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THESIS

Protein Changes of Various Types of Milk as Affected by High Hydrostatic Pressure Processing

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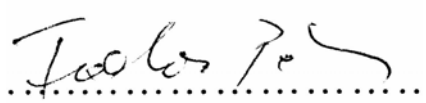

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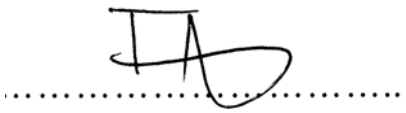

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Protein Changes of Various Types of Milk as Affected by High Hydrostatic Pressure Processing

Introduction

New nutritional concepts require the development and application of technologies which can

(1) preserve or improve the overall quality of the raw materials and their physicochemical functionality;

(2) maintain or enhance the nutritional and physiological value of the end-product;

(3) increase the safety of the product.

Minimal processing may be employed successfully to meet these requirements. The goal of the minimal processing concept is to maintain the natural properties of foods and increase the product's shelf life as well as decreasing processing costs and environmental impact, without compromising product safety.

The introduction of the minimal processing concept was made possible by the development and application of novel non-thermal and thermal food processing technologies which are often less invasive than the conventional methods used in the food industry. One of these novel non-thermal techniques is preserving food by high hydrostatic pressure. As this technology has been introduced to food processing only over the last two decades, basic research is still needed to better understand kinetics and mechanisms underlying the effects observed, and more experimental data are required to assure regulatory approvals.

Prompted by the lack of sufficient data in the field of HP processing of foods and by the fact that I had access to a lab-scale HHP equipment at the Department of Refrigeration and Livestock Products Technology, my interest was drawn to the potential of this new technology, and I decided to pursue a research project in my field of specialization, dairy science.

Objectives

The goal of my work was to learn more about the effect of HHP on different types of milk, especially on milk proteins. I used both classical methods of proteomics and spectrofluorometry in the investigations to find out whether the more rapid fluorescence spectroscopy, which is not a conventional method for protein investigations, can provide sufficient information about the changes in milk components compared to the information obtained using the classical methods.

A very important aspect of the introduction of new food processing techniques is whether the new processing method affects the allergenic potential of a food product, since novel foods can be potential allergens. It is necessary to assess the risk of creating or activating hitherto unseen or not bioavailable immunoreactive structures by the application of new food processing technologies. Thus a further objective of my research was to detect the effect of HHP on the immunoreactivity of milk proteins in different milk types.

Materials and Methods

The following materials were included in the research project: human milk, whole and skimmed bovine milk, whole ewe's milk, whole goat milk, whole mare's milk, and bovine whey.

The samples were treated at different pressure levels and for different holding times. The pressure progressed from 100 MPa to 800 MPa gradually increased by 100 MPa increments, and holding times were 5, 10, 20, 30 and 40 mins. The methods applied in the investigations:

- Polyacrylamide gel electrophoresis (SDS PAGE and native PAGE, two-dimensional PAGE);
- Immunoblotting (Western blotting);
- Spectrofluorometry.

In the spectrofluorometric measurements, whole bovine milk, goat milk and bovine whey were examined. Half of the samples were pressurized and the rest of them underwent heat treatment (from 70°C to 100 °C in 10 °C increments, and from 5 to 30 mins in 5mins increments). The effect of the two preservation methods were compared. Tryptophan emission and retinol emission and excitation intensities were measured. During the tryptophan emission acqusition, wavelength of the excitation was 290 nm and emission was detected between 305 and 450 nm. During the scanning of emission spectra of retinol, intensity of the emitted light was recorded within the wavelength range of 350-500 nm, and the wavelength of excitation was 321 nm. When the excitation spectra of retinol were scanned, the emission wavelength was set to 410 nm, and the excitation wavelengths were recorded between 380 and 600 nm.

In this study, the effect of HHP on different milk types, primarily on their milk proteins, was investigated. In the protein examinations, classical methods used in proteomics, and spectrofluorometry were applied and compared. Investigation of retinol was conducted only

by fluorescence spectroscopy. Potential changes in the immunoreactivity of milk proteins as a result of HHP treatment were detected by immunoblotting.

