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ORIGINAL PAPER

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Effect of high hydrostatic pressure on physicochemical and biochemical properties of milk

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Abstract Interest in high hydrostatic pressure (HHP) applications on milk and dairy products has recently increased as HHP offers a new technology for food preservation to the food industry. Although HHP-induced microbial destruction, rennet or acid coagulation of milk and increase in cheese yield has been reported, the actual effect of HHP application on milk constituents still remains to be unexplained. Therefore, we have analyzed the effect of HHP on physicochemical and biochemical properties such as turbidity, pH and especially protein micelle surface hydrophobicity of milk proteins. To serve for this purpose, milk samples with different fat contents were pressurized from 110 to 440 MPa at 25 °C for 10 and 20 min. Turbidity decreased with pressure increase and there was a slight change in pH. In order to measure the extent of exposure of hydrophobic groups of proteins to HHP, the method described by Bonomi et al. [1], based on use of a fluorescent probe, was utilized. In the light of the results obtained, it can be concluded that HHP has an effect on non-covalent interactions and especially hydrophobic bonds in milk. As the pressure is increased from 110 to 440 MPa, the micelles possibly decompose into sub-micelles and the embedded hydrophobic areas inside these micelles re-position in such a way that they can readily interfere with the fluorescent marker, ANS. These results may lead to practical applications of HHP treatment in the dairy industry to produce microbiologically safe, minimally processed products with high nutritional and sensory quality and novel texture.

Keywords High pressure · Milk · Micelles · ANS · Surface hydrophobicity

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Introduction

Technological developments have resulted in demonstration of industrial scale feasibility of high pressure processing (HHP). These technologies are of specific interest for the food industry, not only as they provide attractive alternatives to conventional methods of thermal processing which often produce undesirable changes in food hampering the balance between high quality and safety (i.e. color, flavor, functionality), but also they do offer opportunities for creating new ingredients and products due to the specific actions on biological materials and food constituents. HHP also allows redesigning of existing processes and creating entirely new ones alone or in combination with conventional processes (e.g. pressure–temperature combinations) [2]. As a result, various researchers have investigated the use of HHP treatment on milk at different temperatures and interesting effects on the physicochemical characteristics have been reported. For example, 450 and 600 MPa treatments for 30 min up to 40 °C led to an increase in protein hydrophobicity, decrease in lightness and average diameter of particles [3]. Hinrichs et al. [4] reported irreversible changes and reduction in size of casein micelles around 230 MPa.

Whey proteins can undergo partial, but fully reversible, unfolding of their native molecular structures under suitable pressures up to 300 MPa. Rademacher et al. [5] showed that pressures above 150 MPa produced significant denaturation of β-lactoglobulin in milk, which is also reported to be relatively more pressure sensitive than α-lactalbumin [6]. The decrease of non-sedimentable serum nitrogen and non-casein nitrogen after pressure treatment of milk has been attributed to denaturation of whey proteins [7].

Nakai and Li-Chan [8] concluded that the conformational changes on the structure of proteins indicate increased exposure of hydrophobic groups, which may also alter the functional properties of the system. Hence, the foaming, emulsifying, gelling and water binding capacities of the proteins may be influenced. If that is the case, this could lead to the development of a range of functional food

ingredients prepared from milk proteins by controlled unfolding of their structure. As an example, the pressure-induced increase in viscosity of solutions containing whey proteins, with increased water binding capacity, can be advantageous in yogurt manufacture [5] and dairy products with novel textures can be processed [9].

Besides hydrophobic properties, the crystallization of milk fat can be accelerated, enforced or initiated because of the shift in the phase transition temperature caused by application of HHP. In this respect, Buchheim et al. [10] reported that the application of HHP to fat containing food systems in general can induce fat crystallization and shorten the time required to achieve a desirable solid fat content.

The effect of HHP on enzymes in general and especially on milk clotting by rennet has been extensively studied in literature [11–14] and increase in the curd rigidity at a pressure range from 20 to 40 MPa for 90 min have been reported. In another study the greatest rearrangement and compression of casein particles was reported around 700 MPa, resulting in smaller particles with greater hydrophobic character and favoring rennet coagulation [7, 11].

As can be seen from examples, the effect of HHP on various milk constituents is still one of the emerging interest areas. Although the listed studies dealt with the subject, the actual effect of HHP application on milk constituents still remains to be unexplained. Therefore, the aim of this study is to analyze the effect of HHP on physicochemical and biochemical properties such as turbidity, pH and especially protein micelle surface hydrophobicity of milk proteins.

Materials and methods

Milk samples

UHT milk samples with three different fat contents (0.1, 1.5 and 3.2%) were obtained from a local market (Pınar Company, İzmir, Turkey). As UHT milk was already processed, results after pressurization show the combined effect of UHT and HHP. Therefore, to see the effect of pressurization alone, raw whole milk that was obtained daily from a local dairy plant (Atatürk Orman Çiftliği-AOÇ, Ankara, Turkey) was used as control.

