Supercritical carbon dioxide spray drying for the production of stable dried protein formulations

Nuchuchua, O.

CHAPTER 4

Critical processing parameters of carbon dioxide spray drying for the production of dried protein formulations: a study with myoglobin

O. Nuchuchua[1], H.A. Every[2], W. Jiskoot[1]

[1] Division of Drug Delivery Technology, Leiden Academic Centre for Drug Research (LACDR), Leiden University, The Netherlands

[2] FeyeCon Development & Implementation B.V., Weesp,

The Netherlands

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Abstract

The aim of this study was to gain fundamental insight into protein destabilization induced by supercritical CO_2 spray drying processing parameters. Myoglobin was used as a model protein (5 mg/ml with 50 mg/ml trehalose in 10 mM phosphate buffer, pH 6.2). The solution was exposed to sub- and supercritical CO_2 conditions (65-130 bar and 25-50°C), and CO_2 spray drying under those conditions. The heme binding of myoglobin was determined by UV/Vis, fluorescence, and circular dichroism spectroscopy, while myoglobin aggregation was studied by using size-exclusion chromatography and flow imaging microscopy. It was found that pressure and temperature alone did not influence myoglobin's integrity. However, when pressurized CO_2 was introduced into myoglobin solutions at any condition, the pH of the myoglobin formulation shifted to about 5 (measured after depressurization), resulting in heme binding destabilization and aggregation of myoglobin. When exposed to CO_2, these degradation processes were enhanced by increasing temperature. Heme binding destabilization and myoglobin aggregation were also seen after CO_2 spray drying, and to a greater extent. Moreover, the CO_2 spray drying induced the partial loss of heme. In conclusion, pressurized CO_2 destabilizes the myoglobin, leading to heme loss and protein aggregation upon spray drying.

1. Introduction

In previous studies, supercritical carbon dioxide ($scCO_2$) spray drying has been shown to be applicable for drying formulations of several proteins, such as lysozyme, a-lactalbumin, a-chymotrypsinogen A, myoglobin, and monoclonal and polyclonal immunoglobulins. However, depending on the protein and the processing conditions, protein aggregation and denaturation were sometimes observed [1-4]. In particular, myoglobin appeared to be especially sensitive to $scCO_2$ spray drying, as reflected by partial heme loss and the formation of insoluble residues [4]. Moreover, protein powders or protein-containing particles prepared using other supercritical carbon dioxide techniques have also shown instability in protein structure, bioactivity and aggregation, as presented in the review by Cape et al. [5]. Based on these observations, it is apparent that there are some elements of the $scCO_2$ drying processes that compromise the stability of myoglobin.

Myoglobin is a heme protein. The heme group is composed of protoprophyrin IX that surrounds an iron ion, which is embedded in the heme pocket of myoglobin. The heme binding affinity of myoglobin depends on the interactions of the heme group with the proximal histidine and the adjacent hydrophobic amino acids [6, 7]. The covalent bonds between the heme-iron and the imidazole ring of the proximal histidine can destabilize under acidic conditions. Lowering the pH to values in the range of 3.5-4.5 also causes protonation of the proximal histidine buried in the heme pocket, resulting in the unfolding of myoglobin, and a decrease in the heme binding affinity [7, 8]. The heme can actually be extracted in cold acidified organic solvents, such as acetone or methylethylketone, to form the heme-free protein, apomyoglobin [9].

Any changes in heme binding within myoglobin can be detected spectroscopically, as the absorption, fluorescence and circular dichroism (CD) spectra are very sensitive to the heme binding and protein conformation [4, 10-12]. Fig. 1 shows a typical UV/Vis spectrum of a myoglobin/trehalose formulation, with the heme absorption at 409 nm (Soret band) and the protein absorption at 280 nm. The position and shape of the Soret band indicate an oxidized form of myoglobin with Fe(III)protoporphyrin [7]. As was observed in a previous study on $scCO_2$ spray drying of myoglobin, a decrease in the relative intensity of the Soret band (in both UV/Vis and CD spectroscopy) and an increase in the intrinsic fluorescence signal indicate heme loss [4]. However, the factor(s) responsible for the heme loss during the $scCO_2$ spray drying process remained unclear.

The $scCO_2$ spray drying process involves atomization and subsequent drying of a protein solution under pressure with a continuous flow of $scCO_2$. During this process, the protein may undergo irreversible changes in structure and consequently a loss in functionality. Moreover, an aqueous protein formulation may become acidified during spraying, due to the reaction between carbon dioxide (CO_2) and water to form carbonic acid [13, 14]. Furthermore, the CO_2/water interface after the atomization may induce protein denaturation and aggregation [15, 16]. Given the sensitivity of myoglobin, it is hypothesized that one or more of these factors could account for the destabilization that was previous observed for myoglobin formulated with trehalose at 1:10 weight ratio and 10 mM phosphate buffer at pH 6.2. Therefore, the aim of this study was to gain fundamental insight into the effect of the sub- and supercritical CO_2 processing conditions on the stability of myoglobin in that formulation. The individual processing parameters, such as pressure, temperature, exposure to CO_2, and CO_2 spray drying, were investigated separately. In order to evaluate the influence of each processing parameter on the integrity of myoglobin, UV/Vis spectroscopy, fluorescence spectroscopy, CD spectroscopy, high performance size-exclusion chromatography (HP-SEC), and flow imaging microscopy analyses were used. It is anticipated that by understanding these fundamental process-related factors of $scCO_2$ spray drying on the stability of myoglobin, the conditions and/or the formulation can be optimized, in order to protect proteins against process-induced damage.

2. Materials and Methods

2.1 Preparation of liquid myoglobin formulation

A liquid myoglobin formulation was prepared from 5 mg/ml myoglobin from equine skeletal muscle with 50 mg/ml trehalose in 10 mM sodium phosphate buffer, pH 6.2. All chemicals were obtained from Sigma-Aldrich, St. Louis, USA. The liquid formulation was filtered through a 0.22-µm pore cellulose acetate filter (Millex®-GV, Cork, Ireland) before performing the experiments.

2.2 Experimental conditions

The effects of pressure and temperature on myoglobin stability were studied independently by using a high-pressure vessel (TELEDYNE ISCO, Lincoln, USA). In all cases, the tests were performed using 10 ml of the liquid myoglobin formulation. The effect of temperature was studied under atmospheric pressure at 25, 37, and 50 °C (conditions A, B, and C). The vessel was heated by flowing water from a controlled temperature water bath (Lauda C6, LAUDA-Königshofen, Germany)

through a heating jacket that surrounds the vessel. The effect of pressure was studied at 65 and 130 bar at different temperatures (25, 37, and 50 °C, corresponding to conditions D, E, F, G, H, I). The desired pressure was achieved by reducing the volume of the vessel with a piston. The myoglobin/trehalose formulations were kept under the set conditions for two hours. After completing each experiment, the solutions were removed from the vessel and left for an hour to cool down to room temperature prior to analysis.

The effect of CO_2 on myoglobin stability was studied by incubating 10-ml aliquots of the liquid myoglobin formulation with pressurized CO_2 in the high-pressure vessel. Carbon dioxide (grade 3.5) was obtained from Linde Gas (Schiedam, The Netherlands). The solution was exposed to both subcritical and supercritical CO_2 at 65 and 130 bar and 25, 37, and 50 °C (conditions J, K, L, N, O, and P). The CO_2 was brought to the desired pressure with a separate pump system before being introduced into the incubation vessel. The volume ratio of the liquid myoglobin formulation to CO_2 was fixed at 1:10. After forty-minute incubation time, CO_2 was slowly removed by depressurization to atmospheric pressure. The CO_2-incubated liquid myoglobin formulations were kept at 4 °C for 12 hours before analysis.

