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Research Paper

Micronization of Insulin by High Pressure Homogenization

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Purpose. The aim of this study was to establish the high pressure homogenization of proteins in non-aqueous suspension as an alternative method for classical micronization strategies and to investigate the effect of high pressure on protein stability and bioactivity.
Methods. The influence of drug loading, homogenization pressure and cycles on particle size reduction was investigated by experimental design using a Box Behnken matrix with insulin as a model compound. Particle size measurements were performed by laser light scattering. Protein stability was investigated by HPLC and HPLC-MS analysis and the bioactivity of insulin was tested in a chondrocyte proliferation assay. For investigations into the effect of temperature on protein stability, insulin was micronized in molten lipid at 75°C in one cycle at 1,000 bar.
Results. Within one homogenization cycle at 1,500 bar, the particle size of insulin could be reduced from 15.8 to 7.3 μm, six cycles resulted in a particle size of 3.7 μm $d(0.5)$ (50% of the particles are smaller than the indicated value). Evaluation of the response surface diagram revealed that the homogenization pressure had the highest impact on micronization efficiency, followed by the number of homogenization cycles. Protein stability was maintained during the micronization process as well as bioactivity. Micronization at elevated temperature (75°C) had no effect on protein stability.
Conclusion. High pressure homogenization of protein suspensions can be used as an alternative method for the micronization of proteins without affecting protein stability or bioactivity.

KEY WORDS: bioactivity of insulin; Box-Behnken design; high pressure homogenization; insulin; micronization.

INTRODUCTION

The development of controlled release devices for protein drugs is a challenging task as the protein integrity and bioactivity have to be maintained during all preparation steps (1). Dissolved proteins are prone to a loss of bioactivity resulting from denaturation, which is often caused by contact with interfaces (2). Therefore, formulation processes involving systems such as aqueous solutions or emulsions frequently bear the risk of activity loss (3). A highly promising strategy to reduce such detrimental effects on proteins is to formulate them in the solid state (4–6). It has been demonstrated that solid proteins are stable even in non-aqueous environments, such as organic solvents, and less susceptible to a loss of bioactivity, due to a minimization of protein unfolding (7,8).

However, to take full advantage of using solid proteins for the manufacture of drug delivery systems, strategies have to be developed that guarantee an ultrafine distribution of the protein particles within a matrix. Unfortunately, commercially available protein powders do not always exhibit small and narrow particle size distributions. Therefore, it is highly desirable to employ techniques for protein powder micronization (9).

Although numerous micronization techniques, such as crystallization (10), spray-drying (11), spray-freeze drying (12), supercritical fluid methods (13), lyophilization with subsequent mechanical destruction and jet-milling (14), have been used on a range of solids, several criteria should be considered in the selection of a method to use with proteins: good control over particle size and distribution, process efficiency even in small batch sizes, no need for additional stabilizers or organic solvents thus ensuring biocompatibility, and the preservation of both protein stability and bioactivity. Most of the aforementioned techniques do not meet all of these requirements.

Therefore, we decided on the micronization of proteins by high pressure homogenization in non-aqueous media as an alternative method. This technique has been successfully used to prepare nanosuspensions of water-insoluble drugs such as budesonide (15), clofazimine (16) and bupravaquon (17), but to our knowledge it has not been tested for the micronization of protein suspensions.

This study was designed to investigate the applicability of high pressure homogenization for the micronization of

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non-aqueous protein suspensions. Insulin served as a model protein with triglycerides as a suspension medium to determine the influence of micronization conditions on protein stability and bioactivity. The effects of different process parameters, such as homogenization cycles, pressure and drug loading of the protein suspension, on particle size reduction were evaluated using a Box-Behnken design. As an excellent application, we explored the potential of the technique to micronize proteins in lipids that can serve as a matrix material for drug delivery devices. In a single preparation step, the protein is micronized and a homogenous protein-loaded matrix material can be manufactured. This extension of the protein formulation process was investigated by micronizing insulin in molten glycerol tripalmitate. The effect of micronization conditions on particle size reduction and the impact of increased temperature on protein stability during the process were determined.

MATERIALS

Crystalline bovine insulin was a gift from Sanofi–Aventis (Frankfurt, Germany), Miglyol® 812 and Dynasan 116® were provided by Sasol AG (Witten, Germany). Chloroform was purchased from Merck (Darmstadt, Germany). We acquired the HPLC-grade dichlormethane from Roth (Karlsruhe, Germany), ethanol p.a. and HPLC-grade acetonitrile from Baker (Deventer, The Netherlands) and trifluoroacetic acid from Riedel-De-Haen (Sigma Aldrich, Taufkirchen, Germany). Water was double-distilled and filtered through a cellulose nitrate filter (pore size 0.2 μm, from Sartorius, Göttingen, Germany) prior to use. Phosphate buffer solution (PBS), 0.25% trypsin-EDTA solution (Invitrogen, Karlsruhe, Germany) and the components of the cell culture medium (Dulbecco's Modified Eagle Medium with 4.5 g/l glucose containing 10% fetal bovine serum, 0.1 mM non-essential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10 mM Hepes buffer) were purchased from Gibco (Karlsruhe, Germany). Additional components of the medium, such as 50 μg/ml of ascorbic acid and 0.4 mM proline, were purchased from Sigma Aldrich (Seelze, Germany).

