Combined microwave processing and enzymatic proteolysis of bovine whey proteins: the impact on bovine β -lactoglobulin allergenicity

Kamel Eddine El Mecherfi^{1,2,3,4} · Sébastien Curet² · Roberta Lupi⁴ · Colette Larré⁴ · Olivier Rouaud² · Yvan Choiset¹ · Hanitra Rabesona¹ · Thomas Haertlé¹

Revised: 9 October 2018 / Accepted: 19 October 2018 / Published online: 8 November 2018
© Association of Food Scientists & Technologists (India) 2018

Abstract The main aim of this study was to develop a continuous microwave treatment system of whey proteins and then apply this process at 37 °C, 50 °C, 65 °C and 70 °C to achieve pepsinolysis and produce extensively hydrolysed bovine whey protein hydrolysates with low allergenic properties. The microwave process was compared to a conventional thermal treatment with similar temperature set points. Both processes were deeply analysed in terms of the thermal kinetics and operating conditions. The pepsin hydrolysates obtained by the continuous microwave treatment and conventional heating were characterized by SDS-PAGE and RP-HPLC. The allergenicity of the whey protein hydrolysates was explored using a human IgE sensitized rat basophil leukaemia cell assay. The results indicate that extensively hydrolysed whey protein hydrolysates were obtained by microwave only at 65 °C and in a shorter time compared with the conventional thermal treatment. In the same temperature conditions under conventional heating, β -lactoglobulin was resistant to pepsinolysis, and 37% of it remained intact. As demonstrated by an in vitro degranulation assay using specific human IgE-sensitized rat

basophils, the extensively hydrolysed whey protein obtained by microwave showed maximum degranulation values of 6.53% compared to those of the native whey protein isolate (45.97%) and hence elicited no more allergic reactions in basophils. This work emphasizes the potential industrial use of microwave heating specific to milk protein processing to reduce their allergenicity and improve their end-use properties.

Keywords Microwaves · Thermal process · Allergen · β -Lactoglobulin · Enzymatic hydrolysis · Pepsin · Basophil degranulation · Specific IgE

Introduction

Milk and egg allergies are the most frequent food allergies occurring in early childhood, affecting 0.5–6% of infants (Koletzko et al. 2012; Nwaru et al. 2014). Hypoallergenic infant formulas based on extensively hydrolysed milk proteins are used in the diets of cow milk allergic infants (Bøgh et al. 2015). The main bovine milk allergens are caseins (CAS), β -lactoglobulin (BLG), and α -lactalbumin (ALA) (Bu et al. 2013). Less common antigens in cow milk are serum albumin (BSA) and immunoglobulins (O'Riordan et al. 2014). Many food allergens are stable and resistant to digestion by gastrointestinal enzymes or are processed into high molecular weight peptides that retain IgE binding and T cell-stimulating properties, as is the case for bovine whey protein β -lactoglobulin (Breiteneder and Mills 2005; Tsabouri et al. 2014). The molecular basis of alterations in protein allergenicity is the destruction of conformational and linear epitopes. However, methods of cow milk processing may even produce new epitopes (neopeptides) or make hidden epitopes accessible by

✉ Kamel Eddine El Mecherfi
kamel-eddine.el-mecherfi@inra.fr

¹ UR 1268 Biopolymères Interactions Assemblages, Équipe Fonctions et Interactions des Protéines, INRA, 44316 Nantes Cedex 03, France
² CNRS, GEPEA, UMR 6144, Site de la Géraudière, ONIRIS, 44322 Nantes, France
³ Laboratoire de Physiologie de la Nutrition et de Sécurité Alimentaire, Université d'Oran 1, Ahmed Benbella, Algeria
⁴ UR 1268 Biopolymères Interactions Assemblages, Équipe Allergie, INRA, 44316 Nantes Cedex 03, France

