Journal of Food Engineering 91 (2009) 42–48

Contents lists available at ScienceDirect

Journal of Food Engineering

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

High hydrostatic pressure inactivation kinetics of the plant proteases ficin and papain

G.I. Katsaros, P. Katapodis, P.S. Taoukis *

Laboratory of Food Chemistry and Technology, School of Chemical Engineering, National Technical University of Athens, 5 Iroon Polytechniou Street, 15780, Greece

ARTICLE INFO

Article history:
Received 5 June 2008
Received in revised form 31 July 2008
Accepted 1 August 2008
Available online 9 August 2008

Keywords:
Papain
Ficin
High pressure
Enzyme kinetics
Thermal inactivation

ABSTRACT

Papain and ficin are general non-specific plant thiol proteases applied in food processing. Inactivation kinetics of papain and ficin were studied for thermal (50–80 °C, ambient pressure) and high pressure/temperature (500–900 MPa at 50–80 °C) process conditions. The effect of temperature at each pressure and the effect of pressure at each temperature were expressed by the values of activation energy, E_a, and activation volume, V_a. The two enzymes showed the same inactivation under thermal process. At high pressures, at each process temperature, increase of pressure increases the inactivation rate. Up to 60 °C for ficin and 70 °C for papain, inactivation rates at high pressures are higher than the corresponding thermal rates at ambient pressure. At higher temperatures up to certain pressures an antagonistic effect was observed. At 80 °C thermal inactivation rates at all high pressures are lower than the ones at ambient pressure. E_a increased for papain (from 50 to 88 kJ/mol) and decreased for ficin (from 139 to 43 kJ/mol) as process pressure increased, a different effect of pressure on temperature sensitivity. V_a depended on process temperature showing increasing effect of pressure at higher temperatures for papain and decreasing for ficin. The enzymes inactivation rate constant was modeled as a function of both temperature and pressure conditions by a multi-parameter equation. Overall, papain and ficin showed a high thermal and pressure stability requiring intense process conditions for adequate inactivation.

1. Introduction

Plant thiol proteases, such as ficin and papain have been used for various applications in the food industry. Papain (EC 3.4.22.4) and ficin (EC 3.4.22.3) are the major protein constituents of the latex in the tropical plant *Carica papaya* and the fig tree *Ficus* sp., respectively (Drenth et al., 1971). These enzymes belong to the C1 papain family of clan CA and have a catalytic triad of Cys, His, and Asn (Rawlings et al., 2004). The proteinases of the C1 papain family comprise a single polypeptide chain of ~25 kDa, which is folded to form a globular protein with two domains. There is a deep cleft in between the two domains where the substrates can bind, and where the active site cysteine (Cys-25) and histidine (His-159, papain numbering) residues form a thiolate–imidazolium ion pair for catalytic activity. The structures of these enzymes are very similar, and the catalytic mechanism is the same. They can differ, however, in the amino acids lining the substrate-binding pocket, which are responsible for binding amino acid side-chains of the substrate. They could therefore differ in substrate specificity, cleaving different peptide bonds of the substrate proteins (Rawlings and Barrett, 1994; Gosalia et al., 2005).

These enzymes have been used as general non-specific proteases for various applications in the food and drink industry. Applications included use of these proteins as meat tenderizers, removal of chill haze in beer, improvement in the processing quality of cereals and plant milk clotting enzymes for novel dairy products (Wallerstein, 1911; Feldberg and Baker, 1971; Kang and Warner, 1974; Lartigue, 1975; Mustafa and Gomaa, 1990; Fadyloglu, 2000).

Non-thermal technologies such as High Hydrostatic Pressure (HHP) processing and Pulsed Electric Fields (PEF) have potential for food preservation purposes because they can inactivate microorganisms (Hoover et al., 1989; Knorr, 1993) and enzymes (Yeom et al., 1999; Polydera et al., 2004), while provide many advantages and benefits with regards to color, flavor, and nutritional quality (Yen and Li, 1996). PEF technology has been used for inactivation of papain (Yeom et al., 1999). The authors concluded that papain was significantly resistant to PEF inactivation and that the inactivation was related to the loss of α-helix structure. HHP can also modify protein structure (Defaye et al, 1995; Heremans, 1995; Galazka et al., 1996) and thus enzyme activity (Gomes and Ledward, 1996). HHP mechanism for enzyme denaturation is governed by the Le Chatelier principle, which predicts that application of pressure shifts an equilibrium to the state that occupies the smallest volume, so any reaction accompanied by volume decrease, is accelerated by elevated pressures (Cano et al., 1997). The effect of HHP on

* Corresponding author. Tel.: +30 210 772 3171; fax: +30 210 772 3163.
E-mail addresses: gkats@chemeng.ntua.gr (G.I. Katsaros), katapo@chemeng.ntua.g (P. Katapodis), taoukis@chemeng.ntua.gr (P.S. Taoukis).