METHODS

Micronization of Insulin Suspended in Miglyol by High Pressure Homogenization

Miglyol® 812 was filtered using a bottle top filter with a pore size of 0.22 μm (Corning Costar, Bodenheim, Germany). Crystals of bovine insulin were weighed according to the experimental design (see Table I) and suspended in Miglyol® 812 using an Ultraturrax (TP 18/10 equipped with a S 25 NK-19G dispersing tool; IKA Laboratory Technology, Staufen, Germany) for 2 min at 10,000 rpm. The resulting suspensions were subsequently micronized by high pressure homogenization employing an APV Gaulin Micron Lab 40 homogenizer (APV Deutschland GmbH, Germany). Applied pressures ranged from 500 to 1,500 bar over 1–6 homogenization cycles. The insulin content of the suspensions varied from 0.2 to 1 mg/ml (Table I).

Table I. Chosen Levels of the Coded Experimental Factors

Variable	Level −1	Center point	Level +1
X_1: homogenization cycles (cyc)	1	4	6
X_2: homogenization pressure (pre), bar	500	1,000	1,500
X_3: drug loading (dru), mg/ml	0.2	0.6	1

Particle Size Determination

The particle sizes of insulin suspensions before and after the homogenization process were analyzed using a Mastersizer 2000 (Malvern instruments, Herrenberg, Germany). For this analysis, the refractive index (RI) of insulin was determined according to the Becke line method (19) by microscopic observation of light refraction behavior of insulin crystals dispersed in immersion oil (RI = 1.52), trans-cimtaldehyde (RI = 1.62) and mixtures thereof. For the size determination, insulin-miglyol-suspensions containing 2 mg insulin were diluted with miglyol to a final insulin concentration of 0.17 mg/ml. Samples were directly injected into the measurement cell, redispersed several times and subsequently measured three times. The measurements were analyzed applying the Mie scattering theory with a refractive index of 1.544 and an absorption of 0.1. The particle sizes are given as $d(0.5)$ (50% of the particle are smaller than the indicated value, representing the median of the particle size distribution). Additional information on the width of the particle size distribution is described by the span, which is defined as: $(d(0.9)-d(0.1))/d(0.5)$.

Morphology of Micronized Insulin Crystals

For the analysis of the morphology and stability, insulin was micronized (concentration of insulin-miglyol-suspension: 1 mg/ml) at 1,500 bar over six homogenization cycles. Subsequently, 1 ml aliquots of the resulting suspension were centrifuged at 4,000 rpm for 20 min. After removal of the miglyol, the obtained crystals were washed four times with 1 ml ethanol and after each washing step the samples were centrifuged for 10 min at 4,000 rpm. Finally, the samples were dried for 30 min using a speedvac concentrator (Speedvac Plus SC110A; Savant Instruments, New York, USA). The particle morphology was investigated using a scanning electron microscope (DSM-950; Zeiss, Oberkochen, Germany). The particles were mounted on aluminium stubs using conductive carbon tape (LeitTabs; Plannet GmbH, Wetzlar, Germany) and coated with a layer of 1.4 nm gold–palladium (Polaron SC515; Fisons surface systems, Grinstead, UK). Micrographs were taken at 10 kV.

Experimental Design

A randomized Box Behnken Design (18) with 15 runs was performed to investigate the effects of the following process parameters on particle size: homogenization pressure, homogenization cycles and drug loading of the insulin-miglyol-suspension. The parameters were investigated on

three levels (see Tables I and II). For the evaluation of the experimental design, the model was fit using multiple linear regression. The PC software MODDE 7.0 (Umetrics, Sweden) was applied for generating the experimental design as well as analysis thereof. The regression coefficients were calculated based on the quadratic polynomial Eq. (1) for the experimental data:

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_1^2 + b_5x_2^2 + b_6x_3^2 + b_7x_1x_2 + b_8x_1x_3 + b_9x_2x_3 + \varepsilon \quad (1)$$

where y is the predicted response, b_0 the constant term, the values b_1–b_3 the linear coefficients and b_4–b_6 the quadratic coefficients, b_7–b_9 the interaction terms and ε is the pure error. After regression analysis of the model by ANOVA, the model was reduced by removing non-significant coefficients.

Determination of the Chemical Stability of Micronized Insulin by HPLC and HPLC-MS Analysis

The structural stability of micronized insulin was determined by a previously described HPLC method (20) and analyzed using a HPLC System with a degasser (Knauer, Berlin, Germany), LC-10AT pump, FCV-10AT$_{vp}$ gradient mixer, SIL-10Ad$_{vp}$ autosampler, CTO-6a oven, SPD-10AV UV-Detector, RF-551 fluorescence detector and SCL-10A$_{vp}$ controller (all from Shimadzu, Duisburg, Germany). One milligram of micronized insulin was dissolved in 1 ml 0.01 M HCl. One hundred microliter of the insulin solution were injected and analyzed at 37°C using a C_{18}-reversed phase precolumn (LC318, 4.6×20 mm; Supelco, Bellefonte, USA) combined with an analytical C_{18}-reversed phase column (Supelcosil, LC318, 4.6×250 mm; Supelco). A linear gradient was applied (mobile phase A: 90% H_2O, 10% acetonitrile, 0.1% TFA; mobile phase B: 90% acetonitrile, 10% H_2O and 0.1% TFA; flow rate of 1 ml/min) by changing the mobile phase B from 20 to 36% over 22 min with a total run time of 30 min. The chromatograms were recorded using UV detection (210 nm and 274 nm) and fluorescence detection (274 nm excitation and 308 nm emission).

