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Enzymatic catalysis in a whey protein matrix at temperatures in the vicinity of the glass transition

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ABSTRACT

The current study evaluates the effect of temperature on α-glucosidase activity, following incorporation of the enzyme into a whey protein matrix through spray drying. Thermomechanical characterization of the matrix was achieved using the techniques of modulated temperature differential scanning calorimetry and small-deformation dynamic mechanical analysis. As the concentration was raised from 75 to 94% (w/w), denaturation of the protein occurred at increasing temperatures. In contrast, denaturation was not observed in calorimetric scans after spray drying. The glass transition temperature (T_g) measured in the dried particles using dynamic mechanical analysis was approximately 40 °C. An optimized procedure was developed whereby α-glucosidase and its substrate *p*-nitrophenyl α-D-glucopyranoside were incorporated into the whey matrix. The effect of temperature on enzymatic catalysis was investigated and, below 40 °C, activity was low and relatively independent of temperature. However, the rates of product formation markedly accelerated as temperatures were increased beyond T_g. These novel observations strongly emphasize the pronounced effect of mechanical T_g of the protein matrix on enzymatic activity.

1. Introduction

Over recent decades, the transition between glassy and rubbery states in amorphous solid materials has attracted particular attention reflecting its significance as a determinant of the stability characteristics in food and pharmaceutical systems (Slade & Levine, 1995). The glass transition temperature (T_g) is an important characteristic of such materials and is strongly influenced by moisture content. Structural stability of individual components within an amorphous material is high in the glassy state, and these matrices can retain their physicochemical state for many years. This reflects the inhibition of large-scale molecular rearrangements and macroscopic flow at temperatures below T_g.

As the rates of reaction are very low in glassy matrices, these systems depict an increase in chemical stability and this has been described in an early report regarding the Maillard reaction between carbohydrates and proteins (Eichner & Karel, 1972). However, several dynamic processes can still proceed at measurable rates within glassy matrices. Examples are the diffusion of water (Tromp, Parker, & Ring, 1997) as well as other small molecules including gases (Schoonman, Ubbink, Bisperink, Le Meste, & Karel, 2002). In addition, local rearrangements of the carbohydrate molecules result in glassy-state aging of the matrix (Noel et al., 2005).

Enzymes are important in many food systems, as they may be naturally occurring components of ingredients, produced during fermentation or spoilage and also following intentional incorporation into the formulation. In the context of the varied roles of enzymes, both as deteriorative agents and also in enhancing product quality and ease of processing, relatively few researchers have addressed the influence of the glass transition upon the rates of enzymatically catalyzed reactions within amorphous matrices. Recently, we have reported upon the activity of the α-glucosidase/*p*-nitrophenyl α-D-glucopyranoside complex as affected by a glassy polysaccharide matrix (Chaudhary, Small, & Kasapis, 2013).

While carbohydrate systems are widely employed as amorphous matrices in foods and pharmaceuticals, a variety of protein ingredients also have potential for the formulation of added value products. Proteins provide a range of useful functional properties (Shahidi & Han, 1993) and these have been described for gelatin (Draye et al., 1998) and wheat gliadin (Duclairoir et al., 1999). In addition, whey protein gels have been used as pH-sensitive hydrogels for the controlled delivery of biologically active substances (Gunasekaran, Xiao, & Ould Eleya, 2006). Their aggregates have been shown to participate in the formation of hydrogels, gel beads and submicron particles for protecting and controlling the release of bioactive ingredients (Nicolai, Britten, & Schmitt, 2011). Advantages of using whey proteins in controlled-release applications are their association without the need for chemical cross-linking agents and their biodegradability, which are two of the major requirements for use in pharmaceutical, functional food and bioprocessing areas (Gunasekaran, Ko, & Xiao, 2007).

Whey powders are also increasingly used in the development of nutritional preparations where enzyme stabilization is required in the dry state. Several authors have reported and attributed the increased

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stability of β-galactosidase in liquid or solid dairy systems to lactose (substrate) binding protection (Schebor, Burin, Buera, Aguilera, & Chirife, 1997; Stellwagen, Cronlund, & Barnes, 1973; Yang, Marchio, & Yen, 1994). The low molecular mobility of various components in the glassy state appears to govern changes that affect the enzymatic stability in dry amorphous dairy systems including mobility of enzyme side chains for folding processes, reactant (amino-carbonyl) mobility for the Maillard reaction, and lactose mobility for the crystallization process of the matrix. Therefore, the physical properties of the matrices that are used to encapsulate enzymes often exert control by affecting enzyme activity and stability (Burin, Joupilla, Roos, Kansikas, & Buera, 2004).

The increasing interest in the properties of whey proteins as encapsulating agents for the food industry reflects the need for effective and selective delivery of bioactive agents to the site of action. As natural polymers, biomacromolecules provide advantages over synthetic polymers due to their wide availability, low cost, low toxicity and ease of modification (Chasin & Langer, 1990). The aim of the present study has been to evaluate the relationship between the activity of α-glucosidase and T_g using a model matrix prepared by spray drying of whey protein isolate.

2. Materials and methods

2.1. Materials

The standard whey protein isolate (WPI) used was from MG Nutritionals, Murray Goulburn Co-operative Ltd, VIC, Australia. According to the supplier, the composition of the WPI was 91.3% protein (TN × 6.38), 0.7% fat, 3.5% moisture, 3.8% ash and 0.44% lactose. The pH of a 10% (w/w) solution was 6.3, bulk density of the powder was 0.45 g/mL and a standard plate count produced 9900 cfu/g.

