Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 95 (2006) 301–306
Food Chemistry
www.elsevier.com/locate/foodchem

High pressure induced hydrolysis at C-terminus of peptide derivatives yielding bioactive peptides

A. Fernández García *, H. Zöller, P. Butz, J. Stärke, B. Tauscher

Institute for Chemistry and Biology, Federal Research Centre for Nutrition and Food, Haid-und-Neustr. 9, D-76131 Karlsruhe, Germany

Received 26 July 2004; received in revised form 4 January 2005; accepted 4 January 2005

Abstract

If the cyclization of a peptide is associated with a volume reduction, pressure should displace the reaction equilibrium in the direction of a lower volume. Here, results in model solutions are considered, showing a pressure-accelerated transformation of linear dipeptides with reactive C-terminals. The theorised cyclization of dipeptides after hydrolysis of the C-terminal amide H–Leu–Gly–NH_2 or methyl ester groups H–Leu–Gly–OMe and H–Leu–Gly–OtBu was found to be significantly accelerated during application of combined pressure/temperature treatments up to 600–800 MPa and 60–80 °C. Yields were dependent on the nature of the reactive site. Products of those reactions were identified as H–Leu–Gly–OH and cyclo(Leu–Gly), which is a bioactive dipeptide. The dipeptide amide yielded only trace concentrations of the amino acid H–Leu–OH and the linear dipeptide. Steric hindrance prevented a pressure induced cyclisation of a dipeptide with *tert*-butyl ester at the C-terminus, and only the linear peptide H–Leu–Gly–OH was formed.
© 2005 Elsevier Ltd. All rights reserved.

Keywords: Pressure; H–Leu–Gly–OMe; H–Leu–Gly–OtBu; H–Leu–Gly–NH_2; Diketopiperazine; Cyclo(Leu–Gly)

1. Introduction

Treatment with high hydrostatic pressure is an attractive food processing technique because of its gentle effect on sensorial and nutritional characteristics of the processed products (Butz et al., 2003; Cheftel, 1992; San Martin, Barbosa-Canovas, & Swanson, 2002). Furthermore, the pressure-assisted thermal sterilisation procedure exploits the unique effect of pressure-induced adiabatic heating and cooling and, although this technique is still in the research phase, results look encouraging since high-quality foods with shelf-lives of up to 3 years without cooling are made possible (Meyer, Cooper, Knorr, & Lelieveld, 2000).

The main concern is still the substantial equivalence of pressurised foods compared to their non-pressurized counterparts. For example, chemical reactions involving a decrease in volume are generally accelerated under pressure. Those reactions might lead to the formation of undesirable compounds. For instance, it has been reported that high pressure induces the formation of hormone-like substances after cyclization of glutamine at the N-terminus of certain peptides (Fernández García et al., 2003). Furthermore, application of pressure accelerates the formation of diketopiperazines after hydrolysis of the ester bond at the C-terminus of aspartame and Leu–Leu–OMe (Butz, Fernández García, Fister, & Tauscher, 1997; Butz et al., 2002).

* Corresponding author. Present address: IRTA Centre of Meat Technology, Granja Camps i Armet s/n, 17121 Monells, Girona, Spain. Tel.: +49 0 721 6625 516; fax: +49 0 721 6625 503 (Germany), Tel.: +34 972 63 00 52; fax: +34 972 63 03 72 (Spain).

E-mail addresses: veli.fernandez@bfe.uni-karlsruhe.de, avelina.fernandez@irta.es (A.F. García).