For HPLC-MS analysis, the analytical method was carried out in positive ion mode using a Hewlett Packard HPLC system with Series 1100 degasser, binary pump, autosampler, column oven and diode array detector (all from Hewlett Packard, Waldbronn, Germany) coupled with a TSQ7000 electrospray-mass spectrometer (ThermoQuest, San José, CA, USA) with an API2-source (capillary temperature: 350°C, spray voltage: 4.0 kV). The Xcalibur software package (ThermoQuest) was used for data acquisition and analysis. The sample was analyzed with a RP_{18} column (Jupiter, 5 μm, 300 Å, 2×250 mm; Phenomenex, Torrance, USA) and a flow rate of 0.3 ml/min. Insulin and desamidoinsulin were detected in the total ion chromatogram of the mass spectrometer as triple-charged ions.

Determination of Insulin-Bioactivity after Micronization over Six Cycles at 1,500 bar

As previously described, a slightly modified chondrocyte culture assay (21) was used to determine the bioactivity of micronized insulin. Bovine chondrocytes were harvested from knee joints by collecting fresh articular cartilage from the surfaces of the patellar grove under aseptic conditions. The cartilage was cut into small pieces and enzymatically digested by collagenase type II in cell culture medium (21). The cell digest was filtered through a 150 μm filter, centrifuged at 1,200 rpm for 5 min and washed three times with PBS. The cell number was determined by cell counting using a hemocytometer and an inverted phase-contrast microscope. After the third passage, 12,500 cells were seeded in 12-well plates (Corning Costar, Bodenheim, Germany) and placed in an incubator at 37°C, 5% CO_2 and 95% humidity. The cells were cultivated for 3 days in 2 ml cell culture medium with unprocessed or micronized insulin in concentrations of 0.1, 2.5 and 5 μg/ml (n = 5 for each group). One group without insulin supplementation served as control. After 2 days of cultivation, the medium was exchanged. The next day, the cells were washed with 2 ml of PBS and then trypsinized with 1 ml of 0.25% trypsin solution for 30 min at 37°C. The reaction was stopped by the addition of 1 ml cell culture

Table II. Experimental Setup of the Box-Behnken Design and Experimental Results

Run	Pressure [bar]	Cycles	Drug loading [mg/ml]	d(0.5) [μm]	Predicted[a]	Obs–Pred[a]	Conf. int(±)[a]
1	500	1	0.6	10.56	10.62	−0.06	0.42
2	1,500	1	0.6	6.68	6.3	0.38	0.42
3	500	6	0.6	8.22	8.28	−0.06	0.42
4	1,500	6	0.6	3.7	3.96	−0.26	0.42
5	500	4	0.2	8.12	8.16	−0.04	0.42
6	1,500	4	0.2	4.00	3.84	0.15	0.42
7	500	4	1	8.85	8.68	0.17	0.42
8	1,500	4	1	4.09	4.36	−0.28	0.42
9	1,000	1	0.2	6.47	6.67	−0.2	0.42
10	1,000	6	0.2	4.42	4.33	0.09	0.42
11	1,000	1	1	7.07	7.2	−0.13	0.42
12	1,000	6	1	5.09	4.85	0.24	0.42
13	1,000	4	0.6	5.29	5.41	−0.12	0.34
14	1,000	4	0.6	5.62	5.41	0.21	0.34
15	1,000	4	0.6	5.33	5.41	−0.08	0.34
Range	500–1,500	1–6	0.2–1	3.7–10.56			

[a] Prediction of the data with the reduced model.

medium. Cell numbers were determined using a Coulter Counter (Coulter Multisizer II; Beckmann Coulter, Germany) by suspending the cells in 200 ml of isotonic NaCl solution (Coulter Isoton II Diluent; Beckmann Coulter). 2 ml of the cell suspensions were analyzed three times using a capillary with an orifice of 100 μm. Statistical analysis was performed by one-way analysis of variance ANOVA in conjunction with a multiple comparison test (Tukey's test) at a statistical significance level of $p < 0.01$.

Micronization of Insulin Suspended in Glycerol Tripalmitate by High Pressure Homogenization

For micronization of insulin in molten glycerol tripalmitate (Dynasan 116®) of the homogenizer in contact with the product were heated with a heating jacket to 75°C. Glycerol tripalmitate was molten and tempered at 75°C to ensure a constant viscosity of the lipid. 160 mg of bovine insulin crystals were suspended in 32 g molten lipid using an Ultraturrax (heated to 75°C) for 2 min at 10,000 rpm. The resulting suspension was subsequently micronized by high pressure homogenization at 1,000 bar. For determination of particle size and insulin stability, 600 μl of the lipid suspension were transferred to 2 ml Eppendorf cups. The solid insulin/lipid mixture was mixed with 600 μl dichlormethane and centrifuged at 13,200 rpm (Centrifuge 5415R; Eppendorf AG, Hamburg, Germany) for 60 min at room temperature. After Removal of the dichloromethane phase the insulin crystals were washed four times with dichlormethane. For particle size analysis, micronized insulin was suspended in dichlormethane. The measurements were performed as described in section 'Particle Size Determination.'

RESULTS

Morphology of Micronized Insulin

To prove the feasibility of micronizing insulin by high pressure homogenization, an insulin-miglyol-suspension (insulin concentration 1 mg/ml) was micronized using one and six cycles at 1,500 bar. Scanning electron microscopy verified that insulin crystals had been efficiently micronized under the chosen conditions (Fig. 1). The SEM photographs show the insulin crystals before (left) and after six homogenization cycles (right). Compared to the bulk insulin crystals, the micronized insulin crystals have an irregular crystal shape and only fragments with visible cracks of the native crystal structure can be detected.

