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Identifying critical process steps to protein stability during spray drying using a vibrating mesh or a two-fluid nozzle

Niels Grasmeijer, Valeria Tiraboschi, Herman J. Woerdenbag, Henderik W. Frijlink, Wouter L.J. Hinrichs*

Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, the Netherlands

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ABSTRACT

The aim of this study was to identify critical steps to protein stability during spray drying using two different nozzle types: a vibrating mesh nozzle and a standard two-fluid nozzle in a Büchi B-90 spray dryer. L-Lactic dehydrogenase was used as a model protein as it is a heat and shear stress sensitive protein. Trehalose was used as excipient because of its excellent stabilizing capacities. The entire spray drying process was split up into smaller steps and after each step the enzymatic activity of the protein was measured. With the vibrating mesh nozzle in total 78% of activity was lost. About 68% was due to atomizing and heating and 10% was caused by dehydration and circulation of the liquid. With the two-fluid nozzle the total activity loss was only 23%, to which atomization, dehydration, and circulation contributed almost equally. Heating was not an issue, as the two-fluid nozzle could be cooled with water. In conclusion, the type and the configuration of the nozzle used for spray drying are important determinants for maintaining protein stability, as atomizing, heating, ultra-sonication, and recirculation of the feed solution negatively influence it. The possibility to cool the two-fluid nozzle offers an important advantage to the vibrating mesh nozzle in the spray drying process of proteins. In this study, we show that, next to the optimization of the formulation, optimization of the spray drying process should be taken into account to maintain protein stability.

1. Introduction

Therapeutic proteins are increasingly used in clinical pharmacy, usually by parenteral administration. Aqueous protein solutions used for injection are often unstable and may require continuous refrigerated conditions during storage and transport. This so-called cold chain is expensive and logistically troublesome especially in third world countries.

Stable solid formulations of proteins can be prepared by incorporating them in a glassy sugar matrix by spray drying. The matrix stabilizes the protein by vitrification as well as by water replacement (Carpenter and Crowe, 1989; Chang et al., 2005; Chang and Pikal, 2009; Grasmeijer et al., 2013; Hancock et al., 1995; Hinrichs et al., 2001; Sampedro and Uribe, 2004; Tonnis et al., 2015). Spray drying is, however, a delicate process since the protein is in solution during a significant part of it. In the framework of process control and thus for the ultimate product quality, it is important to identify critical steps in the spray drying process that can negatively influence protein integrity.

In the spray drying process a number of subsequent steps can be distinguished: transportation of the liquid solution to the spray head, heating of the liquid solution during transport, atomization of the liquid solution into droplets, drying of the droplets, and collection of the dried particles. During each step, the protein may undergo degradation as a result of various forms of stress of which heat, shear, dehydration, and interfacial stresses are the most important ones (Adler and Lee, 1999; Ameri and Maa, 2006; Maltesen and van de Weert, 2008). The aim of our study was to systematically identify the contribution of each step in relation to the maintenance of protein integrity during spray drying process by splitting it up into the different steps as mentioned above.

Currently popular is the Büchi B-90 spray dryer which employs a vibrating mesh to disperse the protein solution into the heated drying air. From inhalation studies it is known that the formation of aerosols during the dispersion step can be detrimental to proteins in solution (Khatri et al., 2001). To investigate whether the protein stability during the spray drying process can be improved by using a different type of nozzle, a vibrating mesh and a two-fluid nozzle were tested in combination with the B-90 spray dryer. As a model protein, L-Lactic dehydrogenase (LDH) was used because of its high sensitivity to shear and

* Corresponding author.
E-mail addresses: h.j.woerdenbag@rug.nl (H.J. Woerdenbag), h.w.frijlink@rug.nl (H.W. Frijlink), w.l.j.hinrichs@rug.nl (W.L.J. Hinrichs).

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temperature stresses (Adler and Lee, 1999; Hertel et al., 2014; Niven and Brain, 1994; Niven et al., 1994). The primarily focus of this study was to investigate the effect of different spray dryer configurations and not on the underlying mechanisms of protein degradation. Therefore, only the remaining enzymatic activity was considered.

