Food Chemistry 141 (2013) 2334–2342

Contents lists available at SciVerse ScienceDirect

Food Chemistry

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

# Whey protein isolate polydispersity affects enzymatic hydrolysis outcomes

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ARTICLE INFO

*Article history:*
Received 25 February 2013
Received in revised form 19 April 2013
Accepted 9 May 2013
Available online 24 May 2013

*Keywords:*
Heat-treatment
Whey protein isolate
Enzymatic hydrolysis
Peptide analysis
Essential amino acids

ABSTRACT

The effects of heat-induced denaturation of whey protein isolate (WPI) on the enzymatic breakdown of α-La, caseinomacropeptide (CMP), β-Lg A and β-Lg B were observed as hydrolysis proceeded to a 5% degree of hydrolysis (DH) in both unheated and heat-treated (80 °C, 10 min) WPI dispersions (100 g $L^{-1}$). Hydrolysis of denatured WPI favoured the generation of higher levels of free essential amino acids; lysine, phenylalanine and arginine compared to the unheated substrate. LC–MS/MS identified 23 distinct peptides which were identified in the denatured WPI hydrolysate – the majority of which were derived from β-Lg. The mapping of the detected regions in α-La, β-Lg, and CMP enabled specific cleavage points to be associated with certain serine endo-protease activities. The outcomes of the study emphasise how a combined approach of substrate heat pre-treatment and enzymology may be used to influence proteolysis with attendant opportunities for targeting unique peptide production and amino acid release.

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## 1. Introduction

The functional applications for whey proteins, both biological and technological, are of significant current interest. Whey as a commercial ingredient can be concentrated as whey protein isolate (WPI) which is high in branched chain amino acids, a source of health benefits (Marshall, 2004; Nilsson, Holst, and Björck, 2007). The amino acid profile of whey also contains higher levels of essential amino acids compared to most other proteins (Schaafsma, 2006). The desire to find novel functional attributes to whey has led to the development of processes other than standard WPI or WPC production. One such process, enzymatic hydrolysis, has been much utilised to improve the functional characteristics of the parent protein (Gauthier & Pouliot, 2003).

The susceptibility of whey proteins to heat-induced structural changes has the potential to alter hydrolysis by exposing previously inaccessible amino acid residues to cleavage (Mullally, Mehra, & FitzGerald, 1998; Reddy, Kella, & Kinsella, 1988). In a mixed protein material the normal course of heat-induced denaturation is affected by intra- and inter-protein interactions (Dalgleish, Senaratne, & Francois, 1997; Havea, Singh, & Creamer, 2002) and, with heat-treatment, the covalent linkages which contribute to stabilise tertiary structures will be affected (Bowler, 2007). The continued application of heat can result in irreversibly aggregated protein which is prevented from returning to the native form by thermodynamic barriers (Kauzmann, 1959; Mulvihill & Donovan, 1987).

The kinetics of enzymatic hydrolysis may also benefit from improved accessibility of active sites in proteins. The use of pre-hydrolysis heat-treatments to induce structural changes in the protein substrate can influence hydrolysis reaction rates. Therefore, endo-protease activity can be affected by the degree of protein denaturation (Panyam & Kilara, 1996) where a positive influence on hydrolysis reaction rates has been demonstrated in individual protein fractions (Guo, Fox, Flynn, & Kindstedt, 1995; Schmidt & van Markwijk, 1993) and heterogeneous concentrates (Kim et al., 2007a). Kim et al. (2007a) reported that a pre-hydrolysis heat-treatment (100 °C for 10 min) increased peptic and tryptic hydrolysis of WPC, while heat-treatments >70 °C for 15 min also have a positive effect on the rate of hydrolysis of WPI that may be associated with differing aggregation phenomena (O'Loughlin, Murray, Kelly, FitzGerald & Brodkorb, 2012).

