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Research Article

Nano-coating of β-galactosidase onto the Surface of Lactose by Using an Ultrasound-assisted Technique

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Abstract. We nano-coated powdered lactose particles with the enzyme β-galactosidase using an ultrasound-assisted technique. Atomization of the enzyme solution did not change its activity. The amount of surface-attached β-galactosidase was measured through its enzymatic reaction product D-galactose using a standardized method. A near-linear increase was obtained in the thickness of the enzyme coat as the treatment proceeded. Interestingly, lactose, which is a substrate for β-galactosidase, did not undergo enzymatic degradation during processing and remained unchanged for at least 1 month. Stability of protein-coated lactose was due to the absence of water within the powder, as it was dry after the treatment procedure. In conclusion, we were able to attach the polypeptide to the core particles and determine precisely the coating efficiency of the surface-treated powder using a simple approach.

KEY WORDS: lactose; nano-coating; protein; ultrasound; β-galactosidase.

INTRODUCTION

Lactose is a widely used excipient in various pharmaceutical solid dosage forms (1,2). During the past few decades, however, inclusion of lactose in pharmaceuticals has been criticized due to an increased number of people being unable to digest milk sugar. Although lactose-containing tablets and capsules clearly cause some symptoms for individuals suffering from severe lactose intolerance, the significance of lactose of pharmaceutical origin is still under debate, and reports in this field are controversial (2).

A powdered particle control design is a popular area of pharmaceutical research since solid dosage forms are the most common preparations for drug administration. Coating of the particle surface can be performed to change the superficial nature of powder and improve the dispersion, dissolution, and flow rate of bulk materials (3–6). However, the coating of powdered particles is challenging due to their small size, irregular shape, and high adhesion and cohesion, and thus, the agglomeration of individual species.

The manufacturing of complex macromolecular compounds (e.g., proteins) presents more difficulties due to their labile structure. Proteins suffer both physical and chemical instability and are therefore very sensitive to the processing conditions (7). Spray drying, spray freeze drying, and lyophylization are the most common methods for the dry powder preparation of protein pharmaceuticals (8,9). However, these techniques have some drawbacks, such as thermal stress, shear force, low yield, and long processing time that can affect the stability of polypeptides (10,11). The spray coating of recombinant human deoxyribonuclease onto the microcarriers by means of top and bottom fluid bed techniques was successfully performed to evaluate the stability and integrity of the protein layer (12,13). The results were compared with the spray-dried product. Both techniques caused the partial loss of protein activity during the drying stage, whereas spray drying was less damaging. On the other hand, spray coated with the nano-sized layer, the micro-sized vaccine preparation formulated for epidermal powder immunization remained entirely active (14).

The successful coating of powdered particles depends on the droplet size of the protein solution. Smaller droplets reduce the risk of agglomerate formation and result in a powder formulation of better performance (14). Atomization of the solution by means of an ultrasound nebulizer produces a cold mist, where the size of droplets is less than 5 μm. However, such devices increase the solution temperature by 10-15°C, which may cause denaturation of the bioactive molecules. In addition, foaming of the protein solution during nebulization might provoke unfolding of their native structure and the aggregation of macromolecules, with a subsequent loss of activity (15). Atomized droplets possess a large air-liquid interface that might also affect protein stability (16–18).

The goal of this study was to improve the pharmaceutical utilization of lactose-containing pharmaceutical formulations for a population with adverse reactions to milk sugar. We deposited the protein substance β-galactosidase onto the surface of lactose carriers and determined the increase in the amount of the surface-attached enzyme. Briefly, the coarse lactose was coated with a 1% (m/V) lactase solution that was nebulized by ultrasound-assisted technique developed in our earlier study (19). The idea was to avoid

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overwetting of the powder mass and prevent activation of the enzyme, both of which are difficult to achieve using more complex devices such as the fluid bed technique. The quantity of the surface-coated protein was measured through its reaction product D-galactose using a standardized method.