Protein composition of different milk types (human milk, bovine, goat, ewe's and mare's milk) was compared using SDS and 2D PAGE.

Results and Discussion

In the case of control samples (raw milk), the two albumin milk types were clearly differentiated from the other milk types belonging to the casein milk group. 2D-PAGE gels showed clearly the differences in the amounts of casein fractions between the milk samples. Several spots, indicating the presence of casein, appeared on the gels for goat milk and bovine milk and most of them had higher intensities than spots in the other milk types. As the literature has suggested, no β-Lg was found in human milk. The amount of α-La was less in goat milk and bovine milk than in human or mare's milk samples. In the analysis of ewe's milk, four, rather than two well separated bands appeared on the gels in the position where α-La was expected.

In the next series of examinations, the effect of HHP on different milk proteins was investigated using gel electrophoretic methods. The parameters of pressurization were 600 MPa and 5 mins holding time. We found that the proteins in milk samples reacted in different ways to pressure treatment.

In human milk, compared to the control sample, very slight or no decrease was observed in the intensity of the casein fractions. A slight decrease was found in the α-La fraction as a result of HP treatment.

High pressure had minimal effect on proteins present in mare's milk. Negligible changes occurred in the intensity of the casein fraction. Intensity of β-Lg increased the most, but not significantly. Intensity of the α-La bands increased merely by ~5%.

Protein fractions of goat milk reacted to HP treatment in different ways. Among the two peaks of α-La on the densitograms, the first one (lower Rf value) didn't change, while the second one increased notably (approx. 34%). On the other hand, the two peaks corresponding to β-Lg, showed a significant reduction (~55%).

In bovine milk, only a minimal decrease could be observed in the protein fraction of α-La. Intensity of the two β-Lg bands changed significantly. The rate of decrease was ~50%, close to that of goat milk.

Since in Hungary, compared to other types of milk, production and consumption of bovine milk is of the greatest importance, the effect of HP on its proteins was investigated in more detail. Bovine milk samples were treated at different pressures (from 100 MPa to 800 MPa) for 10 mins, and for different holding times (5, 10, 20, 30 and 40 mins) at constant pressure, thus the effect of the level of pressure and of the length of holding time could be studied.

Most apparent changes occurred in the β-Lg fraction. According to the intensity of the bands, β-Lg content of pasteurized milk (72°C, 40 s) was approximately the same as the intensity of the sample that had been treated by 300 MPa for 10 mins. By increasing pressure, β-Lg gradually denatured. In the samples pressurized to 800 MPa, this fraction was hardly visible. The bands of proteins, having higher molecular weights, showed an increasingly diffuse distribution indicating aggregation. A small amount (~10%) of native β-Lg remained after HP treatment at 800 MPa for 20 mins. β-Lg appeared on the gels in two bands representing the two isoforms of this protein. The two isoforms reacted in different ways to pressure, β-Lg B denatured first. In gradient gel, the intensity of casein bands increased in pressurized samples. No significant changes in α-La content of the different pressurized samples could be observed.

Holding time of HHP treatment affected milk proteins as well. The longer the applied treatment time was, the more the intensity of the β-Lg bands decreased. Again, β-Lg B proved to be more sensitive to pressure than β-Lg A. Length of holding time didn't seem to affect significantly the intensities neither of casein nor of α-La bands based on the separation methods used in this study.

To examine the interactions between proteins and lipids, the patterns of molecular weight separation of proteins were examined, both in control samples and in samples of pressurized skim milk (0.21 g/100g fat content) and whole milk (4.37 g/100g fat content).

Intensity of protein bands changed in a different way in whole and skim milk. Decided differences appeared in the intensities of β-Lg fractions of skim and whole milk samples at 600 and 800 MPa, respectively. The intensity of β-Lg fractions in skim milk decreased more significantly at these pressures than in whole milk. The densitograms showed that a ~4% difference in fat content caused about 40% lower intensity of the β-Lg bands in the skim milk sample at the pressures applied. This suggested a baroprotective effect of fat on proteins. This effect might be explained by the lipid-protein interaction during HP treatment.