denaturing the native allergen (cryptotopes) (Taheri-Kafrani et al. 2009). Therefore, the use of processing technologies to enhance the functional properties of proteins needs to be well controlled. Novel technologies continue to be explored in the objective of altering the characteristics of proteins: high pressure, ultrasound, microwave heating, or pulsed-electric field (Augustin and Udabage 2007; Madadlou et al. 2011; Uluko et al. 2014; Verhoeckx et al. 2015; Ketnawa and Liceaga 2017). Several studies have reported that microwaves could enhance the effect by creating accelerated organic and chemical reactions (ElAshry et al. 2005; Kappe and Dallinger 2009). George et al. (2008) studied the impact of microwaves at 2.45 GHz on protein unfolding using surface plasmon resonance sensing. They found that the denaturation and unfolding of a bovine crystalline were higher under microwave heating than with conventional heating at the same temperature. However, the impact of microwaves on the denaturation and unfolding processes of protein remains controversial. Damm et al. (2012) performed tryptic digests of three model proteins (bovine serum albumin, cytochrome c and β -casein) at 37 °C and 50 °C using both microwave and conventional heating, applying accurate internal fibre-optic probe reaction temperature measurements. The authors reported that the tryptic digestion of the three proteins and the subsequent analysis of the corresponding cleavage products by MALDI-TOF provided virtually identical results from 2.45 GHz microwave irradiation and conventional heating. Nevertheless, in these studies, sparse information is provided on the operating system of the microwaves, and generally, the water bath immersion used as a conventional heating control does not reproduce the same temperature and energy profile in the sample; hence, any comparison could be biased.

In previous studies, in batch conditions at the lab scale (El Mecherfi et al. 2011, 2015), we demonstrated that microwaves could be an alternative to achieving hydrolysis of β -lactoglobulin in a few minutes compared to the longer time necessary for efficient conventional processing.

Moreover, the hydrolysates obtained with pepsin by a microwave presented a low allergenicity when studied with human IgE immunoreactivity and when using an animal model *ex vivo*.

Milk protein hydrolysates currently available for allergic children are derived from processes that include conventional heat treatments. To our knowledge, microwave heating has not been included in the production of milk protein hydrolysates on an industrial scale. This could be made possible by combining microwave treatment with a heat exchanger to accurately control the thermal parameters and to scale up the operation.

The main objective of this study lies in the optimization of the pepsinolysis of bovine whey proteins under dynamic

microwave irradiation and the comparison of the allergenic properties of the end products with those produced by a conventional heating process. For this purpose, we set up a semi-pilot experimental microwave device to control the process conditions, such as temperature and process duration. With this device, hydrolysis was performed under microwaves with process conditions close to the ones encountered in the industry for tubular heat exchangers.

Regardless of other publications related to the microwave effect on whey protein solutions at the lab scale (Gomaa et al. 2013, 2016), this study also emphasizes detailed operating conditions (temperature, mean residence time distribution, microwave forward power, and biochemistry analysis) so that microwave processing could be efficiently compared to a conventional heating treatment under a similar thermal history.

Materials and methods

Microwave treatment

The microwave device consisted of a solid-state microwave generator (Alter SG524 MKS, USA) able to provide a monochromatic wave in the frequency range of 2.4–2.5 GHz (± 1 MHz) with an adjustable output microwave forwarded power from 9 to 450 W (Fig. 1a). The microwave signal was transmitted directly into the TE₁₀ rectangular waveguide (transverse rectangular section of 86 × 43 mm). The waveguide was made of aluminium. The system was followed by a 3 stub tuner, which was used to minimize the microwave reflected power from the load (impedance matching).

The microwave applicator consisted of two concentric quartz tubes crossing the wide side of the TE₁₀ rectangular waveguide. Two metallic chimneys were added at both large sides of the waveguide (near both the liquid inlet and outlet regions) so that no microwave leakages could be detected in the air surrounding the medium.

The liquid consisting of the whey protein concentrate flowed continuously within a closed loop through the inner quartz tube (6/8 mm diameter) with an adjustable residence time under the microwave. The system was also designed in such a way that compressed cold air coming from a Vortex tube (ExairTM Corporation, OH, USA) could be injected within the outer quartz tube during the microwave treatment (Fig. 1b).

From a practical point of view, the liquid was first pumped out from a feeding tank (50 mL) with a peristaltic pump (MasterFlex, Cole-Parmer, USA). The neoprene tubing circuit was connected to the waveguide chimney and the inner quartz tube. The peristaltic pump, insuring sample circulation, was set at a rate of 2.61 L/h so that the

Fig. 1 Microwave processing device. General device description (a). Schematic representation of the inner and outer quartz tubes and the optical fiber position (b). Conventional heat exchanger device (c)

mean residence time of the liquid in the microwave was in the range of 2 s per cycle. The total experiment lasted 30 min, so 43 microwave cycles were considered.

