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Research paper

Integrity and stability studies of precipitated rhBMP-2 microparticles with a focus on ATR-FTIR measurements

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Abstract

A major obstacle in the development of protein drug formulations is the need to maintain the native, active protein structure both during the formulation process and upon long time storage. Controlled precipitation was evaluated for its potential to supply stable microparticulate formulations of bone-regenerating recombinant human Bone Morphogenetic Protein-2 (rhBMP-2). Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR) did provide insight into the protein formulation and stability. Temperature dependent ATR-FTIR measurements and DSC measurements allow for the study of changes in the protein structure during melting. To address the question of isomerization, peptide mapping was performed, and protein aggregation was monitored by size exclusion chromatography (SEC). It could be demonstrated by ATR-FTIR that controlled precipitation did not harm the protein and the process is fully reversible. DSC measurements further confirmed these findings. No changes in the transition temperature and process were observed after precipitation and redissolution. Upon storage, isomerization and aggregation could be detected, but to a lower extent in the precipitated formulation as compared to a solution reference. Thus, controlled precipitation of rhBMP-2 is fully reversible and has the potential as alternative formulation tool for the generation of a microparticulate drug delivery system.

Keywords: Recombinant human Bone Morphogenetic Protein-2; Precipitation; ATR-FTIR; Protein stability; Protein integrity; Microparticles

1. Introduction

Whereas precipitation is generally considered as an undesirable degradation pathway in protein pharmaceuticals [1], it is a well-known classical tool for protein purification and separation. For example, reversible precipitation is utilized for the fractionation of plasma proteins [2] and in traditional salting-out procedures [3,4]. Additional proof for the usefulness of precipitation can be found by Stratton and co-workers. They demonstrate for lactate dehydrogenase and α-chymotrypsin precipitated in a poloxamer gel that the native structure is maintained [5]. Native-like secondary structure was also found by Kendrick et al. [6] for rhIFN-γ precipitated with PEG 8000. The authors outlined the difference between a destructive aggregation mainly caused by destabilizers directly influencing the conformation of the protein and salt induced precipitation based on the reduced solubility of the macromolecule due to the altered solvent properties.

Recently, the formation of microparticles by controlled precipitation of rhBMP-2 was demonstrated. Microparticles were generated by combining an acidic protein solution with a salt stock solution, leading to an artificial physiological environment after mixing [7]. Under these conditions rhBMP-2 shows reduced solubility and therefore precipitates [8]. The formed microparticle suspension features a bimodal particle size distribution with maxima at approx. 7 and 35 μm [9]. RhBMP-2 has proven to be effective in the field of bone growth and regeneration. For an

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optimized therapeutic response, prolonged presence at the site of healing is suggested [10,11]. Precipitated microparticles could fulfil this demand by sustained redissolution, making rhBMP-2 an ideal candidate for such a formulation. The objective of this study was to evaluate precipitation as an alternative formulation strategy, focusing on the proteins structural integrity as the fundamental prerequisite for the development of a successful delivery system.

In a first step, the secondary structure of native liquid, precipitated and resolubilized rhBMP-2 was examined by ATR-FTIR. Temperature dependent measurements were performed to determine any possible changes in the secondary structure that might indicate instabilities, and the data were correlated with results obtained by DSC measurements.

In a second step, both physical and chemical stability was tested after 6 month storage and the ability of ATR-FTIR to reveal structural changes in stored rhBMP-2 formulations was evaluated.

2. Materials and methods

2.1. Materials

RhBMP-2 in formulation buffer (0.5% sucrose, 2.5% glycine, 5.0 mM glutamic acid, 5.0 mM sodium chloride, and 0.01% polysorbate 80; pH 4.5) was obtained from Wyeth BioPharma, Andover, MA, USA. For buffer exchange, the protein was dialysed against 1 mM HCl using a Spectra/Por®7 membrane (MWCO: 3 kDa; Spectrum Europe B. V., Breda, Netherlands).

For storage stability, two different formulations were prepared and stored at 5 and 25 °C for 6 months: (1) Liquid rhBMP-2 in formulation buffer: 1 mg protein in 400 μl formulation buffer, stored in stoppered 2 ml vials. (2) Suspended rhBMP-2 microparticles: equal volumes of precipitation buffer [100 mM phosphate; pH 7.4; $I = 308$ mM (KCl)] and rhBMP-2 solution (in 1 mM HCl; $c = 2.0$ mg/ml) were mixed via vortex and equilibrated for 1 h at room temperature. The microparticles were concentrated by centrifugation and removal of the supernatant, leading to a final volume of 100 μl with a total protein content of 1 mg. For the analysis of isomerization and aggregation, the microparticle suspensions were redissolved by adding 900 μl formulation buffer to 100 μl suspension, leading to a concentration of 0.4 mg/ml.