Changing the conformation of the substrate pre-hydrolysis leads to a novel hydrolysis reaction which differs from that obtained with un-treated/native protein substrate (Meisel, 1998). The folded structure of native whey proteins provides resistance to hydrolysis by digestive enzymes (Schmidt, Meijer, Slangen, &

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0308-8146/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.foodchem.2013.05.056

Van Beresteijn, 1995) – a feature that is increasingly exploited for techno-functional and bio-activity opportunities. Previous studies have shown that controlled proteolysis of heat-denatured whey proteins can increase the techno-functionality of WPI (Mutilangi, Panyam, & Kilara, 1996) and improve the bio-functionality of WPC (Kim et al., 2007b). Hydrolysis of the proteins will, in many cases, also release free amino acids as well as peptides and many of these amino acids may have important nutritional and physiological benefits. Free amino acids perform many functions including modulation of insulin release (van Loon et al., 2003) and induction of human growth hormone release (Isidori, Lo Monaco, & Cappa, 1981).

The objective of this study was to determine how the combined approach of substrate heat pre-treatment and enzymology may influence proteolysis particularly with respect to the opportunity to target unique peptide production and amino acid release. In light of the effects of aggregation phenomena previously encountered (O'Loughlin et al., 2012) it was opportune to examine how heat-induced modification of WPI affected the molecular level properties of enzymatic hydrolysates obtained following incubation with Corolase® PP (E.C. 3.4.21.4). This porcine pancreatic enzyme preparation is well documented for its high proteolytic activity in whey due to its three serine-protease activities; trypsin, chymotrypsin and elastase, as well as exo-peptidases such as carboxypeptidase (CYP) zymogens CYP A and CYP B (Mullally, O'Callaghan, FitzGerald, Donnelly & Dalton, 1994) which favour the production of non-bitter hydrolysates (Uhlig, 1998). The study was also an opportunity to determine how specific cleavage points associated with certain serine endo-protease activities present in Corolase® PP were affected by the polydisperse conditions created by heat-induced changes to WPI prior to hydrolysis.

## 2. Materials and methods

### 2.1. Materials

Whey protein isolate (Isolac®) was provided by Carbery Food Ingredients, (Ballineen, Co. Cork, Ireland). The powder contained 89.3% protein by Kjeldahl [N × 6.38 (Merrill & Watt, 1973)] comprising 56.5% β-Lactoglobulin (β-Lg) (29.3% β-Lg A, 27.2% β-Lg B), 14.3% α-Lactalbumin (α-La), 10.3% glycosylated caseinomacropeptide (CMP), 8% non-glycosylated CMP, and 1.7% bovine serum albumin (BSA). Denatured material amounted to 15.4% of total protein as determined by urea denaturing RP-HPLC.

The digestive-enzyme complex Corolase® PP (E.C. 3.4.21.4.) was sourced from AB Enzymes GmbH, Darmstadt, Germany, and had a minimum activity of 2,500 units with haemoglobin as a substrate (UHb) and 220,000 Lohlein-Volhard units (LVU) $g^{-1}$ at pH 8.0.

### 2.2. Heat treatment and characterization of WPI solutions

WPI (33.59 g) was dissolved in 300 mL $dH_2O$ (giving a 100 g $L^{-1}$ protein dispersion) and the solution allowed to hydrate overnight at 4 °C. A thermostatically controlled water bath was brought to 80 °C and allowed to pre-equilibrate for 30 min. Samples were adjusted to pH 8 with NaOH and dispersions for heat-treatment were then heated for 10 min under stirring followed by cooling in ice-water.

The conformational state of the major proteins in WPI were characterised by high performance liquid chromatography (HPLC) and the loss in native protein quantified as previously described (O'Loughlin et al., 2012).