2. Materials and methods

2.1. Materials

L-lactic dehydrogenase (Type XI from rabbit muscle, 600–1200 units/mg of protein), bovine serum albumin, sodium pyruvate, and β-nicotinamide adenine dinucleotide, reduced disodium salt hydrate (NADH) were purchased from Sigma-Aldrich Co. (St. Louis, Missouri). Disodium hydrogen phosphate and sodium dihydrogen phosphate were purchased from Merck (Darmstadt, Germany). Sodium dodecyl sulfate was provided by BUFA B.V. (Uitgeest, The Netherlands). Trehalose was obtained from Cargill B.V. (Amsterdam, The Netherlands). Experiments were performed with either Millipore water, type 1 or demineralized water.

2.2. Spray drying process

Spray drying experiments were performed using a B-90 spray dryer (Büchi Labortechnik AG, Flawil, Switzerland) in open configuration. A solution of 0.01 wt% LDH and 2.5 wt% trehalose in 10 mM phosphate buffer pH 7.5, filtered with a 0.22 µm PVDF filter was used for all experiments. For each experiment, 10 ml of protein solution was prepared of which about 6 ml was spray dried for 30 min. During the spray drying process, the beaker containing the protein solution was sealed with parafilm to minimize evaporation of water. The spray dryer was used with either the standard vibrating mesh nozzle or a two-fluid nozzle taken from the B-290 spray dryer (Büchi Labortechnik AG, Flawil, Switzerland) (Figs. 1 and A.1).

The vibrating mesh nozzle was used in conjunction with a 4 µm perforated mesh spray cap. The liquid feed was circulated through the vibrating mesh nozzle using the built-in peristaltic pump at a pump setting of 1. Apart from the short tygon tubing inside the peristaltic pump, all tubing to and from the nozzle consisted of PTFE to minimize protein adsorption. The spray was set at 100%, resulting in an average atomizing flow rate of 0.2 ml/min. Samples were collected after 30 min

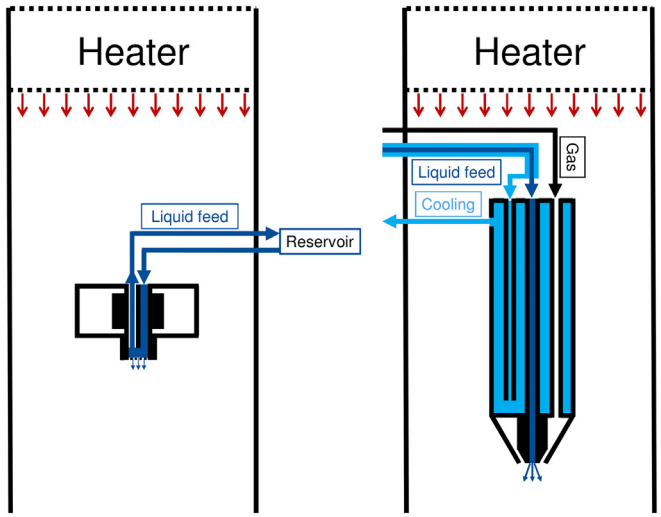

Fig. 1. Schematic overview of the vibrating mesh nozzle (left) and two-fluid nozzle (right) in the B-90 spray dryer. Note that the liquid feed of the two-fluid nozzle is cooled with the cooling water. In addition, with the vibrating mesh nozzle, the liquid feed is only partially atomized and the remainder is recirculated.

of circulating without heating (pump), circulating with heating (pump + heat), circulating and atomizing without heating (pump + spray), and after undergoing the entire drying process (pump + heat + spray (powder)). As the vibrating mesh nozzle also comprises a recirculating feed system, samples were taken from the liquid feed solution after atomizing without heating (pump + spray (feed)) and after the entire spray drying process (pump + heat + spray (feed)) as well. After each step that involved heating, the liquid feed tubing was flushed with a solution containing sodium dodecyl sulfate, and subsequently thoroughly rinsed with demineralized water.