In order to exclude the influence of CO_2 density on myoglobin integrity, a series of experiments at a constant CO_2 density of 0.77 g/cm^3 were conducted. The conditions for these tests were 77 bar and 25 °C, 130 bar and 37 °C, and 188 bar and 50 °C (conditions M, O, Q). The incubation time, depressurization rate and storage conditions were the same as for the experiments above.

The effect of CO_2 spray drying was investigated at 65 and 130 bar and 25, 37, and 50 °C (condition R, S, T, U, V, and W). A 4-liter spray drying module (F54250S model from Separex, Champigneulles, France) was used in this study. The drying vessel was filled with the pressurized CO_2 and brought to the desired temperature and pressure before feeding in the liquid myoglobin formulation via a high-pressure syringe pump maintained at 25 °C (TELEDYNE ISCO, Lincoln, USA). The solution was atomized by the pressurized CO_2 stream through a coaxial converging nozzle (Spraying Systems Co. B.V., Ridderkerk, The Netherlands). The diameter of the nozzle orifices was 0.16 cm and 0.04 cm for the CO_2 and the liquid, respectively. The solution and CO_2 flow rates were kept constant at 0.2 and 500 g/min, respectively. The spraying time was 40 minutes, followed by 30 minutes post-drying step with fresh CO_2, to remove any residual moisture from the vessel and consequently the product. After depressurization, the dried myoglobin formulations were collected from a paper filter (Whatman® qualitative filter paper,

Grade 1, 25 mm diameter, Diegem, Belgium). The product was then stored in a desiccator at room temperature prior to analysis. In addition to these experiments, one test was performed at 130 bar and 37 °C without the post drying step, in order to study the effect of post-drying (condition V-).

All experimental conditions are summarized in Table 1. Each experimental condition was repeated three times (n=3).

2.3 Protein analysis

2.3.1 Sample preparation for myoglobin analysis

If necessary, liquid myoglobin formulation samples were diluted in 10 mM phosphate buffer (PB), pH 6.2. For the dried myoglobin/trehalose formulations, an amount of powder corresponding to 5 mg myoglobin and 50 mg trehalose was accurately weighed and dissolved in 1 ml of water, taking into consideration the amount of phosphate salts and the residual water content after spray drying [3]. The powdered products were dissolved overnight at room temperature to complete the dissolution of powders. The solutions were subsequently diluted in 10 mM PB, pH 6.2. The diluted samples were filtered through a 0.22-μm pore cellulose acetate filter (Millex®-GV, Cork, Ireland) before protein structural analysis by UV/Vis, circular dichroism and fluorescence spectroscopy. The sample concentrations for each analysis are mentioned below.

2.3.2 Protein recovery and recovery of bound heme

The protein solutions were diluted to approximately 0.1 mg/ml prior to measurement. The protein content was determined by using a UV spectrophotometer (Agilent 8453, Agilent Technologies, Santa Clara, USA). The spectra were collected from 190 nm to 1000 nm. The myoglobin concentration was calculated using a molar extinction coefficient of 3.45 x 10^4 $M^{-1}cm^{-1}$ at 280 nm [17]. The absorbance ratio between 409 and 280 nm (A409/A280) from each spectrum represents the absorption of heme group bound to the proximal histidine in the heme pocket of myoglobin [18]. The percentages of protein content recovery of the treated myoglobin (see Eq. 1) were compared to those of the untreated liquid myoglobin formulation at 25°C (condition A).

$$Protein\ recovery(\%) = \left[\frac{A280\ (processed)}{A280\ (nonprocessed)}\right] \times 100 \qquad \text{(Eq. 1)}$$

Table 1. Experimental conditions and the corresponding protein recovery and heme absorption using UV/Vis spectroscopy.

Conditions[a]	Pressure (bar)	Temperature (°C)	CO_2	CO_2 density (g/cm^3)	Spray drying	Post drying	Protein recovery[e] (%wt)	A409/A280[f]
Effect of temperature[b,g]								
A	atmospheric	25	-	-	-	-	100	5.50 ± 0.03
B	atmospheric	37	-	-	-	-	100.0 ± 4.7	5.47 ± 0.01
C	atmospheric	50	-	-	-	-	104.3 ± 1.4	5.56 ± 0.01
Effects of pressure and temperature[b]								
D	65	25	-	-	-	-	97.1 ± 3.0	5.67 ± 0.01
E	65	37	-	-	-	-	96.0 ± 10.7	5.50 ± 0.01
F	65	50	-	-	-	-	104.6 ± 2.0	5.54 ± 0.01
G	130	25	-	-	-	-	100.1 ± 2.0	5.55 ± 0.01
H	130	37	-	-	-	-	98.1 ± 6.4	5.66 ± 0.00
I	130	50	-	-	-	-	100.2 ± 4.1	5.74 ± 0.01
Effects of carbon dioxide[c]								
J	65	25	+	0.72	-	-	91.1 ± 6.3	5.43 ± 0.12*
K	65	37	+	0.18	-	-	95.2 ± 3.6	5.38 ± 0.15*
L	65	50	+	0.15	-	-	85.6 ± 7.7	4.98 ± 0.52*
M	77	25	+	0.77	-	-	93.4 ± 5.8	5.56 ± 0.04
N	130	25	+	0.86	-	-	94.4 ± 3.2	5.42 ± 0.16*
O	130	37	+	0.77	-	-	95.8 ± 0.6	5.28 ± 0.13*

P	130	50	+	0.64	-	-	85.6 ± 0.6	4.99 ± 0.35*
Q	188	50	+	0.77	-	-	86.6 ± 5.0	5.26 ± 0.22
Effects of spray drying and post drying[d]								
R	65	25	+	0.72	+	+	89.0 ± 5.0	5.23 ± 0.10*
S	65	37	+	0.18	+	+	85.2 ± 2.0	4.87 ± 0.36*
T	65	50	+	0.15	+	+	79.1 ± 0.3	4.44 ± 0.45*
U	130	25	+	0.86	+	+	90.4 ± 1.4	5.24 ± 0.17*
V-	130	37	+	0.77	+	-	82.7 ± 2.8	5.05 ± 0.12
V	130	37	+	0.77	+	+	79.5 ± 1.6	4.93 ± 0.18*
W	130	50	+	0.64	+	+	76.2 ± 4.0	4.71 ± 0.20*

[a] Formulation of 5 mg/ml myoglobin and 50 mg/ml trehalose in 10 mM phosphate buffer at pH 6.2 was used for all experiments.
[b] Incubation time was two hours.
[c] The ratio of myoglobin solution to carbon dioxide was 1:10 (v/v), forty-minutes incubation time.
[d] Forty minutes spray drying with (+) and without (-) thirty minutes post drying.
[e] Data were obtained from the absorbance at 280 nm by UV/Vis spectroscopy (see methods).
[f] Data were obtained from the ratio of the absorbances at 409 and 280 nm by UV/Vis spectroscopy (see methods).
[g] Reference solutions.
* A significant reduction ($p < 0.05$) of A409/A280 ratio found comparing to the untreated samples at conditions A, B, C.

2.3.3 Circular dichroism spectroscopy

In order to study the structure of myoglobin, far-UV (190-250 nm) and near-UV/Vis (350-450 nm) CD spectroscopy measurements (J-810 Spectropolarimeter, JASCO Inc., Easton, USA) were performed at 25 °C. Parameters were set at a sensitivity of 100 mdeg, with a data pitch of 10 nm, a bandwidth of 2 nm, and a scanning speed of 100 nm/min. Samples were freshly prepared with a myoglobin concentration of 0.1 mg/ml and placed in a 0.1-cm and 1-cm quartz cuvette for far-UV CD and near-UV/Vis CD measurements, respectively. CD spectra of six sequential measurements were averaged and corrected for the blank. The CD signals were converted to molar ellipticity per amino acid residue (θ). The signal of the heme specific site at 409 nm of the samples was also compared to that of reference solution (25 °C), and shown as a relative molar ellipticity.

