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Effect of ultrasound treatment on particle size and molecular weight of whey proteins

Anet Režek Jambrak [a,*], Timothy J. Mason [b], Vesna Lelas [a], Larysa Paniwnyk [b], Zoran Herceg [a]

[a] Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb Pierottijeva 6, Croatia
[b] Sonochemistry Centre, Faculty of Health and Life Sciences, Coventry University, Priory Street, Coventry CV1 5FB, UK

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A B S T R A C T

The aim of this study was to observe the effect of ultrasound on particle size and molecular weight of whey proteins. In this work high-intensity ultrasound (20 kHz probe and 40 kHz bath) were used. 10 wt.% protein model suspensions of whey protein isolate (WPI) and whey protein concentrate (WPC-60) were treated with ultrasound probe (20 kHz for 15 and 30 min) and ultrasound bath (40 kHz for 15 and 30 min). The results of particle size distribution have shown that, after treatment with an ultrasonic probe of 20 kHz, ultrasound caused a decrease in particle size, narrowed their distribution, and significantly increased the specific free surface in all samples. After treatment with ultrasonic bath of 40 kHz, there was a significant reduction in the size of particles. After treatment with probe of 20 kHz there was a significant decrease in molecular weight and protein fractionation. Ultrasonic bath treatment with 40 kHz ultrasound also showed significant changes in the composition of the molecular weight of protein fractions. Prolonged treatment of WPI with ultrasonic bath of 40 kHz encourages the formation of aggregates of molecules.

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1. Introduction

Ultrasound frequency can mainly be classified into two fields: high frequency low energy diagnostic ultrasound in the MHz range, and low frequency high-energy power ultrasound in kHz range. The high frequency ultrasound is usually used as an analytical technique for quality assurance, process control and non-destructive inspection, which has been applied to determine food properties, to measure flow rate, to inspect food packages, etc. (Floros and Liang, 1994; McClements, 1995; Mason et al., 1996; Mason, 1990).

The beneficial use of sound is realised through its chemical, mechanical, or physical effects on the process or product (Suslick, 1988). When particles of material in a liquid suspension are subjected to sonication a number of physical and mechanical effects can result (Mason, 1998). The cavitational effects, which are the basis of sonochemistry, are also the reason for the extremely effective uses of ultrasound (Mason et al., 1996). Application of the low frequency high-energy power ultrasound in the food industry has been explored for last 10 years. Various areas have been shown to be great potential for future development, e.g. microorganisms and enzymes inactivation, crystallization, drying, degassing, extraction, filtration, homogenisation, meat tenderization, oxidation, sterilization, etc. (Gennaro et al., 1999; Mason, 1998; Mason, 1990; McClements, 1995).

Ultrasound is used in food processing for a number of applications that are related to food preservation, modification of molecules, degassing and foam control, mixing, emulsification, meat tenderization, etc. Ultrasound has been used for many years in the study of proteins (Owen and Simons, 1957; Conway and Verral, 1966; Pavlovskaya et al., 1992; Suzuki et al., 1996). These studies have been used to estimate protein hydration and to infer changes in protein conformation. Chandrapala et al. (2011) studied the sonication-induced changes in the structural and thermal properties of proteins in reconstituted whey protein concentrate (WPC) solutions. The enthalpy of denaturation decreased when WPC solutions were sonicated for up to 5 min. Prolonged sonication increased the enthalpy of denaturation due to protein aggregation. Overall, the sonication process had little effect on the structure of proteins in WPC solutions which are critical to preserving functional properties. These parameters may be related to functional properties of proteins in foods such as solubility, foaming capacity and flexibility (Gekko and Yamagami, 1991). Guzey (2001) reported that high-intensity ultrasonic processing improves emulsifying properties of whey protein isolate.

Arzeni et al. (2012) studied and comparatively explored the impact of high intensity ultrasound (HIUS) on the functionality of some of the most used food proteins at the industrial level: whey protein concentrate, soy protein isolate and egg white protein.

* Corresponding author. Tel.: +385 14605035.
E-mail address: arezek@pbf.hr (A.R. Jambrak).