0308-8146/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2005.01.010

Carboxylic esters hydrolyse to the parent carboxylic acid and an alcohol. If the hydrolysis of a carboxylic ester at the C-terminal of a dipeptide is induced under pressure, the carbonyl group could react with the N-terminal free electron pair of the amino group, leading to the formation of a 2,5-dioxopiperazine. The same product would be expected after pressure-induced hydrolysis of an amide group at the C-terminus of a dipeptide amide. Indeed 2,5-dioxopiperazines are not uncommon by-products, probably arising from nonenzymatic cyclisations, in fermentative broths, cultures of yeast, lichens and fungi, or after food processing (Prasad, 1995). Those are relevant, since they might be toxic, as is cyclo(Asp–Phe) from aspartame (Walton, 1986), or play a physiological role, as many cyclic dipeptides are bioactive or have regulatory activity in vivo (Prasad, 1995). For example, cyclo(Leucyl-Glycyl) (CLG) avoids the development of tolerance to opiates in animal tests probably via dopamine receptors, and could have therapeutic properties. No enzymatic system capable of synthesising this cyclic peptide is known in mammals; however, the synthetic molecule passes from the blood into the central nervous system after intravenous application (Bhargava, Walter, & Ritzmann, 1980).

This work is part of an extensive study on the effects of high pressure and temperature on reactions leading to the formation of hormone-like substances. Those reactions might have a certain relevance for food processing, since the formed compounds might cause physiological disorders. Here, the cyclizations of two dipeptide esters (H–Leu–Gly–OMe and H–Leu–Gly–OtBu), and one dipeptide amide (H–Leu–Gly–NH_2), have been investigated under combined pressure/temperature treatments at neutral pH.

2. Materials and methods

2.1. Materials

Standard compounds H–Leu–Gly–OH, H–Leu–Gly–OtBu · HCl and H–Leu–Gly–NH_2 · HBr were purchased from Bachem [Merseyside, UK]. Chemical solvents used to carry out the analytical studies by High Performance Liquid Chromatography (HPLC) were obtained from Merck [Darmstadt, Germany], and were of LiChrosolv grade.

2.2. Esterification of H–Leu–Gly–OH

Esterification of the carboxylic group of H–Leu–Gly–OH took place following the method described by Fraenkel-Conrat and Olcott (1945). In resume, 150 mg H–Leu–Gly–OH were suspended in 19 ml methanol gradient grade and mixed with 0.16 ml 37% hydrochloric acid. They were allowed to react for 24 h at room temperature with occasional shaking. Two peaks could be observed by HPLC, one of them showing a retention time corresponding to the native substance H–Leu–Gly–OH, and one product peak. The two peaks were identified by LC–MS, the unidentified product showing the mass spectrum of H–Leu–Gly–OMe. After running preparative chromatography, both isolated peaks were lyophilised and H–Leu–Gly–OMe was used for further experiments under pressure.

2.3. Specifications for HPLC

2.3.1. General

All peptides (H–Leu–Gly–OMe, H–Leu–Gly–OtBu, H–Leu–Gly–NH_2, H–Leu–Gly–OH and CLG) were eluted using a Lachrom high performance liquid chromatograph with a pump L-6220 and a detector L-4200 UV–Vis from Merck-Hitachi [Darmstadt, Germany].

2.3.2. H-Leucyl-Glycine methyl ester

A reverse phase Prodigy ODS3 C18 column, 100 mm length, 4 mm ID, 3 μm pore size from Phenomenex [Aschaffenburg, Germany] was used for separation.

Effects of pressure on the cyclization of H–Leu–Gly–OMe (approx. 5 mM) were followed in 50 mM Tris/HCl buffer, pH 7. Detection was at 215 nm; elution rate was 0.75 ml/min. The mobile phases were: (a) 0.1% trifluoro acetic acid in water; (b) 1.0% trifluoro acetic acid in water (40%) mixed with CH_3CN (60%) (all mixtures v/v). A gradient was run from 100% (a) to 100% (b) in 20 min. Injection volume was 10 μl.

2.3.3. H-Leucyl-Glycine tert-butyl ester

A reverse phase Nucleosil 100 C18 column, 250 mm length, 4 mm ID from Knauer [Berlin, Germany] was used for separation.