HHP equipment

HHP treatments were performed in a designed and constructed lab-scale unit (capacity: 30 cm^3, maximum pressure: 500 MPa, maximum temperature: 95 °C). The rate of pressure increase and pressure release was approximately 5–10 s for the designed system. A mixture of water and glycol was used as pressure transmitting medium. The equipment consists of a pressure chamber of cylindrical design (internal diameter 5 cm; length 35 cm), two end closures, a means for restraining the end closures, a pressure pump, and a hydraulic unit to generate high pressure for system compression and also a temperature control device. The liquid was heated prior to pressurization to the desired temperature by an electrical heating system surrounding the chamber. Pressurization time reported in this study did not include the pressure increase and release times.

HHP treatment of milk samples

During pressurization, cryovials (Simport Plastic, Canada) were filled with milk samples avoiding as much air bubbles as possible. Then cryovials were placed inside the cylindrical vessel of HHP equipment, the chamber was closed and the samples were kept for 1 to 2 min for temperature equilibration. The time–temperature combination for equilibration was determined earlier.

The milk samples with different fat content were pressurized at 110, 220, 330 and 440 MPa at 25 °C for 10 and 20 min. Duplicate vials were used for each treatment. After pressurization the vials were immediately removed from the system and placed in an ice bath. Unpressurized milk samples were used as controls. Experiments and measurements were duplicated.

Turbidity and pH measurement

All the samples (pressurized and control) were diluted to 1/100 before measuring the turbidity as described by Needs et al. [15]. The sample turbidity was measured at 320 nm (Novaspec II, Pharmacia LKB, Cambridge, UK) and the results were reported with a precision of ±0.0005. The pH of the samples was measured by a pH meter (Jenway 3010, Jenway Co. Ltd., UK) in accordance with the method described by Johnston and Murphy [16].

Relative fluorescence intensity

In order to measure the extent of exposure of hydrophobic groups of proteins to HHP, the method described by Bonomi et al. [1], based on use of a fluorescent probe, was utilized. The main aim in this procedure is to reach a maximum fluorescence, which would show a saturated fluorescent marker binding. The fluorescent marker used was 1,8-naphthaleresulfonic acid (ANS, Merck, Germany). The samples were measured with a normal glass cell, at λ_{ex}=390 nm and λ_{ex}=480 nm. The emission and excitation slits were set to 10 and 5 nm bandwidth, respectively. Three different concentrations (1, 5 and 10 mM) of ANS solutions were prepared. First, the fluorescence of milk sample was measured by a spectrofluorometer (Shimadzu RF-5000, Japan). Then 10 µl of 1 mM ANS solution was added into sample and the fluorescence was measured again. Then 25 and 50 µl of the solution were added into the sample and the fluorescence was measured. This procedure was repeated also for 5 and 10 mM of ANS solutions. Final concentration of ANS in the milk sample was between 0 and 136 µM. All values were calculated as the average of four different kinetic approaches; Lineweaver-Burk, Hanes-Woolf, Woolf-Augustinsson-Hofstee and Eadie-Scatchard.

Table 1 Change in turbidity of milk by HHP[a]

Pressure (MPa)	Whole milk time (min)		Semi-skimmed milk time (min)		Skimmed milk time (min)		Raw whole milk time (min)	
	10	20	10	20	10	20	10	20
Control	1.63∗	1.63	1.30	1.30	0.75	0.75	1.18	1.28
110	1.60	1.59	1.27	1.23	0.75	0.71	1.10	1.21
220	1.58	1.58	1.16	1.17	0.52	0.51	0.97	1.10
330	1.55	1.50	1.06	1.07	0.31	0.44	0.73	0.77
440	1.57	1.56	1.11	1.08	0.33	0.31	0.84	0.87

[a]Mean values obtained from two experiments

∗Significant correlations at $p<0.01$ level with a standard deviation of ±0.0005

Table 2 Change in pH of milk by HHP[a]

Pressure (MPa)	Whole milk time (min)		Semi-skimmed milk time (min)		Skimmed milk time (min)		Raw whole milk time (min)	
	10	20	10	20	10	20	10	20
Control	6.38∗	6.38	6.55	6.55	6.33	6.33	6.48	6.48
110	6.40	6.42	6.59	6.66	6.38	6.39	6.50	6.54
220	6.41	6.44	6.72	6.71	6.39	6.41	6.61	6.60
330	6.46	6.47	6.71	6.71	6.30	6.42	6.60	6.61
440	6.45	6.46	6.70	6.70	6.27	6.37	6.58	6.60