2.2. Methods

2.2.1. Controlled precipitation

Microparticles were generated as described elsewhere [7]. Briefly, a defined volume (0.5–10 ml) of precipitation buffer (100 mM phosphate; pH 7.4; $I = 308$ mM (KCl)) is quickly added manually with a pipette into a tube containing an equal volume of an already turbulent rhBMP-2 solution (in 1 mM HCl; $c = 2.0$ mg/ml). The turbulent field is created by vortexing at half-maximum speed (Vortex Genie®, Scientific Industries, Bohemia, NY, USA), and mixing is continued for 10 s after combination. After equilibration at room temperature for 1 h, microparticle suspensions featuring bimodal particle size distributions with maxima at approx. 7 and 35 μm were obtained, and no significant difference in the particle size distribution could be observed over time after equilibration and after storage for 6 months at 5 and 25 °C. The residual protein solubility in the supernatant of the microparticle suspension was 0.04 mg/ml.

2.2.2. FTIR spectroscopy

Measurements were conducted with the Confocheck™, a dedicated protein FTIR system (Bruker Optik GmbH, Ettlingen, Germany), at constant temperatures, an acquisition time of 30 s and a resolution of 4 cm^{-1}. All depicted spectra were prepared by vector normalization between and 1740 and 1600 cm^{-1} and second derivative calculation and generated in the attenuated total reflection mode with the BioATRII™ cell (with silicon crystal, 10 μl sample volume). Approx. 10 μl of either the liquid sample or the microparticulate suspension was directly pipetted onto the crystal of the cell. The corresponding blank reference was measured in the same manner for baseline correction. Single measurements were performed at a fixed temperature of 25 °C, and temperature dependent spectra were acquired from 25 to 94 °C in steps of 3 °C at an effective heating rate of approx. 0.7 °C/min. Secondary structure elements were quantified by comparison of the native transmission protein spectra acquired at a constant temperature of 25 °C with a protein transmission spectra library, using a partial least squares algorithm. For these measurements the Confocheck™ was equipped with the AquaSpec™ flowthrough transmission cell (6 μm pathlength, 1 μl sample volume). Data were analyzed with the OPUS™ software. Origin®7.0 software was used to obtain the point of inflection (applying a sigmoid fit according to the Boltzmann model and subsequent differentiation of the fitted curve). The concentrations of the samples vary within 10–20 mg/ml. Protein-free reference material was obtained by centrifugal filter units equipped with a microporous membrane (Ultrafree® Biomax 0.5 centrifugal filter and tube, MWCO: 10K; Millipore Corporation, Billerica, MA, USA).

2.2.2.1. Precipitated rhBMP-2. Precipitation was performed as described above. Eight hundred microliters of the microparticle suspension was 20-fold concentrated by centrifugation (5G for 5 min at room temperature) and removal of the supernatant, resulting in a protein concentration of approx. 20 mg/ml. The supernatant was cleared off residual soluble protein with centrifugal filters.

2.2.2.2. Dialysed rhBMP-2. rhBMP-2 was dialysed into 1 mM HCl. A volume of 500 μl containing 0.4 mg protein was concentrated by ultracentrifugation to approx. 20 μl

with a concentration of approx. 20 mg/ml. The resulting filtrate (480 μl) serves as background.

2.2.2.3. Resolubilized material. Thirty micrograms of the 20-fold concentrated microparticle suspension was resolubilized with 470 μl formulation buffer. Concentration with centrifugal filters delivers approx. 30 μl protein solution with a theoretic concentration of 20 mg/ml and approx. 470 μl protein-free solution for the background measurement.

The stored precipitated samples were redissolved with formulation buffer to a concentration of 0.4 mg/ml. One hundred microliters was further diluted with formulation buffer to a volume of 500 μl and concentrated with centrifugal filter devices to a volume of 10–20 μl, resulting in a calculated concentration of 5–10 mg/ml. The native liquid reference formulation and the liquid storage stability samples were prepared in analogy, with the filtrates being used as baseline references.