Effects of pressure on the cyclization of H–Leu–Gly–OtBu (approx. 40 mM) were followed in 50 mM Tris/HCl buffer, pH 7. Mobile phases were: (a) 80% 0.5 M sodium phosphate buffer, pH 2.1, in 20% methanol; (b) 20% 0.05 sodium phosphate buffer, pH 2.1, in 80% methanol (all mixtures v/v). A gradient was run from 100% (a) to 50% (b) in 20 min. Injection volume was 10 μl.

2.3.4. H-Leucyl-Glycine amide

A reverse phase Prodigy ODS3 C18 column, 500 mm length, 4.6 mm ID, 5 μm pore size from Phenomenex [Aschaffenburg, Germany] was used.

Effects of pressure on the cyclization of H–Leu–Gly–NH_2 (approx. 7.8 mM) were followed in 50 mM Tris/HCl buffer, pH 7. Detection was at 215 nm; elution rate was 0.5 ml/min. The mobile phases were: (a) 0.1% trifluoro acetic acid in water; (b) 1.0% trifluoro acetic acid in water (40%) mixed with CH_3CN (60%) (all mixtures v/v). A gradient was run from 100% (a) to 100% (b) in 40 min. Injection volume was 10 μl.

2.3.5. Peak quantification

Peak areas were measured by integration software Kroma System 2000 from Kontron [Milan, Italy].

2.3.6. Peak identification

H–Leu–Gly–OH was identified by cochromatography with authentic standards. Newly formed peaks (H–Leu–OH, H–Leu–Gly–OH and CLG) were identified using a liquid chromatograph coupled to a mass spectrometer (HP 1100 series), with an atmospheric pressure electrospray ionisation mode (API-ES) for mass identification. For experiments with LC–MS, columns and gradient conditions were used as described above, however, with lowering TFA of concentration to 0.05% (v/v) in buffer A and B to improve detection threshold of LC–MS.

2.4. Pressure treatments

Samples were kept in ice-water before and after pressure treatment. For experiments at 600 MPa, a high pressure multi-vessel system (with 5 autoclaves of 20 ml each for pressures up to 700 MPa and temperatures up to 80 °C) manufactured by aad GmbH (Frankfurt, Germany), described at Butz et al. (2004), was used. Pressure rise was automatically controlled and settled at 90 s to achieve 600 MPa, temperature rise, measured with thermocouples inside the autoclaves, was not higher than 8° during pressure build-up. Pressure experiments up to 800 MPa were conducted in a hydraulic press, U101, from the High Pressure Research Centre, Polish Academy of Science [Warsaw, Poland] for up to 1100 MPa. U101 is a manually operated twin piston hydraulic press with an automatic piston return (80 mm piston movement; 100 mm piston length) equipped with specialised accessories for pressurising vessels. The vessels were continuously heated using a Polystat thermostat from Huber [Offenburg, Germany]. In the U101 system, the effects of adiabatic heating were minimised due to the long pressure rise necessary to achieve the highest pressures (at least 150 s above 700 MPa) and by continuous temperature control in the vessel using a thermostat coupled to the cylinder. Samples were pressurised in polyethylene ampoules (250 μl) and sealed with a Teflon band. To assure complete isolation, the ampoules were introduced in a polyethylene coated aluminium bag which was heat sealed.

3. Results

3.1. General pressure effects

Among others, cyclization reactions involving short chain peptides are generally governed by the Le Chatelier principle and therefore could be accelerated under pressure. They are of greatest interest since products might cause off-flavours in foods, as is known in the case of glutamine (Shallenberger, Pallesen, & Moyer, 1957), or be physiologically relevant (Prasad, 1995).

