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Pressure/Temperature Combined Treatments of Precursors Yield Hormone-like Peptides with Pyroglutamate at the N Terminus

AVELINA FERNÁNDEZ GARCÍA,*,† PETER BUTZ,† BERNHARD TRIERWEILER,† HEIKE ZÖLLER,† JOACHIM STÄRKE,† EBERHARD PFAFF,§ AND BERNHARD TAUSCHER†

Institute of Chemistry and Biology, Federal Research Centre for Nutrition, Haid-und-Neustrasse 9, D-76131 Karlsruhe, Germany, and Institute of Immunology, Federal Research Centre for Virus Diseases of Animals, Paul-Ehrlichstrasse 28, D-72076 Tübingen, Germany

Peptides containing the cyclic product of glutamine at the N terminus are usually biologically active. If the cyclization of glutamine was associated with a volume reduction, pressure should displace the equilibrium in the direction of the lower volume. Here, results in model solutions and in whey are discussed, showing that the theorized cyclization of glutamine in Gln-His-ProNH$_2$ or Gln-Leu-ProNH$_2$ is significantly accelerated during the application of heat and even more strongly when elevated temperature and pressure combinations are used. The reaction rate depended on the intensity of the pressure treatment, the pH, and the nature of the amino acids adjacent to glutamine. The products of the reaction were identified as thyrotropin-releasing hormone (TRH) and [Leu(2)]TRH. The reported reactions could affect the naturally balanced concentration of short-chain peptides in foods and therefore induce unpredictable biological effects.

KEYWORDS: Pressure; pyroglutamate; thyrotropin-releasing hormone; TRH; TRH analogue

INTRODUCTION

Within minimal food-processing technologies, a treatment with high hydrostatic pressure is an attractive technique because of its gentle effect on the sensorial and nutritional characteristics of the processed products (*1*). Despite this, chemical reactions governed by the Le Chatelier principle are usually influenced by a high-pressure treatment. Those reactions might lead to the formation of undesirable compounds and, therefore, should not be disregarded. For instance, it has been reported that high pressure accelerates the formation of a diketopiperazine after hydrolysis of the ester bond at the C terminus of certain peptides (2, *3*) and induces the cyclization of the amino acid glutamine (*4*) under neutral or basic pH.

Structures containing the cyclic product of glutamine at the N terminus (pyroglutamate) are not uncommon in nature. Those peptides have usually an endocrine and/or regulative function in the body and are of highest interest because their concentration in the blood will influence the synthesis pathways of important hormones. Here we investigate the stability of glutamine-containing peptides under combined pressure and temperature conditions. If pressure induced chemical changes in Gln-His-ProNH$_2$ or Gln-Leu-ProNH$_2$, the newly formed structures would probably have the sequence pGlu-His-ProNH$_2$ or pGlu-Leu-ProNH$_2$ as a consequence of the cyclization of glutamine. The first compound, pGlu-His-ProNH$_2$ [= thyrotropin-releasing hormone, or TRH (see **Figure 1**)], is normally produced in the hypothalamus and regulates the metabolism of thyrotropin-stimulating hormone (TSH), prolactin, triiodothyronine (T3), and thyroxine (T4). After the oral intake of TRH, changes in the concentration of TSH, T3, and T4 have been reported (*5*). The second compound, pGlu-Leu-ProNH$_2$ [= thyrotropin-releasing hormone analogue, or [Leu(2)]TRH],

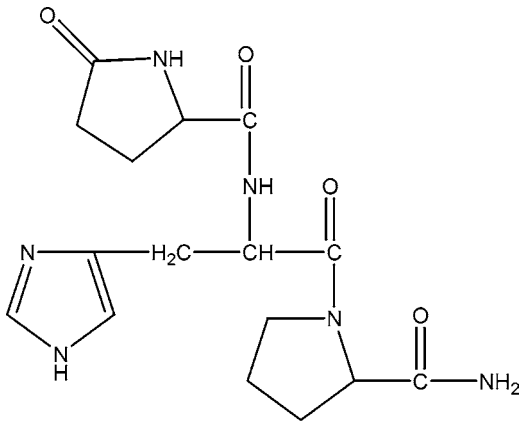

Figure 1. Chemical structure of pGlu-His-ProNH$_2$ (= thyrotropin-releasing hormone, or TRH) with pyroglutamate at the N terminus.