The two-fluid nozzle used was equipped with a 0.7 mm diameter nozzle tip. The liquid feed was transported to the nozzle using a NE300 syringe pump (New Era Pump Systems Inc., Wantagh, NY, USA) set at a flow of 0.2 ml/min. PTFE tubing was used to connect the syringe to the tip of the two-fluid nozzle. The two-fluid nozzle included a cooling mantle, which enabled cooling of the solution to be spray dried during feeding. Water of 4–8 °C was circulated through the cooling mantle by using the built-in peristaltic pump of the B-90 spray dryer at speed setting 2. The temperature of the cooling water exiting the spray dryer was about 30 °C within a few minutes after starting the process (pump + heat + spray) and remained so thereafter. The atomizing airflow was provided by a B-290 spray dryer and set at 50 mm (600 l_n/h). Samples were collected after feeding the liquid without heating (pump), after atomization without heating (pump + spray), and after the entire spray drying process (pump + heat + spray (powder)) for 30 min. Due to a lack of feed circulation in this configuration, no (pump + heat) samples could be taken as was done with the vibrating mesh nozzle configuration.

To avoid any possible activity loss during storage, the enzymatic activity was measured the same day the samples were taken. In addition, collected samples were stored at 4–8 °C until analysis using the enzymatic activity assay.

A 2.5 wt% solution of trehalose without protein was spray dried using both nozzles to compare the particle size distribution of powders produced by the two methods.

All experiments were performed in triplicate with an inlet temperature of 120 °C and a drying air flow of 150 l/min.

2.3. Separating the process into steps

To calculate the activity loss in the separated process steps: transportation, heating, atomization, dehydration, and collecting one has to differentiate between cumulative activity losses and average activity losses. For readability, a detailed explanation and discussion of this calculation can be found in the Appendix.

2.4. Laser diffraction analysis

The particle size distribution of the spray dried trehalose was measured with laser diffraction analysis. Measurements were performed using a HELOS laser diffraction sensor with an R1 (0.1/0.18–35 µm) lens, and a RODOS for powder dispersion (Sympatec GmbH, Clausthal-Zellerfeld, Germany) at 3 bar. Laser diffraction data were based on the Fraunhofer theory. Samples from single spray dry experiments were analyzed in duplicate.

2.5. Enzymatic activity assay

LDH enzymatic activity of collected samples was determined as described by Bergmeyer and Bernt (1974), modified for a 96-wells plate. Shortly, the conversion of pyruvate into lactate is catalyzed by LDH. In the process, NADH is oxidized to yield NAD, which can be measured spectrophotometrically at 340 nm. Measurements were performed on a BioTek Synergy HT Multi-Mode Microplate Reader (BioTek Instruments Inc., Bad Friedrichshall, Germany). As the control, a sample of the protein solution was taken immediately before spray

Table 1
Laser diffraction results for powders produced by spray drying with either the vibrating mesh or the two-fluid nozzle (n = 2, < 5% deviation between duplicate measurements).

Nozzle type	x10 (μm)	x50 (μm)	x90 (μm)	Span[a]	A/V
Ultrasonic	0.42	0.97	2.02	1.66	7.67
Two-fluid	0.38	1.05	2.25	1.78	8.04

[a] Span was defined as (x90 − x10) / x50.

drying (starting solution) and normalized to 100% activity. All samples were analyzed in triplicate.

2.6. Statistical analysis

The difference in enzymatic activity was analyzed by a student *t*-test. Values were considered significantly different when $p < 0.05$.

3. Results

3.1. Particle size and morphology

The volume particle size distribution of the trehalose solution, spray dried using the vibrating mesh and the two-fluid nozzle, was measured with laser diffraction. The results for both nozzles were comparable (Table 1). The volume median diameter (x50) of powders was close to 1 μm.