2.3.4 High performance size-exclusion chromatography

Samples (50 µl of 1 mg/ml protein concentration) were analyzed by HP-SEC with a Discovery® BIO Gel Filtration column (300 Å pore size) (Sigma-Aldrich, St. Louis, USA). The mobile phase consisted of 150 mM phosphate buffer, pH 7.0, and was filtered through a 0.2-µm filter prior to use. The flow rate was set at 0.7 ml/min. The chromatograms were recorded by a UV detector (Agilent 1100 VWD, Santa Clara, USA) and a fluorescence detector (Agilent 1200 FLD, Santa Clara, USA). The absorbance of each spectrum at a wavelength of 280 nm was used to calculate the percentages of myoglobin monomer, aggregates, and fragments. The monomer recovery of the CO_2 incubated (conditions J, K, L, N, O, P) and CO_2 spray dried (conditions R, S, T, U, V, W) myoglobin was calculated as percentage of the monomer peak area relative to that of the untreated sample (condition A). The fluorescence emission intensity was collected at 350 nm with the excitation at 295 nm. For each chromatogram, the total peak area of the fluorescence signal intensity was compared to the absorbance at 280 nm, and shown as I_{350}/A_{280}. Apomyoglobin, which lacks the heme group, was used as a control.

2.3.5 Flow imaging microscopy

The presence of sub-visible microparticles was determined by flow imaging microscopy using an MFI5200 instrument (Protein Simple, California, USA). The measurements were controlled and the data analyzed by MVAS software version 1.3. Prior to analysis, the formulations

were diluted to a myoglobin concentration of 1 mg/ml. One milliliter of solution was introduced at a flow rate of 6 ml/min into a flow cell with the dimensions of 100 and 1016 µm in depth and diameter, respectively, illuminated by a blue LED. Images of the particles were captured by an optical camera. Pictures were obtained with a resolution of 1280x1024 pixels. Total particle concentrations are reported for particle sizes larger than 1, 10, 25 and 50 µm.

2.4 Statistical analysis

The data were analyzed by a two-way ANOVA via the GraphPad Prism to test the myoglobin integrity as a function of processing parameters (untreated, pressurized with and without CO_2, CO_2 spray drying) and temperatures/pressures. Values were considered significant when *p* was less than 0.05. The data of each experimental condition (n=3) are shown as average ± standard deviation.

3. Results and discussion

In a previous study, the destabilization of myoglobin after CO_2 spray drying was observed, as reflected by the presence of insoluble residues and heme loss in the resultant product [4]. However, the drying was carried out in the presence of an organic solvent, which was thought to contribute to the destabilization [4]. Upon repeating these experiments in the absence of organic solvent, however, insoluble residues and heme loss were still detected (unpublished results), which implies that one or more of the other factors within the process also contribute to the destabilization of myoglobin. In order to understand the influence of the CO_2 spray drying processing parameters on the instability of myoglobin, a systematic study of each parameter on the protein's stability was conducted.

The processing parameters related to the CO_2 spray drying were differentiated into four factors: temperature (25-50°C), pressure (atmospheric and 65-130 bar), presence of CO_2, and spray drying in a CO_2 environment at 25-50°C and 65-130 bar. No organic solvents were used in these experiments. Changes in myoglobin structure, in terms of heme loss and protein aggregation, were monitored to explain how these parameters influence myoglobin stability.

To analyze heme-bound myoglobin, UV/Vis analysis was conducted. Myoglobin shows absorption band centered around 409 nm (Fig. 1), related to absorption of the heme group, which indicates the

coordination of heme with the nitrogenous base of the proximal histidine in the heme pocket [18]. The absorption at 280 nm reflects the aromatic amino acid residues the protein. The signal of near-UV/Vis CD at 409 nm (Fig. 2) will shift and/or decrease depending on the heme π-π^* electronic dipole transition moments with proximal aromatic amino acid residues in the heme pocket of myoglobin [19, 20]. Myoglobin's tryptophan fluorescence signal is highly quenched by the bound heme group [21, 22]. Therefore, any increase in the fluorescence emission intensity (Fig. 3) is directly related to heme loss, leading to an increase in the concentration of apomyoglobin. As shown in the previous study, heme loss from myoglobin was not only accompanied by an increase in the fluorescence emission intensity, but also resulted in a reduced absorbance at 409 nm (presented by the ratio of 409 nm to 280 nm), and molar ellipticity at 409 nm (by near-UV/Vis CD) [4]. In addition, soluble and sub-visible myoglobin aggregates were analyzed by HP-SEC and flow-imaging microscopy, respectively. The results are discussed below.

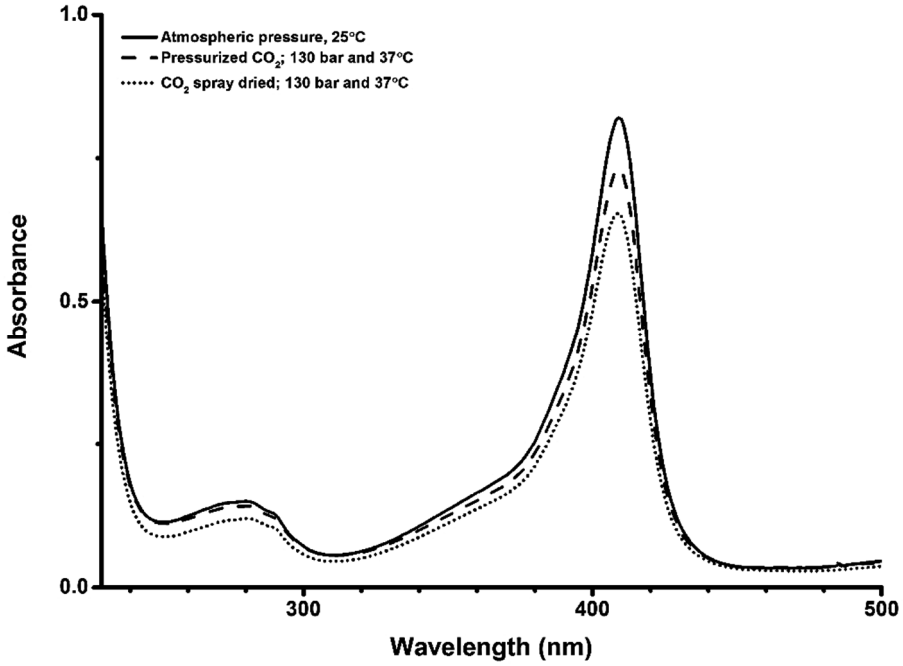


Fig. 1 UV/Vis spectra of CO_2-incubated myoglobin and reconstituted solution of spray dried myoglobin formulations (both conducted at 130 bar and 37 °C, conditions O and V), compared to that of untreated myoglobin formulation (condition A).