* Corresponding author [telephone + 49 (0) 721 6625 516; fax + 49 (0) 721 6625 503; e-mail veli.fernandez@bfe.uni-karlsruhe.de].
† Federal Research Centre for Nutrition.
§ Federal Research Centre for Virus Diseases of Animals.

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is known to have anticataleptic activity and to enhance the secretion of acetylcholine (*6*) but is inactive in the regulation of TSH release. Oral intake of [Leu(2)]TRH increased the endogenous levels of TRH, showing an inhibition of the metabolism of this substance (*7*).

This study was carried out to explore the effects of pressure and temperature on reactions that are possibly leading to the formation of hormone-like substances. These reactions might have a certain relevance for food processing, because the formed substances might cause physiological disorders. Experiments at different pH values were performed in model solutions, in water, and in whey.

MATERIALS AND METHODS

The standard substance pyroglutamyl-histidyl-proline-NH_2 was purchased from Calbiochem (Schwalbach, Germany) and the standard compound pyroglutamyl-leucyl-proline-NH_2 from Sigma-Aldrich Chemie (Taufkirchen, Germany). The glutamine-containing tripeptides glutaminyl-histidyl-proline-NH_2 and glutaminyl-leucyl-proline-NH_2 were synthesized at EMC Microcollections (Tübingen, Germany). All other chemicals used to carry out the analytical studies by high-performance liquid chromatography (HPLC) were obtained from Merck (Darmstadt, Germany) and were of LiChrosolv grade.

HPLC Specifications for TRH Detection. The effects of pressure on the cyclization of Gln-His-ProNH_2 (~2 mM) were examined in water, buffered systems, and whey. Buffered experiments were carried out with Tris-HCl buffers (50 mM) adjusted to pH 7 or 9. Experiments in whey were performed as follows: previous to the pressure experiments, 50 mL of milk (1.5% fat) was centrifuged at 9000*g* in a Sigma 2 K15 centrifuge (Sigma Laborzentrifugen, Osterrode, Germany) at 4 °C, and the supernatant (mainly the whey fraction) was filtered for HPLC through a 0.45 μm pore size filter from Schleicher & Schuell (Dassel, Germany). The pH of the whey fraction remained unaltered and slightly above 7. Afterward, a certain amount of the tripeptide was mixed with the supernatant to achieve a concentration of approximately ~2 mM. Baselines of chromatograms became noisy due to the presence of proteins in the whey fraction, which, however, did not interfere with the identification of additives by retention times and cochomatrography with standards.

Gln-His-ProNH_2 and pGlu-His-ProNH_2 were detected in a high-performance liquid chromatograph Lachrom pump with a detector L-4200 UV−vis from Merck. A reverse phase Prodigy ODS3 C18 column, 250 mm length, 4.6 mm i.d., 5 μm pore size, from Phenomenex (Aschaffenburg, Germany) was used. Detection was at 220 nm, and the elution rate was 1.0 mL/min. The injection volume was 10 μL.

Data on the decay of the glutamine and the increase in the concentration of the pyroglutamate-containing tripeptides were analyzed. Calibration was done by comparing peak areas after the collection of glutamine- and pyroglutamate-containing peaks.

HPLC Specifications for Detection of [Leu(2)]TRH. Gln-Leu-ProNH_2 and pGlu-Leu-ProNH_2 were detected in a high-performance liquid chromatograph Lachrom pump with a detector L-4200 UV−vis from Merck. A reverse phase Nucleosil 100 C18 column, 250 mm length, 4 mm i.d., from Knauer (Berlin, Germany) was used.

Effects of pressure on the cyclization of Gln-Leu-ProNH_2 (~1.7 mM) were followed in Tris-HCl buffer, pH 7. Detection was at 220 nm, and the elution rate was 1.0 mL/min. The injection volume was 30 μL.

Calibration of [Leu(2)]TRH was done by comparing peak areas with the standard chemical purchased from Sigma. Only the data on the increase in the concentration of the pyroglutamate-containing tripeptide were analyzed.