MATERIALS AND METHODS

Materials

β-galactosidase powder (Lactase 14-DS) from *Aspergillus oryzae* with an enzyme activity of no less than 14 U/mg (pH 4.5) was received as a gift from Amano Enzyme Inc. (Nagoya, Japan) and used without further purification. Lactose α-monohydrate (Pharmatose® 80 M, MW 360.32 g/mol) was obtained from DMV (the Netherlands). The standardized reagents of the ultraviolet (UV) method for the determination of lactose and D-galactose in food-stuffs and other materials were purchased from R-Biopharm (Darmstadt, Germany).

Coating Procedure

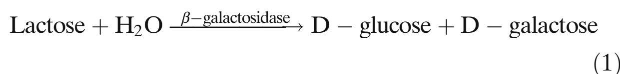
The processing system has been illustrated and described in our earlier paper (19). A prefiltered yellowish solution of the 1% (m/V) β-galactosidase was aerosolized using a commercially available nebulizer (Ultrasonic Nebulizer NE-U17, Ultra Air, Omron, the Netherlands). The produced mist was applied onto the lactose carrier particles delivered by a vibratory feeder (Laborette 24, Germany). A partial loss of enzyme mist to the environment took place due to the open setup of the technique. The coating procedure was reiterated 30 times with the same solid material. Occasional mixing was performed to prevent the formation of liquid bridges between core particles. The batch size of lactose was 33 g. A 5-g sample was taken every fifth cycle for subsequent analysis. Prior to the container closing, the just surface-modified powder was equilibrated for 1 h at 22±1°C and 35±6% relative humidity, and the water content of the untreated and treated powders was analyzed. The moisture content of the samples was measured by using an AquaLab water activity meter (AquaLab 3 TE, Decagon Devices, Inc. Washington, District of Columbia, USA). Table I illustrates the process parameters applied. The entire coating procedure was conducted twice to evaluate the repeatability of the method.

Table I. Summary of Processing Conditions

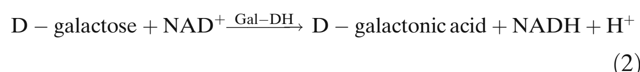
Number of cycles	0–30
Air flow (L/min)	17
Flow rate of mist (g/min)	2.8
Supplement rate of β-galactosidase (mg/min)	28
pH of enzyme solution	5.9
Droplet size of water mist (μm)	4.4
Feeding rate of powder (g/min)	2.3
Rotation rate of stirrer (rpm)	240
Time between cycles (min)	7
Yield (%)	90

Enzyme Activity and Retention of β-galactosidase Activity

To measure the activity of enzyme in solution, a standardized spectrophotometry method (20) was used based on the following reactions:



$$\text{Lactose} + H_2O \xrightarrow{\beta-\text{galactosidase}} \text{D} - \text{glucose} + \text{D} - \text{galactose} \quad (1)$$



$$\text{D} - \text{galactose} + \text{NAD}^+ \xrightarrow{\text{Gal-DH}} \text{D} - \text{galactonic acid} + \text{NADH} + \text{H}^+ \quad (2)$$

In the presence of water, β-galactosidase cleaves disaccharide bonds of lactose by Eq. (1). The reaction product D-galactose is oxidized at slightly basic pH by nicotinamide-adenine dinucleotide (NAD) in the presence of the enzyme β-galactose dehydrogenase, producing a reduced form of NAD by Eq. (2). The amount of NADH measured at 340 nm is stoichiometric to the amount of lactose and D-galactose.

The standard curve for D-galactose determination (Fig. 1) was obtained by dissolving 200 mg of lactose monohydrate and 200 mg of lactase powder in 100 mL of purified water. The optimal conditions for the maximum activity of enzyme with respect to solution pH (4.5) and reaction temperature (55°C) were not used due to difficulties in establishing these conditions. The solution (1.9 g/l of lactose) was incubated for 5 h at 22±1°C and pH 7.1 to insure a full cleavage of lactose. The moisture content of the raw lactic sugar measured by an infrared apparatus (Sartorius MA 100, Sartorius AG, Germany) was 0.16±0.02%. A complete enzymatic degradation of lactose in solution is supposed to yield 0.998 g/L of D-galactose. The stock solution and nine intermediate diluted solutions were prepared and assayed according to the standardized test-combination procedure using a UV/VIS spectrophotometer (Pharmacia Ultrospec III, Pegasus Scientific Inc., Rockville, Maryland, USA).

