratio of the intensity of fluorescence measured at 350 nm and 330 nm (F350/F330) and the temperature derivative of (F350/F330) recorded for HEWL solutions in the presence of butylurea (BU). The arrows indicate the increase of the concentration of BU from 0 mM to 500 mM with the interval of 100 mM.

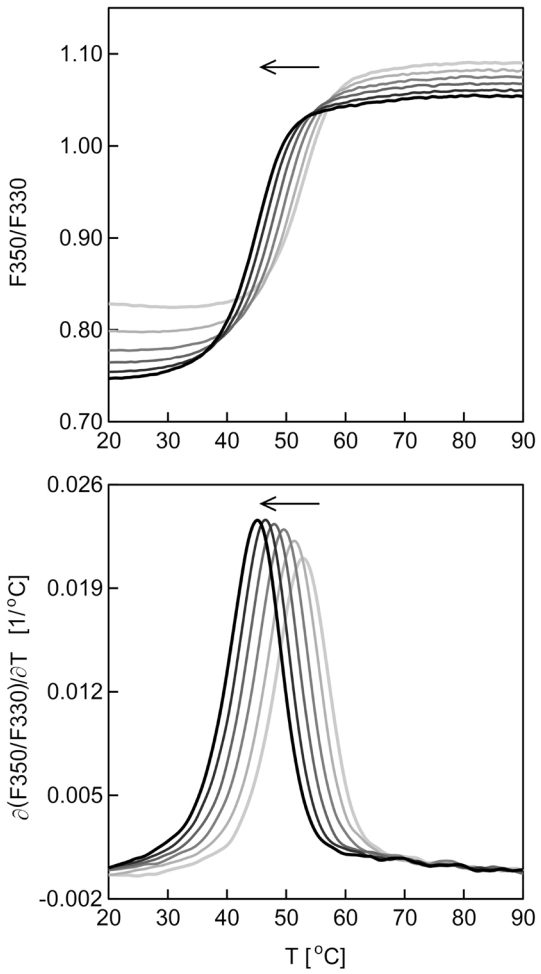

Fig. 9. The ratio of the intensity of fluorescence measured at 350 nm and 330 nm (F350/F330) and the temperature derivative of (F350/F330) recorded for HEWL solutions in the presence of *N,N,N′,N′*-tetramethylurea (TMU). The arrows indicate the increase of the concentration of TMU from 0 mM to 500 mM with the interval of 100 mM.

open whether the other hydrophobically hydrated compounds shown similar behaviour to BU. It is also unknown if the results could be applied to different proteins. More studies are needed to unravel the general patterns.

4. Conclusions

The results of the present work are summarized in Fig. 10. It shows the comparison of the protein fibrillation in solution without additives and in the presence of high concentration of butylurea and tetramethylurea.

The novelty of this study lies in the use of the differently hydrated low molecular weight substances to modulate the amyloidogenesis. Our previous works proved that the hydration properties of butylurea and tetramethylurea are distinctively different [12,18,19]. Butylurea is hydrophobically hydrated compound and strengthens the structure of water. The hydration sphere formed around BU is voluminous but weakly compressible. Tetramethylurea has a structure breaking properties. Hydration sphere for TMU is voluminous with weak water-water and water-solute interactions which leads to the gain in compressibility of solvent. It is also plausible that void around the solute is largely responsible for the properties of the system containing TMU. Our study shows that the above mentioned properties of BU and TMU, most likely along with the specific interactions, significantly affect the HEWL amyloidogenesis.

Table 2
The denaturation temperature of HEWL in the presence of butylurea or *N,N,N′,N′*-tetramethylurea and without additives obtained from nanoDSF measurements.

Additive concentration [mM]	T_m 1 [°C]	T_m 2 [°C]
Butylurea		
0	52.8	
100	49.7	
200	46.2	
300	42.6	
400	38.5	56.3
500	35.1	55.5
N,N,N′,N′-tetramethylurea		
100	51.3	
200	49.6	
300	47.9	
400	46.4	
500	45.1	

One of the main new finding resulting from this study is that the hydrophobically hydrated butylurea exerts greater impact on HEWL and its fibrillation than the tetramethylurea. Both of the studied compounds reduce the induction time of amyloidogenesis (lag-time). In the case of TMU this phenomenon is, however, not accompanied by the