For measuring the particle size distribution of insulin with laser light scattering, the refractive index of insulin had to be determined. In the literature only an approximated value of 1.515 for insulin was reported (22,23). Analyzing samples with laser light scattering using this estimated refractive index resulted in mean size values significantly different from values obtained experimentally by microscopic observations. Therefore, the refractive index (RI) of insulin was evaluated. The best match to the refractive index of insulin was observed in mixtures with an RI of 1.541–1.544:

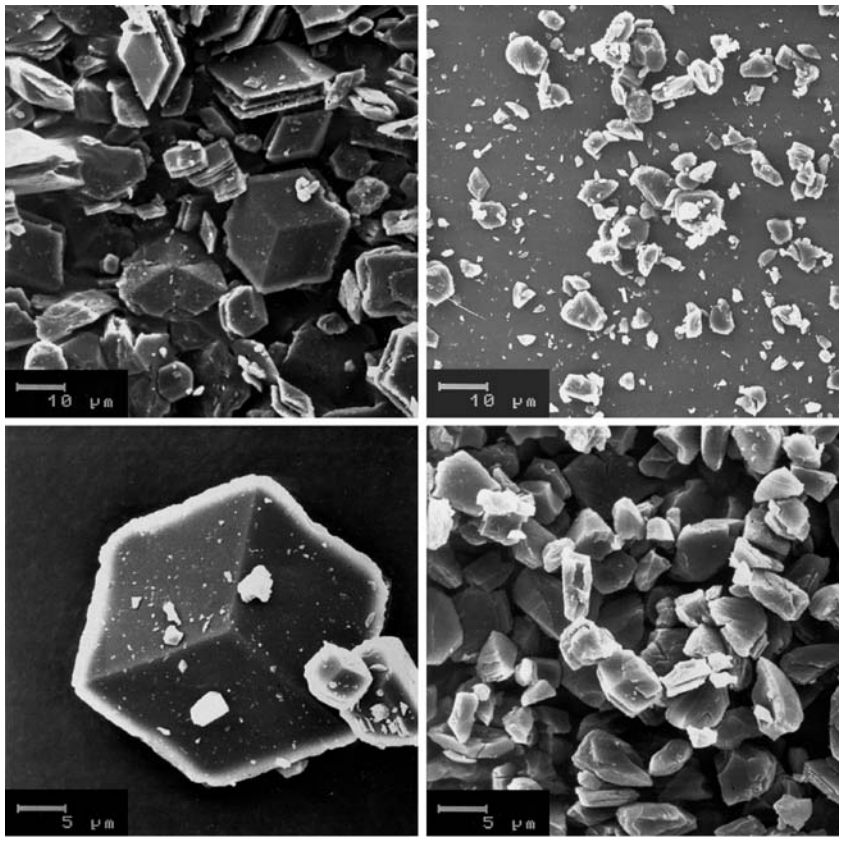

Fig. 1. SEM-pictures of bovine insulin before (*left*) and after six cycles of high pressure homogenization at 1,500 bar (*right*).

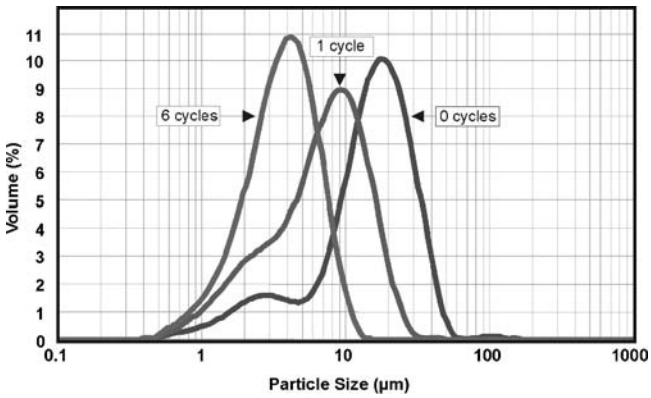

Fig. 2. Particle size distribution of insulin crystals dispersed in Miglyol (span 1.78), insulin crystals homogenized by one cycle at 1,500 bar (span 1.87) and insulin crystals homogenized six times at 1,500 bar in Miglyol (span 1.48) analyzed by laser diffraction (Mastersizer 2000, Malvern Instruments UK). The Span is defined as $(d(0.9)-d(0.1))/d(0.5)$.

the movement of the Becke line by lowering the objective stage was no longer detectable and the lowest contrast (relief) at the edges of the crystals was observed, thus indicating that the refractive index of the dispersion medium was identical to that of the crystal. Analysis of the light scattering raw data and the calculated curve fitting of the particle size measurements showed that applying a refractive index of 1.544 resulted in a close match with particle sizes as observed by scanning electron microscopy. Therefore, all measurements were calculated with a RI of 1.544 and an absorption value of 0.1.

The particle size distribution of the resulting insulin suspensions revealed that the unprocessed insulin crystals with 50% of the particles smaller than 15.8 µm ($d(0.5)$) were reduced to 7.3 µm after one cycle. Applying six cycles led to an even smaller particle size of 3.7 µm (Fig. 2). A reduction in the width of the particle size distribution (span) from 1.78 to 1.48 could be observed as well. The micronization of insulin suspended in miglyol efficiently reduced the particle size. Therefore, in the second step of the investigation the influence of drug loading, homogenization pressure and cycles were investigated by experimental design.