Summarizing the results we found, that intensities of protein fractions in the electrophoretic pattern of HHP treated milk samples decreased with increasing pressure and

holding time. The extent of the decrease varied depending on the milk types, and the milk protein fractions reacted to pressure in different ways, too.

In the higher pressure ranges, decrease in the intensity of the protein fractions, first of all of β-Lg, was smaller in the whole milk samples, than in skim milk.

Decrease in the amount of detectable proteins can be explained by the (partial) denaturation/aggregation of milk proteins under HP. Thus applying HP can significantly decrease their solubility. Whether the non-thermal, mostly reversible denaturation/aggregation of protein fractions produces advantageous or disadvantageous changes in the conformation and biological activity of milk proteins has yet to be determined.

However, there are no available data on the potential risks of high pressure processing of foods, but it is important to clarify the role of HHP in this respect as well. For this reason we included into our research tests to determine the immunoreactivity of proteins in the control and pressurized milk samples.

In the control samples, immune responses were the strongest in the protein fractions corresponding to casein. Ewe's, goat and bovine milk gave more intensive responses than the other two milk types. β-Lg showed immunoreactivity in each milk of animal origin. The weakest responses were given to α-La by human and mare's milk. In the other three milk types, immunoreactivity caused by this protein fraction could be detected. Two active bands were present. However, when milk positive human serum from another patient was used in the examinations, the results were different.

After pressure treatment, the most promising results were obtained for mare's and goat milk. We found the least changes in immunoreactivity took place in bovine milk.

Antigen-antibody complexes were investigated in pressurized bovine milk by using anti-β-lactoglobulin antibody IgG developed in rabbit, and human sera for IgE, respectively. Results obtained with the different antibodies were not identical. When anti-β-lactoglobulin antibody IgG was used, no differences were found in the immunoreactivity of casein and α-La fractions in control and pressurized samples. Decrease in immunoreactivity of β-Lg corresponded to the decrease in the intensity of this protein. Three hundred MPa treatment affected β-Lg B in a different way than β-Lg A. At this pressure the intensity of β-Lg B was about half of the original intensity, but β-Lg A showed only a very slight decrease. At 600 MPa the intensity of both β-Lg isoforms showed similar values.

When immunochemical reactions with milk positive human serum were studied, casein fractions gave definite responses. High pressure decreased the immunoreactivity of these fractions, but the rate of decrease reached its maximum at 400 MPa treatment, no further

reduction was obtained at higher pressures. The other protein fractions didn't show immunochemical reactions, most likely because the human serum originated from a patient who was sensitive only to casein.

Decrease in immunoreactivity could be noticed only in skim milk but not in whole milk under the applied conditions of the experiment.

HP seemed to decrease the immunoreactivity of certain protein fractions in the different milk types. According to the separation and immunoblotting methods used, the extent of the decrease was not significant, except for mare's milk. Thus HP treatment alone did not prove to be useful to produce hypoallergenic milk or milk products.

Heat treated and pressurized bovine milk, bovine whey and goat milk samples were included in the fluorescence investigations. Intensities of tryptophan emission, retinol emission and excitation were measured and compared.

Irrespective of the material investigated and the type of spectra (emission or excitation), the overall tendency was in each case was that the fluorescence intensity increased with higher temperature of the treatment and decreased with increasing pressure.

In the Trp emission measurements, bovine whey showed the lowest intensity values followed by whole bovine milk. Whole goat milk had the highest intensity values. These differences resulted from the composition of the milk types and whey. Whey contains only whey proteins. Casein, the highest protein fraction in milk, can not be found in it. Goat milk contains more protein than bovine milk. The maximum of the emission spectrum of control whey was located at 334 nm and of the 100°C/30 mins sample at 341,7 nm, thus a marked red shift could be observed. More pronounced changes took place in the Trp emission in bovine milk than in whey. The emission peak in raw bovine milk was found at 342 nm. Goat milk had the highest intensity values and this type of milk reacted to heat and to pressure the most, because its emission intensity changed in a slightly higher degree, than that of bovine milk. About 1 nm red shift could be noticed in goat milk samples as an effect of heat treatment.