Mobile Phases. Mobile phases for both experiments were (A) trifluoroacetic acid (0.1%) in water and (B) trifluoroacetic acid (1%) in water, mixed with acetonitrile, ratio 2:3. A gradient was run from 100% A to 50% B in 20 min. Peak areas were measured by the integration software Kroma System 2000 from Kontron (Milan, Italy).

Peak Identification. Peaks containing pyroglutamate were identified by cochromatography with authentic standards and by means of a liquid chromatograph coupled to a mass spectrometer (HP 1100 series), using an atmospheric pressure electrospray ionization mode (API-ES). Peaks were preparatively collected after HPLC loads and rechromatographed in the LC-MS running an isocratic gradient (100% A). Injection volume was 10 μL.

High-Pressure Treatment. Experiments up to 600 MPa were conducted in a high-pressure device consisting of a series of thermostated microautoclaves (i.d. = 16 mm, ~10 mL, 700 MPa) connected by valves. Pressure was generated by an air-driven pump in combination with a pressure intensifier. The pressure-transmitting medium was a mixture of water and glycol (80:20).

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 11000 bar. U101 is a manually operated twin-piston hydraulic press with an automatic piston return (80 mm piston movement; 100 mm piston length) equipped with specialized accessories for pressurizing vessels. The vessel is a cylinder made of steel with an inside diameter of 16 mm and a 150 mm height. The piston position is monitored with a linear transformer transducer, and the pressure-measuring unit is an in-vessel manganin pressure gauge; both are digitally displayed. The transmitting medium was petroleum ether (80−100 °C boiling point). Pressure rise required ~120 s.

Samples were pressurized in polyethylene ampules (250 μL) and sealed with Teflon. To ensure complete isolation, the ampules were introduced in a polyethylene-coated aluminum bag, which was heat sealed. The temperature in the vessels was controlled by a thermostat Polystat from Huber (Offenburg, Germany).

RESULTS

TRH. The concentration of the glutamine-containing tripeptide Gln-His-ProNH_2 (2 mM in Tris-HCl, pH 7) decreased rapidly when the buffered samples were heated under pressure at 600 or 800 MPa (upper panels of **Figures 2** and **3**, respectively). Parallel to the decay in the concentration of Gln-His-ProNH_2, a new product was detected and identified as TRH (lower panels of **Figures 2** and **3**) by LC-MS [molecular weights $[(362.1 + H)^+$ and $(362.1 + Na)^+$ in standard and synthesized] and retention times were identical]. The identified pyroglutamate-containing tripeptide is the product of the cyclization of the amino acid glutamine situated at the N terminus of Gln-His-ProNH_2. Furthermore, among processing time and treatment conditions, the molar concentrations of TRH were a symmetrical reflection of the degradation lines of Gln-His-ProNH_2 and indicated an equimolar reaction. No other new peak could be identified at this wavelength.

The logarithmic representation of C/C_0 (where C is the concentration of Gln-His-ProNH_2 at time x and C_0 is the Gln-His-ProNH_2 concentration at time 0) versus the time (in minutes) decayed following a linear function ($r^2 > 0.98$ in **Table 1**), and the reaction constant (k) could be calculated from the slope. Results presented in **Table 1** revealed that the higher pressure intensities used, combined with the higher temperatures, were able to accelerate the rate of conversion of glutamine into pyroglutamate: 800 MPa was more effective than 600 MPa, and k for the degradation of Gln-His-ProNH_2 increased when the treatment intensity was increased. Remarkable is the yield of ~80% TRH after reaction for 30 min at 800 MPa and 80 °C. Moreover, 120 min under the highest pressure/temperature intensities led to a roughly complete conversion into TRH. High-pressure sterilization of foods requires the application of high temperatures to inactivate spores: the interpolation of values at 10 min, a holding time typically required during commercial pressurization treatments to achieve a convenient inactivation of microorganisms, would indicate a decrease of >30% in the concentration of the initial Gln-His-ProNH_2 if samples were pressurized at 800 MPa and 80 °C.