3.2. Enzymatic activity loss with only pump, pump + heat, pump + spray, and pump + heat + spray

Each spray dryer configuration, using either the vibrating mesh or the two-fluid nozzle, reduced the enzymatic activity of LDH, although the reduction using pump or pump together with spray in combination with the two-fluid nozzle was not significant (Fig. 2). With the vibrating mesh nozzle, circulation of the feed through a tube inside the non-heated drying chamber without spraying (pump) resulted in a cumulative activity loss of about 14%. When the spray dryer was also heated (pump + heat), the cumulative activity loss increased to about 36%. When the circulating feed was only sprayed but not heated (pump + spray), about 43% of LDH activity was lost in the atomized droplets and about 19% in the feed solution. The latter, however, was not significantly higher than the loss found when only the pump was used. When the spray dryer was both heated and the feed sprayed

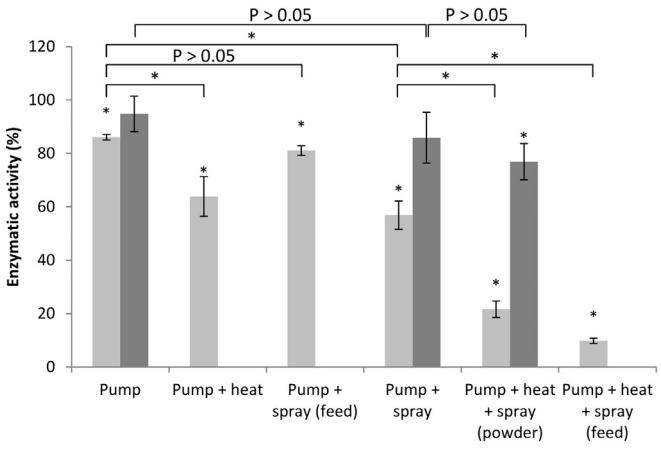

Fig. 2. Remaining enzymatic activity of LDH after specific spray drying process steps with a vibrating mesh (light grey) or a two-fluid (dark grey) nozzle. Data shown as averages, n = 3 ± SD. A student's *t*-test was used to determine significance (* = $p < 0.05$).

(pump + heat + spray), about 78% of the initial LDH activity was lost in the powder and about 90% in the feed solution.

Using the two-fluid nozzle, the activity loss of LDH was significantly lower than with the vibrating mesh nozzle in comparable process configurations. Pumping the feed solution through the non-heated nozzle without spraying (pump) resulted in an activity loss of about 5%. When the solution was subsequently sprayed (pump + spray), the activity loss increased to about 14%, although the loss was still not significant. After the entire spray drying process (pump + heat + spray), the activity loss was about 23%, a significant loss compared to the control. With both nozzles approximately equal yields (40 to 65%) were obtained.

After 30 min of processing with the vibrating mesh nozzle, the spray head reached a temperature of about 54 °C under "pump + spray" conditions, 75 °C under "pump + heat" conditions, and 108 °C under "pump + heat + spray" conditions, whereas the two fluid nozzle could be cooled. During the latter conditions, the outlet temperature inside the spray dryer after 30 min was 54 °C with the vibrating mesh nozzle and 48 °C with the two-fluid nozzle.

3.3. Activity loss in the separate process steps

To discriminate between the effects of the different forms of stress involved on LDH activity loss, the processes of pump, heat, and spray were separated and presented in terms of the process steps: transportation, heating, atomization, dehydration, and collecting in Fig. 3. A detailed explanation of the calculation used to obtain these results can be found in Appendix 1.

For the vibrating mesh nozzle the most critical steps appeared to be heating and passing over the vibrating mesh, and atomizing, resulting in 53% and 15% average activity loss, respectively. Circulation of the protein solution alone and the combination of dehydrating and collecting accounted for 7%, and 2% activity loss, respectively. Using the two-fluid nozzle, pumping, atomizing, and the combination of dehydrating, heating, and collecting resulted in 5%, 9% and 9% activity loss, respectively.

4. Discussion

The most critical process steps when spray drying protein solution depended heavily on the choice of nozzle. With the vibrating mesh nozzle, the heating and atomization steps caused the highest activity loss during spray drying. However, switching to the two-fluid nozzle decreased the activity loss in these steps to a point where no critical steps could be distinguished anymore. Overall, compared to the vibrating mesh nozzle, the two-fluid nozzle performed considerably better in each spray drying step. However, the methods used and results found do require further discussion.