Evaluation of the Experimental Design—Influence of Micronization Conditions on Particle Size Reduction

Statistical evaluation of the obtained experimental data (Table II) was performed with the MODDE 7.0 software and used to generate the fitted polynomial equation (2) for the response by calculating the scaled and centered coefficients (Table III):

$$y = 5.63 - 2.14x_1 - 1.17x_2 + 0.26x_3 + 1.19x_1^2 + 0.47x_2^2 - 0.34x_3^2 - 0.17x_1x_2 - 0.16x_1x_3 + 0.01x_2x_3 + \varepsilon \quad (2)$$

The significance and validity of the model was estimated by analysis of variance (ANOVA) (data not shown). Due to model validity, the evaluation of the significance of the investigated factors and their interactions on the particle size reduction was performed by an *F*-test. The influence of the scaled and centered coefficients of the modeled polynomial Eq. (2) were investigated at a confidence level of 0.95 (data not shown). The linear and quadratic coefficients of the factors significantly affected the response, whereas no significant interaction of the investigated factors on the response was detected. Therefore the interaction terms b_7–b_9 were removed from the model and the regression coefficients were recalculated based on the quadratic polynomial Eq. (3):

$$y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_4x_1^2 + b_5x_2^2 + b_6x_3^2 + \varepsilon \quad (3)$$

The experimental data were analyzed with the reduced model to generate the fitted polynomial Eq. (4):

$$y = 5.63 - 2.16x_1 - 1.17x_2 + 0.26x_3 + 1.19x_1^2 + 0.47x_2^2 - 0.34x_3^2 \quad (4)$$

Analysis of variance (ANOVA) was applied for determining the significance and validity of the revised model (Table III). The regression model fit to the experimental raw data with a goodness of fit (R^2, variation explained by the model) of 0.991 and a high predictive power (Q^2, predicted variation) of 0.964 (18,24). The value of R^2 adjusted (0.983)

Table III. Results of the Analysis of Variance for the Response $d(0.5)$ Significant at Level $p < 0.05$ of the Reduced Model

$d(0.5)$	DF[a]	SS[b]	MS[c] (variance)	F[d]	P	SD[e]
Total	15	639.618	42.641			
Constant	1	582.804	582.804			
Total Corrected	14	56.813	4.058			2.014
Regression	6	56.277	9.379	139.789	**0**	3.063
Residual	8	0.537	0.067			0.259
Lack of Fit (Model Error)	6	0.472	0.079	2.432	**0.32**	0.280
Pure Error(Replicate Error)	2	0.065	0.032			0.180
N[f] = 15		Q2 = 0.964[g]		Cond. no. = 4.383		
DF = 8		r^2 = 0.991		Y-miss = 0		
		r^2 Adj.[i] = 0.983		RSD[j] = 0.259		

[a] Degrees of freedom, [b] sum of squares, [c] mean square, [d] *F*-distribution value, [e] standard deviation, [f] number of experiments, [g] Q2 is the fraction of variation of the response that can be predicted by the model, [h] *R*2 is the percentage of the variation of the response explained by the model, [i] *R*2 Adj is the fraction of variation of the response explained by the model adjusted for degrees of freedom; [j] RSD residual standard deviation.

Table IV. Coefficient List Scaled End Centered of the Reduced Model

Coded in Eq. (1)	$d(0.5)$	Coeff. SC	Std. Err.	P	Conf. int(±)
b_0	**Constant**	5.63	0.15	3.43e-010	0.35
b_1	**pre**[a]	−2.16	0.09	1.11e-008	0.21
b_2	**cyc**[b]	−1.17	0.09	1.31e-006	0.21
b_3	**dru**[c]	0.26	0.09	0.02	0.21
b_4	**pre*pre**	1.19	0.13	2.15e-005	0.31
b_5	**cyc*cyc**	0.47	0.14	0.01	0.33
b_6	**dru*dru**	−0.34	0.13	0.04	0.31

All coefficients represent significant effects at a confidence level of 0.95.
Coefficients that represent significant effects at a confidence level of 0.95 are printed in bold.
[a] Pressure, [b] homogenization cycles, [c] drug loading.

suggests that 98% of the total variation of $d(0.5)$ can be attributed to the independent variables and only 2% of the variation cannot be explained by the model. To determine the significance of the model, an F-test using a significance level of 5% was performed revealing a high significance ($p < 0.005$). The lack of fit measures the failure of the model to represent data in the experimental domain by comparing the model error with the replicate error. As the before mentioned errors are of the same magnitude ($p = 0.320$) the model has no lack of fit.

The effect of the factors on the micronization was investigated by analysis of variance of the coefficients (Table IV). All linear and quadratic coefficients of the factors significantly affected the response. The highest impact on the particle size reduction had the applied pressure in the micronization process followed by the number of homogenization cycles. Drug loading had a lower impact on particle size than applied pressure and numbers of homogenization cycles. Higher drug loading resulted in a lower effective particle size reduction. To visualize the influence of changing the levels of the factors on the response, three-dimensional plots for the measured response were generated. The resulting plots for different drug loading showed no great differences in orientation, therefore only one plot for the highest drug loading is exemplarily shown (Fig. 3). The surface diagram exhibited no local minimum and by choosing the highest levels of the factors with a homogenization pressure of 1,500 bar and six homogenization cycles, the most efficient particle size reduction can be obtained. Additionally, no twisting in the surface stemming from interactions of the investigated factors could be detected, supporting the statistical analysis that no significant contribution of the interaction related coefficients was present. The influence of drug loading on the micronization process was more easily discerned within the 2-D contour plots for the different levels of homogenization pressure (Fig. 4). A reduced micronization efficiency using higher drug loading can be detected at lower homogenization pressure of 500–1,000 bar (Fig. 4), whereas this effect was negligible at higher homogenization pressure.

