Colloids and Surfaces B: Biointerfaces 146 (2016) 700–706

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Effect of mechanical denaturation on surface free energy of protein powders

Mohammad Amin Mohammad[a,b,*], Ian M. Grimsey[a], Robert T. Forbes[a,c], Ian S. Blagbrough[d], Barbara R Conway[e]

[a] School of Pharmacy, University of Bradford, Bradford, BD7 1DP, UK
[b] Faculty of Pharmacy, University of Damascus, Damascus, Syria
[c] School of Pharmacy and Biological Sciences, University of Central Lancashire, PR1 2HE, UK
[d] Department of Pharmacy and Pharmacology, University of Bath, Bath BA2 7AY, UK
[e] Department of Pharmacy, University of Huddersfield, Queensgate, Huddersfield, HD1 3DH, UK

ARTICLE INFO

Article history:
Received 5 March 2016
Received in revised form 9 June 2016
Accepted 4 July 2016
Available online 5 July 2016

Keywords:
Protein denaturation
β-Galactosidase
Lysozyme
Conformational change
Inverse gas chromatography
Surface free energy

ABSTRACT

Globular proteins are important both as therapeutic agents and excipients. However, their fragile native conformations can be denatured during pharmaceutical processing, which leads to modification of the surface energy of their powders and hence their performance. Lyophilized powders of hen egg-white lysozyme and β-galactosidase from *Aspergillus oryzae* were used as models to study the effects of mechanical denaturation on the surface energies of basic and acidic protein powders, respectively. Their mechanical denaturation upon milling was confirmed by the absence of their thermal unfolding transition phases and by the changes in their secondary and tertiary structures. Inverse gas chromatography detected differences between both unprocessed protein powders and the changes induced by their mechanical denaturation. The surfaces of the acidic and basic protein powders were relatively basic, however the surface acidity of β-galactosidase was higher than that of lysozyme. Also, the surface of β-galactosidase powder had a higher dispersive energy compared to lysozyme. The mechanical denaturation decreased the dispersive energy and the basicity of the surfaces of both protein powders. The amino acid composition and molecular conformation of the proteins explained the surface energy data measured by inverse gas chromatography. The biological activity of mechanically denatured protein powders can either be reversible (lysozyme) or irreversible (β-galactosidase) upon hydration. Our surface data can be exploited to understand and predict the performance of protein powders within pharmaceutical dosage forms.

1. Introduction

In the pharmaceutical field, there is considerable interest in the use of globular proteins for their therapeutic effects. During pharmaceutical processes, protein powders are often subjected to mechanical stresses. For example, milling has been used to prepare protein particles suitable for pulmonary delivery and protein-loaded microparticles in industrial quantities [1,2]. The mechanical stresses applied during the milling can partially or completely denature the proteins and change their bulk properties [3]. In recent years, denatured globular proteins have found extensive applications as excipients in pharmaceutical formulations [4,5]. Denatured globular proteins have been used to prepare emulsion systems designed to enhance the absorption of insoluble drugs and to form nanoparticles for drug delivery and targeting [4]. Globular proteins have also been successfully used to formulate controlled drug delivery tablets, which delay drug release in gastric conditions by forming a gel-layer stabilized by intermolecular–beta sheets of denatured globular proteins [5].

Surface energies of powders are critical properties to be considered during formulation and development of dosage forms in the pharmaceutical industry. Surface energy has significant effects on pharmaceutical processes such as granulation, tableting, disintegration, dissolution, dispersibility, immiscibility, wettability, adhesion, flowability, packing etc. Resultant data from recent deter-

* Corresponding author at: School of Pharmacy, University of Bradford, Richmond Road, Bradford, BD7 1DP, UK.
E-mail addresses: m.a.mohammad7@bradford.ac.uk, mam2014uk@gmail.com (M.A. Mohammad), i.m.grimsey@bradford.ac.uk (I.M. Grimsey), r.t.forbes@bradford.ac.uk (R.T. Forbes), prsisb@bath.ac.uk (I.S. Blagbrough), b.r.conway@hud.ac.uk (B.R. Conway).

http://dx.doi.org/10.1016/j.colsurfb.2016.07.010
0927-7765/

mination of surface energies have been used to reduce the time of formulation development and enhance the quality of the final product [6–8].

The effect of the protein denaturation on their surface chemistry has been determined using time-of-flight secondary ion mass spectrometry [9]. However, the effect of mechanical denaturation on the surface energies of globular proteins has not been reported and these effects must be understood to exploit the full potential of globular proteins in pharmaceutical industry both as therapeutic agents and excipients. Inverse gas chromatography (IGC) is a useful verified tool for surface energy measurements [10]. IGC has been used to measure the surface free energy of lyophilized protein particles, detecting lot-to-lot variations in the amorphous microstructure of lyophilized protein formulations [11].