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The size of aggregates suffered an overall reduction for WPC and SPI. HIUS affected the studied functional properties differently depending on the size and nature of the protein. Jambrak et al. (2008) studied the effect of low-intensity ultrasound (500 kHz) and high-intensity ultrasound (20 kHz probe and 40 kHz bath), on solubility, emulsifying and foaming properties of whey protein isolate (WPI), whey protein concentrate (WPC) and whey protein hydrolysate (WPH). They observed that solubility increased significantly for all samples for 20 kHz probe, 40 kHz and 500 kHz baths except for WPC. Sağlam et al. (2013) studied heat stability and rheological properties of concentrated whey protein particle dispersions in different dispersing media. Whey protein particles were formed using a combination of two-step emulsification and heat induced gelation. Heat treatment did not significantly alter the zeta potential of the particles, whereas the size of the particles increased after heating due to swelling. The results show that swelling of the particles plays a significant role in the heat stability and rheological properties of these dispersions. Martini et al. (2010) studied power ultrasound (US) to decrease the turbidity of whey suspensions. This research shows an approximately 90% decrease in turbidity when US was applied. The greatest decrease in turbidity was observed when US was applied for 15 min using 15 W of electrical power at 60 °C.

Whey are widely used as ingredients in foods due to their unique functional properties, i.e. emulsification, gelation, thickening, foaming, and fat and flavour binding capacity (Bryant and McClements, 1998; McClements, 1995; Morr and Ha, 1993). They are used because of their high nutritive value and GRAS status (Bryant and McClements, 1998).

The aim of this study was to observe the effect of ultrasound on particle size and molecular weight of whey proteins as a function of ultrasound intensity and frequency of ultrasound, and also to compare sonication and its effects at a frequency of 40 kHz and 20 kHz.

2. Materials and methods

2.1. Materials

Protein powders were purchased as declared by manufacturer (Table 1):

Whey protein isolates (WPI, BiPRO®, Davisco Foods International, USA); Whey protein concentrates (WPC, »Meggle« GmbH, Wasserburg, Germany, WPC-60).

2.2. Sample preparation

The model systems marked as WPI or WPC were aqueous suspensions of powdered whey protein isolate and whey protein concentrate containing 10.0% of dry matter. For this purpose appropriate amount of WPI or WPC powder sample were dispersed in distiled water in volume of 100 mL. Each was dissolved in distiled water (temperature was 23 °C) by gentle magnetic stirring for 30 min to provide a 10% powder (w/w) dispersions and allowed to stand overnight at 4 °C for complete hydratation until homogenous suspensions were obtained. By measuring solubility it is possible to predict the protein/solvent, protein/protein and solvent/protein interactions which determine the functionality of whey proteins (Webb et al., 2002). The high values for solubility in water of control samples under investigation (89.2 g/100 g for WPC and 96.6 g/100 g for WPI, respectively) revealed the high proportion of native whey proteins warranted the broad commercial usage of investigated samples. The protein content is known as declared by manufacturer (Table 1). Temperature of samples was measured before and after ultrasound treatments.

Table 1
Protein powder specification declared by manufacturer.

Composition (%)	WPI	WPC
Protein	95	60
Fat	1	6
Carbohydrate–lactose	1	25
Ash	1	6
Moisture	2	3

2.3. Ultrasound treatment

2.3.1. Ultrasound treatment with 20 kHz probe

Samples for ultrasound treatment with probe (20 kHz) were placed in 100 mL flat bottom conical flask. Samples were treated for 15 and 30 min with power ultrasound, high intensity and low frequency, 20 kHz probe (Sonics & Materials Inc., Danbury, CT., USA, Model: V1A, power 600 W) attached to the transducer so that high power intensity can be obtained (Jencons Scientific Ltd. – Ultrasonic processor). Probe has a vibrating titanium tip 1.2 cm and is immersed in the liquid and the liquid is irradiated with an ultrasonic wave directly from the horn tip. In this ultrasonic experiment the ultrasonic intensity was 43–48 W/cm^2, as measured by calorimetry by thermocouple Hanna Instruments, model: HI 9063.