Chemical Stability and Bioactivity of Insulin under Micronization Conditions

For determining if the chemical stability of insulin was affected during the micronization process, insulin micronized by six homogenization cycles at 1,500 bar was subjected to HPLC-MS analysis (Fig. 5) and compared with unprocessed insulin (data not shown). In the total ion chromatogram (TIC), two peaks could be detected (Fig. 5a). In the mass spectra of the peak with the retention time of 23.9 min (Fig. 5b), the multiple charged ions $(Ins+3H)^{3+}$ and $(Ins+4H)^{4+}$ were detected. The resulting calculated mass of the triple charged ions of 5,734.2 Da was equivalent to that

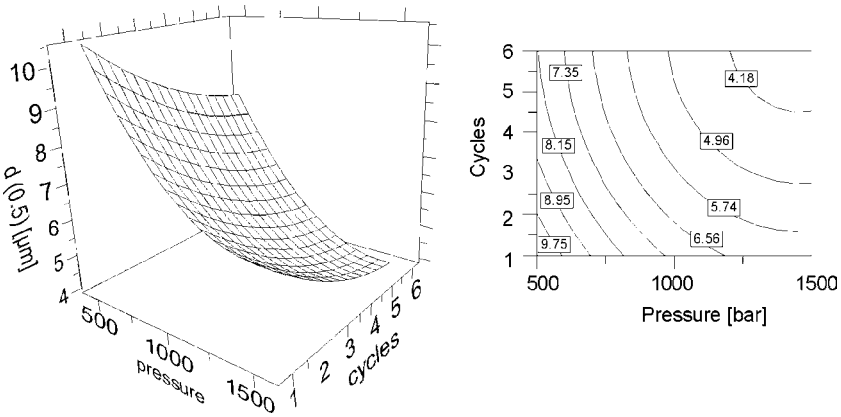

Fig. 3. Response surface diagram (*left*) and contour plot (*right*) of micronization process for particle size $d(0.5)$, as a result of varying the homogenization pressure from 500 to 1,500 bar and applying one to six cycles for a drug loading of 1 mg/ml.

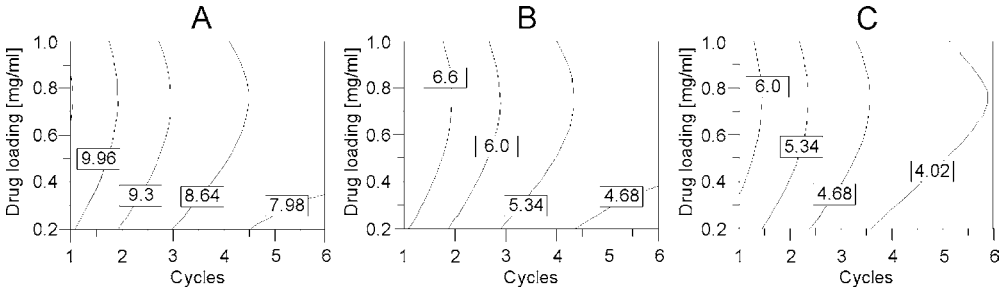

Fig. 4. Contour plot for particle size $d(0.5)$ of micronized insulin at 500 bar (A), 1,000 bar (B) and 1,500 bar (C). Analysis of the contour plot for 500 bar showed that higher drug loading resulted in a reduced micronization efficiency.

of native insulin. From the multiple charged ions detected in the mass spectra for the peak with the retention time of 24.4 min (Fig. 5c), a mass of 5,735.1 was calculated and matched the desamidoinsulin peak found in the bulk substance. The desamidoinsulin content of the bulk material and the micronized insulin was quantified from HPLC investigations to 1.9 and 1.8%, respectively, by calculating the desamidoinsulin/insulin ratio by area. ANOVA analysis revealed that there was no significant increase in desamidoinsulin content caused by the homogenization process. The bioactivity of insulin depends on its chemical integrity as well as secondary and tertiary structure. Therefore, the bioactivity