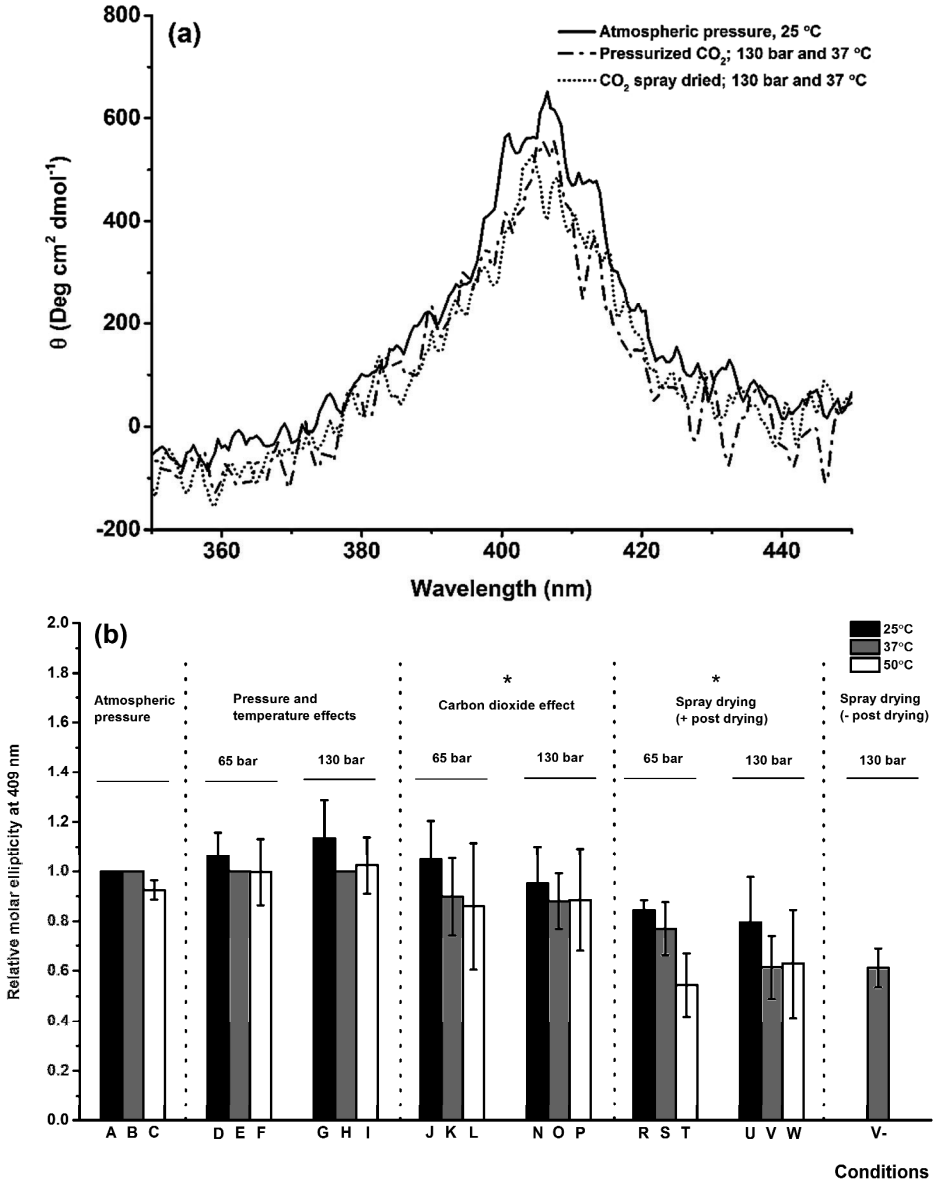


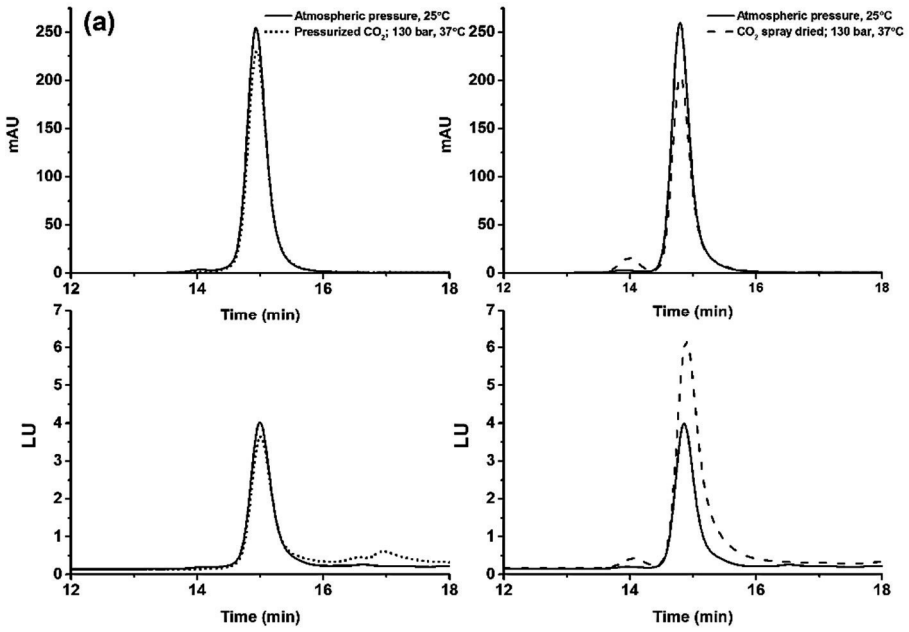
Fig. 2 Near/Vis-CD spectra (**graph a**) of CO_2-incubated myoglobin and reconstituted solution of spray dried myoglobin formulations (both conducted at 130 bar 37 °C), compared to that of untreated myoglobin formulation. Relative molar ellipticity (**graph b**) at the Soret band (409 nm) for myoglobin formulations at atmospheric pressure (conditions A, B, C), pressurized at 65 bar (conditions D, E, F) and 130 bar (conditions G, H, I), CO_2-incubated myoglobin formulations at 65 bar (conditions J,

K, L) and 130 bar (conditions N, O, P) at 1:10 volume ratio of myoglobin formulation to carbon dioxide, and reconstituted spray dried myoglobin formulations with half an hour post drying produced at 65 bar (conditions R, S, T) and 130 bar (conditions U, V, W). The experiments for the atmospheric pressure, 65 and 130 bar were done at 25, 37, and 50 °C. A significant difference (*) in the relative molar ellipticity at the heme specific site found in the experimental group of CO_2 incubation (conditions J, K, L, N, O, P) and CO_2 spray drying (conditions R, S, T, U, V, W), comparing to the untreated samples at conditions A, B, C with $p < 0.05$.

3.1 Effects of temperature 25-50°C at atmospheric pressure (1 bar) and 65-130 bar without carbon dioxide

The $scCO_2$ spray drying process takes place under pressurized conditions at a given temperature. It was first necessary to evaluate whether temperature and pressure alone affect myoglobin's structure. Therefore, pressures at 65-130 bar and temperatures at 25-50 °C used for the CO_2 spray drying conditions were studied. The results of protein recovery, the ratio of A409/A280 (Table 1), molar ellipticity (as a measure of myoglobin's secondary structure (data not shown)), circular dichroism at the heme specific site (Fig. 2), and fluorescence intensity (Fig. 3b) indicate that the heme binding site was not perturbed over the pressure and temperature range studied. In addition, the range of pressures and temperatures studied did not induce any myoglobin aggregation, as shown by HP-SEC (Fig. 4) and flow imaging microscopy (Fig. 5, conditions A-I). Both untreated myoglobin and pressurized myoglobin solutions contained very low numbers of sub-visible particles. Therefore, myoglobin was stable under these conditions.

These results are in line with a study by Doster and Gebhardt [23], who found that a myoglobin solution at pH 7 was only denatured when exposed to pressures above 3 kbar, as indicated by the optical spectral properties of the heme group. Under these high-pressure conditions, only 40% of the a-helix structure of myoglobin was recovered.