2.3.2. Ultrasound treatment with 40 kHz bath

Samples were placed in 100 mL flat bottom conical flask for ultrasound treatment with bath (40 kHz). Samples were treated for 15 and 30 min, where Erlenmeyer flask was immersed into a 40 kHz bath (Sonomatic, Model SO375T, HF-Pk-power 300 W – overall dimensions: 370 × 175 × 250 mm; internal dimensions: 300 × 150 × 150 mm). An ultrasonic transducer is attached to the outer surface of the liquid container and the liquid is irradiated with an ultrasonic wave from the surface of the liquid container. A standing wave of an ultrasonic wave is formed inside the liquid. The typical acoustic amplitude in a standing-wave type sonochemical reactor is much smaller than that in a horn-type sonochemical reactor (Tuziuti et al., 2002). In this ultrasonic experiment the ultrasonic intensity was 1 W/cm^2, as measured by calorimetry by thermocouple Hanna Instruments, model: HI 9063.

2.3.3. Ultrasound power measurements

Ultrasonic power, which is considered as mechanical energy, would partly lose in the form of heat when ultrasound passes through the medium (Thompson and Doraiswamy, 1999). Since the ultrasonic irradiation of a liquid produces heat, recording the temperature as a function of time leads to the acoustic power estimation (in W) by the equation (Margulis and Maltsev, 1969; Margulis and Margulis, 2003).

$$P = m \cdot Cp \cdot (dT/dt)_{t=0} \quad (1)$$

where m is the mass of the sonicated liquid (g), Cp its specific heat at a constant pressure (J/gK), and dT/dt is the slope at the origin of the curve.

It is expressed in watts per unit area of the emitting surface (W/cm^2), or in watts per unit volume of the sonicated solution (W/cm^3).

Treatments were labelled:

No ultrasound (A); 20 kHz probe – 15 min (B1); 20 kHz probe – 30 min (B2); 40 kHz bath – 15 min (C1); 40 kHz bath – 30 min (C2).

2.4. Temperature changes

Before and after each treatment, temperature of samples has been measured with thermometer and then calculated average

increase in temperature after treatment. During ultrasound treatment temperature has been controlled by thermocouple Hanna Instruments, model: HI 9063.

2.5. Particle size distribution

The particle size distribution was determined directly after ultrasound treatment when the particles were still dispersed in the collection fluid. Particle size was determined by laser light scattering (Malvern Mastersizer 2000 equipped with a 100 mm lens, Malvern Instruments Limited, Malvern – Worcestershire, UK with Hydro MU sample dispersion unit). Results were analysed with, Mastersizer 2000 software, using a Mie scattering model for the analysis of the raw data.

2.6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

The protein fractions were analysed by SDS–PAGE (sodium dodecyl sulphate–polyacrylamide gel electrophoresis) with a Mini-PROTEAN 3 Electrophoresis System (Bio-Rad Laboratories, Inc., USA), with modified procedure described by Laemmli (1970). Protein model solutions (1 μL/mL protein) of 10 mL volume were dialyzed against several changes of phosphate buffer 10 M (pH = 7) and final dialysis against water. After dialyze the volume of solutions was <10 mL so electrophoresis was conducted, otherwise if the volume would have been >10 mL samples should have been freeze dried and again re-dissolved. So prepared samples were mixed with reducing sample buffer (12.5 mL 1.5 M Tris pH = 8, 10 mL glycerol, 0.5 g SDS, 0.25 mL 2-mercaptoethanol, 0.5% bromophenol blue solution) in the ratio 1:1 in an eppendorf tube and placed in a boiling water bath for 3 min to be denatured (lids off) and then microfuged at high speed (5000*g*) for 2 min (Model 5414, Brinkmann Instruments Inc., Westbury, NY). 20 μL of sample (10 μL + 10 μL sample buffer) was prepared for each sample. Samples (20 μL) were then loaded onto a Tris-acrylamide gel (Bio-rad, Hercules CA; 12% Tris–HCl, 10 wells). Standards were ProteMix Protein Standard, ANAMED (Anamed Elektrophorese GmbH, Darmstadt, Germany) and Prestained Protein Marker, Broad Range (BioLabs Inc., New England, UK).

Gels were run at 150 V, $I > 20$ mA for 45 min in buffer (1.5 M, Tris–HCl, pH = 8; Tris–Glycine SDS Buffer (0.025 M Tris; 0.192 M Glycine; 0.1% SDS)), stained with Coomassie Blue R250 (0.15 g Coomassie brilliant blue, 60 mL methanol, 15 mL glacial acetic acid, 75 mL distiled water) and distained (10% methanol // 10% acetic acid). Gel pictures were taken by ChemiDoc XRS System and PDQuest Basic 2-D Analysis Software (Bio-Rad Laboratories, Inc., USA).