### 2.3. Enzymatic hydrolysis of WPI solutions

Degree of hydrolysis (DH) represents the number of peptide bonds cleaved (h) as a percentage of total peptide bonds ($h_{tot}$) and can be related to the consumption of base as hydrolysis releases protons according to Eq. (1) (Adler-Nissen, 1986):

$$\mathrm{DH}\% = 100 \times B \times \mathrm{N}_B \times \frac{1}{\alpha} \times \frac{1}{\mathrm{MP}} \times \frac{1}{h_{tot}} \quad (1)$$

where $B$ is the volume of base, $N_B$ is the normality of the base, $1/\alpha$ is the average degree of dissociation of α-$NH_2$ residues (=1.13 at pH 8, 50 °C), MP is the mass of protein and $h_{tot}$ is the total number of peptide bonds given in meq $g^{-1}$ protein based on Kjeldahl nitrogen (N) and a conversion factor ($f_N$) of 6.38. The $h_{tot}$ for whey protein concentrates is 8.8.

For hydrolysis experiments, 300 mL solutions of WPI (100 g $L^{-1}$ protein) were hydrolysed to a target DH of 5% with Corolase® PP. Hydrolysis was performed at an enzyme:substrate ratio of 1:100 ($ww^{-1}$) on a protein equivalent basis and the hydrolysis conditions were 50 °C and pH 8 which were controlled throughout the reaction. The pH was controlled by titration with 2 N NaOH using a Metrohm 842 Titrando dosing unit (Metrohm Ltd., Herisau, Switzerland) and the reaction was agitated utilising an over-head stirrer at 300 rpm. The reaction was terminated by heating the enzyme at 85 °C for 20 min. All hydrolysis experiments were conducted in triplicate.

The hydrolysates were then lyophilized prior to molecular level analysis. For analysis of the changes in protein distribution and peptide formation taking place during the course of hydrolysis, aliquots of the reaction were withdrawn at different DH points and the enzyme activity was stopped by dilution in 0.1% ($vv^{-1}$) trifluoroacetic acid (TFA) instead of heat-inactivation.

### 2.4. Solubility and high performance liquid chromatographic analysis of hydrolysates

Solubility was determined through centrifugation and subsequent protein determination of supernatant according to the method of O'Loughlin et al. (2012). Solubility experiments were conducted in duplicate.

Reversed-phase HPLC profiles were generated utilizing a Phenomenex Jupiter C18 (4.6 mm × 250 mm, 5 μM, 300 Å, Phenomenex, Cheshire, UK) column under linear gradient elution conditions; Solvent B: from 0% to 100% in 30 min, 100% for 5 min, 100% to 0% in 5 min, and 0% for 5 min at a flow-rate of 1 mL $min^{-1}$ where Solvent B is 80% acetonitrile (MeCN) containing 0.1% TFA ($vv^{-1}$). Diluted protein solutions (20 μL of 2.5 mg $mL^{-1}$) were loaded onto the column.

Size exclusion chromatography (SEC) was carried out on a TSK Gel G2000SW, 7.8 mm × 600 mm, (Tosoh Bioscience GmbH, Stuttgart, Germany) on an isocratic gradient of 30% MeCN containing 0.1% TFA ($vv^{-1}$) with a run-time of 60 min. The samples were diluted in 30% MeCN containing 0.1% TFA ($vv^{-1}$). 20 μL of 2.5 mg $mL^{-1}$ Protein solutions (20 μL of 2.5 mg $mL^{-1}$) were also loaded onto the column. Commercial α-La, β-Lg A and B, BSA, lactoferrin, and CMP (Sigma–Aldrich, Dublin, Ireland) were used as standards with Ribonuclease A, Cytochrome C, Aprotinin, Bacitracin, His-Pro-Arg-Tyr, Leu-Tyr-Met-Arg, Bradykinin, Leu-Phe, Tyr-Glu (Bachem AG, Bubendorf, Switzerland) used as molecular weight standards. Samples and standards were pre-filtered through 0.22 μm low protein binding membrane filters (Sartorius Stedim, Surrey, UK) prior to application to the column. All solvents were filtered under vacuum through a 0.45 μm high velocity filters (Millipore (UK) Ltd., Durham, UK).

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