The enzyme used (α-D-glucoside glucohydrolase (EC 3.2.1.20), product number G3651) from *Bacillus stearothermophilus* was from Sigma-Aldrich (St. Louis, MO, USA). The lyophilized powder (3.2 mg, containing potassium phosphate buffer salt) was dissolved in potassium phosphate buffer (10 mL, 67 mM, pH 6.8 at room temperature) prior to use. Substrate (*p*-nitrophenyl α-D-glucopyranoside; *p*NPG), potassium phosphate and L-glutathione (acting as stabilizer) were also purchased from Sigma-Aldrich and sodium carbonate was from BDH, Port Fairy, VIC, Australia. Milli-Q water from Millipore was used in all experiments.

2.2. Sample preparation

2.2.1. Preparation of whey protein for thermal studies

WPI dispersions of 30% (w/w) solids were prepared by mixing the powder in Milli-Q water at room temperature. Dispersions were stirred for ~2 h using a magnetic stirrer to ensure maximum dissolution. In order to achieve thorough hydration and to remove air bubbles, dispersions were stored overnight at 4 °C. On the following day, a rotary vacuum evaporator was used at 40 ± 1 °C to remove water and prepare a series of solutions at concentrations of 75%, 77.5%, 80%, 82.5% and 85% (w/w). A spray dried sample with 94% solid content was also subjected to thermal analysis.

2.2.2. Sample preparation for dynamic mechanical analysis, scanning electron microscopy and enzymatic studies

WPI dispersions of 10% (w/w) solids were prepared by mixing the powder in Milli-Q water and holding at 4 °C overnight to ensure thorough hydration. Following optimization of the spray drying procedure, the dispersion was treated using a Lab Plant SD Basic FT30MKIII spray drier (Keison products, Chelmsford, Essex, UK) to produce microcapsules for dynamic mechanical analysis and scanning electron microscopy.

Microcapsules for the enzymatic assay were also produced by following the same procedure after mixing the WPI dispersion (50 g in 500 mL Milli-Q water) with potassium phosphate buffer (25 mL, 67 mM), glutathione (3.32 mL, 3 mM), enzyme solution (1.67 mL, containing 42 units) and *p*NPG (20 mL, 20 mM), with the mixture being constantly stirred while spray drying. The microcapsules were produced in triplicate and stored at −30 °C for subsequent analysis.

2.3. Methods

2.3.1. Calorimetric measurements

Samples consisting of WPI (75% to 94% solids, w/w) were hermetically sealed in T_{zero} pans and subjected to temperature modulated differential scanning calorimetry (MDSC) measurements (DSC Q2000, TA instruments, New Castle, DE). To calibrate the heat flow signals, a traceable indium standard ($\Delta H_f = 28.3$ J/g) and a sapphire standard were used. Samples of 8 mg were heated to 90 °C, cooled to −90 °C and reheated to 90 °C, at a modulation amplitude of 0.53 °C for each period of 40 s. Temperatures down to −90 °C were achieved by using a refrigerated cooling system attached to the calorimeter. An empty T_{zero} pan was used as a reference, nitrogen purge gas was at a flow rate of 50 mL/min, and controlled scanning was carried out at 2 °C/min.

2.3.2. Rheological measurements

Estimates of the glass transition temperature of spray dried WPI (94% solids) were obtained using a dynamic mechanical analyzer (DMA 8000) from Perkin Elmer (Waltham, MA, USA) with liquid nitrogen as the coolant. The sample was subjected to deformation mode by compression within a temperature ramp of −100 °C to 90 °C, scan rate of 2 °C/min and frequency of 1 rad/s. Results reported for thermal and rheological experiments are of individual traces selected as representative of three replicates, which were effectively superposing curves.

2.3.3. Scanning electron microscopy (SEM)

Images of WPI particles produced by spray drying were obtained using an FEI Quanta 200 ESEM instrument (Hillsboro, OR, USA). Samples were gold-coated and imaged using high-vacuum mode at an accelerating voltage of 30 kV, pressure of 0.54 mbar and spot size of 5. Regarding the moisture content of our materials, these were analyzed gravimetrically by oven-drying at 100 °C for 24 h.

2.3.4. Spectrophotometry and enzymatic assay

In 10% (w/w) WPI solution (500 mL) we added 1.67 mL enzyme equivalent to 42 units and 20 mL of *p*NPG (20 mM concentration). This solution was spray dried, and to analyze the enzymatic activity in spray dried WPI (94% solids), containing the above enzyme–substrate complex, a spectrophotometric approach from Sigma Chemical Co (2012) was adapted and used. For this, samples were individually packed in small containers (0.1 g each) and stored at −30 °C to prevent enzymolysis until experimentation commenced. In doing so, the spray dried powders were equilibrated at various temperatures (25 °C, 30 °C, 35 °C, 40 °C, 45 °C, 50 °C or 55 °C) selected to extend the range of observations above and below the mechanical T_g value of 40 °C established using DMA. Five containers with 0.1 g sample each were stored at the aforementioned temperatures for 1 h before commencing the "incubation" and taking readings at 0, 5, 10, 15 and 20 min.

At each time, the enzyme was inactivated by addition of sodium carbonate solution (8.0 mL, 100 mM) and vortexing vigorously for 1 min. Absorbance of the resultant solution was recorded at 400 nm on a Lambda 35 UV–visible spectrophotometer (Perkin Elmer, Singapore) for each time interval of 5 min. Measurements of enzyme activity at each experimental temperature were carried out in triplicate, and average values are reported in units of nanokatals (nkat) per g of matrix.