Whole bovine milk samples were stored overnight at refrigerator temperature. Intensity of emission spectra of samples measured directly after the treatments were compared to the intensity values of stored samples. Trp emission intensity of stored samples was lower than the intensity of "fresh" samples. Not only the intensity, but also the intervals between the spectral curves of stored samples were smaller. This indicated that structural re-arrangement, primarily partial refolding of milk proteins, first of all β-Lg, took place during storage, and it was equivalent to conformational changes caused by an approx. 20°C drop in temperature.

The tendency, observed in the fluorescence behaviour of Trp under pressure, can be explained as follows. Crystallographic studies have shown that the polarity of Trp environment correlates well with the energy of the fluorescence emission. At higher pressures the native environment of the Trp is replaced by one of considerably greater polarity. Water molecules penetrate the interior of the protein and they cluster close to the Trp residues. Thus strong interaction with the field of the dipole fluorophore becomes possible. Structures of native and HP treated proteins are different. As an effect of HP, the Trp containing region in the hydrophobic part of the protein gets closer to the core of the molecule and is shielded from the environment. Cavities inside the protein either are filled off under high pressure, or the protein is so heavily compressed that the gaps disappear. This results in a loss of the protein's functional abilities and in a stabilisation of the hydrophobic regions.

Proteins reacted in the opposite way to heat than to pressure. Structural changes brought about by temperature are such, that the Trp side chains become more exposed to the surface of the protein, primarily β-Lg molecule, and therefore, to the solvent. This indicates an expanded structure. As a result of heat treatment, proteins (partially) unfold and the hydrophobic regions, containing Trp, loose their shielding effect and Trp is released gradually to the environment.

Emission and excitation intensity of retinol was also measured in the two milk types and whey. The same tendencies appeared in this case as in the Trp investigations: the intensity of emission and excitation increased with increasing temperature, and decreased with increasing pressure.

In heat treated bovine milk the maximum difference in retinol emission was observed between the control and the sample heated to 70°C. Among pressurized samples, the biggest interval in intensity was found between the samples subjected to 400 and 600 MPa pressures. When excitation intensities were examined, the maximum difference was registered between the samples processed at 70°C and 80°C, respectively. The biggest difference in excitation intensities resulted between the control and the sample treated at 200 MPa. The excitation spectra of samples pressurized to 400 and 600 MPa almost overlapped each other, that is, pressures higher than 400 MPa didn't cause any more changes.

Both emission and excitation intensities were lower in goat milk than in bovine milk, due to the smaller retinol content of goat milk. The emission maximum in bovine milk was located at 407 nm and in goat milk at 409 nm. The shape of the excitation spectra differred from the emission spectra. It had one peak and two shoulders at lower wavelengths. The excitation maximum in both milk types was measured at 319 nm. The biggest differences

both in emission and excitation intensities were found in goat milk between the control and the sample heated to 70°C, and the control and the sample kept at 200 MPa pressure.

The increase in the intensity of the excitation and emission curves was caused by the release of retinol from the fat globules by heat treatment. High pasteurization temperatures denature the cryoglobulins in the fat globule membrane, and aggregation of the fat globules and creaming are impaired or prevented. Severe heat treatments remove lipids and proteins from the fat globule membrane, partially denude the fat globules and may cause them to coalesce and form large fat clumps. Thus retinol, solved in the fat clumps, with destroyed membranes, is more exposed to the exciting light, since it is shielded less than in its initial position inside the intact fat globule.

High pressure processing had the opposite effect on retinol fluorescence than heat treatment. HP induced fat crystallisation, and the solid fat content is higher in HP treated cream and milk, than in the untreated one. Fluorescence shows up less effectively in a solid phase. Besides, the fact that the amount of lipolytic products doesn't increase in HP treated milk it indicates, that HP does not damage the milk fat globule membrane and so the milk fat globules are not disrupted. Thus, the retinol remains in the fat globule and stays better shielded from the environment. Additionally, the fat globules were more compact after the pressure treatment, resulting in a better shielding effect of retinol fluorescence.