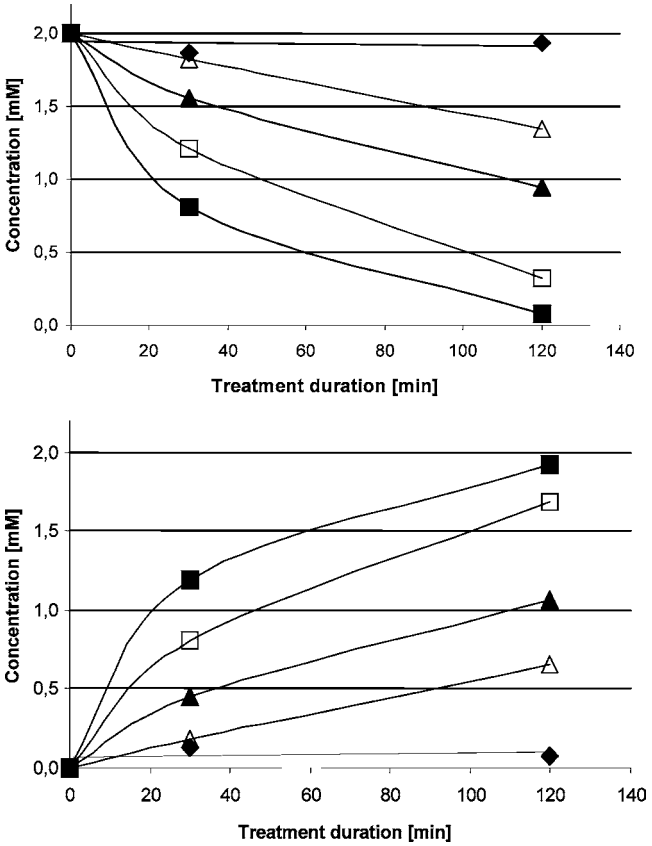

Figure 2. (**Top**) Decay of 2 mM Gln-His-ProNH$_2$ in 50 mM Tris-HCl buffer, pH 7; (**bottom**) formation of TRH (pGlu-His-ProNH$_2$) from 2 mM Gln-His-ProNH$_2$ in 50 mM Tris-HCl buffer, pH 7: (◆) 600 MPa, 20 °C; (△) 0.1 MPa, 60 °C; (▲) 600 MPa, 60 °C; (□) 0.1 MPa, 80 °C; (■) 600 MPa, 80 °C. Data points are means of three independent replications; the average standard deviation was always <10%.

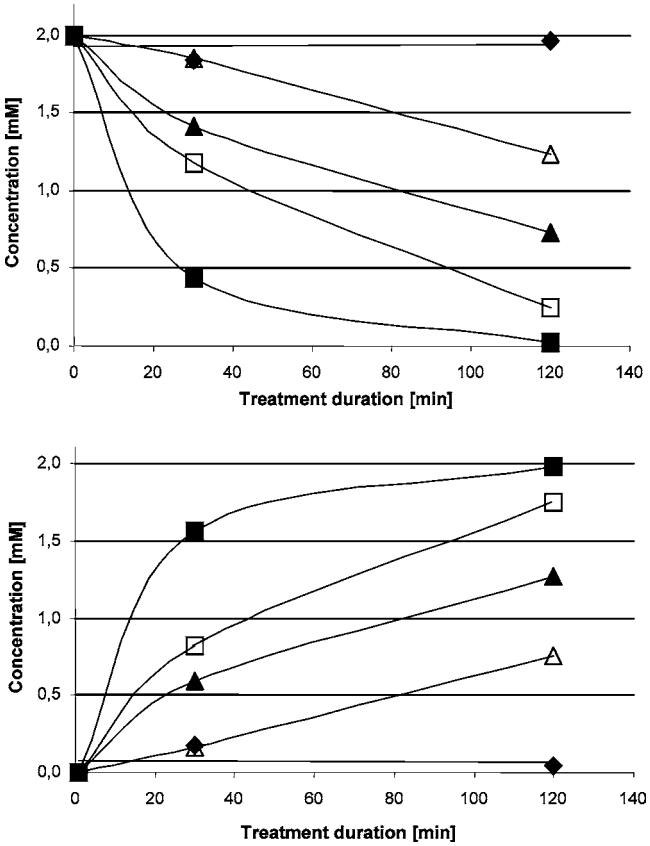

Figure 3. (**Top**) Decay of 2 mM Gln-His-ProNH$_2$ in 50 mM Tris-HCl buffer, pH 7; (**bottom**) formation of TRH (pGlu-His-ProNH$_2$) from 2 mM Gln-His-ProNH$_2$ in 50 mM Tris-HCl buffer, pH 7: (◆) 800 MPa, 20 °C; (△) 0.1 MPa, 60 °C; (▲) 800 MPa, 60 °C; (□) 0.1 MPa, 80 °C; (■) 800 MPa, 80 °C. Data points are means of three independent replications; the average standard deviation was always <8%.