To obtain the resonant cavity conditions and appropriate standing wave patterns within the microwave applicator, the waveguide termination consisted of a sliding short with an adjustable moving piston.

The first trials consisted of finding the optimal cavity size by moving the piston upward or downward to obtain the lowest microwave reflected power (impedance matching). If the sliding short did not allow for obtaining zero microwave reflected power, the operation consisted of

triggering the 3 stub tuner to find the lowest reflection coefficient. The automatic frequency tuning option of the solid-state microwave generator also enabled modification of the microwave frequency in the range 2.4–2.5 GHz to find the best impedance match of the single mode cavity throughout the experiments.

In the case of the inner quartz tube, two thermocouples connected to a datalogger (OM-CP QuadTemp 2000, OMEGA, France) were placed at both the liquid inlet and outlet so that the electric field did not disturb the temperature measurement. In the case of the outer quartz tube, two

thermocouples were also placed at both the inlet and outlet for airflow.

An optical fibre sensor was used to monitor the temperature of the liquid at the centre of the inner quartz tube (NeoptixTM ReflexTM, Canada). This sensor was connected to the input of a PID controller (Eurotherm 2408/2404, UK), which in turn triggered the microwave forward power of the solid-state generator according to the target temperature.

The operating conditions were set up to reach target temperatures of 37 °C, 50 °C, 65 °C and 70 °C at the center of the quartz tube. The PID controller was tuned to reach this target temperature at 25 °C/min, starting from the ambient temperature. Once the target temperature was reached, the controller allowed the maintenance of this target temperature throughout the microwave treatment (30 min). Nevertheless, to ensure constant microwave irradiation during the holding phase at the target temperature, the inner quartz tube was continuously cooled with compressed cold air flowing through the outer quartz tube.

All fluid temperatures and the microwave forward power were recorded with a data logger (Datalog, AOIP France) connected to a personal computer.

Conventional heat exchanger

The second set up consisted of mimicking the operating conditions encountered during the microwave heating process, but with an external heat flux from hot water (Fig. 1c). The heat exchanger consisted of the same inner quartz tube as described previously (150 mm length and 6/8 mm diameter). The tube was inserted into a metallic enclosure where hot water was circulating within an external closed loop connected to a water bath (Julabo, F32, Germany). The system was thermally insulated from the air surrounding medium with insulated foam at the external surfaces.

The peristaltic pump was set at the same rate as for the microwave processing conditions (2.61 L/h) to mimic a similar residence time distribution in both processes.

Two thermocouples were placed at both the inlet and outlet regions of the quartz tube. Another thermocouple was placed directly into the centre of the tube to precisely measure the inner temperature. All temperature measurements were recorded with a data logger (OM-CP Quad-Temp 2000, OMEGA, France). The temperature of the external fluid was adjusted manually according to the target temperature at the center of the quartz tube.

Preparation of the samples and proteolysis

A native whey protein concentrate solution (WPC80: 80% protein in dry matter and 16% lactose) was dissolved in a

glycine buffer (50 mM, pH 2) at a concentration of 1.25% (w/v) under gentle mixing at 25° until complete solubilization occurred. The total protein concentration was 9 mg/mL, as determined by the BCA method (Brown et al. 1989).

Fifty milliliters of native whey protein solution was added to the sample container for the proteolysis experiments.

The pepsin hydrolysis was performed by microwave and conventional heating by adding 27 U/mg of whey protein, and samples were obtained at 5, 10, 20 and 30 min. The set point temperatures applied were 37 °C, 50 °C, 65 °C and 70 °C. The enzyme was deactivated by raising the pH to 7 with Tris 2 M (pH 8), and samples were stored at – 20 °C.

Protein pattern of the whey hydrolysates

SDS-page

The protein pattern was analysed by SDS-PAGE. The hydrolysates (5 µg per sample) were applied on PAGE under reducing conditions. After protein separation, the proteins were stained with Coomassie Blue Brilliant R250 as described previously (El Mecherfi et al. 2011).