This paper aims to evaluate the effects of mechanical denaturation on the surface energies of globular protein powders using IGC. β-Galactosidase is a hydrolytic enzyme that has been widely investigated for potential applications in the food industry to improve sweetness, solubility, flavor, and digestibility of dairy products. Preparations of β-galactosidases have also been exploited for industrial, biotechnological, medical, and analytical applications [12]. Lysozyme is a naturally occurring enzyme found in bodily secretions such as tears, saliva, and milk and has been explored as a food preservative and pharmaceutical. The isoelectric points (pI) of β-galactosidase from *Aspergillus oryzae* and hen egg-white lysozyme are 4.6 and 11.3, and these proteins were used as models of acidic and basic globular proteins, respectively [13]. Lyophilized powders of these proteins were mechanically denatured by milling. Their surface energies before and after denaturation were compared in order to understand how the surfaces of the globular protein powders respond to the mechanical denaturation.

2. Materials and methods

2.1. Materials

Micrococcus lysodeikticus (Sigma–Aldrich), 2-nitrophenyl β-D-galacto pyranoside (Sigma–Aldrich), lyophilized powders of β-galactosidase from *A. oryzae* (Sigma–Aldrich) and hen egg-white lysozyme (Biozyme Laboratories, UK) were purchased as indicated. The purchased β-galactosidase and lysozyme powders were considered to be unprocessed samples and named UNG and UNL, respectively.

2.2. Preparation of mechanically denatured protein powders

Mechanically denatured powders of β-galactosidase and lysozyme were prepared by manually milling. The milling was achieved by rotating a marble pestle over the powder within a marble mortar at ~45 cycles per minute (cpm). Milling times of 60 min were enough to completely denature the protein powders, and this was confirmed by differential scanning calorimetry (DSC) [3]. The mechanically denatured powders of β-galactosidase and lysozyme were named DeG and DeL, respectively. Three batches (2 g each batch) of the mechanically denatured powders were prepared for each protein.

2.3. Microscopy

A Zeiss Axioplan2 polarizing microscope (Carl Zeiss Vision GmbH; Hallbergmoos, Germany) was used to visualize the samples. The accompanying software (Axio Vision 4.2) was then used to determine the projected area diameters of the powders.

2.4. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC) thermograms were obtained using a Perkin-Elmer Series 7 DSC (Perkin-Elmer Ltd., Beaconsfield, UK). Samples (4–7 mg) were sealed in aluminium pans. The escape of water was facilitated by making a pinhole in the lid prior to sealing. The samples were equilibrated at 25 °C and heated to 250 °C at a scan heating rate of 10 °C/min under a flow of anhydrous nitrogen (20 ml/min). Each sample was analysed in triplicate. The temperature axis and cell constant of the DSC cell were calibrated with indium (10 mg, 99.999% pure, melting point 156.60 °C, and heat of fusion 28.40 J/g).

2.5. FT-Raman spectroscopy

FT-Raman spectra of samples were recorded with a Bruker IFS66 optics system using a Bruker FRA 106 Raman module. The excitation source was an Nd: YAG laser operating at 1064 nm and a laser power of 50 mW was used. The FT-Raman module was equipped with a liquid nitrogen cooled germanium diode detector with an extended spectrum band width covering the wave number range 1800–450 cm^{-1}. Samples were placed in stainless steel sample cups and scanned 200 times with the resolution set at 8 cm^{-1}. The observed band wave numbers were calibrated against the internal laser frequency and are correct to better than ±1 cm^{-1}. The spectra were corrected for instrument response. The experiments were run at a controlled room temperature of 20 ± 1 °C.

2.6. Enzymatic assay

The enzymatic activity of lysozyme samples was measured to determine the ability of lysozyme to catalyze the hydrolysis of β-1,4-glycosidic linkages of cell-wall mucopolysaccharides [14]. Lysozyme solution (30 μl, 0.05% in phosphate buffer, pH = 5.2; 10 mM) was added to *Micrococcus lysodeikticus* suspension (2.97 ml, 0.025% in phosphate buffer, pH = 6.24; 66 mM). The decrease in the absorbance at 450 nm was monitored by using a UV–vis spectrophotometer (PU 8700, Philips, UK). The activity was determined by measuring the decrease in the substrate bacterial suspension concentration with time. Hence the slope of the reduction in light absorbance at 450 nm against the time of 3 min, starting when the protein solutions were mixed with the substrate bacterial suspension, was considered to be the indicator of the lytic activity of lysozyme [15].

The enzymatic activity of β-galactosidase samples was determined using a method relying on the ability of β-galactosidase to hydrolyse the chromogenic substrate *o*-nitrophenyl β-D-galacto pyranoside (ONPG) to *o*-nitrophenol [16]. The results were achieved by adding 20 μl of protein solution (0.05 w/v% in deionised water) to 4 ml of the substrate solution (0.665 mg/ml) in a phosphate buffer (100 mM and pH = 7). The mixture then was incubated for 10 min in a water bath at 30 ± 1 °C. The absorbance at λ = 420 nm was measured to indicate the activity.

The concentrations of the protein solutions had been determined prior to the activity tests using the following equation:

$$[Protein] = Abs_{280nm}/E_{280nm} \quad (1)$$

where [*Protein*] is the concentration of protein in the tested solution w/v%, Abs_{280nm} is the absorbance of the tested protein solution at 280 nm, and E_{280nm} is the absorbance of protein standard solution with concentration 0.05 w/v%. The concentrations of the solutions were diluted to be about 0.05% w/v so as to give an absorbance value of less than 0.8. The activities of all samples were measured relative to that of a corresponding fresh sample, which was considered as the standard solution.