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European Journal of Pharmaceutics and Biopharmaceutics 62 (2006) 260–266
European Journal of Pharmaceutics and Biopharmaceutics
www.elsevier.com/locate/ejpb

Research paper

Evaluation of the potential of air jet milling of solid protein-poly(acrylate) complexes for microparticle preparation

Wolfgang Schlocker, Siegfried Gschließer, Andreas Bernkop-Schnürch *

Department of Pharmaceutical Technology, Institute of Pharmacy, University of Innsbruck, Innsbruck, Austria

Received 29 March 2005; accepted in revised form 1 September 2005
Available online 24 October 2005

Abstract

It was the aim of this study to evaluate the potential of air jet milling for the preparation of protein-loaded microparticles in industrial quantities. The model protein horseradish peroxidase was incorporated via co-precipitation in carbomer (NaC934P) (1:100) and a poly(methacrylate) (Eudragit®L100-55) (1:100) used as carrier matrix. Co-precipitation of the model protein and each polymer in aqueous solution was achieved either by a pH-shift or by the addition of various non-solvents. Dried protein/polymer complexes (desiccator under vacuumization at 4 °C with silica blue gel) were ground with an air jet mill and resulting microparticles were investigated regarding protein load, remaining protein activity, size distribution and shape. Results of this study showed that the polymer used and the method of co-precipitation has a great impact on protein load. Using carbomer a maximum protein load of 60 ± 1% was achieved, whereas in case of Eudragit® L100-55 the maximum was 78 ± 5% (means ± SD; $n = 3$–4). Using petroleum ether, isopropanol or tetrahydrofurane as non-solvents led to significantly higher protein loads than a pH-shift from 7 to 5, 4 and 3.5, respectively. Determination of the remaining protein activity after milling showed, that the grinding air pressure (GAP) has a major impact on protein stability. In case of Eudragit®L100-55 at a GAP of 4.5 bar peroxidase activity was almost completely lost, whereas 42 ± 1% loss in activity was determined at a GAP of 2.5 bar. The mean particle size of protein/carbomer and protein/poly(methacrylate) particles was determined to be 3.6–5.2 and 4.5–8.7 μm at a GAP of 2.5 bar and 2.7–3.1 and 2.4–3.1 μm at a GAP of 4.5 bar, respectively. Generally, 90% of all particles were in the range of 3–16 μm. All particles were of spherical shape exhibiting a non-porous surface.

According to these results, air jet milling seems to represent a novel method for the large-scale production of protein drug loaded microparticles.

Keywords: Air jet mill; Carbomer; Microparticles; Eudragit®; Protein complexation; Micronization

1. Introduction

Continually increasing shares of therapeutic agents are peptides and proteins. Due to a substantial progress in biotechnology and recombinant genetic engineering, the production of therapeutic polypeptides became already feasible in significant economic quantities. Apart from their parenteral administration, the development of non-invasive peptide and protein delivery systems via the pulmonal, nasal and even oral route has received considerable attraction within recent years. A promising strategy to overcome barriers being encountered with mucosal drug delivery such as the absorption and enzymatic barrier is the use of multifunctional polymers displaying enzyme inhibitory, permeation enhancing as well as mucoadhesive properties protecting embedded therapeutic peptides and proteins towards an enzymatic attack and improving drug uptake [1–6]. Moreover, due to the incorporation of therapeutic peptides and proteins in polymeric excipients their storage stability can in many cases be significantly improved [7].

The efficacy of multifunctional polymers for the mucosal administration of therapeutic proteins can be further improved by formulating polymer protein complexes to micro- or nanoparticles. Utilizing particulate formulations, a greater mucosal surface area can be reached [8,9] and a prolonged residence time at the site of drug absorption is provided [10,11].

The production of micro- and nanoparticles, however, is on the one hand in most cases extensive in the realisation, material- and cost-intense and in addition, an inactivation of the active ingredient during the production of micro- and nanoparticles may occur. On the other hand, problems often occur at the scale up process for the production of industrial quantities.