(a)
Atmospheric pressure, 25°C
Pressurized CO2; 130 bar, 37°C
CO2 spray dried; 130 bar, 37°C
mAU
LU
Time (min)

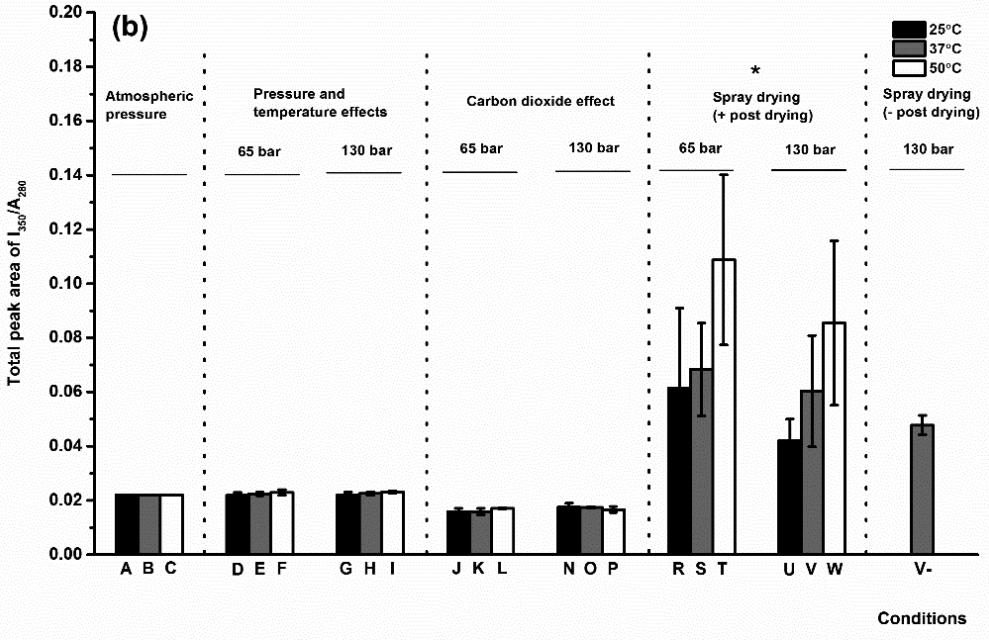
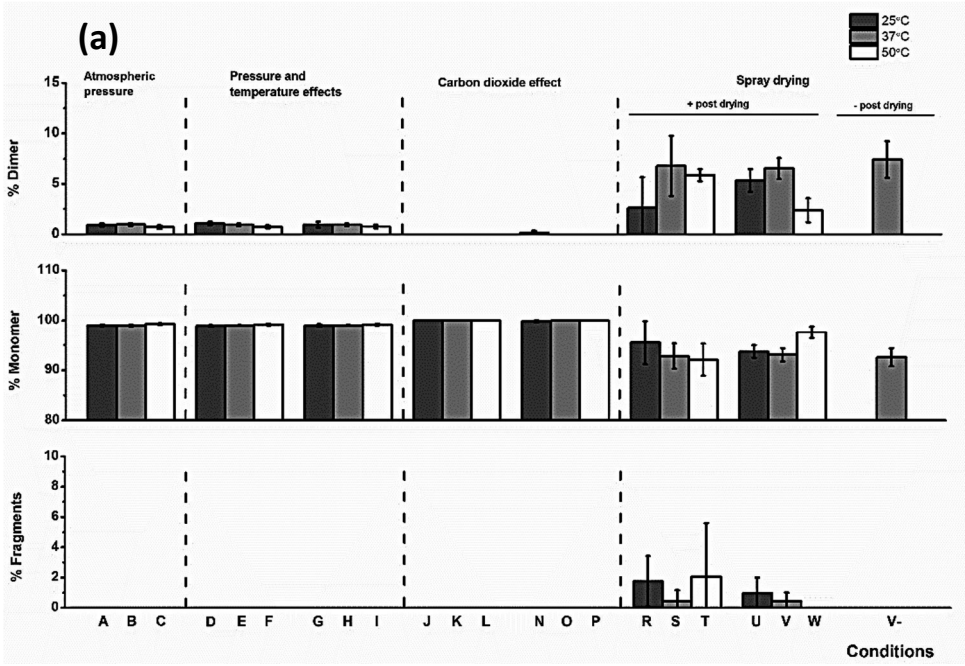

(b)
25°C
37°C
50°C
Atmospheric pressure
Pressure and temperature effects
Carbon dioxide effect
Spray drying (+ post drying)
Spray drying (- post drying)
65 bar
130 bar
Total peak area of I350/A280
A B C
D E F
G H I
J K L
N O P
R S T
U V W
V-
Conditions

Fig. 3 HP-SEC chromatograms (**graph a**) detected by UV absorption at 280 nm (upper panels) and fluorescence emission at 350 nm (lower panels) of CO_2-incubated myoglobin formulation (left panels) and reconstituted spray dried myoglobin formulation (right panels) conducted at 130 bar 37 °C, compared to untreated myoglobin formulation. mAU = milliabsorbance unit; LU = luminescence **units**. The relative total peak area (**graph b**) of the fluorescence emission signal at 350 nm to protein absorption at 280 nm (I_{350}/A_{280}) for reference myoglobin formulations (conditions A, B, C), pressurized at 65 bar (conditions D, E, F) and 130 bar (conditions G, H, I), CO_2-incubated myoglobin formulations under 65 bar (conditions J, K, L) and 130 bar (conditions N, O, P) at 1:10 volume ratio of myoglobin formulation to carbon dioxide, and reconstituted spray dried myoglobin formulations with half an hour post drying produced at 65 bar (conditions R, S, T) and 130 bar (conditions U, V, W). See Table 1. A significant difference (*) of the fluorescence intensity found in the experimental group of spray drying at conditions R, S, T, U, V, W, comparing to the untreated non-pressurized samples at conditions A, B, C with $p < 0.05$.