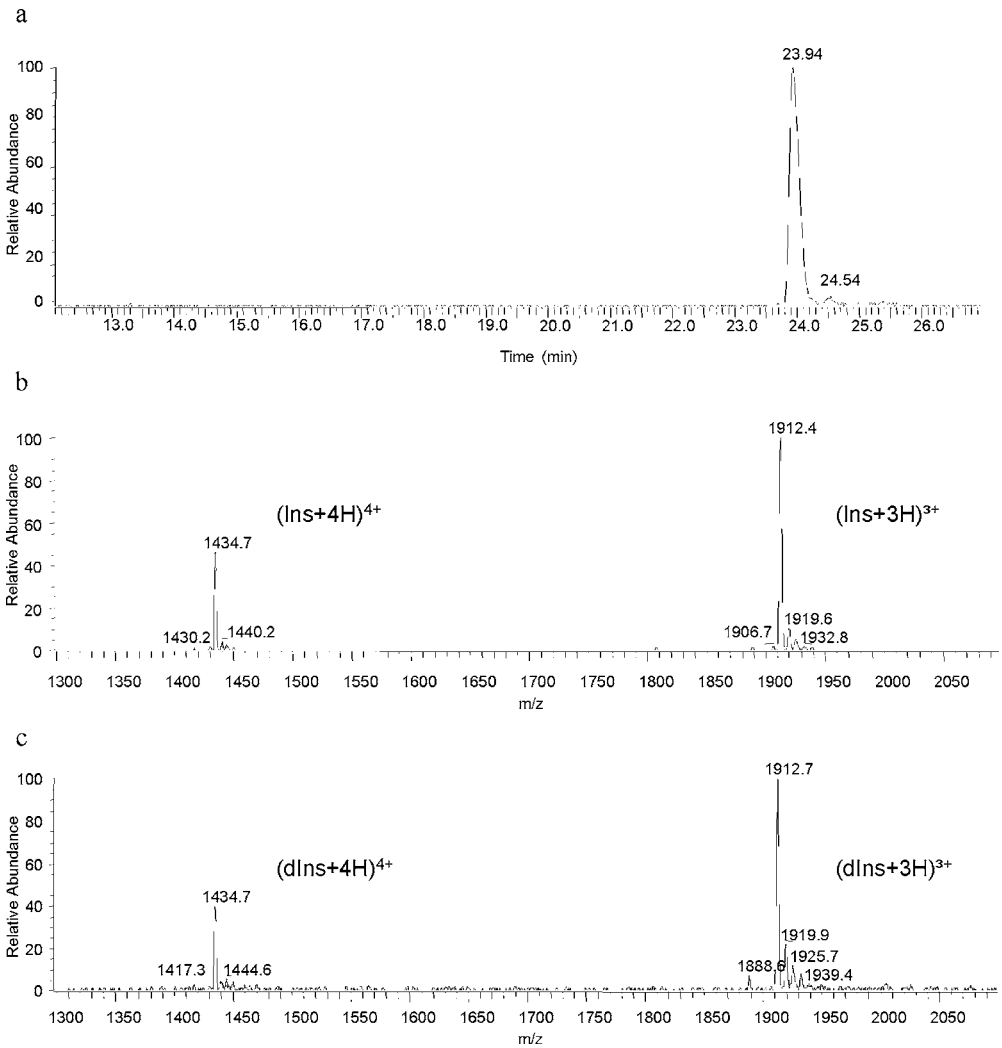

Fig. 5. (a) HPLC-MS total ion chromatogram (*TIC*) of insulin micronized by high pressure homogenization at 1,500 bar over six cycles, (b) electrospray mass spectrum of the peak at the retention time 23.89–24.03 min with a mass of 5,734.2 Da corresponding to intact insulin and (c) electrospray mass spectrum of the peak at the retention time 24.44–24.65 min with a mass of 5,735.1 Da corresponding to desamidoinsulin.

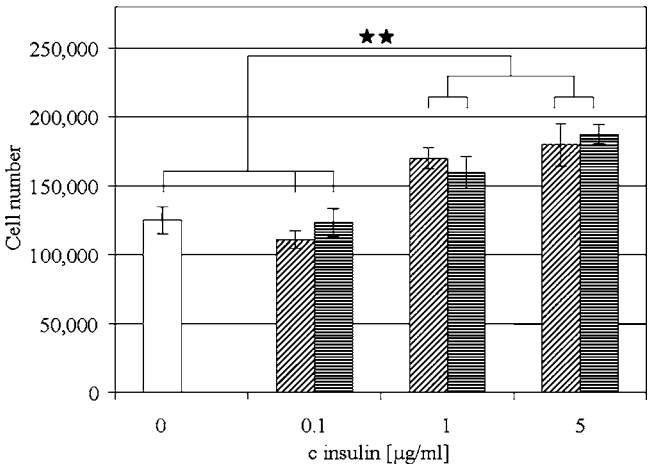

Fig. 6. Determination of bioactivity of insulin before (*diagonally striped bars*) and after high pressure homogenization (six cycles, 1,500 bar; *horizontally striped bars*). The cell number was significantly increased by insulin supplementation >0.1 µg/ml and micronized insulin had the same effects on cell growth as unprocessed insulin (**level of significance $p < 0.01$).

of micronized insulin was tested in a chondrocyte proliferation assay. Micronization of insulin did not lead to any loss of bioactivity in the chondrocyte proliferation assay. While no increase in proliferation was measured in any group supplemented with 0.1 µg/ml insulin, at 1 and 5 µg/ml cell numbers were significantly increased for both unmodified and micronized insulin, as compared to the control group receiving no insulin (Fig. 6). At each insulin concentration, micronized insulin resulted in cell numbers not significantly different from unmodified insulin, indicating the maintenance of bioactivity.

Micronization of Insulin in Dynasan 116

When insulin was micronized in molten glycerol tripalmitate at 75°C by applying 1,000 bar for one homogenization cycle, the particle size analysis showed that the particle size ($d(0.5)$) was reduced from 13.28 to 7.28 µm (Fig. 7). As the stability of insulin might be affected by the homogenization at higher temperature, the micronized insulin was again subjected to HPLC analysis. In the resulting chromatograms,

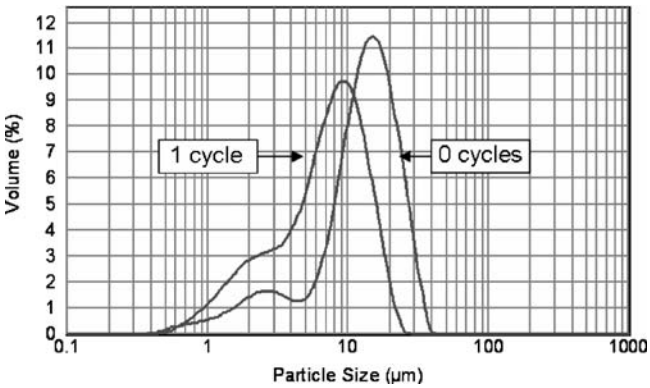

Fig. 7. Micronization of insulin in glycerol tripalmitate. The initial particle size of insulin of 13.28 µm was reduced to 7.28 µm within one cycle of high pressure homogenization at 1,000 bar.