* Corresponding author. Department of Pharmaceutical Technology, Institute of Pharmacy, University of Innsbruck, Innrain 52d, A-6020 Innsbruck, Austria. Tel.: +43 512 507 5371; fax: +43 512 507 2933.
E-mail address: andreas.bernkop@uibk.ac.at (A. Bernkop-Schnürch).

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doi:10.1016/j.ejpb.2005.09.001

It was therefore the aim of this study to develop a simple microparticle production method, which allows the preparation of microparticles for peptide and protein drugs in commercial quantities without loosing activity of the incorporated therapeutic agent during the manufacturing process. As model protein, horseradish peroxidase was chosen for analytical reasons. Drug-loaded solid particles were generated by co-precipitation of the protein with a neutralized carbomer (NaC934P) and a poly(methacrylate) (Eudragit L 100-55), representing the carrier matrix and grinding via an air jet mill [12]. Resulting microspheres were analysed regarding particle size and shape as well as protein activity and drug load.

2. Materials and methods

Unmodified carbomer (Carbopol 934P) and Eudragit L 100-55 (Röhm Pharma, Darmstadt, Germany) were used as received.

2.1. Preparation of the sodium salt of carbomer 934P

Carbomer (Carbopol 934P, Noveon, Raubling, Germany) was neutralized by gradually suspending 10 g of the polymer in 100 ml of 4% (m/m) sodium hydroxide methanolic solution while vigorously agitating with a magnetic stirrer. The resultant sodium salt was separated by filtration, washed with methanol until the pH of the filtrate was found to be neutral, and dried in a desiccator. The neutralized polymer NaC934P was stored at room temperature until use [14].

2.2. Incorporation of the model protein into the neutralized carbomer

First, 2.00 g of NaC934P were hydrated in 50 ml of demineralised water until a homogeneous gel was formed. To this gel, 20 mg of peroxidase (55.000 Units, Sigma Aldrich, St Louis, MO) dissolved in 2 ml of demineralised water was added. Thereafter the peroxidase containing carbomer-gel was precipitated by the addition of the following non-solvents: isopropanol, isopropanol/cyclohexane 3:1, isopropanol/cyclohexane/acetone 3:1:0.5 and tetrahydrofurane, which were chosen because of their non-solvent character for both the polymers and the protein, low toxicity and cost effectiveness.

First, 250 ml of non-solvent were added under stirring. The solution was incubated for 14 h at 4 °C in order to achieve a quantitative co-precipitation of the complex.

The liquid phase was substituted three-times by 250 ml of non-solvent followed by the proceeding as described above. The precipitate was dried in a desiccator under vacuumization at 4 °C with silica blue gel up to the constant weight. The dried model protein/carbomer complex was ground carefully in a mortar to a size of approximately 1.5 mm (the highest particle size for the jet mill) and stored at −18 °C until further use.

2.3. Incorporation of the model protein to the poly(methacrylate)

2.3.1. pH mediated co-precipitation

First, 2.00 g of Eudragit L 100-55 was dissolved in 13 ml of isopropanol. To this solution, 20 mg of peroxidase dissolved in 50 ml of demineralised water were added under strong agitating. Co-precipitation was achieved by the addition of 1 M HCl until a pH value of 3.5, 4.0, and 5.0 was reached, respectively. The co-precipitation product was dried, ground, and stored as described above.

2.3.2. Non-solvent mediated co-precipitation

First, 2.00 g of Eudragit L 100-55 were dissolved in 13 ml of isopropanol. Peroxidase (20 mg) was dissolved in few milliliters of isopropanol/water 40:60 and mixed with the Eudragit solution. Co-precipitation was achieved by the addition of 80 ml of petroleum ether under agitation. The co-precipitation product was dried, ground, and stored as described above.