RP-HPLC

Reverse phase chromatography was performed on a C18 Symmetry column from Waters (3.5 µm, 4.6 mm × 100 mm) using a Waters (Milford, MA) HPLC System Alliance with the following conditions: flow rate 1 ml/min, eluant A (trifluoroacetic acid (0.07%) in water), and eluant B (80% acetonitrile, 20% water and 0.06% TFA). Elution was performed with a two-step linear gradient of eluant B from 0% to 35% over 22 min and then from 35% to 100% over 7 min. The detection was performed with a Waters Dual Wavelength Detector at 214 nm, and the data were analysed using Empower software.

Degree of hydrolysis

The evaluation of the degree of hydrolysis (DH) of the whey protein hydrolysate samples was performed using the OPA method, as described by Nielsen et al. (2001). DH is defined as the percentage of cleaved peptide bonds: $DH\% = h/htot * 100$, where $htot$ is the total number of peptide bonds per protein equivalent and h is the number of hydrolysed bonds. $htot$ is dependent on the amino-acid composition of the raw material ($htot$ whey protein = 8.8) (Nielsen et al. 2001).

The OPA method is based on the reaction of the primary amino nitrogen with ortho-phthalaldehyde (OPA), which forms a compound detectable at 340 nm in a UV–Vis

spectrophotometer (V-650, Jasco Europe, Italy). The OPA reagent was prepared by dissolving Na tetraborate decahydrate, Na dodecyl sulfate (SDS), ortho-phthaldialdehyde 97% (OPA) and dithiothreitol 99% (DTT) in a deionized water solution. A leucine solution was used to prepare a standard curve (0.25–2.5 mM). In each measurement, 3 mL of OPA reagent was added to 400 µL of deionized water (blank), the leucine solution (standard) or the sample.

All measurements, performed in triplicate, were carried out at 25 °C using deionized water as a control after 5 min of reaction. The number of primary amino acids was determined from the absorbance values and used to calculate the hydrolysis degree (HD%).

Basophil activation test

The activation of rat basophil leukaemia (RBL) cells was performed by native and whey protein hydrolysates in the presence of the patient sera. Ten out of 60 patients with confirmed milk allergies were selected based on their total and BLG-specific IgE levels (Table 1). G418-selected RBL-SX38 cells expressing human FcεRI were seeded at 1.2×10^4 cells/mL and cultured at 37 °C in a humidified atmosphere with 5% CO₂. Sera were heated for 45 min at 56 °C in the presence of 4 M glucose to reduce cytotoxicity (Binaghi and Demeulemester 1983) and diluted 1:40 prior to their addition to the cells for 48 h. IgE-sensitized cells were stimulated for 45 min at 37 °C by native BLG. The native whey proteins or their hydrolysates were diluted from 1 to 2×10^5 ng/ml in Tyrode buffer containing 50% deuterium oxide (D₂O, Sigma) (Blanc et al. 2009). All experiments were performed in triplicate. In mast cells and basophils, most of the β-hexosaminidase (pro-inflammatory molecule) is localized in the granules. After exposure to harmless allergens, such as food allergens, for

sensitization, allergen-specific IgE-bound FcεRI were cross-linked and mast cell/basophile granules were released. β-hexosaminidase release was measured as follows: blank and positive controls were obtained with unstimulated cells and after stimulation with mouse anti-human IgE, respectively. The percentage of degranulation was expressed as the ratio between the sample value and the anti-IgE degranulation value, both corrected for the blank. The highest mediator release value (MaxD) was calculated using GraphPad Prism 5.02 for Windows software (GraphPad Software Inc., La Jolla, CA, USA).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.02 for Windows software (GraphPad Software Inc., La Jolla, CA, USA), and *p* values below 0.05 were considered significant. Data are expressed as the mean ± SEM. Data per group were analysed, and differences between medians/means were analysed by ANOVA with a subsequent “Newman-Keuls Multiple Comparison” post-test (more than 2 groups).

Results and discussion

Temperature control

The first objective of this study was to explore the impact of microwave treatments at different temperature conditions on the rate of pepsin hydrolysis of BLG and to then apply the same temperature conditions using conventional heating to compare both process treatments. Hence, it remains of primary concern to accurately perform and control both the thermal history of the processed samples

Table 1 Specific IgE of the major whey protein for the serum samples used in the rat basophil degranulation test (RBL)

Patient	Age (months)	Symptoms	Anti BLG IgE (ng/ml)	Anti ALA IgE (ng/ml)	Total IgE (ng/ml)
35	6	Vom, Diarr	135	1289	393
36	6	Vom, Eczema	188	249	175
37	12	Abd P, Vom	239	529	859
41	10	Diarr, Vom	126	300	148
45	18	Abd P, Diarr	224	325	633
50	22	Abd P	323	429	523
9	5	Diarr	186	257	639
1606	8	Vom, Diarr	370	453	655
1610	7	Abd P	241	214	667
69	13	Abd P, Eczema	100	95	700

Vom, vomiting; Diarr, diarrhoea; AbdP, abdominal pain

and the operating conditions to study the impact of a microwave effect during whey protein hydrolysis.