Molecular protein bands were analysed according to the intensity of pixels and their distances. On the basis of these values in relation to the standards, molecular weight of unknown protein was calculated, as well as certain factions. Protein denaturation with SDS was performed to bind approximately constant mass ratio, and the resulting SDS-protein complexes have a uniform charge density and migrate on the basis of the molecular mass in the electric field. It was found that the relationship is then graphically $\log_{10}$Mr and R_f values (the distance that exceeds the distance that the protein crosses the colour) is strictly linear. To calibrate the gel system, proteins of known molecular weights (standards) that can be separated and then processed along with the samples were used. Molecular weight of target bands were calculated according to standards via software ImageJ, and using a set of macro-commands, which than calculate values from the set of known values.

2.7. Statistical analyses

The whole study was repeated and each value represents the mean of three measurements from three independent ultrasound treatments. The effect of ultrasound treatment on tested parameters was determined by analysis of variance, using statistical analyses with SPSS for Windows version 13.0 (SPSS Inc., Chicago, IL). Analysis of variance (One-Way ANOVA), significant level used was 5% ($\alpha = 0.05$), was carried out to assess whether the different treatments conducted to statistically different results for those variables evaluated. The values statistically different are accompanied with another letter (b), and those not statistically different by the same letter (a).

3. Results and discussion

3.1. Temperature changes

The high local temperatures and pressures that result from cavitation lead to formation of free radicals and other compounds, so ultrasound can induce oxidant species. Measurements of samples temperature are shown in Tables 2 and 3. One can observe that highest temperature of sample is obtained after ultrasound treatment with 20 kHz probe where temperature increased up to 43–45 °C that is significantly lower than denaturation temperature of proteins (Giroux and Britten, 2004). For 40 kHz bath temperature increased up to 25–34 °C. This is logical because highest input of energy is with 20 kHz probe, and excess energy is liberated as increase in temperature. From Tables 2 and 3, one can observe that highest average increase in samples temperature is after ultrasound treatment with 20 kHz probe for 30 min and it is 21.3 °C, than with 40 kHz bath for 30 min (4.0–8.7 °C).

Table 2
Particle size distribution, specific surface area of untreated and ultrasound treated whey protein isolate (WPI) suspensions with 20 or 40 kHz frequency set and temperature changes.

Treatment	Specific surface area ($m^2\,g^{-1}$)	Particle size (μm)			Temperature (°C)
		10% Less than[A]	50% Less than[B]	90% Less than[C]	
A	$0.037^a \pm 0.001$	$126.75^a \pm 3.27$	$192.74^a \pm 4.01$	$508.20^a \pm 6.32$	23 ± 0.02^a
B1	$0.126^b \pm 0.015$	$22.03^b \pm 0.91$	$148.48^b \pm 6.68$	$264.18^b \pm 2.56$	42 ± 0.01^b
B2	$0.195^b \pm 0.013$	$90.29^b \pm 1.11$	$165.01^b \pm 4.53$	$285.56^b \pm 6.54$	43 ± 0.01^b
C1	$0.041^a \pm 0.003$	$116.60^b \pm 4.77$	$226.58^b \pm 2.65$	$334.59^b \pm 4.71$	28 ± 0.01^a
C2	$0.027^b \pm 0.002$	$1030.31^b \pm 13.75$	$148.14^b \pm 5.54$	$313.22^b \pm 4.89$	34 ± 0.03^a

Each value represents the mean of three measurements from three independent ultrasound treatments. The values statistically different are accompanied with another letter (b), and those not statistically different by the same letter (a) as compared to control.
[A] Using Mie's theory it is determined that 10% particles have diameter less that stated.
[B] Using Mie's theory it is determined that 50% particles have diameter less that stated.
[C] Using Mie's theory it is determined that 90% particles have diameter less that stated.

Table 3
Particle size distribution, specific surface area of untreated and ultrasound treated whey protein concentrate (WPC) suspensions with 20 or 40 kHz frequency set and temperature changes.