2.4. Determination of model protein load

The amount of peroxidase incorporated in the dried protein/carbomer and protein/poly(methacrylate) complex was determined via TNBS (2,4,6-trinitrobenzenesulfonic acid) test. First, 5 mg of each protein/polymer complex were hydrated in 500 μl of 0.5% sodium chloride solution. Thereafter, 100 μl of each sample were transferred in the first well of a microtitration plate (Greiner 96, Greiner Bio-one, Vienna) and diluted in 1:2 steps with 0.5% sodium chloride in the following five wells. Then, 100 μl TNBS solution (200 μl 5% TNBS and 9.8 ml 8% $NaHCO_3$—solution) were added to each well. The reaction was allowed to proceed in the dark at 37 °C for 90 min. Afterwards the absorption was measured at 450 nm with a microtitration plate reader (Fluostar/Polarstar Galaxy, BMG Labtechnologies, Vienna). Increasing amounts of peroxidase dissolved in 0.5% sodium chloride solution served as standard curve.

2.5. Preparation of microparticles via air jet milling

The experiments were accomplished on a Hosokawa Alpine Aeroplex spiral jet mill 50 AS (diameter and height of grinding chamber 50 and 4.5 mm; standard blowing out nozzle; number of nozzles 4; nozzle diameter 0.8 mm; nozzle pitch 50°; solid feed rate 0.1–1 kg/h) equipped with a temperature sensor (Lutron, DH-802C, Ming Chuan, Taiwan) [13,15,16]. The injector air pressure was 7.5 bar and the grinding air pressure (GAP) was 2.5 or 4.5 bar, respectively. Dependent on the injector pressure, each 1 g of the protein/polymer complex was ground for 10 min. The mill material was kept at −18 °C until further use.

2.6. Particle size distribution

The particle size distribution was determined using a laser diffraction particles size analyzer (‘analysette 22’ compact

version, Fritsch GmbH, Idar Oberstein). Low viscous silicone oil WACKER AK 10 (viscosity 10 mPas ± 10% (25 °C), Wacker/Hüls, Nünchritz, Germany), was used as dispersing vehicle. The particle suspensions were prepared with an ultrasonic stick (Dr Hielscher GmbH, ultrasonic processor UP200H) prior to analysis. In the measurement cell, the use of a propeller mixer (dispersion equipment, Fritsch GmbH, Idar Oberstein) facilitated the continuous flux of particles. For calculations of particle size determinations the Fraunhofer model was used.

2.7. Determination of particle morphology

The morphology of the samples was visualized with a scanning electron microscope (JSM 5310LV, Jeol, Japan) operating at 25 keV.

2.8. Measurement of peroxidase activity

Peroxidase activity in microparticles was determined in the same way as described for the determination of model protein load. Merely instead of the TNBS solution 150 µl of a peroxidase substrate solution (36 mg of o-phenylendiamine dihydrochloride (Sigma-Aldrich, St Louis; MO) dissolved in 18 ml of 0.1 M phosphate buffers saline (PBS) pH 6.7 and 36 µl 30% hydrogen peroxide) were added. After 5 min incubation at room temperature the enzymatic reaction was stopped by the addition of 50 µl of 2 M HCl. The absorption was measured at 492 nm with the microtitration plate reader.

Increasing amounts of peroxidase dissolved in 0.1 M PBS pH 6.7 and analysed as described above served as standard curve.

In order to evaluate the protective effect of the microparticulate formulation on the stability of peroxidase, the activity loss in simple physical protein/polymer mixtures being micronized as described above was determined in comparison. For this study, 10 mg of peroxidase were homogenized with 990 mg carbomer (Turbula T2A mixer, WAB, Basel, Switzerland; 72 rpm, 10 min), ground via air jet milling with a GAP of 2.5 bar and analysed as described above.