As will be discussed later, the total degradation of BLG by microwave heating was obtained at 65 °C.

The experimental temperatures of the liquid sample recorded at the center of the quartz tube exposed to microwave irradiation at a target temperature of 65 °C are presented in Fig. 2.

The temperature evolution during microwave heating showed a quasi-linear progression until 65 °C and then showed a holding phase around the set point with continuous microwave irradiation. This permanent microwave incident power remained possible by the use of the compressed cold air injection (≈ -8 °C at the inlet) around the outer quartz tube, as described in detail in the microwave processing section. However, during the conventional heating process, the come-up time to reach the set point temperature was significantly higher than during the microwave treatment due to the heat transfer limitation by conduction/convection (Fig. 2). Additional experiments by SDS-PAGE and RP-HPLC were conducted, and they confirmed that the thermal history to reach the set-point temperature of 65 °C for both treatments did not modify the proteolysis rate (data not shown).

Overall, as soon as the holding phase remained the same for both treatments, it could be deduced that the thermal history was similar between the conventional heating process and the microwave treatment.

Whey protein hydrolysate characterization

SDS-PAGE and RP-HPLC results

Figure 3 presents the SDS-PAGE profiles of the whey protein hydrolysates obtained after 5, 10, 20 and 30 min of pepsin hydrolysis by microwave irradiation at different temperatures (37 °C, 50 °C, 65 °C and 70 °C). The native

Fig. 2 Temperature profile recorded during the microwave and conventional heating processes with microwave forward power

whey proteins showed two major bands with molecular weights of 18 and 14 kDa, corresponding to bovine BLG and ALA, respectively. Except at the highest temperature, ALA was degraded after only 5 min of hydrolysis. In contrast, the BLG total degradation was only obtained after 30 min of microwave-assisted pepsinolysis at 65 °C (Fig. 3c). When proteolysis was carried out at 50 °C and 70 °C (Fig. 3b, d), BLG was only partially degraded, and 80% of the intact residual BLG remained in the end product. This could be due principally to the pepsinolysis resistance of BLG until 50 °C, considering that BLG still exhibits a compact structure at this temperature, as demonstrated by circular dichroism studies (Iametti et al. 1996; Manderson et al. 1999). In contrast, at 70 °C, the protein starts to unfold and becomes more susceptible to enzyme degradation (Iametti et al. 1996). On the other hand, at this temperature, the impact of BLG degradation is compromised (lowered) by the deactivation of pepsin, the activity of which is conserved until 60 °C. This observation concerning temperature conditions making BLG more susceptible to pepsin at 65 °C confirms our past results (El Mecherfi et al. 2011). Accordingly, the total degradation of BLG by microwave was achieved at 65 °C under these operating conditions. To verify whether the same effect on BLG pepsin susceptibility could be obtained with conventional heating, similar experiments were carried out with conventional heating at the 65 °C set point. SDS-PAGE analysis (Fig. 4a) shows an incomplete hydrolysis under conventional heating at 65 °C, with a clear resistance of BLG to pepsin hydrolysis. These results were confirmed by RP-HPLC analysis (Fig. 4b) with the absence of the peak corresponding to BLG and eluted at 29 min.

Furthermore, the degree of hydrolysis (DH) of the products obtained by pepsinolysis in microwaves and conventional heating at 65 °C were compared. The DH obtained by microwave was significantly higher ($P < 0.001$) than that of the hydrolysate obtained with conventional heating, reaching 14.1% versus 7.1%, respectively (Fig. 4c).

It is assumed that BLG is very resistant to pepsin digestion, which makes it the major whey protein allergen. The resistance is due to the stability of its globular conformation at an acidic pH and the poor accessibility of the target hydrophobic amino acid residues to pepsin action (Schmidt et al. 1995).