3.2. Pressure effects on dipeptide esters

After the effective esterification of leucyl-glycine and the isolation of the methyl esterified peptide, experiments under pressure were carried out, followed by immediate quantification using HPLC. The initial concentration of the methyl esterified dipeptide H–Leu–Gly–OMe (5 mM in Tris/HCl pH 7) decreased rapidly when the buffered samples were heated under pressure at 600 MPa (Fig. 1); degradation kinetics, calculated with points measured up to 3 min treatment, followed a first order with reaction rate $k = 2 \times 10^{-2}\ \mathrm{min}^{-1}$ ($R^2 = 0.98$). Remarkable is the loss of approx. 55% H–Leu–Gly–OMe after reaction for 30 min at 600 MPa and 60 °C. Parallel to the observed decay in the concentration of H–Leu–Gly–OMe, two new products were detected and identified as H–Leu–Gly–OH [$(188.1 + H)^+$ and $(188.1 + Na)^+$] (22%) and CLG [$(170.1 + H)^+$ and $(170.1 + Na)^+$] (32%) (Fig. 2). The identified

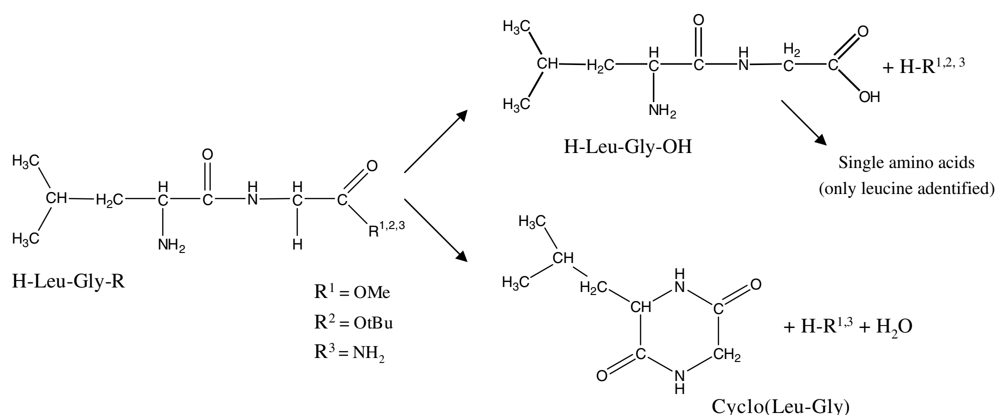

Fig. 1. Major reaction products of the degradation of H–Leu–Gly–R at neutral pH.

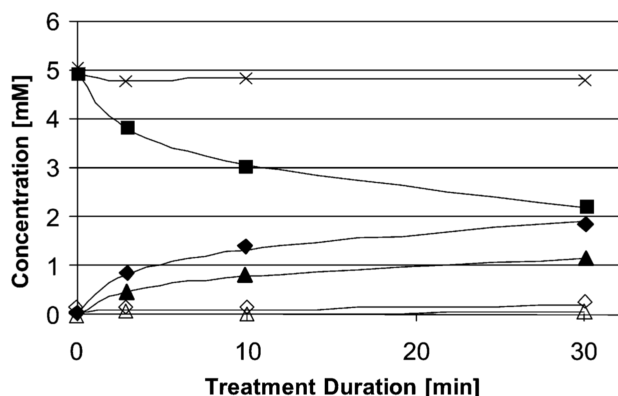

Fig. 2. Pressure treatment (600 MPa, 60 °C) of H–Leu–Gly–OMe (40 mM) in 50 mM Tris/HCl buffer, pH 7. X, H–Leu–Gly–OMe control at 60 °C, 0.1 MPa; ■, H–Leu–Gly–OMe at 60 °C, 600 MPa; open symbols (△ and ◇), respectively, H–Gly–Leu–OH and cyclo-(Leu-Gly) at 60 °C, 0.1 MPa; bold symbols (▲ and ◆), respectively, H–Gly–Leu–OH and cyclo(Leu–Gly) at 60 °C, 600 MPa. Data points are means of three independent replications; the average standard deviation was always below 10%.

2,5-dioxopiperazine CLG is identical with the known biologically active product described above. Furthermore, among processing time and treatment conditions, the sum of the molar concentrations of the newly synthesised substances was a symmetrical reflection of the degradation of H–Leu–Gly–OMe. Further experiments (results not shown) refuted the idea either of the new formed peptides (H–Leu–Gly–OH or CLG) could react further under pressure under the chosen conditions (pH 7, 600 MPa, 60 °C, up to 30 min). Other new peaks were found only in trace amounts.