The concentration of TRH after pressure treatment at pH 7 (lower panels of **Figures 2** and **3**) was comparable to the concentration of TRH after pressurization of 2 mM Gln-His-ProNH$_2$ in whey (**Figure 4**). The reaction rate under those conditions was similar, and no additional matrix effects could be reported. Milk is a complex matrix with several potent buffering systems that seem to be stable under pressure. On the other hand, solutions of 2 mM Gln-His-ProNH$_2$ pressurized in Tris-HCl buffer at pH 9 (**Figure 4**) showed that, surprisingly, the reaction rate for the conversion of the glutamine-containing tripeptide into TRH was slower at basic pH, as the levels of TRH at pH 7 or in whey were not reached. Following the Le Chatelier principle, the achieved equilibrium toward the deprotonated amine should be pressure sensitive. However, it has been probably compensated by the amino acids situated at the second and third positions of the tripeptide.

The experiments explained above were carried out in 50 mM Tris-HCl buffer, pH 7 and 9, a pressure-resistant buffer because of its very small ionization volume, or in whey. However, aqueous solutions of Gln-His-ProNH$_2$ were stable under pressure (**Figure 4**), and the cyclization could not take place, probably due to the self-ionization of water, which is promoted by pressure. Self-ionization of water results in a lowering of the pH and, therefore, the stabilization of the tripeptide by protonation of the N-terminal amine.

In addition, heat treatments at normal pressure triggered the cyclization of glutamine in buffered systems as well. Heating at 60 °C for 30 min helped the reaction of ~6% of 2 mM Gln-His-ProNH$_2$. The values found at 80 °C were remarkable (lower panels of **Figures 2** and **3**) because 40% of 2 mM Gln-His-ProNH$_2$ in Tris-HCl, pH 7, was converted into TRH after 30 min and only 10% of the original substance remained after 120 min. Furthermore, experiments performed in whey under conditions necessary to fulfill a long pasteurization (60 °C, 30 min) showed that at least 10% of the glutamine-containing tripeptide was converted into the hormone-like structure (TRH). Contrary to these results, the amino acid glutamine itself was rather stable when heated at 50 °C (*4*), and only negligible losses have been reported.

Table 1. Reaction Constants (min^{-1}) for the Decay of Gln-His-ProNH$_2$ under Isobaric–Isothermal Conditions in 50 mM Tris-HCl Buffer, pH 7

treatment	k ($\times 10^{-3}$ min^{-1})	r^2
600 MPa, 60 °C	7.6	0.99
800 MPa, 60 °C	8.6	0.98
600 MPa, 80 °C	26.7	0.99
800 MPa, 80 °C	39.1	0.99

[Leu(2)]TRH. The glutamine-containing tripeptide Gln-Leu-ProNH$_2$ (~1.7 mM in Tris-HCl, pH 7) disappeared slowly when the buffered samples were heated under pressure at 600 MPa (**Figure 5**). Only the higher pressure intensities used in combination with the higher temperatures accelerated the rate of conversion of glutamine into pyroglutamate: 800 MPa was considerably more effective, and the reaction rate for the degradation of Gln-Leu-ProNH$_2$ increased with increasing treatment intensity. Remarkable is the loss of ~30% Gln-Leu-ProNH$_2$ after reaction for 120 min at 800 MPa and 80 °C.