[a]Mean values obtained from two experiments

∗Significant correlation at $p<0.01$ levels with a standard deviation of ±0.005

Table 3 Effect of HHP on strength of binding (F_{max}/K_d)[a]

Pressure (MPa)	Whole milk time (min)		Semi-skimmed milk time (min)		Skimmed milk time (min)		Raw whole milk time (min)	
	10	20	10	20	10	20	10	20
Control	4.24∗	4.24	6.62	6.62	10.00	10.00	10.62	10.62
110	3.67	3.81	6.27	6.66	9.48	9.65	11.53	11.56
220	4.14	4.86	7.10	7.34	10.21	11.03	12.08	13.32
330	5.09	4.52	7.98	8.06	10.99	9.98	13.78	13.20
440	5.68	5.05	8.20	8.79	14.09	10.75	13.33	16.20

[a]Mean values obtained from two experiments

∗Significant correlation at $p<0.01$ levels with a standard deviation of ±0.005

Statistical analyzes of the data

Individual experiments and measurements were performed in duplicate, so data represent the mean of two independent assays. Significant differences between means were tested using Duncan's multiple range test with a probability level fixed at $p<0.01$.

Results

Effect of HHP on turbidity and pH

The change in turbidity and pH of milk after HHP treatment for 10 and 20 min are given in Tables 1 and 2, respectively. pH measurements are reported with a precision of ±0.005.

Effect of HHP on surface hydrophobicity

Three parameters were defined as F_{max}, $1/K_d$ and F_{max}/K_d to interpret the effect of HHP on surface hydrophobicity of milk proteins. The results were interpreted as in enzyme kinetics with the following modification in notation; F_{max} instead of V_{max}, K_d instead of K_m and [ANS] instead of [S] were used. Here F denotes the amount of fluorescence created by the number of ANS bonded surface hydrophobic sites on the protein micelle. Thus, F_{max} represents both the maximum fluorescence that could be attained at the given conditions and also the maximum surface allowable for hydrophobic sites that ANS could be bound. Therefore any increase in numeric value of F_{max} means an equal increase in the number of hydrophobic sites on the protein micelles and vice versa. Two possible reasons of increase in F_{max} value could be either those micelles divided into sub-micelles and/or micelles that expand and inside hydrophobic sites come out of the surface.

$1/K_d$ gives the binding affinity of ANS to the milk protein. If $1/K_d$ increases it means the binding affinity of ANS also increases. F_{max}/K_d denotes the strength of binding. Increase in F_{max}/K_d denotes an increase in the strength of binding. From ANS titration curves, F_{max}, $1/K_d$ and F_{max}/K_d values were calculated as described above and F_{max}/K_d values are presented in Table 3. Regarding the data presented, $1/K_d$ seems to be unaffected by pressure, fat content and application time (data not shown). However an increase can be seen in F_{max}/K_d values as the slight decrease is recovered especially at pressures 220 MPa and up (Table 3). Fat amount also affects F_{max}/K_d inversely, with increasing values as fat amount decrease.

The lowest and highest F_{max} values were recorded for UHT whole milk and UHT skimmed milk, respectively (Figs. 1 and 2). As in the case of pH and turbidity, raw whole milk has values close to UHT semi-skimmed milk.

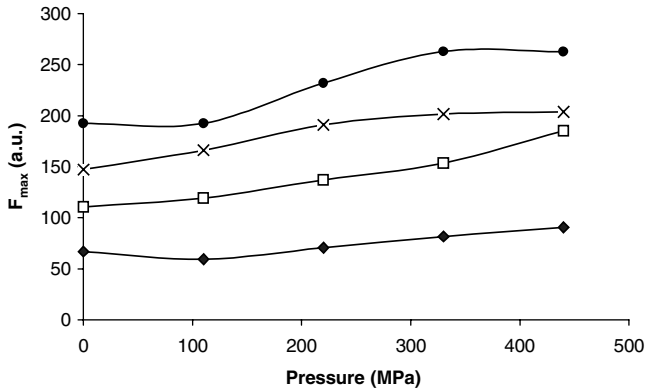

Fig. 1 Change in F_{max} by pressure at 25 °C for 10 min, whole milk (♦), (□) semi-skimmed milk, (●) skimmed milk and (×) raw whole milk