Subsequent experiments were carried out with the linear dipeptide H–Leu–Gly–OtBu, also thought to be a possible subject of a pressure induced deesterification, and a probable precursor of the bioactive peptide cyclo-(Leu–Gly). The standard substance H–Leu–Gly–OtBu (approx. 40 mM in Tris/HCl, pH 7) was rapidly degraded when buffered samples (Tris/HCl, pH 7) were heated under pressure at 600 MPa (Fig. 3); data points delivered up to 3 min treatment followed first order kinetics with reaction rate $k = 1 \times 10^{-2}\ \mathrm{min}^{-1}$ ($R^2 = 0.99$). The reaction exclusively yielded one new product, that could be identified as H–Leu–Gly–OH (Fig. 1). That linear dipeptide is the product of the hydrolysis of the *tert*-butyl ester group situated at the C-terminus of H–Leu–Gly–OtBu, however, the cyclisation previously reported for a dipeptide methyl ester had not taken place under the described conditions, probably for steric reasons. The molar concentrations of the newly synthesised substance were a symmetrical reflection of the degradation of H–Leu–Gly–OtBu. Concentrations of other substances found at this wavelength was negligible, and therefore they were not included in the data analysis.

Contrary to results under elevated pressures, heat treatments at 60 °C in buffered solutions at atmospheric pressure resulted in almost no hydrolysis of the ester groups of either precursor (methyl and *tert*-butyl esters), and detected amounts of degradation products were negligible (Figs. 2 and 3) after 30 min treatment. Indeed, the already described kinetics of aspartame degradation show a relatively slow (could take days) but effective degradation of that molecule when heated at neutral pH (Homler, 1984).

3.3. Pressure effects on dipeptide amides

H–Leu–Gly–NH_2 (approx. 4.8 mM in Tris/HCl, pH 7), a dipeptide containing a primary amide at the C-terminus, was relatively stable at pressure treatments up to 600 MPa and 60 °C for long holding times (up to

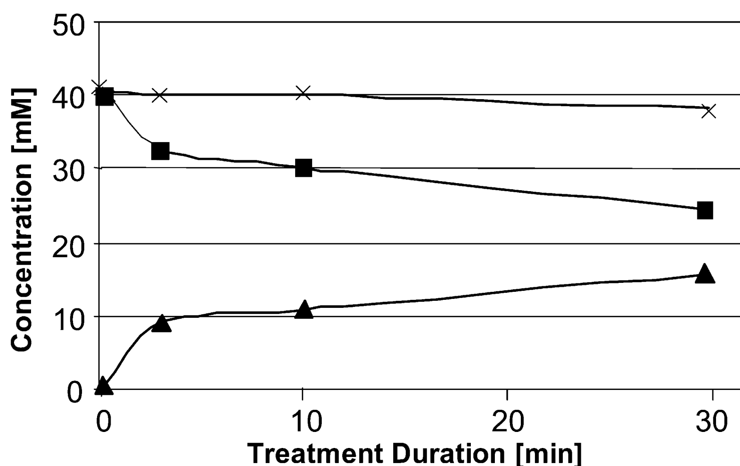

Fig. 3. Pressure treatment (600 MPa, 60 °C) of H–Leu–Gly–OtBu (40 mM) in 50 mM Tris/HCl buffer, pH 7. X, H–Leu–Gly–OtBu control at 60 °C, 0.1 MPa; ■, H–Leu–Gly–OtBu; ▲, H–Gly–Leu–OH. Data points are means of four independent replications; the average standard deviation was always below 10%.