Our results demonstrate that BLG undergoes particularly intense dynamic denaturation under microwave action, which is different from what occurs during conventional heating. In a previous study by El Mecherfi et al. (2015), we demonstrated that the BLG heated by microwaves at 63 °C exhibits different far- and near-UV circular dichroism spectra, suggesting that microwaves at this temperature weaken BLG folding compared to

Fig. 3 SDS-PAGE 12% of the native whey protein (WPC) and whey protein pepsin hydrolysates obtained by microwave (MW) at **a** 37 °C, **b** 50 °C, **c** 65 °C and **d** 70 °C. 1, 2, 3, and 4 represent process

durations of 5, 10, 20 and 30 min of hydrolysis, respectively. CTL: native bovine whey proteins

Fig. 4 **a** SDS-PAGE 12% of the native whey protein (CTL) and whey protein pepsin hydrolysates obtained by microwave (MW) and those obtained by conventional heating (CH) after 30 min of hydrolysis, MM: molecular weight marker. **b** RP-HPLC of the native whey protein (WPC) and whey protein pepsin hydrolysates obtained

by microwave (MW_WPH) and those obtained by conventional heating (CH_WPH) after 30 min of hydrolysis. **c** Degree of hydrolysis of the pepsin whey protein hydrolysates obtained by microwave and conventional heating at 65 °C

conventional heating; consequently, less rigid tertiary and secondary structures are produced, making BLG more susceptible to pepsinolysis. Recently, Gomaa et al. (2016) used spectroscopic methods to study the unfolding of BLG

under microwaves compared with conventional heating. They indicate and confirm a substantial effect of microwave treatment on the thermal unfolding of BLG compared with conventional heating. They also provide supporting

evidence that the observed pepsin susceptibility as a function of the microwave treatment is due to the increased exposure of the pepsin target residues in the microwave-treated protein.

Basophil activation test

To pass from the development phase to clinical application, it is important to evaluate the allergenicity/antigenicity of hydrolysed formulas for their safety. In our study, the residual allergenicity of whey protein hydrolysates produced by microwaves (MW_WPH) and conventional heating (CH_WPH) was investigated by a basophil activation assay, which presents a powerful *in vitro* tool mimicking IgE-mediated reactions *in vivo*. The assay was achieved using a pool of children with a confirmed milk protein allergy. This step mimics what happens physiologically in the effector phase of an allergy.

Fig. 5 Basophil activation assay with human sera. RBL-SX38 cells were stimulated with BLG, native whey protein (WPC), microwave assisted whey protein pepsin hydrolysates (MW_WPH) and conventional heating assisted whey protein pepsin hydrolysis (CH_WPH) at concentrations from 1 to 2×10^5 ng/ml. **a** Degranulation curves for the pool of 10 sera from allergic children. **b** Maximum degranulation (Max D) data calculated from three repetitions in triplicate. Mean $\pm$ SEM and results of statistical tests (ANOVA with a subsequent Newman–Keuls multiple comparison post-test) are presented (*** $p < 0.001$)

Figure 5a shows the percent of β -hexosaminidase released after the cross-linking of allergens with the anti-milk-specific human IgE of sensitized basophils. The native whey protein (WPC) and native BLG used as triggering antigens induced a large release of β -hexosaminidase, reaching maximum values of $46 \pm 5\%$ (Fig. 5b), confirming the strong allergenicity of BLG compared to total whey protein. The stimulation with the whey protein hydrolysates obtained in conventional heating at 65°C still induced the release of β -hexosaminidase, with a MaxD remaining at $25 \pm 3\%$. In contrast, no significant degranulation was observed when the pepsin whey protein hydrolysates generated by microwaves at 65° were used as triggering antigens compared with the native whey proteins and whey protein hydrolysates obtained under conventional heating. These results indicate that no epitope determinants in these hydrolysates were recognized by the pool of anti-whey human IgE, suggesting that pepsin hydrolysis was more efficient under microwave conditions. This microwave process could enhance the accessibility of resistant and buried sites to pepsin proteolysis. These results confirm our previous findings (El Mecherfi et al. 2015) in which the whey protein hydrolysates obtained with pepsin under microwave conditions did not induce any anaphylactic reactions when challenging the intestinal fragment from BLG-sensitized mice in the Ussing chamber.