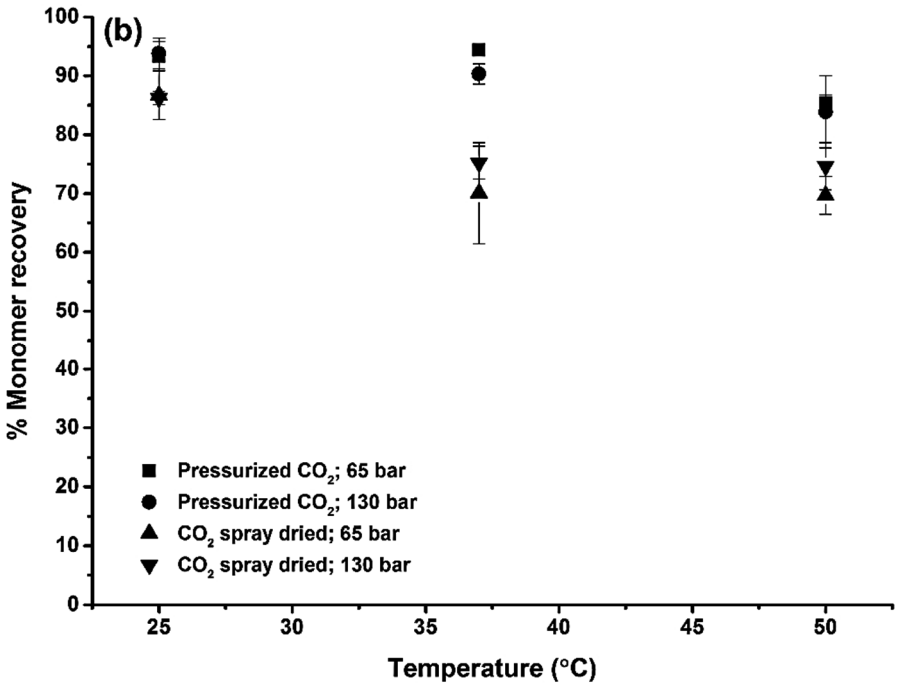
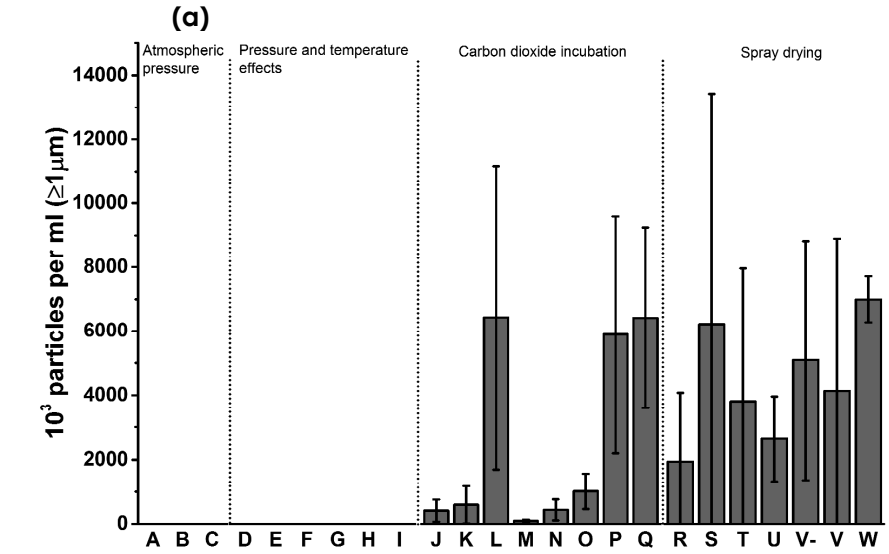
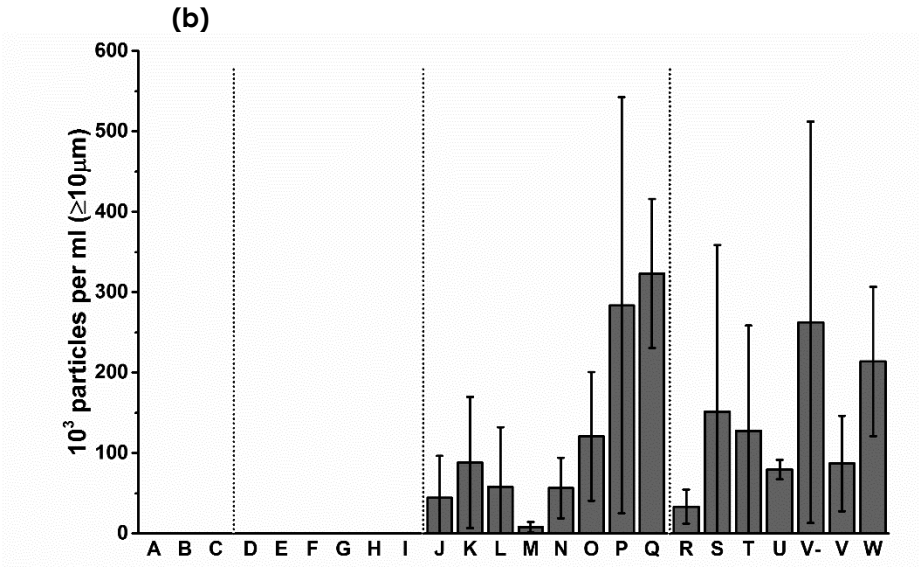


Fig. 4 The percentages of dimer, monomer, and fragments (**graph a**) for reference myoglobin formulations (conditions A, B, C), pressurized at 65 bar (conditions D, E, F) and 130 bar (conditions G, H, I), CO_2-incubated myoglobin formulations at 65 bar (conditions J, K, L) and 130 bar (conditions N, O, P), and reconstituted spray dried myoglobin formulations with post drying produced at 65 bar (conditions R, S, T) and 130 bar (conditions U, V, W), as determined by HP-SEC (absorption at 280 nm). The percentage of monomer recovery (**graph b**) for myoglobin formulations incubated with pressurized CO_2 at 65 bar (conditions J, K, L) and 130 bar (conditions N, O, P), and reconstituted spray dried myoglobin formulations with post drying produced at 65 bar (conditions R, S, T) and 130 bar (conditions U, V, W). See Table 1 for conditions.


(a)
Atmospheric pressure
Pressure and temperature effects
Carbon dioxide incubation
Spray drying
10^3 particles per ml (≥1μm)
0
2000
4000
6000
8000
10000
12000
14000
A B C D E F G H I J K L M N O P Q R S T U V- V W


(b)
10^3 particles per ml (≥10μm)
0
100
200
300
400
500
600
A B C D E F G H I J K L M N O P Q R S T U V- V W

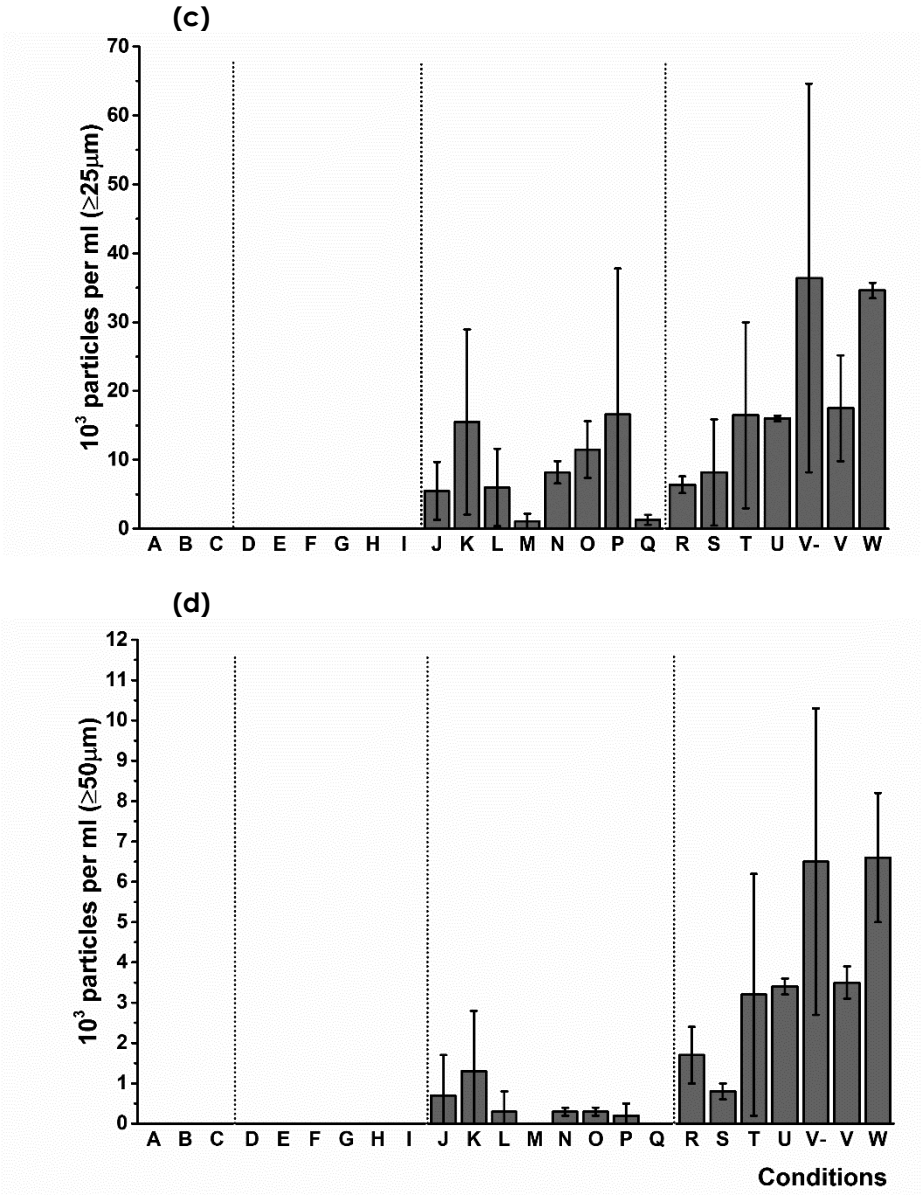


Fig. 5 Particle concentrations larger than 1 µm (a), 10 µm (b), 25 µm (c) and 50 µm (d) in untreated, pressurized without CO_2, pressurized with CO_2 and CO_2 spray drying myoglobin formulations observed by flow imaging microscopy. Volume refers to that of undiluted (reconstituted) liquid myoglobin formulation. See Table 1 for experimental conditions. A significant difference (*) of the particle concentrations (≥ 1 µm) found in the experimental group of CO_2 incubation.