120 min) and only a minimal reactivity could be recorded (less than 1%). However, this peptide disappeared relatively rapidly when the buffered samples were heated at 80 °C while pressurised at 800 MPa (Fig. 4). The higher pressure intensities, in combination with the higher temperatures for the longer times (240 min), produced a considerable hydrolysis of the amine group and a remarkable degradation of the precursor dipeptide amide (approx. 30%). Reaction rate was, however, slower than in the previously reported cases of peptide esters ($k = 2 \times 10^{-3}$ min^{-1}, $R^2 = 0.99$). Contrary to this, heat treatments at 80 °C in buffered solutions at atmospheric pressure had almost no effect on the reaction rate of amide hydrolysis, which normally has a low equilibrium constant even at basic pH. Detected amounts of degradation products were negligible (Fig. 4) also after long heat treatments.

Parallel to the degradation of H–Leu–Gly–NH_2, three new products were detected and identified after LC–MS analysis of the isolated compounds from the mixture. The deamidation was coupled with the cyclisation of the dipeptide and generated mainly the theorised cyclo(Leu–Gly) (Fig. 1): the reaction yielded extremely low amounts of the amino acid H–Leu–OH and the linear dipeptide, H–Leu–Gly–OH, which were not recorded in Fig. 4, but at least 30% CLG [$(170.1 + H)^+$ and $(170.1 + Na)^+$] was synthesised after long heat/pressure treatments (Fig. 4). The cyclic dipeptide has a homologous structure to the well known bioactive peptide CLG and could have physiological relevance as is known for the synthetic molecule. No other new peaks could be identified at this wavelength (215 nm) and the decrease in the concentration of the precursor dipeptide amide was a symmetrical reflection of the products' increase.

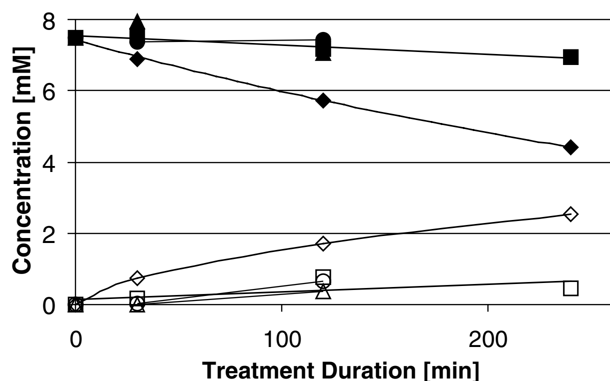

Fig. 4. Pressure treatments (600 and 800 MPa, 60 and 80 °C) of H–Leu–Gly–NH_2 (7.8 mM) in 50 mM Tris/HCl buffer, pH 7. Open symbols: concentration of cyclo(Leu–Gly); bold symbols: concentration of H–Leu–Gly–NH_2. ○,● samples treated at 60 °C; ■,□ samples treated at 80 °C; ▲,△ samples treated at 60 °C, 600 MPa; ◇,◆, samples treated at 80 °C, 800 MPa. Data points are means of three independent replications; the average standard deviation was always below 10%.

4. Discussion

The cyclisation of dipeptides and their stability have been well studied in the case of food additives producing diketopiperazines (Kroyer, Pinger, Washüttl, & Steiner, 1993) under different circumstances, including thermal treatment, in food matrices. Here, the effects of pressure on two dipeptide esters and one peptide amide have been examined.

Dipeptide esters are not expected to be frequent natural food components, but can be used as food additives. As is already known in the case of pressure induced aspartame degradation (Butz et al., 1997), a dipeptide ester widely used as a sweetener, the here-described pressure induced formation of the bioactive dipeptide CLG from the dipeptide H–Leu–Gly–OMe at neutral pH should have been due to a nucleophilic attack of the free electron pair of the alpha-nitrogen of leucine on the carboxyl group of glycine, accompanied by the liberation of methanol. Contrary to this, in the case of the dipeptide H–Leu–Gly–OtBu, the tertiary butyl group hindered the reaction under pressure, potentially due to steric constraints, and the bioactive cyclisation product was not formed; only the degradation into the linear dipeptide took place. Generally, such reactions should be taken into account, especially when intending the pressurisation of food additives containing an ester group at the C-terminus.