2.2.3. DSC

Measurements were conducted with the Nano II Differential Scanning Calorimeter 6100 (Calorimetry Sciences Corp; Lindon, UT, USA). Data acquisition was performed from 25 to 110 °C with a heating/cooling rate of 1 °C/min ($n = 2$). Analysis was performed utilizing the $Cp_{convert}$ program of the instruments analysis software, assuming a two state process for the transition. Samples and background solutions were prepared either by dialysis or (ultra)centrifugation. The concentrations were adjusted to 2.0 mg/ml by dilution with the appropriate buffer. The buffer exchange medium of the last dialysis step serves as background. The preparation of dialysed and precipitated samples was performed in the same manner as the samples for ATR-FTIR. Resolubilized samples: protein pellets of precipitated rhBMP-2 were redissolved either with formulation buffer or 10 mM HCl, followed by dialysis (formulation buffer or 1 mM HCl, respectively) and concentration adjustment to 2 mg/ml.

2.2.4. Peptide mapping

Samples were desalted on a 5 ml HiTrap™ desalting column (Amersham Biosciences Europe GmbH, Freiburg, Germany). Samples containing 50 μg protein were digested with 0.5 μg Endoproteinase Asp-N (Roche Diagnostics, Mannheim, Germany) for 1.5 h at 37 °C. Separation was achieved with a TSK Super-ODS 4.6 mm ID × 10.0 cm (Toso Haas, Japan) on an Agilent HP1100 HPLC system (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) with UV detection at 214 nm.

2.2.5. Size exclusion chromatography

SEC was performed using a Merck-Hitachi L-6200/F 1050 HPLC system with a TSK-Gel®2000WXL column (7.8 mm × 30.0 cm, 5 μm particle size; Toso Haas, Japan) and a arginine–HCl/glutamic acid buffer, pH 4.5, as mobile phase.

3. Results and discussion

3.1. Process stability of precipitation measured by ATR-FTIR

The amide I band is most commonly used to study protein structures since its band shape is very sensitive to the protein's secondary structure. The advantage of second derivative calculation as compared to Fourier self-deconvolution as possible band-narrowing technique lies in the fact that there is no need to choose subjective parameters, and therefore is completely objective [12]. Every band or shoulder in the original spectra results in a negative band in the second derivative.

Fig. 1 shows the normalized second derivative spectra of different rhBMP-2 formulations, all concentrated to approx. 20 mg/ml for the measurement to increase the signal intensity. The native, the precipitated, as well as the resolubilized protein spectra are essentially the same. All show the strong band at 1631 cm^{-1}, which can be assigned to β-sheet structure, and the weaker one at 1680 cm^{-1}, which is associated in the literature with β-turns [13]. Furthermore, a band around 1658 cm^{-1} which is attributed to α-helical structure elements, and a band at 1646 cm^{-1} which reveals unordered structures can be identified [14,15]. Thus, no structural changes are induced by the precipitation process, and the native secondary structure in the precipitated rhBMP-2 microparticles is maintained. In addition, the native structure is preserved after resolubilization, demonstrating that precipitation is fully reversible.

Secondary structure element determination based on measurements performed in the transition mode resulted in 9.5% α-helix and 25% β-sheet. Whereas the value for

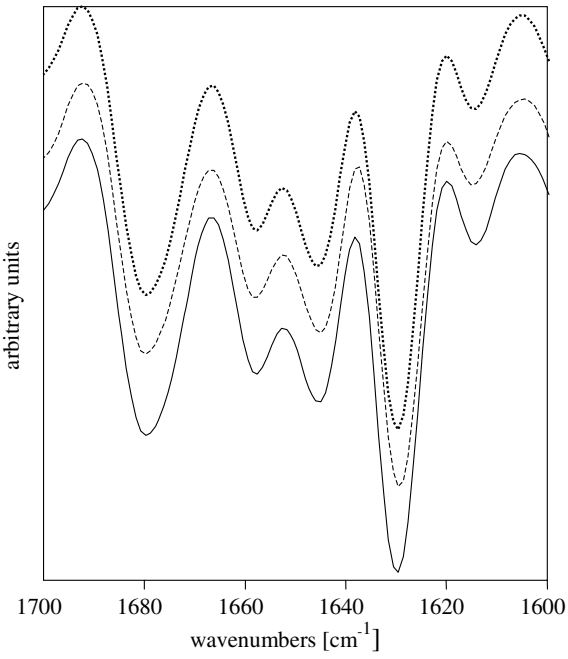

Fig. 1. Area normalized, second derivative spectra of dialysed (···), precipitated (--) and resolubilized (—) rhBMP-2.

the α-helix is in good correlation to data from X-ray crystallography (9.5% vs. 8% α-helix), the calculation for the β-sheet content varies considerably (25% vs. 42% β-sheet structure; [16]). This can be explained by structural differences between the solved and the crystallized form of rhBMP-2 and the fundamental physicochemical difference between the applied techniques.