In addition, the influence of pH on peroxidase activity was determined. For this study, 1 mg of peroxidase was dissolved in 1 ml of 0.1 M PBS (pH 6.7). The pH was adjusted to pH 2.8, 4.0 and 5.0 by the addition of 1 M HCl. Samples were incubated at 37 °C. At predetermined time points aliquots were withdrawn, diluted 1:20 with 0.1 M PBS (pH 6.7) and analysed as described above.

2.9. Statistical data analysis

Statistical data analysis was performed using the Student *t*-test or ANOVA with $P < 0.05$ as the minimal level of significance.

3. Results and discussion

3.1. Influence of the preparation method on microparticle characteristics

3.1.1. Incorporation of the protein during co-precipitation—drug load

As the drug load is an important parameter for microparticle preparation providing valuable information about the efficacy of the preparation method used, it was determined within this study. Orientating studies showed that the inserted drug gets primarily lost during the co-precipitation process, whereas the loss in model protein is marginal during the milling process. Hence, the influence of various parameters on the loss of the model protein during the co-precipitation process was investigated in detail.

Results of this study demonstrated that the polymer used as well as the type of added non-solvent has a great impact on the resulting drug load. Focusing on the influence of the carrier matrix used the results showed that by using carbomer a maximum protein load of 60 ± 1% (mean ± SD; $n = 4$) could be achieved, whereas in case of Eudragit® L 100-55 a maximum protein load of 78 ± 5% (mean ± SD; $n = 3$) was feasible.

Apart from the influence of the polymer also the way of co-precipitation had a significant influence on protein load. In Fig. 1 the impact of petroleum ether- and pH-mediated co-precipitation on the protein/poly(methacrylate) complex is shown. At higher pH values, comparatively more peroxidase was incorporated in the polymer.

As in case of carbomer, a pH-mediated co-precipitation was not feasible, the influence of various organic non-solvents on the drug load was investigated in more detail. Results of this study, however, showed no significant differences in the protein load (data not shown).

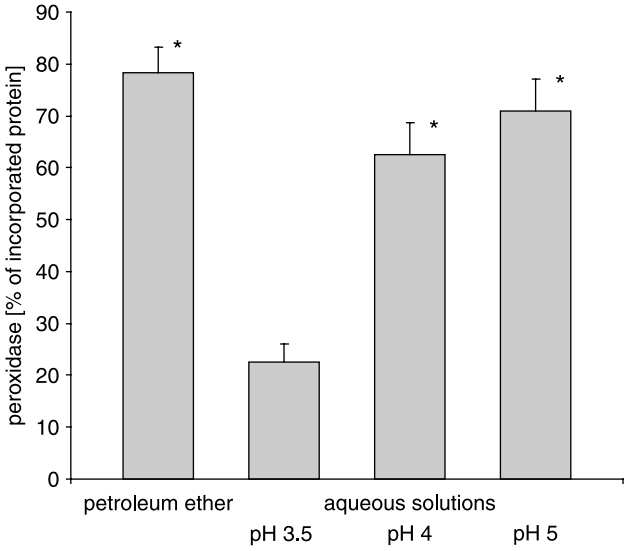

Fig. 1. Influence of non-solvent and pH-mediated co-precipitation process on the amount of protein being incorporated in Eudragit® L 100-55. Indicated values are means ± SD of at least three experiments. *, differs from co-precipitation mediated at pH 3.5 with $P < 0.001$.