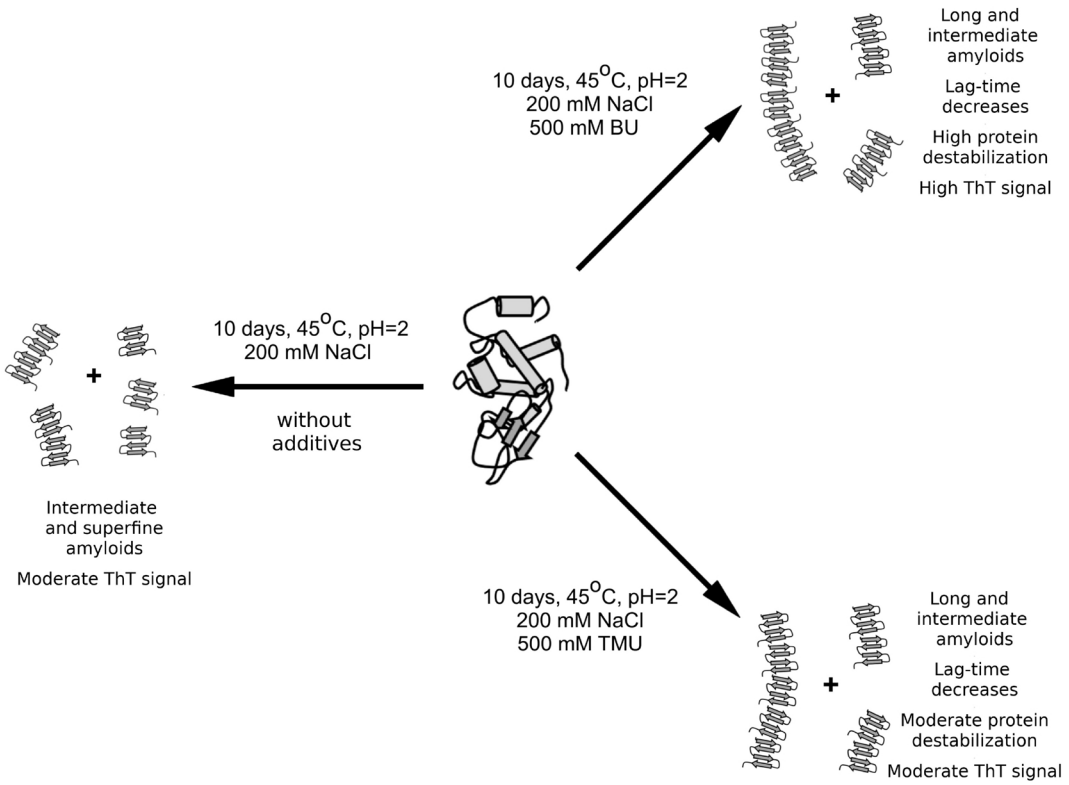

Fig. 10. The influence of butylurea and tetramethylurea on hen egg white fibrillation.

increase of the total amount of the amyloids formed after 10 days of incubation. Previous reports [27–29] show that the shorter lag-time does not necessarily lead to increased efficiency of the fibrillation. The results obtained for BU are consistent with intuition, the shorter lag-time is followed by the increased ThT fluorescence (in comparison to the control sample) at 10th day of incubation.

The atomic force microscopy reveals that morphological differences between amyloids in the samples are not large. It seams, however, that for the highest concentration of urea derivatives (500 mM) the formed fibrils are longer and the superfine fibrils are absent.

Unexpectedly, BU and TMU significantly decrease the HEWL stability, in comparison to the lysozyme solution without additives, but the fibrillation is rather weakly affected. This fact questions the importance of the partial unfolding as a key event in the course of amyloidogenesis.

Probably the most important observation coming from our work concerns the mechanism of the amyloid fibril formation in the conditions of the experiments. The activity of compounds cannot be solely explained by protein destabilization effect. NanoDSF measurements show that both TMU and BU decrease thermal stability of the lysozyme. This effect is larger for BU, what is in agreement with the reported higher impact of butylurea on the investigated process. However, for the systems with equivalent reduction of the HEWL stability (250 mM BU and 500 mM TMU) the not-equivalent increase of the protein fibrillation was recorded (higher for BU). It means that the specific properties of the solution containing BU play important role in acceleration of the hen egg white lysozyme fibrillation.

The infrared spectroscopy studies [21] indicate that BU does not interact directly with the lysozyme (at least as the carbonyl residue of the BU is concerned). In the opposite to butylurea, it was proved that tetramethylurea interacts directly with HEWL but, as the current study shows, the impact of TMU on the HEWL amyloidogenesis is weaker than BU. The observed differences could result from the different hydration properties of the investigated compounds and the influence of the structure of water on amyloidogenesis or the presence of the water mediated interactions. According the alternative hypothesis the alkyl chain of BU interacts with the hydrophobic protein interior [21] and in this way promotes HEWL fibrillation.

The current study may be useful for nano-materials manufacturing [14] or generation of the amyloids for other purposes [15]. It shows how the differently solvated protein destabilizers influence the amyloidogenesis. The butylurea significantly increases fibrillation of hen egg white lysozyme and the process can be carried out at temperature as low as 45 °C.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bpc.2019.106265.

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