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Microwave-assisted digestion of β-lactoglobulin by pronase, α-chymotrypsin and pepsin

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Abstract

The effects of microwave irradiation (MWI) on kinetic parameters for pronase, α-chymotrypsin and pepsin hydrolysis of bovine β-lactoglobulin (β-Lg) were evaluated. The experiments were performed under MWI or conventional heat (CH) at 40 °C. The initial velocity (V_0) of peptide bonds cleavage was measured by *o*-phthaldialdehyde method; the peptide profile was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE). Higher catalytic effectiveness (K_{cat} K_m^{-1}) values were obtained in the pronase and α-chymotrypsin digestions performed under MWI (7793 and 2073 min^{-1} mM^{-1}, respectively) in comparison with the values in the respective CH digestions (1802 and 941 min^{-1} mM^{-1}, respectively). The Michaelis–Menten constant (K_m) for either enzyme was reduced under MWI. Pepsin showed very low activity on β-Lg at pH 4.0 regardless of the heating procedure used. For two enzymes, pronase and α-chymotrypsin, differences in SDS–PAGE profiles were obtained due to the MWI applied during the enzymatic hydrolysis. The combined enzyme/MWI treatments could have a relevant application in the development of β-Lg hydrolysates.

Keywords: Microwave irradiation; β-Lactoglobulin; Pronase; α-Chymotrypsin; Pepsin

1. Introduction

The dairy components of many infant formulas are modified by adding bovine whey to the milk, to imitate the ratio of casein to whey proteins (40:60) of breast milk. However, this could be an unsuitable strategy because human milk does not contain β-lactoglobulin (β-Lg), the major soluble protein in bovine milk. This protein seems to be resistant to gastric digestion and apparently remains intact after passing through the stomach (Yvon, Van Hille, Pelissier, Guilloteau, & Toullec, 1984), and thus its amino-acid components may be nutritionally unavailable for infants. The β-Lg is considered also to be one of the main allergens in bovine milk (Huang, Coleman, & Stanworth, 1985; Otani, 1987; Okamoto, Hayashi, Enomoto, Kaminogawa, & Yamauchi, 1991; Sélo, Negroni, Creminon, Yvon, Peltre, & Wal, 1998).

Enzymatic proteolysis and conventional heat treatment are normally used in the production of partially or extensively hydrolysed formulas to lower the content of β-Lg and other intact proteins, in order to reduce the antigenicity of milk proteins. However, residual allergenicity has been reported in several of these commercial preparations (Restani et al., 1995; Van Berensteijn, Meijer, & Schmidt, 1995; Calvo & Gomez, 2002), which could be due to inaccessibility of some sequential epitopes to proteases, even in the denatured protein.

Microwave irradiation (MWI) could be a useful tool in the process of enzymatic hydrolysis of whey proteins in general, and β-Lg in particular. Numerous studies have dealt with the application of MWI as an alternative method to conventional heating (CH) during enzymatic hydrolysis of food proteins. Enzymatic hydrolysis of β-Lg with the use of this non-conventional energy could accelerate enzymatic

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processes as described by Pramanik et al. (2002) for protein mapping by trypsin digestion. These authors reported the effectiveness of this treatment, since the peptide fragmentation of several biologically active proteins was achieved in minutes using MWI, in contrast to the hours required by CH during incubation. The potential advantages of MWI treatment have been reported also for acceleration of protein hydrolysis in the preparation of samples for amino acid analysis (Chen, Chiou, Chu, & Wang, 1987; Chiou & Wang, 1989; Marconi, Panfili, Bruschi, Vivanti, & Pizzoferrato, 1995), for milk pasteurisation (López-Fandiño, Villamiel, Corzo, & Olano, 1996) without adverse effects on flavour during cold storage (Valero, Villamiel, Sanz, & Martínez-Castro, 2000), and for preparation of samples for atomic absorption analysis (de la Fuente & Juarez, 1995). However, the application of MWI during enzymatic reactions of food proteins remained largely unexplored.