To determine the effect of ultrasound-assisted nebulization on β-galactosidase activity, a 1% (m/V) protein solution was prepared, part of the enzyme solution was used as prepared, and another part was atomized and the mist containing β-galactosidase was collected as a fluid. Both solutions were used to prepare 24 intermediate solutions (100 mL each); 12 of them contained 0.005-0.2 g/L of enzyme from the prepared solution and another 12 were made similarly, but using a nebulized fluid. Each of the intermedi-

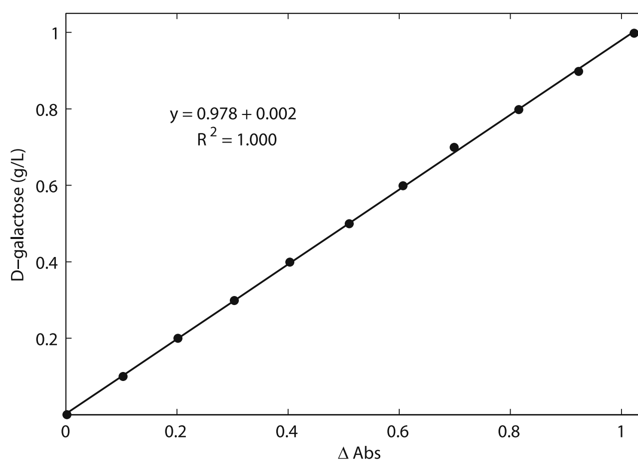


Fig. 1. Standard curve for D-galactose determination

ate solutions contained 1.9 g/L of lactose. When the enzyme and substrate came into contact, the solutions were incubated for precisely 24 h at 22±1°C and assayed for D-galactose. Each solution was analyzed once.

Determination of Coating Efficiency and Stability of the Enzyme Preparation

To measure the change in the amount of surface-attached β-galactosidase, 200 mg of coated powder from each sample was dissolved in 100 mL of purified water. The solutions were incubated at 22±1°C for 2, 5, and 24 h and analyzed for monosaccharide content. It is noteworthy that due to the tiny amount of enzyme coat, no back calculations were done for the amount of D-galactose produced. The stability of the β-galactosidase preparation was tested after 2 weeks and after 1 month of storage, conducting the analysis as outlined above.

To evaluate the potential enzymatic degradation of lactose during the treatment procedure, 200 mg of the final freshly coated powder was dissolved in 100 mL of purified water and assayed immediately for D-galactose without incubation. The same procedure was conducted with raw lactose, and the results were compared. Due to the standardized method, every sample was assayed only once for D-galactose content in the solution.

Determination of Particle Size and Morphology

A Malvern laser diffractometer (Malvern 2600c, Malvern, England) was applied to measure the particle size distribution of the processed powder in a liquid suspension. One hundred milligrams of untreated and treated lactose was added to 5 mL of isopropyl alcohol and mixed for 10 s. Several droplets of each suspension were loaded into a stirred sample cell containing 13 mL of isopropanol. A focal length of 300 nm and a beam length of 10 nm were used to determine the intensity of laser light and calculate the volume diameters d_{10}, d_{50} and d_{90} of the particle distribution. The distribution spread was calculated by Eq. (3):

$$\text{Span} = \frac{d_{90} - d_{10}}{d_{50}} \tag{3}$$

Three parallel measurements were performed for each sample.

Particle size and surface morphology were investigated using a scanning electron microscope (Zeiss DSM 962, Carl Zeiss, Oberkochen, Germany). Powder samples were mounted onto a metal plate using a two-sided adhesive tape and coated with platinum by a vacuum evaporator. Photographs of samples were taken at three magnifications (×50, and ×1,000) using an accelerated voltage of 10 kV.

Solid-state Properties

Calorimetric analysis was carried out on a differential scanning calorimeter (DSC) (Mettler Toledo DSC 823e, Greifensee, Switzerland). The sample size was 9±1 mg in 40-µl aluminum pans with two pinholes. The heating rate was 10°C/min, and the cooling rate approximately 70°C/min.