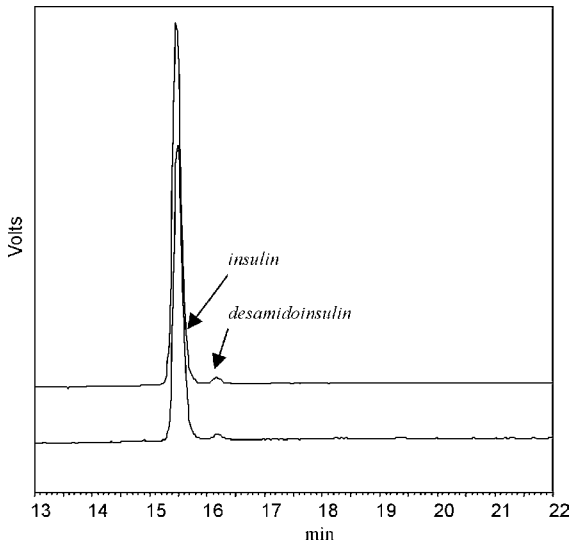

Fig. 8. HPLC chromatogram of unprocessed protein (*bottom*) and insulin micronized with one cycle at 1,500 bar in glycerol tripalmitate (*top*). No changes in the chromatogram related to further degradation could be detected.

only two peaks were detected: insulin (retention time of 15.5 min) and desamidoinsulin (retention time 16.2 min) (Fig. 8).

Substances eluted at these retention times had been previously identified by HPLC-MS (data not shown). The desamidoinsulin content was not significantly increased compared to the bulk insulin.

DISCUSSION

The applicability of micronizing insulin by high pressure homogenization could be demonstrated by the presented experiments. Scanning electron microscopy pictures revealed that the insulin crystals micronized by six homogenization cycles at 1,500 bar were efficiently reduced into small particles. The particle size analysis of micronized insulin particles verified that the crystal size was efficiently reduced from $d(0.5)$ = 15.8 µm to $d(0.5)$ = 3.7 µm accompanied by a decreased span of the size distribution.

For investigating the effect of homogenization pressure, cycle numbers and drug loading on particle size reduction, a factorial design was applied. It revealed that the homogenization pressure had the highest impact on particle size reduction, followed by the number of homogenization cycles. Higher drug loading of the insulin suspension lowered the particle size reduction efficiency when a pressure of 500 to 1,000 bar was applied. This effect resulted from more particles absorbing the disintegration energy, and had been observed in the preparation of nanosuspensions by high pressure homogenization as well (25).

The influence of high pressure homogenization on insulin stability and bioactivity was investigated by HPLC-MS analysis as well as in a chondrocyte proliferation assay. Neither the chemical stability nor the bioactivity of insulin were affected by the micronization at 1,500 bar over six homogenization cycles, the harshest conditions investigated

in this study. Although these results may be surprising, (as it is known that proteins can be denatured by high pressure in hydrophilic solutions by irreversible unfolding or hydration of proteins (26,27) and has been recently considered by the FDA for inactivation of microorganism in food) high pressure does not necessarily result in protein instability, depending on the type of protein, formulation and applied pressure (26). In several studies, high pressure has even been used for crystallization (28), refolding (29) and the dissociation of protein aggregates (30). For some proteins, such as somatostatin and insulin, it has been shown that the chemical integrity of the proteins was preserved under high pressure in the range from 200 to 5,000 bar and a temperature of 20–37°C (31). Furthermore, the bioactivity of insulin incorporated in a w/o emulsion after high pressure homogenization at 500 bar over six homogenization cycles could be maintained (32). The data presented support the assumption that non-aqueous media can maintain the protein stability under high pressure (33).

Further insights into the stability of proteins in lipid material could be obtained by the micronization of insulin by high pressure homogenization of insulin in molten glycerol tripalmitate at 75°C. The particle size ($d(0.5)$) was reduced from 13.3 to 7.3 μm over one cycle at 1,000 bar. To investigate the effect of increased temperature on protein stability under high pressure homogenization, the micronized insulin was analyzed by HPLC. The high temperature did not affect the chemical stability of the protein at high pressure. That proteins in solution can withstand higher temperatures at high pressure results from a volume and enthalpy reduction under high pressure (34). However, there is little known about the effect of high pressure in combination with increased temperature on the stability of solid proteins. Our experiments support the fact that proteins can be stable under high pressure and increased temperature when they are formulated as solids. This is in agreement with a number of related findings described in literature, although little has been published using temperatures as high as 75°C. For example, high pressure is suggested as an alternative method to sterilize insulin powder formulations. Insulin was stable after treatment at 3,000 to 6,000 bar at 25 to 30°C (35). In another investigation, solid lipid nanoparticles loaded with lysozyme were manufactured by high pressure homogenization at 50°C over three cycles at 1,500 bar and it could be demonstrated that the protein stability and bioactivity were maintained (36).

We conclude that high pressure homogenization is a promising alternative for micronizing proteins in small and large scale processes, offering the possibility to micronize proteins in amounts as small as 0.2 mg/ml with little batch-to-batch variation (37) and to transfer the process to industrial batches by using high pressure homogenizers with a capacity up to 1,000 l/h (17).

Furthermore, the presented micronization technique can be used for the direct manufacture of lipid based formulations, processing proteins in the solid state is a straightforward strategy to achieve an ultrafine distribution of the protein particles within a lipid matrix and simultaneously control the protein particle size. This concept can be transferred to s/o/w protein emulsions and suspensions as well as to microencapsulation for preparing microparticles and implants.

CONCLUSIONS

Using insulin as a model protein, we could demonstrate that the high pressure homogenization of protein suspensions is an alternative method to micronize proteins. Effective micronization of insulin could be shown with different combinations of the process parameters: homogenization pressure, homogenization cycles and drug loading. Despite concerns regarding protein stability caused by micronization conditions, we verified that the stability and bioactivity of insulin micronized at 1,500 bar over six cycles was not affected.

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