3.2 Effects of carbon dioxide at 65-130 bar and 25-50°C

In $scCO_2$ spray drying, CO_2 is responsible for the atomization of the solution as well as being the drying medium. The properties of CO_2, such as the density, viscosity and surface tension, depend on the operating pressure and temperature. As myoglobin's integrity was maintained upon varying the pressure and temperature, the next step in this study was to expose the myoglobin solution to CO_2. To this end, CO_2, at given pressures and temperatures, was introduced into a high-pressure vessel containing the myoglobin formulation. This experiment was designed to simulate the exposure of the protein to the CO_2 medium at 65-130 bar and 25-50°C, but without the drying process.

After incubating the myoglobin solutions with pressurized CO_2 at sub- and supercritical conditions (65-130 bar 25-50°C), the pH had shifted from 6.2 to approximately 5.1 (Table 2). The shift in the pH is due to the production of carbonic acid from the interaction between the CO_2 and the water [13, 14]. Varying the CO_2 conditions hardly influenced the final pH of the solution. Furthermore, the original pH (pH 6.2) was not recovered, even 12 hours after completion of the experiment (Table 2). This indicates that the 10 mM phosphate buffer at pH 6.2 is not capable of controlling the pH of the solution upon exposure to pressurized CO_2.

Table 2. The pH of the myoglobin formulations after CO_2 incubation at 25, 37, 50 °C and 65 bar (conditions J, K, L) and 130 bar (conditions N, O, P). The pH was re-measured 12 hours after depressurization. The untreated myoglobin formulations at 25, 37, 50 °C were used as a control.

Pressure conditions	**pH**		
	25°C	**37°C**	**50°C**
Atmospheric pressure	6.27 ± 0.00	6.26 ± 0.00	6.25 ± 0.00
0 h after depressurization			
65 bar	5.20 ± 0.03	5.31 ± 0.07	5.38 ± 0.06
130 bar	5.17 ± 0.01	5.32 ± 0.05	5.45 ± 0.07
12 h after depressurization			
65 bar	5.43 ± 0.04	5.62 ± 0.01	5.86 ± 0.02
130 bar	5.41 ± 0.01	5.50 ± 0.05	5.94 ± 0.16

As the pH could only be measured after depressurization, it is possible that the myoglobin solution was exposed to even lower pH values during the course of the experiments. Hofland et al. [24] found that casein solutions (120 g in 1 liter water) exposed to pressurized CO_2 showed a pH drop from 6.75 (original pH) to 4.82 upon increasing the pressure from 1 to 25 bar. The pH remained stable at 4.82, even with a further increase in pressure. The pH under pressurized conditions was slightly higher for higher temperatures over a range of 25-50°C. From these observations, it can be concluded that myoglobin is exposed to acidic conditions when it comes in contact with high-pressure CO_2, which may contribute to its destabilization, as discussed below.

In this study, the incubation with pressurized CO_2 was also found to affect the integrity and the recovery of myoglobin, as shown by a decrease in the A409/A280 ratio and the absorbance at 280 nm, respectively (see Fig. 1 and Table 1). A further decrease in the A409/A280 ratio was observed with increasing temperature. These results indicate a change in the heme coordination within the heme pocket. It has previously been shown that a decrease in the pH of a myoglobin solution results in the protonation of the proximal histidine in the heme pocket, which causes the disruption of the iron-histidine bond and a change in the heme absorption spectrum [25, 26]. Thus, it is expected that the affinity of myoglobin for the heme will be reduced after the exposure to low pH induced by the pressurized CO_2. Indeed, Hargrove et al. [27, 28] showed that the heme dissociation from myoglobin at pH 5 was $\geq$100-fold faster than at neutral pH at 37°C, as monitored by changes in the Soret band. Consequently, such conditions may lead to heme dissociation when myoglobin is exposed to pressurized CO_2.

In order to determine whether the structure of myoglobin was influenced by CO_2, CD signals at 409 nm for probing the heme-protein interaction was studied. The relative molar ellipticity decreased after exposure to CO_2, with a greater decrease observed at higher temperatures, suggesting a change in the heme specific binding site. However, the results were not significantly different between 65 and 130 bar at constant temperature (Fig. 2).

In addition, the predominantly a-helical secondary structure of myoglobin, after the CO_2 incubation, did not change under the conditions studied (Fig. 6). In contrast, Ishikawa et al. [29] showed that CO_2 bubbling under pressurized conditions resulted in irreversible unfolding of myoglobin. This was achieved at ambient temperatures. However, it should be noted that these experiments were performed for myoglobin solutions that did not contain any excipient. Moreover, it is

possible that the change in myoglobin structure was influenced by the CO_2/water interface because of the gas bubbling method.

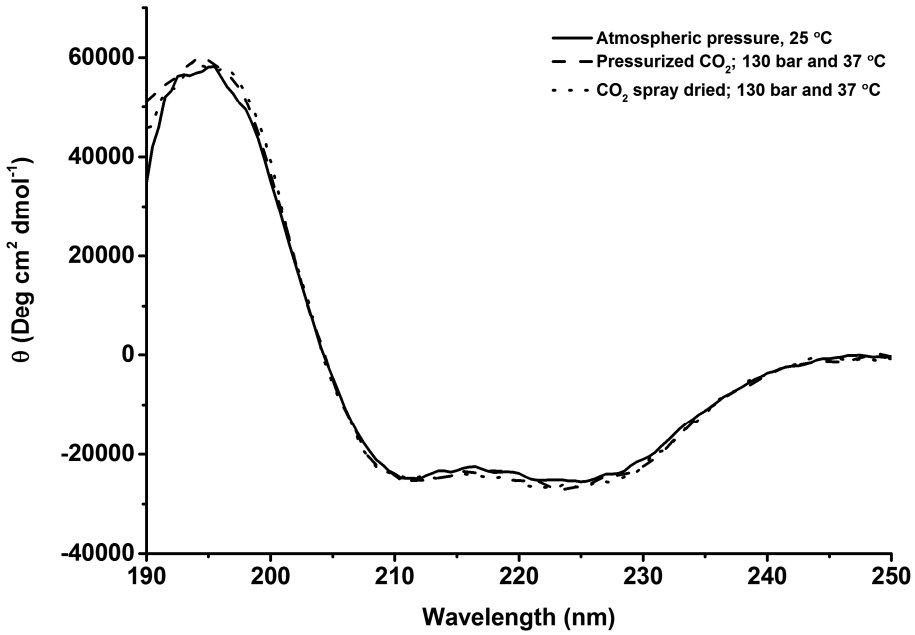


Fig. 6 Far-UV CD spectra of CO_2-incubated myoglobin and reconstituted solution of spray dried myoglobin formulations (both conducted at 130 bar and 37 °C, conditions O and V), compared to that of untreated myoglobin formulation.

The results from UV/Vis and CD spectroscopy showed that the myoglobin structure changes upon increasing temperature. However, the fluorescence intensity of myoglobin after the CO_2 incubation (Fig. 3) gave a constant signal, similar to that of the untreated (conditions A, B, C) and pressurized samples without CO_2 (conditions D, E, F, G, H, I). The ratio between the fluorescence at 350 nm and the absorbance at 280 nm (I_{350}/A_{280}) was determined from the total peak areas observed by HP-SEC. For the samples exposed to elevated temperature and pressure, the ratio of I_{350}/A_{280} was 0.22, and constant in all cases, while apomyoglobin showed a substantially larger I_{350}/A_{280} of 1.19 (not shown). Consequently, the tryptophan fluorescence signal can be used to determine the loss of heme from myoglobin. For the myoglobin

formulations exposed to CO_2, no increase in the fluorescence signal was observed, with the I_{350}/A_{280} values also around 0.22, suggesting that the heme was still embedded in the heme pocket.