The Raman spectra were collected using a Raman spectrometer (PhAT System™, RamanRXN1™, Kaiser Optical System Inc., Ann Arbor, Michigan, USA) equipped with a fiber optically coupled PhAT probe head. The integration time was 3 s for the lactose samples and 1 s for the pure enzyme. All Raman spectra were acquired using HoloGram software (HoloPro™, Kaiser Optical System Inc., Ann Arbor, Michigan, USA).

RESULTS AND DISCUSSION

Protein Mist Coating

Recently, we thin-coated ibuprofen particles with hydroxypropyl methylcellulose polymer to reduce the cohesiveness of the highly charged material and improve the flow properties of powder (21). However, the increase in the thickness of the coating layer was measured with an indirect method, and quantitative analysis of the particle coat was not performed. Thus, the coating efficiency of our technique needed to be determined. There were several reasons for selecting β-galactosidase as a model substance. Firstly, the stability of the nebulized product needed to be checked, and β-galactosidase is a fragile molecule that can lose its activity very easily. Secondly, a precise method exists for the determination of the substrate/product concentration of β-galactosidase, and consequently, the amount of enzyme. The method is well standardized since the quantitative estimation of lactose in dairy products is a common procedure due to the large number of people with lactose intolerance (22–24). Thirdly, to prove that the surface-modified powder remains dry and no degradation product appeared as an enzymatic reaction requires water. Our results suggest that the water activity of the processed samples (α_w=0.308±0.008) did not differ from the water activity of the raw powder (α_w=0.301±0.003), and the parameters were in agreement with the ambient conditions. The proportion of D-galactose produced of the maximally treated powder in the freshly prepared solution was under the detection limit (ΔA<0.005).

Effect of Nebulization on β-galactosidase Activity

It is well known that proteins are stress-sensitive bioactive molecules that can undergo irreversible conformational changes with subsequent activity loss (25–27). According to the enzyme product list (28), the β-galactosidase

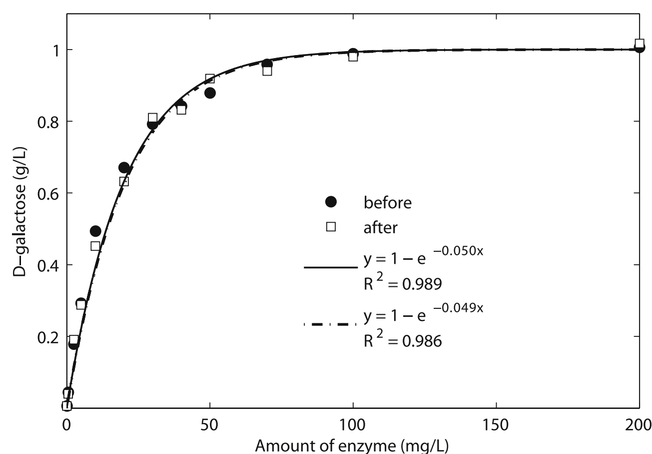


Fig. 2. Retention of β-galactosidase activity before and after nebulization of the enzyme solution. The incubation time of substrate/enzyme solutions is 24 h

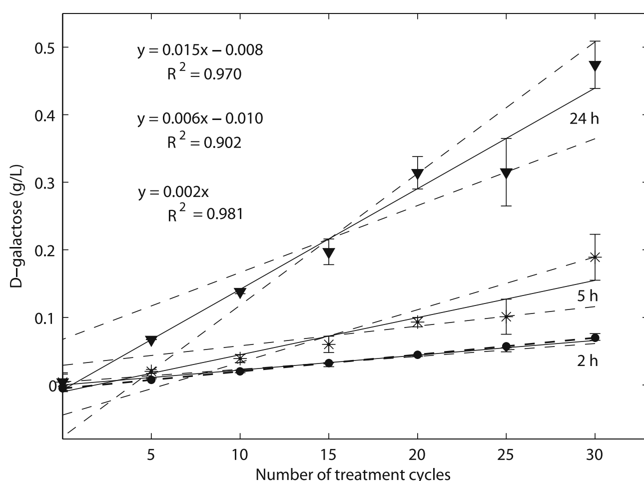


Fig. 3. Coating efficiency, expressed as amount of D-galactose (g/L) produced by progressively increased enzyme coating after incubation of 2, 5, and 24 h of 1.9 g/L unmodified and modified lactose solutions. *Error bars* ± absolute deviation of linear fitting (n=1). The results were fitted within the approximation of error (*broken line*)