3.2. Temperature ramp of precipitated rhBMP-2 microparticles

Fig. 2 shows the temperature induced changes in the secondary structure of precipitated microparticles. Up to 70 °C, only continuous deviations in the intensities of the main bands compared to the previous temperature step appear, but significant changes in the curve patterns are absent. At 73 °C, peak shifts that give evidence to a significant change in secondary structure become obvious. It is accompanied by clear losses of α-helical (1658 cm^{-1}) and β-turn structures (1680 cm^{-1}) and the formation of a novel band at 1690 cm^{-1}, which can be assigned to intermolecular β-sheet structures [17]. At higher temperatures the band intensity at 1690 cm^{-1} further increases and above 91 °C the formation of an additional band at approx. 1622 cm^{-1} is induced.

3.3. Temperature ramp of resolubilized rhBMP-2 microparticles

Fig. 3 illustrates the temperature dependent refolding and unfolding of resolubilized rhBMP-2 microparticles.

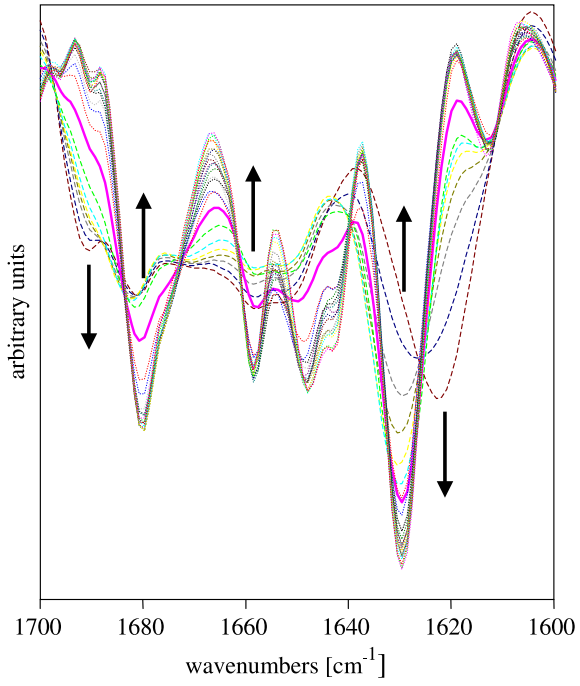

Fig. 2. Second derivative, area normalized amide I spectra of temperature induced re/unfolding of precipitated rhBMP-2 microparticles; the arrows indicate the direction of intensity shift at wavenumbers of interest with rising temperatures from 25 to 70 °C (···), 73 °C (—), 76 to 94 °C (--).

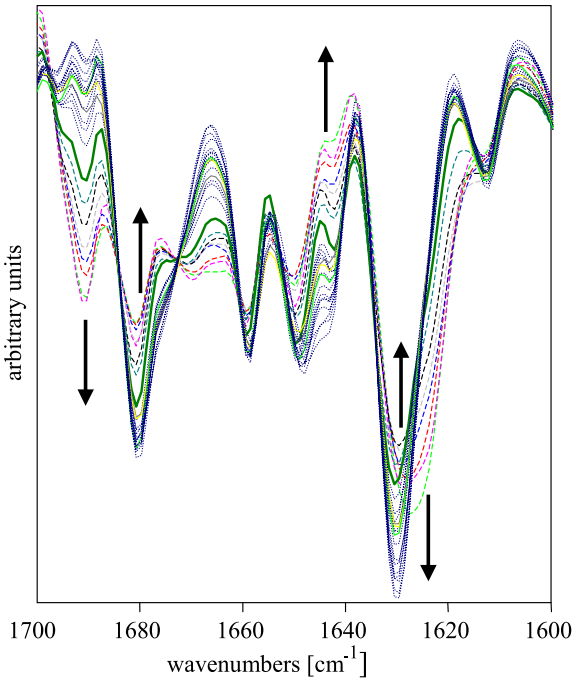

Fig. 3. Second derivative, area normalized amide I spectra of temperature dependent re/unfolding of resolubilized rhBMP-2; the arrows indicate the direction of the intensity shift at wavenumbers of interest with rising temperatures from 25 to 70 °C (···), 73 °C (—), 76 to 94 °C (--).

Essentially the same curve pattern is obtained for the temperature ramp of native liquid rhBMP-2 (data not shown). In contrast to the precipitated material, the band contour between 1640 and 1680 cm^{-1}, which includes α-helical structures at 1658 cm^{-1}, is mainly preserved as the temperature is increased. At higher temperatures, additional bands at 1690 and 1627 cm^{-1} also develop.