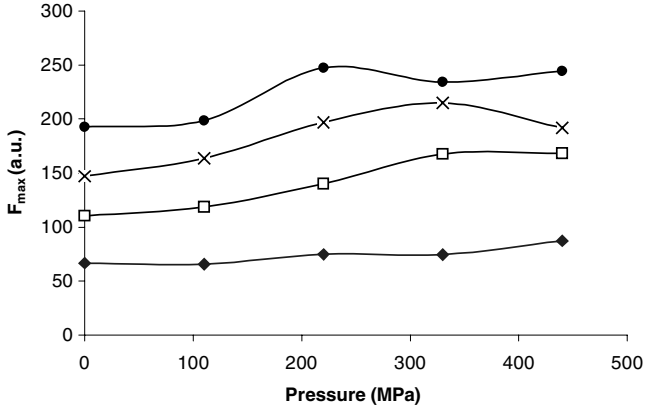

Fig. 2 Change in F_{max} by pressure at 25 °C for 20 min, whole milk (♦), (□) semi-skimmed milk, (●) skimmed milk and (×) raw whole milk

It was seen that pressure has a significant effect on the change of F_{max} within the conditions of the study ($p<0.01$) but pressurization time has found to have an insignificant effect on the change of F_{max} value up to 440 MPa; where F_{max} slightly decreased with increasing pressurization time.

Discussion

Effect of HHP on turbidity of milk

Turbidity, in simple terms is the amount of cloudiness that is found in a sample of liquid. Technically it is the measure of how much light passes through the sample, which is caused by suspended particles that scatter light. Since fat is one of the suspended particles found in milk, increasing fat amount should also increase the turbidity, which is the case observed for unpressurized (control) milk samples. However, the results presented in Table 1 indicate that turbidity decreased with increasing pressure within the conditions of the study. The relation between the turbidity of HHP-treated milk and the change in micelle size was previously reported [7, 11]. Therefore it is highly possible that, casein micelle size and soluble protein fraction in milk changes during HHP treatment [15]. Since micelle size was reduced as the applied pressure is increased, turbidity was also decreased. It is also reported that, at pressures around 230 MPa casein micelle size experiences an irreversible change and reduction in size causing a decrease in turbidity, whiteness and an increase in viscosity [4]. Our results revealed a decrease in turbidity up to 330 MPa. Since turbidity changes with changes in casein micelle size, decreasing turbidity can be an evidence of a decrease in the size of micelles, which is also reported in literature [7].

In addition to milk fat content and applied pressure the presented results also suggest that turbidity is affected by the pressurization time especially at lower range of pressures studied (up to 220 MPa); in this range as application time increases from 10 to 20 min at constant pressure, turbidity decreases.

Effect of HHP on pH of milk

In general there is a slight change in pH of milk within the pressure range studied but this change is not significant ($p<0.01$) (Table 2). Hinrichs et al. [4] showed that between 100 and 1,000 MPa, pH decreases only about 1 unit and Johnston et al. [7] reported that there is no significant change in pH between 200 and 600 MPa with a little increase after 500 MPa. The difficulty of measuring pH changes during HHP treatment prevents one to give more clues about the surface changes of micelles, but the slight differences in pH values of milk samples having different fat contents just after pressurization proves the effect of fat content per se on pH as the applied pressure is increased. It has also been seen that increasing the pressurization time from 10 to 20 min cause a slight increase in the pH of most of the milk samples.

Effect of HHP on surface hydrophobicity of milk proteins

The results have revealed that, as HHP increases from 110 to 220 to 330 and finally to 440 MPa, F_{max} also increases. Milk fat was also found to be effective on hydrophobicity changes with decreasing F_{max} values at all the pressure–time combinations. The affinity of ANS for binding to milk proteins seem to be unaffected by HHP treatment within the conditions studied, but binding strength of ANS showed gradual increase with increasing pressure. This fierce binding of ANS to hydrophobic surfaces on milk protein indicates that these hydrophobic areas are ready for other possible reactions. Nakai and Li-Chan [8] also reported that increased exposure of hydrophobic groups may alter functional properties and as a result foaming, emulsifying, gelling, and water binding capacities may be influenced. Rademacher et al. [5] showed that the pressure-induced

increase in viscosity of solutions containing whey proteins, which is due to the increased water binding capacity of the whey proteins, could be an advantage in yogurt manufacture.

Only a slight irreversible denaturation of milk proteins is expected in this study, as these are primary result of changes in secondary structure which is expected to occur at pressures 700 MPa and above leading to irreversible denaturation [17]. As the selected pressure range is well below this range only change in tertiary structure of protein is expected.

Delayed milk curding above 60 MPa with a rearrangement and compression of casein particles at around 700 MPa resulting with smaller particles with greater hydrophobic character and favoring rennet coagulation was reported in literature [7, 11].

In the light of these results, it can be concluded that HHP has an effect on non-covalent interactions and especially hydrophobic bonds in milk. As the pressure is increased, the micelles possibly decompose into sub-micelles and the embedded hydrophobic areas inside these micelles re-position in such a way that they can readily interfere with the fluorescent marker, ANS. Thus, HHP-treated milk could be used to produce minimally processed dairy products at industrial scale with high nutritional and sensory quality and novel texture.

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