solution (stable pH 4.0-8.0) is thermally stable at least up to 50°C at pH 4.5 and 6.5. However, the protein activity starts to decrease drastically as temperature exceeds 50°C. It means that temperature increase up to 40°C in the ultrasound device supposed to have no influence on the thermal degradation and activity loss of the enzyme. In addition, in the literature reported, denaturation temperature for reconstituted in water β-galactosidase (pH 6.5) performed by high resolution calorimetry is 69.7°C that exceeds almost twice the expected temperature of the nebulizing solution (15).

To evaluate the effect of the processing conditions, the retention of atomized enzyme activity was investigated. The β-galactosidase activity measured before and after nebulization of the enzyme solutions is presented in Fig. 2. The ultrasound nebulization did not affect the catalytic activity of lactase. The amount of enzyme needed to cleave a predetermined amount of lactic sugar was the same for the atomized protein as for the commercial protein.

Table II. Particle Size Descriptors of Untreated and Enzyme-treated Lactose Powder Obtained by the Malvern Laser Diffractometer (Data are Presented as Mean ± SD, n=3)

Sample	Particle size (μm)			Span
	d_{10}	d_{50}	d_{90}	
Lactose, untreated	96±4	226±14	394±14	1.32±0.15
Lactose, 5 cycles	107±5*	241±20	417±10	1.29±0.09
Lactose, 10 cycles	107±5*	228±18	399 ±11	1.28±0.14
Lactose, 15 cycles	117±3*	226±5	410±7	1.30±0.02
Lactose, 20 cycles	117±4*	228±11	391±21	1.20±0.14
Lactose, 25 cycles	120±6*	222±1	404±1	1.28±0.03
Lactose, 30 cycles	115±5*	238±11	389±20	1.15±0.14

*p<0.05, significant difference compared with untreated substance by Student's unpaired t test

Determination of Protein Loading

The quantity of β-galactosidase on the lactose particle surface was determined via its reaction product D-galactose by UV spectrophotometry. The enzymatic reaction in solution was slow, yielding a marked difference in the amount of D-galactose after 24 h of incubation (Fig. 3). The enzyme loading increased gradually with the treatment cycles. The amount of D-galactose produced rose nearly linearly as the coating proceeded, suggesting a uniform increase in the thickness of the coating layer.

The stability of the enzyme preparation was examined after 2 weeks and after 1 month of storage. As seen in Fig. 4, β-galactosidase tended to remain unchanged in its ability to cleave disaccharide bonds. Furthermore, the repeatability of our technique was tested by conducting an analogical coating experiment. The results from the second run did not differ from those of the first (Fig. 4). The technique therefore appears to give rise to an enzyme coat in a reproducible way. The minor deviations of the results could be due to experimental or analytical limitations.

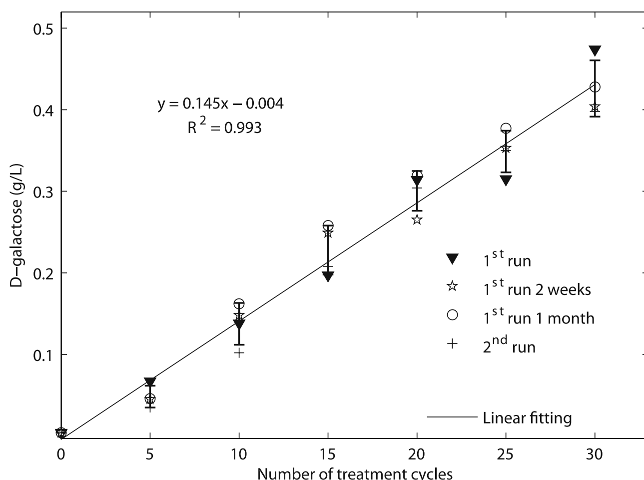


Fig. 4. Stability of β-galactosidase formulation after 2 weeks and after 1 month of storage (first run) and repeatability of the technique (first and second runs). The incubation time of 1.9 g/L unmodified and modified lactose solutions is 24 h. *Error bars* mean ± standard deviation (n=4)