4.1. Droplet size generated with both nozzle types

The particle size distribution measurements of powders produced by spray drying were used to indicate whether the activity loss due to atomizing can be attributed to a difference in the generated droplets, or the atomization mechanism itself. When the protein solution is dispersed as small droplets in the drying air, the interfacial area between air and water is greatly increased. It is well known that proteins tend to denature when exposed at such interfaces. Therefore, after atomization, the chances that the protein will degrade and lose its activity at the interface are also greatly increased. When the droplets generated by both nozzles would be different in size, the interfacial stress would be different as well. Not only that, a different droplet size would also imply a different energy input to the protein solution resulting in a difference in multiple stresses that are not necessarily related to the atomization mechanism itself. Therefore, to enable a direct comparison of the two nozzle types, the droplets that are generated should be of the same size.

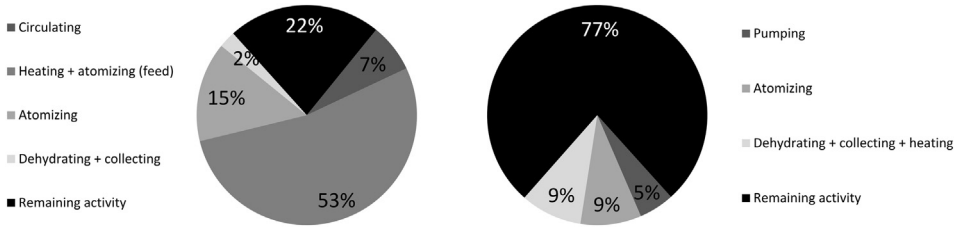

Fig. 3. LDH activity loss and remaining activity after spray drying, caused by the different separate steps that occur during spray drying with a vibrating mesh nozzle (left) and two-fluid nozzle (right). It has to be noted that activity loss due to heating could not be determined separately with the two-fluid nozzle.

This is shown to be the case in this study by measuring the particle size distribution of powder produced with both nozzle types (Table 1). Therefore, the difference in activity loss between both nozzle types in the atomizing step is not attributed to a difference in interfacial stress, but mainly to the difference in atomization mechanism of the vibrating mesh and two-fluid nozzle.

4.2. Chosen spray drying conditions

Because it was not the intention to optimize the spray drying process for protein stabilization in this study, aggressive spray dryer settings were used to make the critical process steps and the difference between nozzle types more apparent. This implies that the activity losses due to heating encountered during this study are not necessarily an indication of the activity loss found during a spray drying process with optimized conditions. However, what it does show are the critical process parameters during spray drying and more importantly, that the critical process steps depend heavily on the chosen nozzle type. It is tempting to conclude that the large difference in activity loss between the vibrating mesh and two-fluid nozzle is exacerbated by the high inlet temperature of 120 °C. Indeed, the chosen condition would normally not be used when spray drying protein solutions, especially those that are thermally sensitive. Even more so, lowering the inlet temperature would most certainly lower the difference in activity loss between the vibrating mesh and two-fluid nozzle. However, even when the activity loss due to heating could be reduced to zero, the estimated activity loss due to atomizing would still be much higher with the vibrating mesh nozzle than with the two-fluid nozzle. Because the atomizing step results in an estimated activity loss of 37% of the remaining activity when the protein passes through the nozzle, the average activity loss due to atomizing will increase from 15% to a maximum of 37% when the activity loss prior to atomizing is reduced to zero (Fig. 3 and Appendix, Fig. A.2).

4.3. Circulation is bad - cumulative vs average activity loss with the vibrating mesh nozzle

From the results it is clear that the circulation inherent to the design of the vibrating mesh nozzle has a detrimental effect on the process stability of LDH, and most likely many other proteins as well. In addition, it made the assessment of process and product quality unnecessarily complex. As shown in the Appendix, Fig. A.2, circulation results in activity losses that are time dependent, which were calculated with the aid of Eq. (A.1).