Subsequently, a clinical study of the use of microwave heated milk on the efficiency of oral immunotherapy (OIT) was conducted (Takahashi et al. 2016). The authors demonstrated that when children with milk allergies were given milk heated in a microwave at 500 W (60°C) during oral food challenge (MH-CM-OFC), the threshold (minimum of milk volume administered to provoke clinical reactions) for MH-CM-OFC significantly increased compared with that of the fresh cow milk oral food challenge (CM-OFC). In addition, the severity of the allergic symptoms in MH-CM-OFC was significantly smaller than that observed in the case of CM-OFC. This could be related to the enhanced digestibility of the milk proteins heated under microwave conditions. In addition, oral immunotherapy was achieved with success when children received the microwave heated milk.

Conclusion

In conclusion, according to our observations, the application of a microwave heating process in concomitance with enzymatic proteolysis improved the susceptibility of resistant proteins (BLG) to pepsinolysis and represents a new way for improving the bioactive properties of milk

proteins, which could be integrated easily into an industrial set-up.

In addition, the extensively hydrolysed whey protein hydrolysates generated under microwaves did not elicit the allergic reactions as did those produced by applying conventional heating processes.

From an industrial point of view, microwave processing for liquid food products highly improves the flexibility of the system compared to a conventional heating treatment (low inertia and potential energy savings).

The microwave-assisted proteolysis of dairy products is thus a promising technique that could be scaled-up and integrated for industrial applications.

Acknowledgements This work was performed in the framework of the IAFAPOM project (Improvement of Acceptability of Foods of Animal and Plant Origin by the combination of Microwaves with proteolytic treatment) and with financial support from the Region Pays de la Loire—France (RFI “Food for Tomorrow”). We would like to thank Mr. Christophe Couëdel for the conception and development of the microwave processing unit and his technical assistance.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