Whereas pressure and temperature without CO_2 did not influence the total myoglobin concentration, a decrease in the protein content was found after the CO_2 incubation at higher temperatures (Table 1), with only 85% protein recovery at 65 bar and 130 bar at 50 °C (conditions L and P). This appears to be due to aggregation of myoglobin, because a decrease in the monomer content was found under these conditions (Fig. 4b). Moreover, an increase in concentration of particles larger than 1 µm was observed by flow-imaging microscopy (Fig. 5). Interestingly, the dimer content was decreased after incubation with CO_2 (Fig. 4a). Since no oligomers were detected by HP-SEC and the monomer content was reduced, the dimers proably were converted into micron-sized aggregates. Altogether, from these results it can be concluded that the aggregation is most likely the result of the shift in pH.

For the CO_2 incubation tests, different temperatures and pressures were used, which gave rise to different CO_2 densities. In order to evaluate whether the CO_2 density has an influence on the stability of myoglobin, several conditions were chosen that have the same CO_2 density (0.77 g/cm^3, conditions M, O, and Q). As was observed for the CO_2 incubation tests, the results also showed a reduction in the heme absorption and protein content as the processing temperature increased (Table 1). Moreover, these values obtained at the same temperature but different pressure (and therefore different density) are almost the same (Table 1). From this, it can be concluded that changes in the myoglobin integrity can be attributed to the increase in temperature, and not the differences in CO_2 density.

Overall, it can be concluded that exposing the myoglobin formulation to pressurized CO_2 caused acidification, resulting in a change in the heme binding and aggregation of myoglobin (monomer loss and high particle concentrations). However, the heme was not removed, as the tryptophan fluorescence signal did not increase. In addition, the destabilization of myoglobin was promoted by increasing the processing temperature.

3.3 Effects of spray drying using carbon dioxide at 65-130 bar and 25-50°C

Having studied the influence of temperature, pressure and CO_2 exposure, the next series of experiments evaluated the influence of the spraying process on myoglobin integrity.

Upon reconstituting the spray-dried myoglobin, a greater reduction ($p < 0.05$) in heme absorbance was found (decreasing to approximately 85% at both 65 and 130 bar and 50 °C) when compared to the CO_2-incubated myoglobin solutions (as seen in Table 1 and Fig. 1). The reconstituted myoglobin formulation did not show a significant difference in the secondary structure (Fig. 6). However, a further decrease in the molar ellipticity at heme specific site (by CD) was observed with increasing temperature (Fig. 2). However, the data were not significantly different as compared to the CO_2-incubated group. Furthermore, the fluorescence signals also increased significantly, which was not observed in the case of the CO_2-incubated myoglobin solutions (Fig. 3). As an increase in the fluorescence signal corresponds to the formation of apomyoglobin, these results indicate that a partial loss of heme occurs during spray drying, at both subcritical and supercritical CO_2 conditions. In addition, the loss of heme slightly increased with increasing temperature.

Lower protein recoveries were observed for the reconstituted spray dried products with less than 80% recovery at the highest temperatures, as can be seen in Table 1. Furthermore, higher amounts of dimers (2.5-7.5%) and additionally some fragments (0.5-2%) of myoglobin were present after spray drying (Fig. 4a) when compared to the untreated myoglobin formulation (condition A). A further loss of monomer in the reconstituted solutions was also found, when compared to the CO_2-incubated samples (Fig. 4b). The CO_2 spray drying process also induced the formation of larger myoglobin aggregates, as shown in Fig. 5. Moreover, the total particle content was higher in the reconstituted solutions of spray-dried myoglobin powders when compared to the myoglobin solutions incubated with CO_2. In these cases, the sub-visible particles were also larger in diameter (Fig. 5).

Upon reconstitution of the spray-dried myoglobin/trehalose formulation produced at 130 bar and 37°C, the pH was 5.12 ± 0.06. In a similar manner to the CO_2 incubated solutions, these results suggest that the atomized myoglobin solution was acidified by the pressurized CO_2. In addition, it has been observed before that residual CO_2 can be present in powdered products after depressurization [30], which may explain the decrease in the pH upon reconstitution.

In this study, it was concluded that acidification causes a weakening of the heme binding of myoglobin. When this is combined with the spray drying process, some of the myoglobin molecules loose the heme. For the spray drying tests, the spray time was 40 minutes, which was typically followed by 30 minutes of post-drying. One experiment was also conducted without post drying. The results showed that the ratio of A409/A280 and relative molar ellipticity of myoglobin (at the heme specific site) treated with or without the post drying were not significantly different (Table 1 and Fig. 2, condition V and V-). Myoglobin aggregation, with and without the post-drying, were also similar in terms of dimer content and sub-visible particle sizes, as shown in Fig. 4 and Fig. 5. This suggests that the heme removal and myoglobin aggregation occurred during the 40-minute spray drying, when the myoglobin/trehalose formulation is atomized and dehydrated in the pressurized CO_2 environment.

While the acidification by CO_2 mainly affects the heme binding affinity and aggregation, other parameters of the CO_2 spray drying method may interfere with the myoglobin structure. The atomization step subjects the solution to a large CO_2/water interface, which may lead to protein denaturation and aggregation. Moreover, CO_2 is non-polar and therefore the protein may reorient so that the hydrophobic components are exposed to the surface, which may initiate the unfolding of the protein. Any of these factors, combined with the lowering of the heme affinity due to acidification by CO_2, could cause the destabilization of myoglobin, thus leading to the removal of heme and an increase in myoglobin aggregation. However, it is difficult to independently study atomization and drying parameters of the CO_2 spray drying method.

3.4 Effect of protein formulation

It is anticipated that this mechanistic study of the influence of the CO_2 spray drying process on myoglobin destabilization could be useful for the future development of protein formulations for protein drying. What is apparent from this study is that for pH-sensitive proteins like myoglobin, the formulation should be carefully selected to avoid a pH shift during CO_2 spray drying. In order to evaluate this hypothesis, the concentration of the phosphate buffer pH 6.2 was increased up to 150 mM. Under these conditions, it was found that the A409/A280 ratio showed nearly 100% recovery of the heme (CO_2 spray dried at 130 bar and 37°C) (data not shown). Such high buffer concentrations may not be ideal, e.g., when the formulation has to be kept isotonic, but could provide a solution if high-concentration protein formulations are used

that can be diluted before administration. Also, one could use a higher buffer pH than 6.2, such as 7.0, which in particular for phosphate would further increase its buffering capacity.

4. Conclusion

In this study, the influence of the critical CO_2 spray drying processing parameters on the stability of dried myoglobin formulations was investigated. This was done by monitoring the effect of temperature, pressure, contact with CO_2 and the spray drying steps on myoglobin structure. In the range of 65-130 bar and 25-50 °C, pressure and temperature alone did not influence the myoglobin integrity. After incubation with pressurized CO_2, the pH of the myoglobin solution was about 5, which compromised the heme binding in myoglobin and induced myoglobin aggregation. Spray drying with pressurized CO_2 resulted in the loss of heme and a further increase in myoglobin aggregation. Moreover, this was exacerbated by an increase in temperature but not affected by increasing pressure. Overall, the results indicate that exposure of myoglobin to CO_2 destabilizes the myoglobin, which eventually leads to heme loss and protein aggregation during CO_2 spray drying.

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