To be able to directly compare the results of the vibrating mesh nozzle with those of the two-fluid nozzle, a distinction had to be made between cumulative activity loss in the liquid feed and the average activity loss in samples collected after atomizing. This distinction is most noticeable in the difference between activity loss in the liquid feed and the powder when running the whole process using the vibrating mesh nozzle (pump + heat + spray, Fig. 2). The activity loss in the liquid feed was higher than the activity loss in the dried powder (Fig. 2, 90% and 78%, respectively), because the former was cumulative, whereas the latter was an average activity loss. During the circulation, the feed was repeatedly subjected to the heat from the drying chamber and to ultra-sonication, which resulted in a significant decrease of the LDH activity in the feed over time (cumulative). Therefore, LDH that was dried at the start of the process had a higher activity than LDH that was dried at the end, and the remaining activity of the dried and collected LDH powder is an average of the activity of all powder collected over time. This average activity will be higher than the remaining activity of the feed, especially considering the low activity loss due to dehydration and collection relative to the total activity loss in the feed.

Another implication of this time dependent activity loss is that it suggests that when only a small fraction of the liquid feed is spray dried or the processing time is shortened, the activity loss would be much lower. However, this is not a realistic scenario, since a prepared protein solution will always be spray dried wholly and the liquid feed flow that is atomized can only be increased by decreasing the mesh size of the perforated mesh, thereby also increasing the particle size of the resulting powder.

Because the two-fluid nozzle lacks recirculation of the feed, activity losses will not be time dependent. Therefore, apart from the standard activity losses during storage in the liquid feed and in the collector, with the two-fluid nozzle the processing time and thus batch size do not have to be taken into account when optimizing the spray drying process.

4.4. Activity loss due to pumping

Pumping resulted in a relatively small average activity loss of 5–7% with both nozzle types. This loss can be attributed to a combination of shear and adsorption.

4.5. Activity loss due to heating and passing over the vibrating mesh with the vibrating mesh nozzle

Two methods were used to calculate the average activity loss due to heating and passing over the vibrating mesh (Appendix, Table A.1). The average activity loss that was found with the whole process was much higher than when only heating or spraying were used (53% and 15%, respectively). The difference suggests that there is a synergistic effect on degradation between heating and atomizing. This will in part be due to the increased temperature inside the spray head of 108 °C when both heating and spraying compared to 75 °C and 54 °C when only heating or spraying, respectively. Using the calculated heat loss from the entire process meant that activity lost due to heating or passing over the vibrating mesh could not be separated. However, this loss (53%) is a much more accurate estimate of the activity loss due to heating (and passing over the vibrating mesh) than the loss calculated from the partial process (15%).

4.6. Separating heating from dehydrating and collecting with the two-fluid nozzle

With the two-fluid nozzle, it was impossible to separate the influence of heating from dehydrating and collecting (Fig. 3). Due to the design of the nozzle, no sample can be safely collected while only heating as with the vibrating mesh nozzle, because the liquid is not recirculated. However, during spray drying the liquid feed and the nozzle itself were cooled with water of 4–8 °C. After passing through the heated drying chamber, upon leaving the spray dryer, the temperature

of the cooling water was measured at around 30 °C within a few minutes after starting the process (pump + heat + spray) and remained constant thereafter. Because this was only 5 °C above the room temperature, the influence of the heating step on the activity loss of the protein is expected to be negligible over the activity loss that might occur at room temperature in the starting solution. Furthermore, it is expected that the activity loss due to dehydrating and collecting is similar with both nozzles, because the outlet conditions are similar as well (48 °C and 54 °C, with the two-fluid and vibrating mesh nozzle, respectively). Although the average activity loss with the vibrating mesh nozzle shown in Fig. 3 is very low (2%), it is effectively about 10% of the remaining activity after atomizing. With the two-fluid nozzle also 10% of the remaining activity is lost due to dehydrating, collecting and heating, further indicating that heating indeed does not play a role with the cooled two-fluid nozzle.