References

- Augustin MA, Udabage P (2007) Influence of processing on functionality of milk and dairy proteins. In: Taylor S (ed) *Advances in food and nutrition research*, 1st edn. Elsevier, Amsterdam, pp 1–38. [https://doi.org/10.1016/s1043-4526\(07\)53001-9](https://doi.org/10.1016/s1043-4526(07)53001-9)
- Binaghi RA, Demeulemester C (1983) Influence of the medium on the heat and acid denaturation of IgE. *J Immunol Methods* 65:225–233
- Blanc F, Adel-Patient K, Drumare M-F, Paty E, Wal J-M, Bernard H (2009) Capacity of purified peanut allergens to induce degranulation in a functional in vitro assay: Ara h 2 and Ara h 6 are the most efficient elicitors. *Clin Exp Allergy* 39:1277–1285
- Bøgh KL, Barkholt V, Madsen CB (2015) Characterization of the immunogenicity and allergenicity of two cow milk hydrolysates—a study in brown norway rats. *Scand J Immunol* 81:274–283
- Breiteneder H, Mills ENC (2005) Molecular properties of food allergens. *J Allergy Clin Immunol* 115:14–23
- Bu G, Luo Y, Chen F, Liu K, Zhu T (2013) Milk processing as a tool to reduce cow milk allergenicity: a mini-review. *Dairy Sci Technol* 93:211–223
- Brown RE, Jarvis KL, Hyland KJ (1989) Protein measurement using bicinchoninic acid: elimination of interfering substances. *Anal Biochem* 180:136–139
- Damm M, Nussold C, Cantillo D, Rechberger GN, Gruber K, Sattler W, Kappe CO (2012) Can electromagnetic fields influence the structure and enzymatic digest of proteins? A critical evaluation of microwave-assisted proteomics protocols. *J Proteom* 75:5533–5543
- El Mecherfi KE, Saidi D, Kheroua O, Boudraa G, Touhami M, Rouaud O et al (2011) Combined microwave and enzymatic treatments for β -lactoglobulin and bovine whey proteins and their effect on the IgE immunoreactivity. *Eur Food Res Technol* 233:859
- El Mecherfi K-E, Rouaud O, Curet S, Negaoui H, Chobert J-M, Kheroua O et al (2015) Peptic hydrolysis of bovine β -lactoglobulin under microwave treatment reduces its allergenicity in an ex vivo murine allergy model. *Int J Food Sci Technol* 50:356–364
- ElAshry ESH, Ramadan E, Kassem AA, Hagar M (2005) Microwave irradiation for accelerating organic reactions. Part I: three-, four- and five-membered heterocycles. In: Scriven E (ed) *Advances in heterocyclic chemistry*. Elsevier, Staffordshire, pp 1–110
- George DF, Bilek MM, McKenzie DR (2008) Non-thermal effects in the microwave induced unfolding of proteins observed by chaperone binding. *Bioelectromagnetics* 29:324–330
- Gomaa AI, Sedman J, Ismail AA (2013) An investigation of the effect of microwave treatment on the structure and unfolding pathways of β -lactoglobulin using FTIR spectroscopy with the application of two-dimensional correlation spectroscopy (2D-COS). *Vib Spectrosc* 65:101–109
- Gomaa AI, Nsonzi F, Sedman J, Ismail AA (2016) Enhanced unfolding of bovine β -lactoglobulin structure using microwave treatment: a multi-spectroscopic study. *Food Biophys* 11:370–379
- Iametti S, Gregori B, Vecchio G, Bonomi F (1996) Modifications occur at different structural levels during the heat denaturation of β -lactoglobulin. *Eur J Biochem* 237:106–112
- Kappe CO, Dallinger D (2009) Controlled microwave heating in modern organic synthesis: highlights from the 2004–2008 literature. *Mol Divers* 13:71
- Ketnawa S, Liceaga AM (2017) Effect of microwave treatments on antioxidant activity and antigenicity of fish frame protein hydrolysates. *Food Bioprocess Technol* 10:582–591
- Koletzko S, Niggemann B, Arato A, Dias JA, Heuschkel R, Husby S et al (2012) Diagnostic approach and management of cow's-milk protein allergy in infants and children: ESPGHAN GI committee practical guidelines. *J Pediatr Gastroenterol Nutr* 55:221–229
- Madadlou A, Sheehan D, Emam-Djomeh Z, Mousavi ME (2011) Ultrasound-assisted generation of ACE-inhibitory peptides from casein hydrolyzed with nano encapsulated protease. *J Sci Food Agric* 91:2112–2116
- Manderson GA, Creamer LK, Hardman MJ (1999) Effect of heat treatment on the circular dichroism spectra of bovine β -lactoglobulin A, B, and C. *J Agric Food Chem* 47:4557–4567
- Nielsen PM, Petersen D, Dambmann C (2001) Improved method for determining food protein degree of hydrolysis. *J Food Sci* 66:642–646
- Nwaru BI, Hickstein L, Panesar SS, Roberts G, Muraro A, Sheikh A (2014) The EAACI food allergy and anaphylaxis guidelines group, prevalence of common food allergies in Europe: a systematic review and meta-analysis. *Allergy* 69:992–1007
- O'Riordan N, Kane M, Joshi L, Hickey RM (2014) Structural and functional characteristics of bovine milk protein glycosylation. *Glycobiology* 24:220–236
- Schmidt DG, Meijer RJGM, Slangen CJ, Beresteijn ECH (1995) Raising the pH of the pepsin-catalyzed hydrolysis of bovine whey proteins increases the antigenicity of the hydrolysates. *Clin Exp Allergy* 25:1007–1017
- Taheri-Kafrani A, Gaudin J-C, Rabesona H, Nioi C, Agarwal D, Drouet M et al (2009) Effects of heating and glycation of β -lactoglobulin on its recognition by IgE of Sera from cow milk allergy patients. *J Agric Food Chem* 57:4974–4982
- Takahashi M, Taniuchi S, Soejima K, Hatano Y, Yamanouchi S, Kaneko K (2016) Two-weeks-sustained unresponsiveness by oral immunotherapy using microwave heated cow milk for

- children with cow milk allergy. *Allergy Asthma Clin Immunol* 12:44
- Tsabouri S, Douros K, Priftis K (2014) Cow milk allergenicity. *Endocr Metab Immune Disord Drug Targets* 14:16–26
- Uluko H, Liu L, Li H, Cui W, Zhang S, Zhao L, Xue H, Lv J (2014) Effect of power ultrasound pretreatment on peptidic profiles and angiotensin converting enzyme inhibition of milk protein concentrate hydrolysates: improving milk protein concentrate hydrolysates functionality using ultrasound. *J Sci Food Agric* 94:2420–2428
- Verhoeckx KCM, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S et al (2015) Food processing and allergenicity. *Food Chem Toxicol* 80:223–240