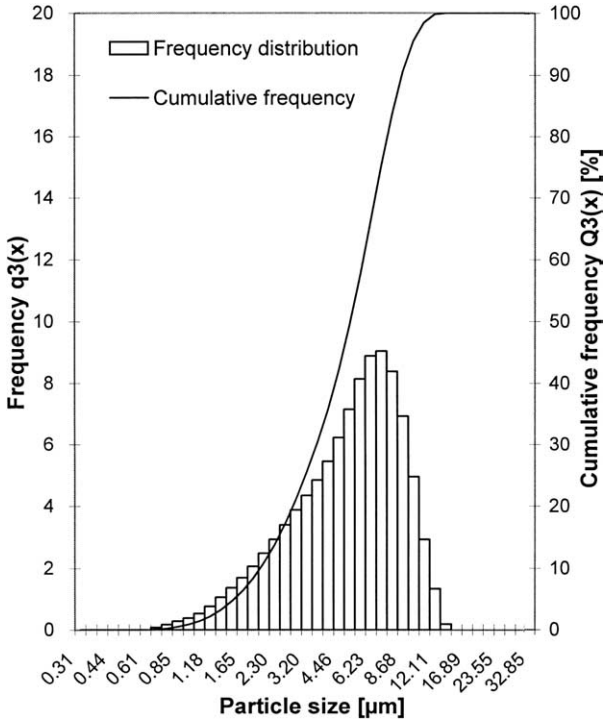

Fig. 2. Cumulative frequency and histogram of frequency distribution of protein/NaC934P complex precipitate in isopropanol, prepared via jet mill GAP 2.5 bar.

3.1.2. Preparation of microparticles

Small particle sizes with a narrow particle size distribution can be achieved by the use of air jet mills [12]. The grinding air pressure (GAP) is thereby the dominating parameter for the resulting particle size [13]. Results obtained within this study are in good accordance with this theory. Analyses of microparticles revealed that a higher GAP leads to a more pronounced fineness and a narrower particle size distribution. In Fig. 2, a representative size distribution of microparticles obtained via jet milling is shown. Even the products ground with a GAP of 2.5 bar led to particles of a mean size <10 µm as listed in Tables 1 and 2. The influence of GAP is shown in Tables 1 and 2 as well. In case of the protein/poly(methacrylate) complex the GAP had a greater impact on the resulting particle size than in case of the protein/carbomer complex.

Table 1
Influence of non-solvents and grinding air pressure on particle size distribution of NaC934P/peroxidase microparticles

Non-solvent	GAP (bar)	Mean particle size $D_{50}\pm$SD (µm), $n=3$	$\bar{S}_r$
Isopropanol	2.5	5.2±0.2	2.3
	4.5	2.7±0.1	1.6
Isopropanol/ cyclohexane 3:1	2.5	3.6±0.4	2.0
	4.5	2.9±0.3	1.7
Isopropanol/ cyclohexane/acetone 3:1:0,5	2.5	4.1±0.9	2.1
	4.5	3.1±0.1	1.8
Tetrahydrofurane	2.5	3.8±0.2	2.2
	4.5	2.7±0.1	1.7

$\bar{S}_r$ mean standard deviation of the particle size distributions.

Table 2
Influence of non-solvents and grinding air pressure on particle size distribution of Eudragit L 100-55/peroxidase microparticles

Non-solvent		GAP (bar)	Mean particle size $D_{50}\pm$SD (µm), $n=3$	$\bar{S}_r$
Aqueous solution	pH 3.5	2.5	8.3±1.4	2.9
		4.5	2.4±0.3	1.6
	pH 4.0	2.5	8.7±0.4	3.0
		4.5	3.1±0.2	1.8
	pH 5.0	2.5	7.2±0.3	2.8
		4.5	2.8±0.1	1.7
Petroleum ether		2.5	4.5±0.6	2.3
		4.5	2.8±0.2	1.6

$\bar{S}_r$ mean standard deviation of the particle size distributions.

The sizes listed in Tables 1 and 2 were confirmed by scanning electron microscopy. As shown in Fig. 3 microparticles showed a non-porous, smooth surface and were of spherical shape. Utilizing a comparatively small jet mill as described within this study allows the grinding of 1000 g per hour, which is already in an industrial production range given that the upstream complexation process is optimized accordingly.