Amidation of the C-terminus is, however, a common feature, protecting peptides against degradation by peptidases. Thus, those peptides acting as hormones or transmitters usually prolong their medium life by blocking their C-terminus. Moreover, enzymes with amidative activities are able to cleave dipeptides from oligopeptides of different lengths (Bleakman & Smyth, 1987). Therefore, a certain amount of dipeptide amides could be expected to be present in foods. Those peptides might be the substrate of deamidating reactions as is the one reported above. The pressure-induced deamidation might alter the proportion of bioactive peptides by increasing the accesibility of the otherwise protected C-terminus then available to peptidases in the body. Pressurising of pharmaceuticals containing those reactive groups would induce the loss of bioactive compounds with therapeutic properties. Furthermore, the generated 2,5-dioxopiperazines, after the pressure-induced cyclisation of dipeptides, often have hormone-like activity. An increased concentration of those substances due to the cyclisation of non-bioactive peptides might lead to an unbalanced presence of bioactive peptides in food, with unknown physiological implications, and should be taken into consideration during risk assessment.

Typical parameters necessary for a pressure pasteurisation range from 400 to700 MPa, at temperatures not higher than 40–50 °C. Under such conditions, the

described degradation of dipeptides, and the subsequent cyclisation reactions, would have only minor importance, depending more on the hydrolysed group and the nature of the amino acids adjacent to the reactive site, as has already been reported for the case of the cyclisation of glutamine at the N-terminus of oligopeptides under pressure (Fernández García et al., 2003). Thus, among all analysed groups, methyl esters seem to be the most reactive, leading to the highest loss of precursor peptides. In fact, the loss of dipeptide esters was comparable to the loss of aspartame and H–Leu–Leu–OMe reported previously (Butz et al., 1997; Butz et al., 2002), which also generated a high amount of 2,5-dioxodiketopiperazines (cyclo(Asp–Phe) and cyclo-(Leu–Leu)). Subsequent synthesis of cyclopeptides under pressure occured when no steric hindrance conditioned the process, which must be the reason for the exclusive generation of a linear dipeptide in the case of the *tert*-butyl ester. Much slower is the hydrolysis of the amide at the C-terminal of H–Leu–Gly–NH_2, and the parameters investigated during this work which are generally required for a pasteurisation under pressure (600 MPa, up to 60 °C), seemed not to be sufficient to accelerate this reaction.

Effects of pressure on peptide esters were found relevant at combined mild temperature/pressure intensities and they were not further studied under more extreme conditions. However, the slow deamidation induced under conditions necessary to achieve a pasteurisation was remarkably accelerated at the highest temperature/pressure intensities, and the cyclisation of the dipeptide H–Leu–Gly–NH_2 occurred relatively more rapidly. It is then possible that under pressure-assisted sterilisation conditions, otherwise slow reactions, occuring at neutral pH, might be accelerated in food, for example in highly proteolytic environments, yielding bioactive peptides (in considerable amounts), which are products of cyclisation reactions of precursors with a sensitive group at the C-terminus. In fact, continuous pressure or pressure pulses up to 1000–1200 MPa at temperatures around 115 °C are absolutely mandatory to achieve the sterilisation of foods or pharmaceuticals under pressure (Meyer et al., 2000). Notable is the fact that the impact of such reactions will depend on the reactive groups and the amino acids close to the C-terminus, and the reaction rate of the different amino acids should be studied independently under pressure. Indeed, we expect the deamidation reported here not to be significant when sterilisation takes place in only a few minutes. However, faster reaction rates may occur when other amino acids are involved.

The destabilising effects of pressure and heat, when applied together, have broadly been reported for chemical reactions (Pfister & Dehne, 2001) and enzyme inactivation (Weemaes, Ludikhuyze, VandenBroeck, & Hendrickx, 1998). Therefore, we can include that the effects of pressure/temperature combinations on peptides with a reactive C-terminus are not negligible and need to be considered during risk assessment in high pressure food processing and high pressure sterilisation of food and pharmaceuticals.