The gradual intensity shift at 1642 cm^{-1} seen in both Figs. 2 and 3 indicates a loss of β-sheet structure which is not completely resolved by band narrowing from the underlying unordered structures at 1645 cm^{-1}. Both samples show the formation of additional peaks at 1690 and 1622 cm^{-1} (for the precipitated sample) and 1627 cm^{-1} (for the resolubilized sample), respectively. According to the literature, it is a common feature of thermally unfolded proteins to exhibit a low-frequency indicator band around 1620 cm^{-1} and a weaker high-frequency band around 1685 cm^{-1}, assigned to intermolecular antiparallel β-sheet structures [18]. As these wavenumbers were determined in D_2O, slightly higher values will result in H_2O [12]. Therefore, the change in band structure fits into the scheme of thermal unfolding. Interestingly, the observed structural losses were not accompanied with an increase in the peak at 1645 cm^{-1} representing unordered structures. The degree of β-sheet structure loss (1630 cm^{-1}) in favour of antiparallel intermolecular β-sheet formation is more pronounced in the precipitated microparticle formulation, and α-helical structures are partially conserved in the resolubilized sample. The gradual overall shift of the intensities can be explained by the conformational motility of proteins

and the assumption that not all protein molecules react to the altered conditions at the same time. Consequently, a loss of intensity at a certain wavenumber is caused by a structural loss of a certain population of protein molecules, whereas other molecules still exhibit the native conformation. With increasing temperatures, the balance between native and denatured protein molecules is further pushed into the direction of the denatured fraction.

3.4. Unfolding temperatures of ATR-FTIR measurements

Chechin et al. [19] use the intensity ratio decreasing to increasing band to determine the transition temperature T_m from infrared spectroscopic measurements. In our studies, all bands with decreasing intensities show also slight shifts in their maxima at higher temperatures, suggesting that they are not solely due to structural losses. To estimate T_m from the temperature induced unfolding spectra, the intensity of the new arising band at 1690 cm^{-1} is plotted against temperature (Fig. 4). Sigmoid curve fitting and differentiation deliver the point of inflection. The corresponding temperature at the inflection point is considered to be the transition temperature T_m. For the precipitated sample, T_m was determined to be 75 °C as compared to a slightly higher, but not significantly different transition temperature of 77.6 °C for the resolubilized sample.

3.5. Differential scanning calorimetry

To confirm the findings of temperature induced unfolding measured with ATR-FTIR, DSC measurements were performed. It is well known from the literature that several factors such as formulation composition, pH, concentration and measuring conditions can affect T_m of a given protein [20]. At first, the influence of formulation, pH, and composition was investigated. It could be shown that samples containing between 1 and 5 mg/ml native rhBMP-2 in formulation buffer, T_m is 64.3 °C without statistically significant differences. Subsequently, all measurements were performed at 2 mg/ml to avoid any possible influence on T_m due to concentration effects. As the controlled precipitation of rhBMP-2 to form microparticles is partly caused by an increase of pH to physiological values, the influence of pH on the transition temperature of rhBMP-2 is of special interest. While increasing the pH from 3.0 to 5.5, the transition temperature rises from 48.1 to 70.2 °C (Table 1). In parallel to the T_m shift, the calorimetric enthalpy increases. The observed T_m shift can be explained by repulsive forces present in the molecule. RhBMP-2 is a basic protein with an isoelectric point of 8.5. At low pH values, the carboxylic acid side chains are preferentially uncharged, whereas the dominating fraction of basic groups carries positive charges. This is energetically unfavourable due to repulsive forces. With increasing pH values, the net positive charge diminishes and so do the repulsive forces, leading to an increase in T_m. The excipients also affect the transition temperature. For example, two different formulations of the same pH vary significantly in their unfolding behaviour. Whereas rhBMP-2 in 1 mM HCl shows a T_m of 54.8 °C, the T_m is more than 6 °C lower in formulation buffer (both at pH 3.0). Thus, comparison and interpretation of T_m determined by DSC must take the pH and excipient conditions of the protein into consideration.