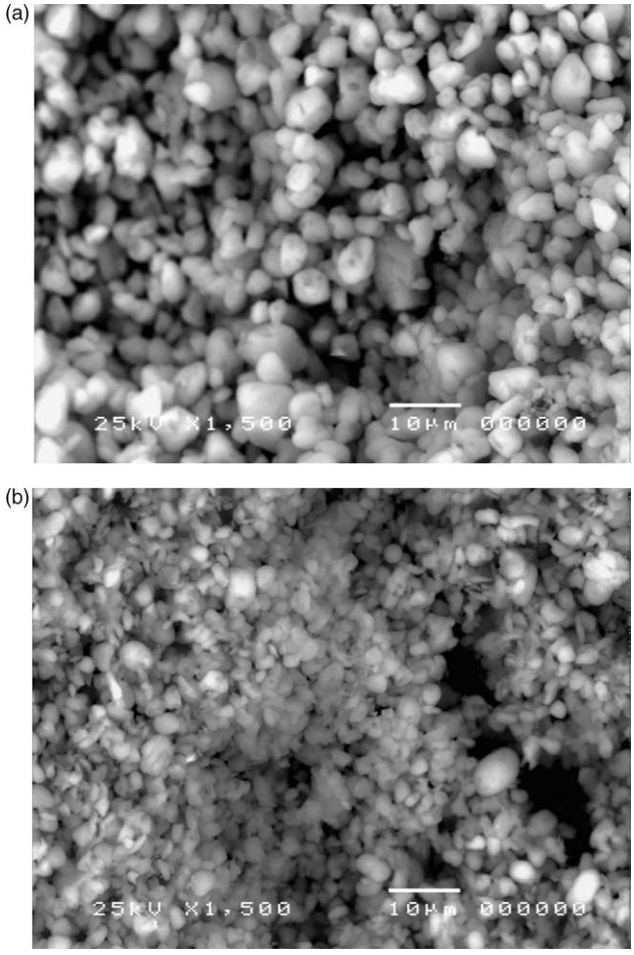

Fig. 3. SEM of the protein/NaC934P complex ground in a jet mill at GAP 2.5 bar (a) and GAP 4.5 bar (b).

The most frequently used alternative techniques for production of large scale protein drug loaded micro- and nanoparticles are over all based on inverse (w/o) emulsion polymerization, co-precipitation [18], spray drying [17], precipitation polymerization in an organic solvent [19–21] and water in oil emulsification solvent evaporation techniques [22].

Previous studies demonstrated that a controlled drug release out of poly(acrylate) microparticles having been produced in different ways can be easily achieved by making use of ionic interactions between the polymeric excipient and the embedded protein drug. Leitner et al., for instance, showed a controlled release of human growth hormone out of poly(acrylate) microparticles within 6 h [23]. Utilizing these microparticles for in vivo studies, their potential for non-invasive protein delivery could already be demonstrated. A significantly improved nasal uptake of this peptide drug was achieved [23]. This effect could even be strongly further improved by utilizing thiolated poly(acrylic acid) instead of the unmodified version. An explanation for this further improved effect can be given by the comparatively higher permeation enhancing and mucoadhesive properties of the thiolated polymer [24]. The microparticle production method described here should also allow the large-scale production of protein-loaded microparticles being based on thiolated polymers.

3.2. Influence of the preparation method on protein integrity

Peroxidase is a relatively unstable protein. In the present study, it could be shown that peroxidase degrades in solution over time. This effect is even more pronounced in the acidic environment. After 90 min incubation in aqueous solution at pH 2.8, almost no activity was remaining (data not shown). This sensitivity of peroxidase to high proton concentrations seems to be the reason for the comparatively high activity loss in case of pH-mediated co-precipitation of the protein/poly(methacrylate) complex as shown in Fig. 4. The more acid the pH value of the precipitating agent was, the lower was the remaining activity. Merely at a co-precipitation at pH 5.0 a significant protein activity could be maintained.