Microwaves are electromagnetic waves and the heating of proteins is accomplished by the absorption of microwave energy, rotation of the bipolar water molecules and oscillatory migration of the ionic components of the proteins (Ohlsson & Bengtsson, 2001). Prior information about kinetic properties of the enzyme in question under MWI is essential for designing an enzymatic process, since the kinetic parameters are enzyme, substrate and environment specific (Beg, Saxena, & Gupta, 2002).

The purpose of this work was to establish whether MWI treatment of β-Lg during its digestion by pronase, chymotrypsin and pepsin would affect the kinetic parameters of the enzymes, as well as the nature of the end products.

2. Materials and methods

2.1. Materials

β-Lactoglobulin (β-Lg) from bovine milk, pronase from *Streptomyces griseus* (E.C. 3.4.24.31), α-chymotrypsin type I-S from bovine pancreas (E.C.3.4.21.1), and pepsin from porcine stomach (E.C. 3.4.23.1) were obtained from Sigma Chemical Company, (St. Louis, MO, USA). *O*-phthaldialdehyde (OPA) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

2.2. Hydrolysis experiments

The enzymatic digestions were carried out separately with three different proteases, pronase, α-chymotrypsin and pepsin. Fifty microlitres of enzyme solution (0.056 mM) and 20–100 μL of β-Lg solution (2.8 mM) in water were added to sufficient buffer (50 mM sodium phosphate at pH 8 for pronase and chymotrypsin, and 50 mM sodium citrate at pH 4 for pepsin) to make the total volume 500 μL. The proteolysis reactions were carried out at 40 °C for 3 min under MWI or CH. Blanks were also included using the same conditions without enzyme. Each digestion experiment was replicated six times. After incubation, the hydrolysates were heat treated (80 °C, 5 min) and stored at −20 °C until analysis.

2.3. Microwave irradiation and conventional heat treatment

The samples (hydrolysis reactions) were subjected to MWI in a single beam microwave applicator Synthewave 402 (Prolabo, Fontenay Sous-Bois, France), with a maximum power of 300 W, using a non-contact infrared continuous feedback temperature system; only 5–10% of available power was used during the enzymatic reactions. The sample vial containing the substrate dissolved in the buffer was preheated to 40 °C, and immediately after adding the enzyme the vial was placed in a one-hole Teflon insertion rack, which in turn was lowered into the irradiation chamber. The temperature of the apparatus was programmed to ensure that the reaction solution temperature was 40 °C immediately after 3 min of MWI treatment. As a control, hydrolysis was performed under CH at 40 °C in a thermostatic water bath in an identical fashion but without MWI.

2.4. Assessment of the extent of proteolysis

The degree of hydrolysis was determined by quantification of cleaved peptide bonds from TCA (5%, w/v, final concentration) soluble extracts of samples using the *o*-phthaldialdehyde (OPA) spectrophotometric assay described by Peñas, Préstamo, and Gómez (2004). An aliquot of 100 μL of the extracts was added to 3.0 mL of OPA reagent in a 3.5 mL quartz cuvette; the solution was swirled by inversion, incubated for 2 min at room temperature, and the absorbance (340 nm) was measured using a 4049 LKB Biochrom spectrophothometer (Biochrom Ltd., Cambridge, UK). Duplicate determinations were performed.

2.5. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The electrophoretic analysis of hydrolysates was performed by reducing SDS–PAGE using precast gradient gels of 4–20% polyacrylamide-Tris–HCl (Bio–Rad, Richmond, CA, USA; Laemmli, 1970), at constant current (15 mA/gel) for 1.5–2 h. Heat treated samples were mixed with the buffer (1:5, v/v), consisting of 62.5 mM Tris–HCl, glycerol (10%), SDS (2%), bromophenol blue (0.0025%), and β-mercaptoethanol (2.5%), pH 6.8, and then heated at 95 °C for 5 min. To detect the protein digestion products from 20 μg of β-Lg, 20 μL of the buffer treated samples were applied to the gels. The bands were stained with 0.1% PhastGel Blue R (Pharmacia, Dübendorf, Switzerland) in methanol:water:acetic acid (20:70:10) and destained with this solvent.