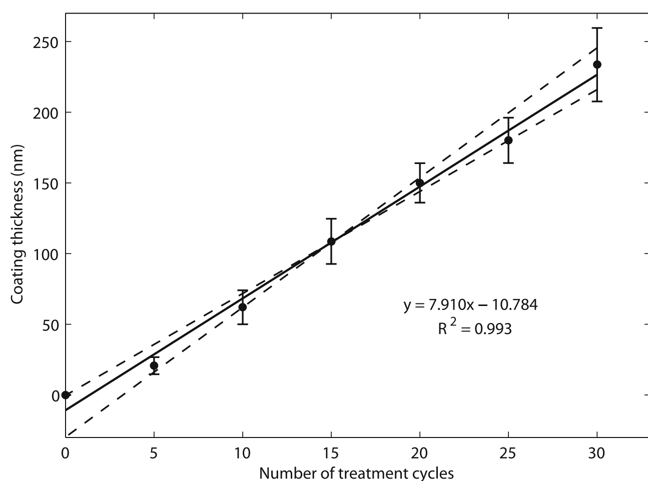


Fig. 5. Calculated thickness of the coating layer, n=4. *Error bars* mean ± standard deviation. The results were fitted within the approximation of error (*broken line*)

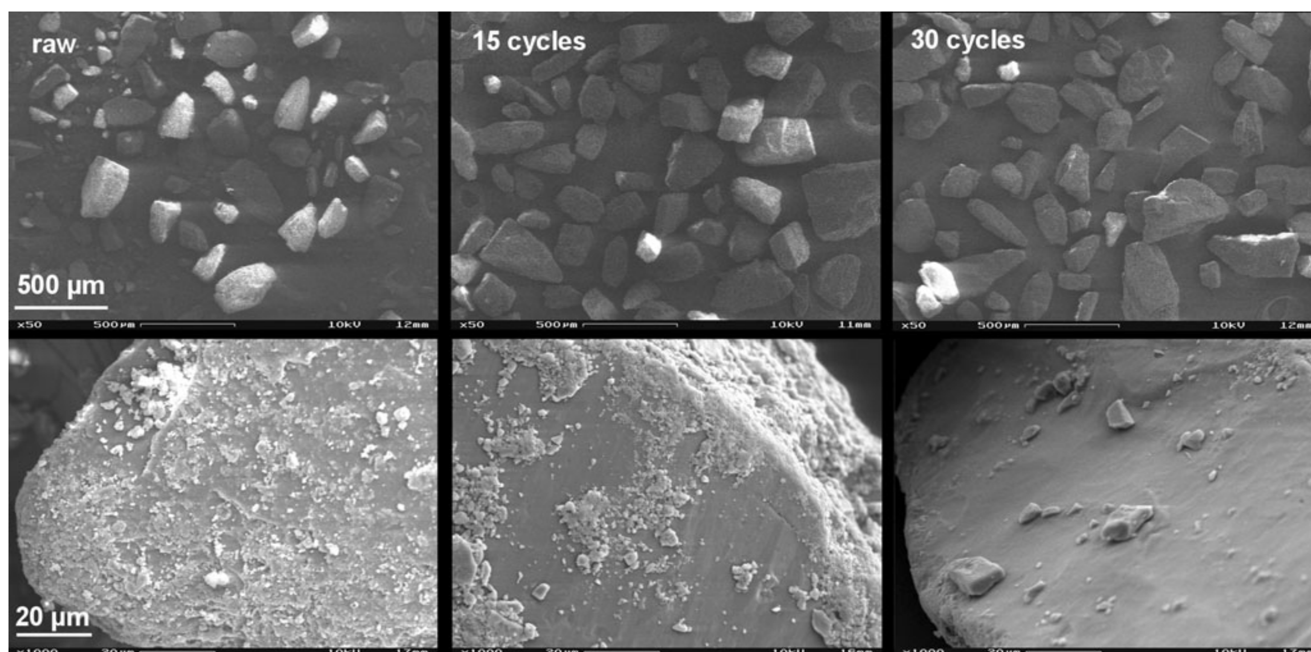


Fig. 6. Scanning electron micrographs of untreated and β-galactosidase-coated lactose powder at magnifications of ×50, and ×1,000