References

Bhargava, H. N., Walter, R., & Ritzmann, R. F. (1980). Development of narcotic tolerance and physical dependence: effects of Pro–Leu–Gly–NH_2 and cyclo(Leu–Gly). *Pharmacological and Biochemical Behaviour, 12*(1), 73–77.

Bleakman, A., & Smyth, D. G. (1987). Sequential processing reactions in the formation of hormone amides. *European Journal of Biochemistry, 167*, 161–165.

Butz, P., Fernández García, A., Fister, H., & Tauscher, B. (1997). Influence of high hydrostatic pressure on Aspartame: instability at neutral pH. *Journal of Agricultural and Food Chemistry, 45*(2), 302–303.

Butz, P., Fernández García, A., Lindauer, R., Dieterich, S., Bognar, A., & Tauscher, B. (2003). Influence of high pressure processing on fruit and vegetable products. *Journal of Food Engineering, 56*, 233–236.

Butz, P., Fernández García, A., Schneider, T., Stärke, J., Tauscher, B., & Trierweiler, B. (2002). The influence of high pressure on the formation of diketopiperazine and pyroglutamate rings. *High Pressure Research, 22*, 697–700.

Butz, P., Serfert, Y., Fernández García, A., Dieterich, S., Lindauer, R., Bognar, A., et al. (2004). Influence of high pressure treatment at 25 and 80 °C on folates in orange juice and model media. *Journal of Food Science, 69*(3), 117–121.

Cheftel, J.-C. (1992). Effects of high hydrostatic pressure on food constituents. An overview. In C. Balny, R. Hayashi, K. Heremans, & P. Masson (Eds.). *High pressure research and biotechnology* (Vol. 224, pp. 195–209). Colloque INSERM/John Libbey Eurotext Ltd.

Fernández García, A., Butz, P., Zöller, H., Stärke, J., Pfaff, E., & Tauscher, B. (2003). Pressure/temperature combined treatments yield hormone-like peptides with pyroglutamate at N-terminus. *Journal of Agricultural and Food Chemistry, 51*, 8093–8097.

Fraenkel-Conrat, H., & Olcott, H. S. (1945). Esterification of proteins with alcohols of low molecular weight. *Journal of Biological Chemistry, 161*(1), 259–268.

Homler, B. E. (1984). Properties and stability of aspartame. *Food Technology*(July), 50–55.

Meyer, R., Cooper, K. L. L., Knorr, D., & Lelieveld, H. L. M. (2000). High pressure sterilisation of foods. *Food Technology, 54*(11), 67–72.

Kroyer, G., Pinger, R., Washüttl, J., & Steiner, I. (1993). Stabilität und Wechselwirkungen der künstlichen Süßstoffe Aspartam und Acesulfam-K in Lebensmitteln. *Ernährung/Nutrition, 17*(10), 546–549.

Pfister, M. K. H., & Dehne, L. I. (2001). High pressure processing. An overview of chemical changes in food. *Deutsche Lebensmittel-Rundschau, 97*(7), 257–268.

Prasad, C. (1995). Bioactive cyclic dipeptides. *Peptides, 16*(1), 151–164.

San Martin, M. F., Barbosa-Canovas, G. V., & Swanson, B. G. (2002). Food processing by high hydrostatic pressure. *Critical Reviews in Food Science and Nutrition, 42*, 627–645.

Shallenberger, R. S., Pallesen, H. R., & Moyer, J. C. (1957). Formation of pyrrolidoncarboxylic acid during the production of beet puree. *Food Technology*(February), 92–93.

Walton, R. G. (1986). Seizure and mania after high intake of aspartame. *Psychosomatics, 27*, 218–220.

Weemaes, C., Ludikhuyze, L., VandenBroeck, I., & Hendrickx, M. (1998). High pressure inactivation of polyphenoloxidases. *Journal of Food Science, 63*(5), 873–877.