4.7. Advantage of combining the two-fluid nozzle with the B-90 spray dryer

Besides the possibility to cool the nozzle and the lack of circulation, there are a few other advantages of using the two-fluid nozzle over the vibrating mesh nozzle in combination with the B-90 spray dryer. Although the combination of the two-fluid nozzle with the B-290 spray dryer is able to produce a powder with a similar particle size distribution, the yield will be lower due to a lower collection efficiency of a cyclone, even when using the high performance cyclone, as compared to an electrostatic collector (data not shown). In addition, the electrostatic collector that is included in the B-90 spray dryer is better able to collect smaller batch sizes efficiently. Although not evident from the results described in this paper, even when a total of 50 mg of protein and excipients dissolved in 10 ml of water was spray dried, a yield of up to 35 mg (70%) was obtained, which has never been possible with the B-290 spray dryer (data not shown). This is especially useful for expensive and potent proteins, which for research purposes are often only needed in small amounts. Finally, with the two-fluid nozzle, the liquid feed and atomizing gas flow (and thus particle size) can be varied freely, whereas with the vibrating mesh nozzle there are only three perforated mesh sizes available that can be used to change the particle size, each with their fixed liquid feed flow. This makes it much easier to optimize the process and to control the outlet conditions, especially the outlet temperature and humidity, when the two-fluid nozzle is used.

4.8. Effect of protein and sugar

LDH is highly sensitive to heat and shear (Adler and Lee, 1999; Hertel et al., 2014; Niven and Brain, 1994; Niven et al., 1994). In our study, it was found that with the vibrating mesh nozzle as much as 78% of the enzymatic activity of LDH was lost during the entire spray drying process, which was mostly due to heat and atomization stresses. In contrast, when the two fluid nozzle was used, only a moderate loss of enzymatic activity was found (23%). These results clearly indicate that the vibrating mesh nozzle imposes much more heat and shear stresses to the protein solution than the two fluid nozzle. Obviously, when a protein would have been spray dried, which is less sensitive to heat and shear stresses, the difference in activity loss as found for LDH between the two nozzles, would have been smaller.

In our study, trehalose was selected as excipient as it is generally considered to be the gold standard for protein stabilization during drying processes (Balcão and Vila, 2015; Manning et al., 2010). However, in some exceptional cases other sugars perform better as protein stabilizers. E.g. in a study of Liao et al. (2002), it was found that the secondary structure of lysozyme during spray drying was better maintained using sucrose instead of trehalose as stabilizer. We therefore cannot exclude that when another sugar would have been used, the loss of enzymatic activity of LDH after spray drying would have been less. Obviously, less well performing sugars would result in increased loss of activity. However, we do not expect that the use of other sugars would change the major conclusions of this study. As sugars act as a stabilizer in a nonspecific manner, i.e. by vitrification and water replacement, it is unlikely that one sugar would better protect the protein during spray drying against a particular type of stress e.g. heat stress while another sugar provides protection against another type of stress e.g. dehydration stress. In other words, when using another sugar, we expect that the loss of enzymatic activity of LDH would have changed proportionally to the same extent when using the vibrating mesh or the two fluid nozzle.

5. Conclusions

This study shows that every different process step in the spray drying of proteins may result in a loss of protein activity. However, the extent to which the different steps add to the total process stability of the protein may significantly differ for different process equipment used and for different proteins. With regard to the process equipment and configuration, the avoidance of any time dependent process was found to be essential to maintain the highest possible fraction of the protein intact; especially during upscaling this may be an aspect of utmost importance. With the model protein LDH in this study we show that the choice of the droplet generating principle (the nozzle) determines to a large extent the process stability of the protein. Heating of the feed solution (due to the use of a non-cooled nozzle), the exposure to ultrasonication, as well as the recirculation of fluid through the nozzle should be prevented. This makes the two-fluid nozzle used in our study much better suited for spray drying of proteins than the vibrating mesh nozzle tested.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejps.2018.11.027.

Declarations of interest

None.

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