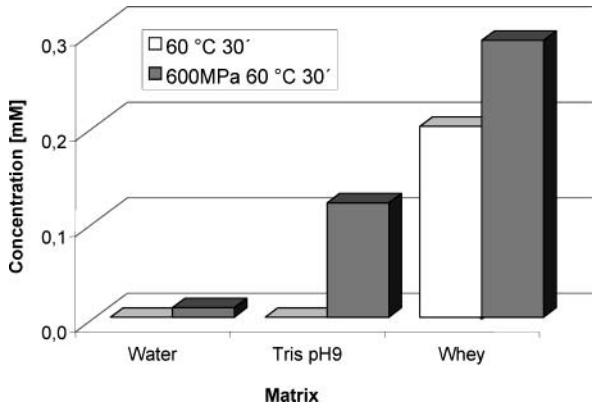

Figure 4. Formation of pGlu-His-ProNH$_2$ from 2 mM Gln-His-ProNH$_2$ in water, 50 mM Tris-HCl buffer, pH 9, and whey. Data points are means of three independent replications; the average standard deviation was always <5%.

Parallel to the degradation of Gln-Leu-ProNH$_2$, a new product was detected and identified as [Leu(2)]TRH [molecular weights [(338.2 + H)$^+$ and (338.2 + Na)$^+$ in standard and synthesized] and retention times were identical]. The pyroglutamate-containing tripeptide would be the product of the cyclization of the amino acid glutamine situated at the N terminus of Gln-Leu-ProNH$_2$. No other new peak was identified at this wavelength.

DISCUSSION

Generally, any reaction in food to which the principle of Le Chatelier applies is of interest under hyperbaric conditions. According to this principle, under equilibrium, a process associated with a decrease in volume will be favored by pressure. Among others, cyclization reactions involving short-chain peptides are generally governed by the Le Chatelier principle and are of highest interest because the cyclization products might cause off-flavors in foods, as is known in the case of glutamine (*8*), or have a certain physiological relevance (*9*).

The accelerated cyclization of the amino acid glutamine at high temperatures has been known for a very long time (*10*). Here we communicate that two new molecules have been rapidly generated when glutamine-containing tripeptides were heated while pressurized in buffers at neutral or basic pH and in a natural matrix. Those molecules were identified as TRH and [Leu(2)]TRH. Conditions approximating parameters necessary for a pressure pasteurization or sterilization (600 or 800 MPa at 60 or 80 °C) would result in the accelerated formation of two hormone-like structures with probable endocrine and regulatory functions. The expected results showed that heating the samples at mild temperatures (60 or 80 °C) at neutral or basic pH would also lead to a considerable enhancement in the production of such bioactive peptides. Other authors (*11*) have identified pyroglutamate-containing short-chain peptides after hydrolysis of protein-rich preparations. They concluded that certain (nonspecified) processing conditions would induce the formation of the nondigestible pyroglutamate at the N-terminal glutamine. We have shown here that the application of heat alone, and of heat accompanied by high pressure, might greatly accelerate the cyclization of the N-terminal glutamine at neutral pH.

Yields of glutamine cyclization under pressure were determined by the nature of the amino acids situated at positions close to the α-amine. The glutamine at the N terminus of Gln-His-ProNH$_2$ was rapidly cycled in buffers at neutral pH and in whey but more slowly at basic pH. Contrarily, glutamine itself had proven to be more stable to pressure-induced cyclization at neutral than at basic pH (*4*). The tripeptide illustrates an effect probably marked by the presence of the imidazole group of histidine at the second position. The imidazole is weakly acid at basic pH, thus negatively charged, and might weaken the nucleophilic character of the free amine at the N terminus by competing with the available electron par, avoiding cyclization. In the same way Gln-Leu-ProNH$_2$ was rather pressure stable at neutral pH, and only pressures of up to 800 MPa applied at 80 °C for at least 120 min were able to induce a remarkable reaction to pyroglutamate, showing that the lateral chain of leucine, an unpolar aliphatic amino acid, might protect the adjacent glutamine from cyclization. The influence of pressure treatments on other peptides containing glutamine at the N terminus remains to be investigated and will be the subject of forthcoming works. However, and in contrast to the results presented here, Butz et al. (*3*) showed that peptides containing glutamine at the C terminus are stable after application of pressure combined with temperature, in the same way as they are generally stable in solution, and therefore regularly used as glutamine supplementation.