Figures 2 and 4 allow us to calculate the precise amount of surface-deposited enzyme material using a fitted exponential function. There was on average 0.5 and 5.7 mg of β-galactosidase coat per 1 g of lactose powder after five and 30 cycles, respectively. This means that every coating cycle increased the amount of surface-attached enzyme by 0.16± 0.03 mg/g of treated lactose monohydrate.

From the ratio (α) between the weight of the enzyme coat and the weight of lactose powder, assuming that lactose particles were spheres, the thickness of the coating layer can be calculated according to Eq. (4):

$$r_2 = r_1 \times \sqrt[3]{\frac{\alpha \times \rho_1 + \rho_2}{\rho_2}}, \tag{4}$$

where, r_1 and r_2 are the radii of uncoated and coated lactose particles, and ρ_1 and ρ_2 are the true densities of pure lactose and enzyme powder, respectively. From the literature, ρ_1 is 1.545 g/cm^3 (2), and ρ_2 is 1.42 g/cm^3 (27). The volume median diameter of raw lactose particles was measured to be 226 µm (Table II).

The calculated thickness of the β-galactosidase layer was up to 234 nm, increasing nearly linearly with every treatment cycle (Fig. 5). The uniform increase in the quantity of the surface-attached protein is important for the homogeneous distribution of the active pharmaceutical ingredient in the powder. Nano-coating of powdered particles offers some advantages relative to the widely applied mechanical mixing; with the latter method, problems are experienced with demixing due to cohesion of particular components, segregation of powder and formation of "dead spots" inside the blender.

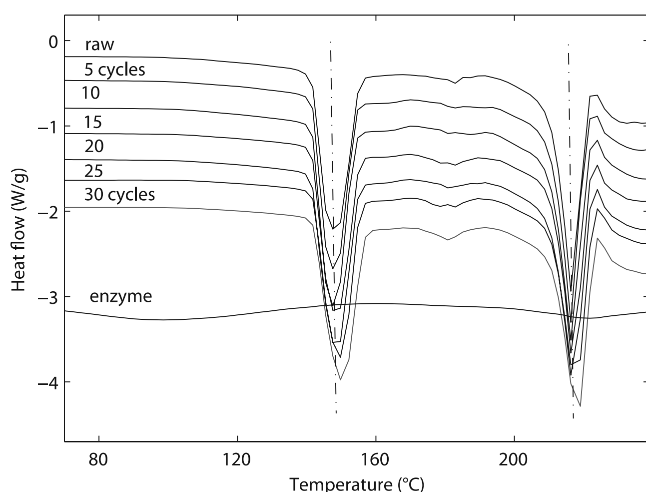


Fig. 7. Differential scanning calorimetry thermograms of pure β-galactosidase, untreated and enzyme-treated lactose powder

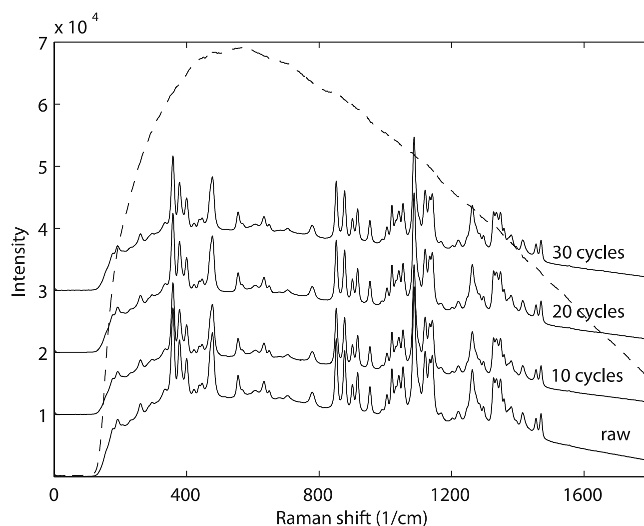


Fig. 8. Raman spectra of pure β-galactosidase (*broken line*), untreated and enzyme-treated lactose powder