β-Lg seem to play an important role in the accumulation of retinol in milk. β-Lg was shown to bind retinol. During heat treatment the native structure of β-Lg is denatured. The loss of the secondary, tertiary and quaternary structure of the protein can result in an irreversible structural change of the central calyx. Therefore retinol can not bind any longer to the protein, and it is released to the environment. As a result, denaturation of β-Lg has a synergistic effect on the increase of the emission and excitation intensity of retinol fluorescence.

Summary

Based on the above results we can state that the differences in the degree of intensity changes, and in the measure of red shift, indicate that applying high pressure affected milk, primarily milk proteins and milk fat, to a lesser extent than applying heat. Thus HHP treatment of milk, considering its effect on the main components, seems to be a milder processing method, than heat treatment.

The results obtained by the two different techniques, gel electrophoresis and spectrofluorometry, were in good agreement with each other. Using gel electrophoresis, intensity of the bands of the different pressurized milk protein fractions was decreasing with increasing pressure and holding time, indicating a loss in the fractions. In accordance with these results, intensities of Trp and retinol emission and excitation spectra were also decreasing with increasing pressure and holding time. The similar tendencies found by the two different methods support the assumption that spectrofluorometry can be a a viable alternative in protein research. However, the type of information provided by PAGE and spectrofluorometry is different. The classical methods of proteomics can not be replaced by fluorometric measurements, only in specific aimed situations, but where applicable, fluorescence spectroscopy affords rapid, reliable, well-reproducible results in contrast to the time-consuming electrophoretic methods.

Based on these findings, I would like to pursue my research both by concentrating on the spectrofluorometric approach and by broadening my interest towards the application of HHP to the processing of other dairy products (e.g. fermented products such as yoghurt or cheese. Regarding spectrofluorometry, I endeavour to compile a database of milk by systematically adjusting treatment parameters and by measuring the pertinent fluorescence intensities. With the help of an appropriately large data collection, compiled using mathematical statistical methods, such as principal component analysis and discriminance analysis, identification and classification of an unknown sample would be possible. One could also determine, what kind of treatment was used and whether the product underwent an adequate treatment or not. Thus, spectrofluorometry could be made a new and more efficient method of quality control in the dairy industry. Regarding the application of HP treatment to dairy products other than milk, there is still much to learn about the effect of HHP on their coagulation, texture, ripening, and functional characteristics of proteins.

Theses (new scientific results)

1. Higher fat content of milk exerts a protective effect on milk proteins, primarily β-Lg, against pressure. The baroprotective effect of milk fat on milk proteins was confirmed by the examinations on immunoreactivity as well.
2. To my knowledge no research was carried out and published on the treatment of mare's milk by HHP. Immunoreactivity of mare's milk was completely eliminated by

the application of 600 MPa pressure for 5 mins. Thesis: HP treated mare's milk could be a good alternative for patiens suffering from cow's milk allergy.

3. Immunoreactivities of the two isoforms of β-Lg reacted differently to pressure. Thesis: to decrease the immunoreactivity of β-Lg, pressures higher than 300 MPa are needed.
4. Heat and pressure exerts opposite effects on the tryptophan emission in the materials tested. Intensity of Trp emission increases with increasing temperature and decreases with increasing pressure while maintaining constant holding time. Intensity of Trp emission increases with increasing holding time at constant temperature and decreases with increasing holding time at constant pressure.
5. Heat and pressure exerts opposite effects on the retinol emission and excitation in the materials tested. Intensity of retinol emission and excitation increases with increasing temperature and decreases with increasing pressure while maintaining constant holding time. Intensity of retinol emission and excitation increases with increasing holding time at constant temperature and decreases with increasing holding time at constant pressure.
6. In whole goat milk, the biggest changes in the intensity of retinol emission and excitation take place between the control sample (raw milk) and the samples that were treated to the least extent. Higher treatment parameters cause only very slight differences. Initial changes are the biggest under the applied conditions.

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