0260-8774/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jfoodeng.2008.08.002

Nomenclature

A	enzyme catalytic activity at time t (μeq H^+ min^{-1} ml^{-1})
A_o	initial enzyme catalytic activity (time zero of processing) (μeq H^+ min^{-1} ml^{-1})
a	constant that expresses the effect of pressure on V_a (ml mol^{-1} K^{-1})
b	constant that expresses the effect of temperature on E_a (MPa^{-1})
E_a	activation energy (kJ/mol)
E_{a_o}	activation energy at P_{ref} (kJ/mol)
k	first order inactivation rate constant (min^{-1})
k_o	first order inactivation rate constant at a reference temperature or pressure (min^{-1})
k_T	inactivation rate constant at a temperature T (min^{-1})
k_P	inactivation rate constant at a pressure P (min^{-1})
P	pressure (MPa)
P_{ref}	reference pressure (MPa)
R	universal gas constant (8.314 J/mol K)
t	time (min)
T	temperature (°C)
T_{ref}	reference temperature (°C)
V_a	activation volume (ml/mol)
V_{a_o}	activation volume at T_{ref} (ml/mol)

the enzymes can be attributed to the fact that HHP affects hydrogen bonds and alters the three-dimensional configuration of the molecules. The effect of HHP on enzyme inactivation has been shown to be strongly dependent on the type of enzyme, pH, nature of the medium in which the enzyme is dispersed, temperature and treatment time (Cheftel, 1992; Kunugi, 1992; Seyderhelin et al., 1996; Goodner et al., 1998; Hendrickx et al., 1998; Nguyen et al., 2002). The effect of pressure on the activity of enzymes important to food quality such as phenolases, pectinases and peroxidases has been studied and reported in several publications (Suthanthangjai et al., 2005; Rodrigo et al., 2007; Polydera et al., 2004; Guiavarc'h et al., 2005). Proteases of plant origin such as papain and ficin have not been adequately studied. Inactivation of papain after processing at 800 MPa and 60 °C was reported by Gomes et al. (1997). No studies on high pressure inactivation of ficin have been published.

Mathematical models have been used to describe pressure–temperature dependence of inactivation rate constants of enzymes (Stoforos et al., 2002; Polydera et al., 2004). Such models can be a useful tool in designing and optimizing high pressure processes.

The objective of this work was to investigate thermal and pressure inactivation of papain and ficin during combined HHP (200–900 MPa) and temperature (30–80 °C) treatments. Thermal inactivation (40–80 °C) at atmospheric pressure was also investigated. Mathematical description of the effect of process parameters on enzyme inactivation using the appropriate kinetic models was undertaken.

2. Materials and methods

2.1. Enzymes and substrates

Ficin (EC 3.4.22.3) and papain (EC 3.4.22.2) used for the experiments and the appropriate substrate pGlu-Phe-Leu *p*-nitroanilide (GPLNA) were obtained from Sigma Chemical Co. (MI, USA). All chemicals used were analytical grade. The enzymes were activated before treatments. Solutions containing 0.2 M phosphate buffer (pH 7.0, 25 μL), 0.1 M L-cysteine (100 μL), 0.1 M EDTA (10 μL) and deionised water (865 μL) thermally treated at 40 °C for 20 min were used to activate the enzyme.

2.2. Enzyme activity assay

The hydrolytic activity of ficin and papain, A, was determined using GPLNA (pGlu-Phe-Leu *p*-nitroanilide) as a low molecular weight substrate (Filippova et al., 1984). The enzymatic activity was calculated from the initial rate of GPLNA hydrolysis by determining pNA liberation within the given period of time. Hydrolysis of GPLNA was carried out in 50 mM phosphate buffer, pH 7.0 at 40 °C using a SPECTRAmax 250 Microplate Spectrophotometer (Molecular Devices, USA). Reaction after temperature equilibration was initiated by the addition of 50 μl solution of activated enzyme (ficin or papain) (0.05 mg/ml) in a total volume of 250 μl. The substrate concentration ranges used were: 0.1–2 mM for GPLNA. The release of pNA was monitored spectrophotometrically at 410 nm, recorded every 20 s over 5 min. Self-hydrolysis of GPLNA was determined by substituting the enzyme with buffer solution per substrate concentration. Dimethylsulfoxide (DMSO) was always added at 5% (v/v). The molar absorptivity ($\varepsilon^{410\ nm}$) of *p*-nitroanilide of 9800 (M^{-1} cm^{-1}) was used to calculate the activity. All experiments were repeated at least three times. The decrease of enzyme catalytic (hydrolytic) activity expressed as a fraction of the initial activity, A/A_o, defines the degree of enzyme inactivation.

2.3. Thermal treatment

The effect of temperature treatment on the ficin and papain stability was examined. The enzyme solutions (0.05 mg/ml) were activated at 40 °C in 0.05 M phosphate buffer at pH 7.0, and subsequently 0.5 ml aliquots in thin-walled glass test-tubes were placed in water-baths (WB/OB 7–45, MEMMERT GmbH + Co., KG, Schwabach, Germany) at temperatures in the range 40–80 °C for various periods of time. During thermal treatment temperature was monitored and recorded at 2 s intervals in a multichannel datalogger (CR10X, Campbell Scientific, Leicestershire, UK) by a type T thermocouple placed inside a capillary used as a temperature indicator. After heating, the samples were quickly cooled and assayed for their enzymatic activity at 40 °C. Storage before the assay at 0 °C (from 30 min to 48 h) did not alter the measured activities significantly.

2.4. High pressure treatment

Papain and ficin enzyme solutions were placed into 2 ml pouches (PP film) for HHP experiments. HHP inactivation experiments were conducted in triplicate at various combinations of pressure (200–900 MPa) and temperature (40–80 °C) for appropriate processing times. The high pressure unit (Food Pressure Unit FPU 1.01, Resato International BV, Roden, Holland), comprised a pressure intensifier and a multivessel system consisting of six vessels of 45 mL capacity each, with a maximum operating pressure and temperature of 1000 MPa and 90 °C. The pressure transmitting fluid used was polyglycol ISO viscosity class VG 15 (Resato International BV, Roden, Holland). Process temperature in the vessels was achieved by liquid circulation in the outer jacket controlled by a heating-cooling system. The desired value of pressure was set and after pressure build up (20 MPa/s), the pressure vessels were isolated. This point defined the time zero of this process. Pressure