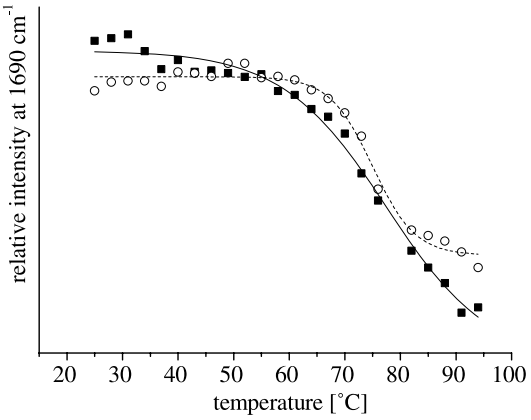

Fig. 4. Transition temperatures of precipitated (○) and resolubilized (■) rhBMP-2 based on the intensity shift at 1690 cm^{-1} (sigmoid fit: $R^2 = 0.990$ (—), $R^2 = 0.988$ (···)).

Comparison of rhBMP-2 before precipitation in aqueous HCl (pH 3.0) and after precipitation, redissolution and dialysis into the same formulation shows the same transition temperatures (54.8 vs. 54.6 °C, Fig. 5a). In addition, rhBMP-2 precipitated, redissolved and dialysed against formulation buffer results in the same T_m as the starting material in the same buffer (64.3 vs. 63.9 °C, Fig. 5b). These data support the finding that the precipitation process itself does not induce any permanent changes and is reversible. Further increase of pH, phosphate and KCl concentration leads to rhBMP-2 precipitation and a further increase in the transition temperature to 70 °C. Thus, the precipitated state of the protein goes hand in hand to improved thermal stability. In the precipitate, the associated protein molecules interact in a stabilising manner with each other. To unfold their native structure, higher temperatures are necessary. A closer look at the calorimetric enthalpy further supports the hypothesis that precipitation is reversible. Within the accuracy of the method, the calorimetric enthalpies of the 1 mM HCl samples are nearly identical (107.2 ± 7.0 vs. 103.4 ± 0.7 kcal/mol). For the rhBMP-2 samples analyzed in formulation buffer, the difference is more pronounced (107.0 ± 1.6 vs. 86.7 ± 1.3 kcal/mol). In general, calorimetric enthalpies below 100 kcal/mol indicate weak transitions, and results based on integration of such transitions were accompanied by higher errors. Therefore, interpretations should be done with care [21]. The precipitated formulation exhibits the highest value in calorimetric enthalpy of all samples tested, arguing for its enhanced stability.

Table 1
Transition temperatures and calorimetric enthalpies of different rhBMP-2 formulations

Sample description	pH	T_m (°C)	Calorimetric enthalpy (kcal/mol)
rhBMP-2 in 1 mM HCl	3.0	54.8 ± 0.05	107.2 ± 7.0
Resolubilized microparticles in 1 mM HCl	3.0	54.6	103.4 ± 0.7
rhBMP-2 in formulation buffer	4.5	64.3	107.0 ± 1.6
Resolubilized microparticles in formulation buffer	4.5	63.9 ± 0.05	86.7 ± 1.3
Precipitated microparticles in physiologic phosphate buffer	7.4	70.0 ± 0.05	165.1 ± 4.4
rhBMP-2 in formulation buffer	3.0	48.5 ± 0.05	52.5 ± 1.9
rhBMP-2 in formulation buffer	5.5	70.2	126.0 ± 4.5

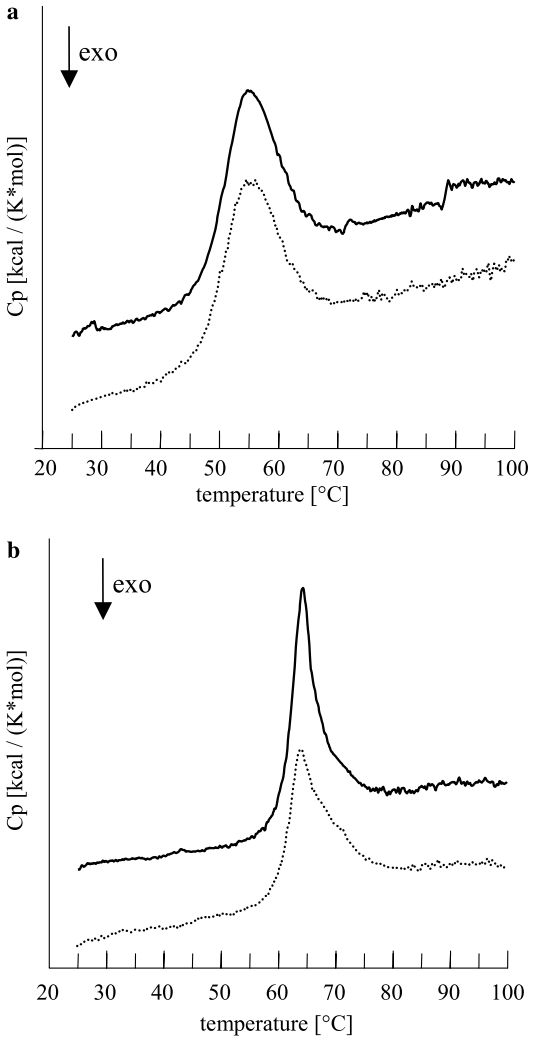

Fig. 5. DSC curves of rhBMP-2 before precipitation (—) and after precipitation and resolubilization (···) in 1 mM HCl (a) in formulation buffer pH 4.5 (b).