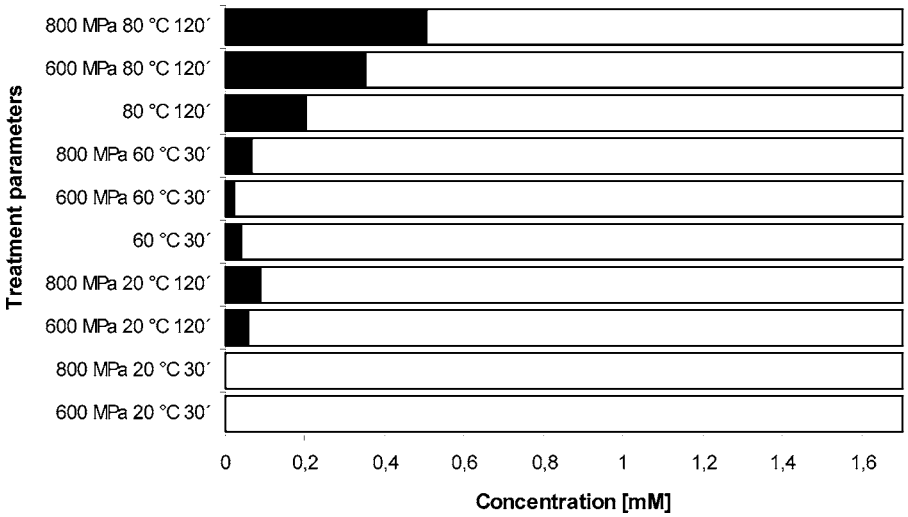

Figure 5. Formation of [Leu(2)]TRH (pGlu-Leu-ProNH$_2$) from 1.7 mM Gln-Leu-ProNH$_2$ in 50 mM Tris-HCl buffer, pH 7: (white) residual concentration of Gln-Leu-ProNH$_2$; (black) concentration of pGlu-Leu-ProNH$_2$ after treatment. Data points are means of three independent replications; the average standard deviation was always <5%.

The typical parameters necessary for a pressure pasteurization range from 400 to 700 MPa, at temperatures not higher than 40−50 °C. Under those conditions we expect that the related reactions would have only a negligible importance. However, pressure/temperature combinations to achieve sterilization parameters use continuous pressure or pressure pulses up to 1000−1200 MPa at temperatures of ∼115 °C. In this case a rapid yield of bioactive peptides containing pyroglutamate at the N terminus could be expected in foods at neutral pH or in highly proteolytic environments.

The principal question arising from the discussed results is whether the target peptides could be naturally available in foods and, therefore, whether the formed hormone-like substances could be relevant in the natural matrices. Indeed, the generation of short-chain peptides with glutamine at the N terminus should not be disregarded because glutamine and glutamic acid are, for example, the major amino acids in milk, quantified as ∼20% of all amino acids in milk proteins (*12*). For instance, the concentration of pyroglutamate has been acceptably correlated to the ripening state of hard-cooked cheeses (*13*). On the other hand, TRH and TRH derivatives have been reported to exist naturally in milk (*14*). Those compounds are usually the product of enzymatic activity in the matrix, meaning that the precursor molecules are present in the food. Consequently, the formation of precursor peptides during food processing should be a question of probability.

Roberts et al. (*15*) concluded that short peptides generated in the diet can be absorbed intact through the intestine and probably play a role in the modulation of organ functions and disease. Moreover, evidence has been reported on the resistance to degradation during digestion of forms containing pyroglutamic acid at the N terminus (*16*). Because of that, the products of the investigated reactions could have effects at the metabolic level. On the one hand, the pyroglutamate ring present at the N terminus is not digested by regular aminopeptidases, with the consequent reduction in the concentration of free glutamine and the possible effects on the bioavailability of this amino acid, relevant for persons with intestinal disorders. If TRH was absorbed in the gut, concentrations found in the blood might have, for example, consequences on the liberation of TRH derivatives. Indeed, an oral intake of TRH and TRH analogues, among them pGlu-Leu-ProNH_2, raises the levels of TRH in the spinal cord and the brain, whereas the levels of cyclo(His-Pro) decrease (*7*). The physiological effects might also be positive, as, for example, pyroglutamic acid has been found to improve age-associated memory impairment (*17*).

The increment in the concentration of pyroglutamate-containing peptides in buffers and in a natural matrix discussed throughout this paper would therefore affect the naturally balanced concentration of short-chain peptides when occurring in foods and might induce unpredictable biological effects that should be further studied in natural peptides contained in food matrices.

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