Apart from the influence of the pH also the non-solvent used had a great impact on the protein activity. In case of protein/poly(methacrylate) complexes a comparatively much higher activity could be maintained by using petroleum ether as non-solvent than acidic aqueous solutions. Precipitation with petroleum ether led to a remaining activity of $67 \pm 4.7\%$ ($n = 3$). This observation might be attributed to the insolubility of peroxidase in petroleum ether. In comparison, the protein dissolves to a relative high extent in water. The activity of unground protein/carbomer complexes derived from four different precipitation methods are shown in Fig. 5. Isopropanol as best precipitant leads to a remaining activity of approximately 90%.

Apart from this pH- and solvent dependent instability, peroxidase is also sensitive to higher temperatures. Orientating studies demonstrated, for instance, that the protein is completely inactivated by heating aqueous protein solutions up to 100 °C for 2 min. The influence of thermal stress during

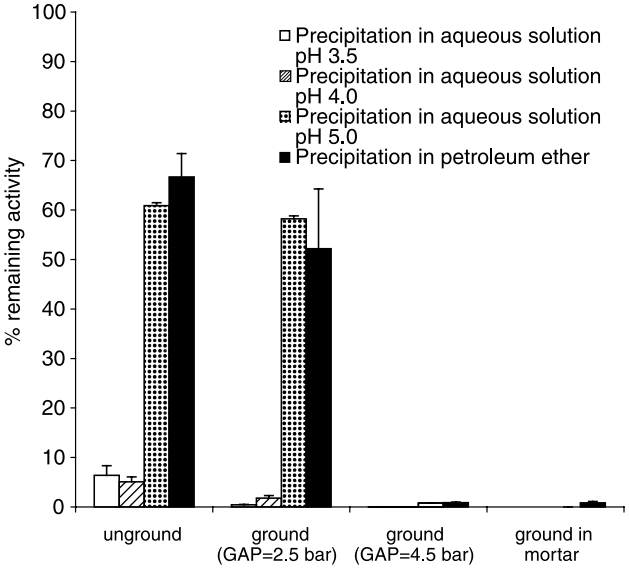

Fig. 4. Activity of peroxidase/poly(methacrylate) complexes, unground, ground in a jet mill and ground in a mortar for 10 min. Indicated values are means ± SD of at least three experiments.

the milling process on peroxidase activity was therefore investigated. It was assumed that through the collision of the particles during the grinding process high temperature tops—designated hot spots—arise [25]. The significant reduction of the model protein activity during the grinding process in particular of unprotected peroxidase in the physical powder mixture as shown in Fig. 6 would support this theory. Temperature measurements during the milling process, however, revealed that the temperature inside the milling chamber even decreased by 2 °C. This observation can be

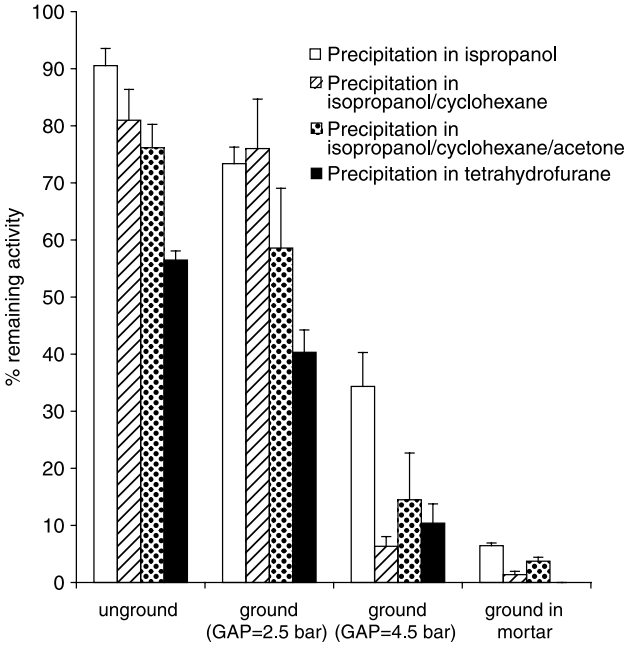

Fig. 5. Activity of peroxidase/carbomer complexes, unground, ground in a jet mill, and ground in a mortar for 10 min. Indicated values are means ± SD of at least three experiments.