3.6. DSC vs. ATR-FTIR

The unfolding temperature of precipitated rhBMP-2 calculated from the intensity shift of the ATR-FTIR temperature ramp is slightly higher than the corresponding value determined by DSC ($T_{m(spectroscopy)} = 75$ °C compared to $T_{m(calorimetry)} = 70$ °C). The greater difference in T_m for the resolubilized samples ($T_{m(spectroscopy)} = 77.6$ °C compared to $T_{m(calorimetry)} = 63.9$ °C) is caused by the method of sample preparation. For the ATR-FTIR samples, the resolubilized protein was not dialysed after redissolution so that residual excipients of the precipitation process (phosphate buffer salts and KCl) are present. The consequently slightly higher pH and the presence of phosphate explain the higher $T_{m(spectroscopy)}$. Minor differences between T_m acquired with ATR-FTIR and DSC are expected, as the two techniques show fundamental differences in their way of sample heating and data analysis. In the spectroscopic approach, the sample is equilibrated at the measuring temperature and kept constant during the measurement with an effective heating rate of approx. 0.7 °C/min, whereas the sample is heated continuously in the calorimeter. In addition, $T_{m(spectroscopy)}$ is calculated as the temperature where the rate of intermolecular β-sheet formation is maximal, whereas the temperature of maximal heat flux (at peak maximum in the thermogram) is considered to be $T_{m(calorimetry)}$.

3.7. Storage stability after 6 months

It can be shown by ATR-FTIR measurements that the precipitation process does not alter the secondary structure of the protein and that it is maintained when redissolution is performed within a few hours. To study whether aging occurs and the protein is harmed upon long time storage in the precipitated state, samples were stored for 6 months at 5 and 25 °C and analyzed by both ATR-FTIR and classic protein analysis including peptide mapping (to determine any chemical modification) and SEC (to unclose the physical instability of high molecular weight species formation). Fig. 6 shows the ATR-FTIR spectra of the stability samples. No major difference can be detected between the samples stored for 6 months and the control spectra of native rhBMP-2. With peptide mapping, it is possible to monitor isomerization of aspartic acid (Asp) within the rhBMP-2 molecule [2]. Significant formation of isoAsp is observed in the pure liquid formulation, whereas isomerization is greatly reduced in the precipitated rhBMP-2 formulation (Fig. 7). As expected, isomerization is accelerated at higher temperatures. Furthermore, 6 month storage leads to higher aggregate contents in both formulations as detected by SEC (Fig. 8). However, the increase is less pronounced in the precipitated state as compared to the liquid formulation. These findings support the hypothesis

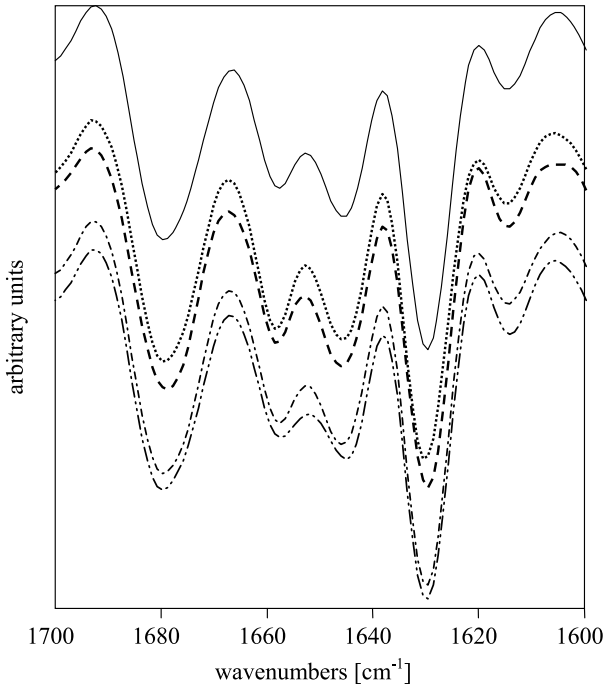

Fig. 6. Spectra of native, unstressed liquid rhBMP-2 (—), redissolved microparticles after storage as precipitated microparticle suspension at 5 °C (···) and 25 °C (- -), and liquid rhBMP-2 reference formulation stored at 5 °C (-·-·-) and 25 °C (—··—) for 6 months.