Morphology of Enzyme-coated Lactose Carriers

Particle irreversible adhesion to each other can take place during the coating procedure (13). This leads to greater particle size distribution. Agglomerated particles behave as granules and lose their native powder characteristics. Therefore, prevention of particle agglomeration is highly desirable. Particle size analysis of β-galactosidase-coated lactose samples reveals the absence of granule formation (Table II). The d_{10} descriptors of the surface-modified powders compared with the original lactose particles increased slightly, whereas the median particle size parameters (d_{50}) showed no significant differences. Obviously, dissolution of fine species and their subsequent deposition together with enzyme material onto the coarser lactose particles occurred, especially during the early stage of coating. In addition, some fine particles could be captured by the stream of mist. This caused a slight increase in the d_{50} and d_{90} values of processed powder in comparison with raw material. However, the width of the distribution became narrower for enzyme-coated lactose particles (Table II). The high standard deviation of the results can be due to difficulties in obtaining a homogeneous dispersion of dense lactose particles during measurements with a laser diffractometer.

Photographs from a scanning electron microscope confirmed the results obtained with particle size analysis (Fig. 6). The more uniform particle size distribution can be seen on the micrographs of the modified lactose samples, whereas the raw material has a fraction of fine particulate. In addition, a lack of agglomerates and the presence of individual particles were observed for all samples. Figure 6 show that the lactose carriers before enzyme coating had a rough surface. During progressive treatment, the surface of modified lactose particles became smoother. After the last coating cycles, the superficial nature of core particles appears to be visually flat. Evidently, a smooth enzyme coat formed on the surface of lactose particles. However, it is not possible to recognize individual coating layers due to the nano-coating procedure.

Solid State Properties of Enzyme-coated Lactose

The DSC thermograms for untreated and enzyme-treated lactose powders were typical for α-lactose monohydrate (Fig. 7). The two peaks corresponded with the elimination of hydrate water at 147.6°C and the melting of crystalline α-lactose at 216.3°C (29). However, after 25 and 30 treatments, the dehydration peaks of lactose powder shifted to the right to 149.8°C and the melting point appeared to be at 219.1°C for maximally coated lactose particles. Perhaps, the shift in the dehydration peak was due to the presence of a tiny amount of monosaccharides in the powder as the melting temperatures of glucose and galactose are 148-155°C and 167°C, respectively (30). However, the traces of glucose and galactose were under the detection limit by UV method ($\Delta A < 0.005$) as mentioned above. The DSC trace of pure β-galactosidase showed an endothermic peak at 221.8°C. This event may be responsible for the shift of lactose melting point to a higher temperature. The second endothermic event of pure enzyme powder was attributed to water elimination, as the powder is very hygroscopic and contains up to 10% moisture (drying loss).

Analysis of the Raman spectra showed that unmodified, enzyme-coated and pure enzyme powders fluoresced, with protein fluorescing to the greatest extent (Fig. 8). However, the Raman scattering patterns of processed and original materials were identical, with the highest intensity in the spectrum of raw material. In conclusion, solid state characteristics of nano-coated lactose powder remained unchanged compared with untreated material.

CONCLUSIONS

Homogeneous deposition of β-galactosidase onto coarse lactose particles was successfully carried out. The thickness of the protein layer increased progressively, whereas the quantity of the surface-attached enzyme was 0.2-5.7 mg/g of lactose powder. A tiny amount of the surface-coated lactase did not cause significant changes in the crystal structure of α-lactose monohydrate. Such pre-treatment of lactose may be useful for formulating pharmaceutical dosages for patients with lactose intolerance. In addition, this coating technique could be applied, e.g., in production of dosage forms in which uniformity of the content of active pharmaceutical ingredient is critical. However, further studies are required to demonstrate the pharmaceutical processability of the nano-coated lactose excipient.

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