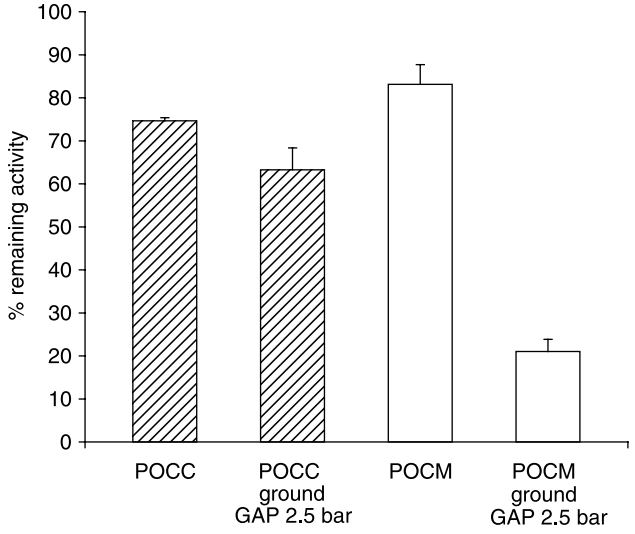

Fig. 6. Comparison of peroxidase activities in peroxidase/carbomer complex (POCC) and physical mixtures of peroxidase/carbomer (POCM) before and after grinding via air jet mill at a GAP of 2.5 bar.

explained by the expansion of air compensating the share-forces derived heating process. As hot spots could nevertheless not be excluded, a decrease in peroxidase activity during the milling process due to temporary and local high temperatures is possible.

Another parameter strongly influencing protein activity is the grinding air pressure during jet milling as illustrated in Fig. 5. Generally, peroxidase activity decreased during the grinding process. Utilizing isopropanol for co-precipitation and a GAP of 2.5 bar the remaining activity, for instance, was determined to be $75 \pm 3\%$, $n = 3$. At higher GAP, the measured loss in activity was even higher. This is demonstrated with a remaining activity of approximately $35 \pm 6\%$ ($n = 3$). Protein/carbomer microparticles, which were manufactured in the air jet mill, showed higher activity than those microparticles, which were obtained in the mortar by grinding where a remaining activity of $6 \pm 1.7\%$ ($n = 3$) was determined.

In case of protein/poly(methacrylate) complexes the influence of the GAP was even more pronounced. As shown in Fig. 4, independently from the preparation method the activity of peroxidase was completely lost by applying a GAP of 4.5 bar during the jet milling process. In addition, no activity remained by grinding in the mortar for 10 min.

4. Conclusions

Within this study, the potential of jet milling for large-scale protein-loaded microparticle production has been evaluated. Results demonstrated that the way of co-precipitation has a much greater impact on protein stability than the milling process itself. In particular at a low grinding air pressure of 2.5 bar no activity at all was lost during the milling process, when peroxidase was incorporated in carbomer via co-precipitation using an isopropanol/cyclohexane solution as non-solvent. According to these results, the microparticle preparation method described here might be a new tool for the production of protein loaded particulate drug delivery systems.

Acknowledgements

The Austrian Nano-Initiative co-financed this work as part of the Nano-Health project (no. 0200), the sub-project NANO-0204 being financed by the Austrian FWF (Fonds zur Förderung der Wissenschaftlichen Forschung) (Project no. 0204-NAN).

The authors acknowledge Dr A. Saxer, Institute of Betonbau, Baustoffe and Bauphysik for the SEM micrographs.

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