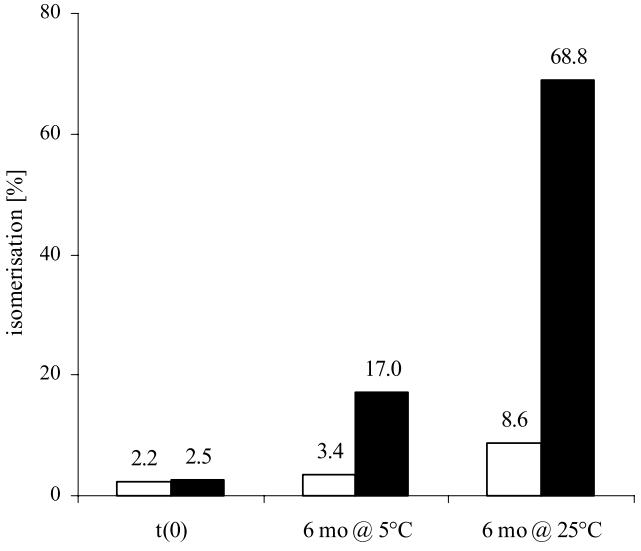

Fig. 7. Isomerization of rhBMP-2 formulations after 6 month storage, detected with peptide mapping: the white bars represent the microparticle suspension, the black bars the liquid formulation.

of a stabilising influence of precipitation. The inability of ATR-FTIR to disclose these changes suggests that both isomerization and aggregation of rhBMP-2 do not coincide with changes in the secondary structure. Another reason might be a lack of sensitivity of ATR-FTIR to unclose the storage induced modifications. The great advantage of FTIR, as compared to other techniques such as circular dichroism, Raman spectroscopy or chromatographic techniques, is its ability to provide structural information independently from the physical state of the sample, enabling insight into the precipitated state of a protein.

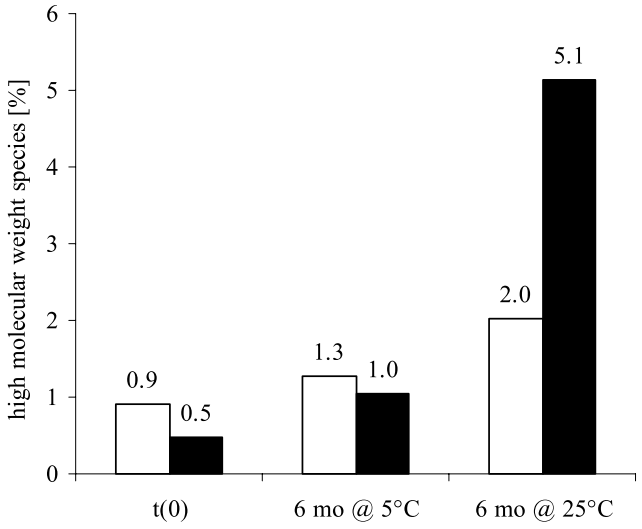

Fig. 8. Formation of high molecular weight aggregates of rhBMP-2 formulations after 6 month storage, analyzed with SEC: the white bars represent the microparticle suspension, the black bars the liquid formulation.

4. Conclusion

ATR-FTIR measurements allow structural insight into precipitated protein particles. The measurements demonstrate that the native structure of rhBMP-2 is maintained in the precipitated state. T_m determination via DSC further supports the finding that the precipitation process is reversible and does not harm the protein. In the precipitated state, rhBMP-2 exhibits a higher transition temperature, as well as higher values for the calorimetric enthalpy, indicating greater thermal stability towards unfolding. Results comparing the liquid formulation with the precipitated microparticles indicate that precipitation has a stabilising effect. Isomerization occurs to a much higher degree in the liquid formulation, and the potential to minimize aggregation at low storage temperatures is also higher in the precipitated state. The information obtained from T_m determination is consistent with the results from the stability study. ATR-FTIR failed to disclose isomerization and HMW aggregate formation as compared to standard analytics. Possibly, isomerization in the side chain did not induce structural changes that lead to detectable band shifts, or the changes were below the limit of detection.

The presented findings suggest that precipitation has the potential as alternative formulation strategy for rhBMP-2. The usefulness of ATR-FTIR to provide structural information of a protein in the precipitated state and its relevance for stability testing and formulation development is obvious.

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