Treatment	Specific surface area ($m^2 g^{-1}$)	Particle size (μm)			Temperature (°C)
		10% Less than [A]	50% Less than [B]	90% Less than [C]	
A	$2.24^a \pm 0.01$	$0.74^a \pm 0.01$	$103.68^a \pm 1.82$	$324.08^a \pm 2.13$	23 ± 0.01^a
B1	$13.5^b \pm 0.84$	$0.20^b \pm 0.02$	$0.44^b \pm 0.01$	$6.98^b \pm 0.02$	42 ± 0.02^b
B2	$20.1^b \pm 0.97$	$0.17^b \pm 0.01$	$0.31^b \pm 0.03$	$1.04^b \pm 0.01$	45 ± 0.03^b
C1	$14.7^b \pm 0.56$	$0.19^b \pm 0.01$	$0.55^b \pm 0.02$	$4.76^b \pm 0.02$	25 ± 0.03^a
C2	$15.3^b \pm 0.34$	$0.18^b \pm 0.01$	$0.48^b \pm 0.01$	$1.68^b \pm 0.01$	31 ± 0.02^b

Each value represents the mean of three measurements from three independent ultrasound treatments. The values statistically different are accompanied with another letter (b), and those not statistically different by the same letter (a) as compared to control.
[A] Using Mie's theory it is determined that 10% particles have diameter less that stated.
[B] Using Mie's theory it is determined that 50% particles have diameter less that stated.
[C] Using Mie's theory it is determined that 90% particles have diameter less that stated.

3.2. Particle size distribution

Physical and structural properties of proteins can sometimes change mechanically. High pressure and ultrasonic vibrations cause partial denaturation that significantly affects the physical and structural properties of proteins. Previous studies have shown that ultrasound, except the destruction of agglomerates can also significantly change the structural properties of whey proteins (Bryant and McClements, 1998; Jambrak et al., 2008). The results of particle size distribution (Tables 2 and 3) show that, after treatment with an ultrasonic probe of 20 kHz, ultrasound caused a decrease in particle size, narrowed their distribution, and significantly increased the specific free surface ($p < 0.05$) in all samples. After treatment with ultrasonic bath of 40 kHz, there was a significant ($p < 0.05$) reduction in the size of particles, but not to the extent as treatment probe. Treatment with 20 kHz probe is more effective (in terms of molecular weight reduction) for 15 min for samples of whey proteins. When whey protein concentrate were treated with ultrasound there have been primarily a reduction of fat globules as a result of the effects of ultrasound, as is observed in the study (Villamiel and de Jong, 2000). Ultrasound-induced structural changes in proteins are associated with partial cleavage of intermolecular hydrophobic interactions, rather than peptide or disulphide bonds. When the particles are subjected to shear stress, and increases the speed of aggregation, there is increase in collision. As the ultrasound treatment increases, the particle size is reduced and the particle size distribution narrows. Sonication has no effect on surface charge, but creates a new surface and reduces the size of the particles. By reducing the size of particles the free surface of the material increases. In this case, the particles are reduced because of the forces of cavitation. This includes the destruction of agglomerates and aggregates. Ultrasonic cavitation is very effective in breaking up agglomerates, aggregates, and even smaller particles, violating the van der Waals's forces.

After treatment with ultrasonic bath of 40 kHz, there was a significant ($p < 0.05$) reduction in the size of particles, but not to the extent as treatment with probe. When treatment with bath is used there is more prevalent effect of turbulence and shear stress, but cavitation. There is greater the surface area of a source of mechanical vibrations, but not very high intensity as in the action directly immersed probe. In this ultrasonic experiment the ultrasonic intensity did not exceed 1 W/cm^2. The most effective treatment is 30 min in samples of whey proteins. For WPI there has been a partial aggregation after treatment with an ultrasonic bath of 40 kHz for 30 min (Table 2). When whey protein concentrate were treated with ultrasound baths of 40 kHz for 15 and 30 min there was a significant ($p < 0.05$) reduction in the particle size (Table 3). Sufficiently high intensity ultrasound, is causing friction and turbulence, because it generates a negative pressure that is strong enough to lead to an implosion of bubbles and cavitation. Thus, it acts on the surface of material that is subjected to treatment. Due to the very small power levels, low intensity ultrasound does not cause physical and chemical changes in the properties of the material through which the wave passes. In this ultrasonic experiment the ultrasonic intensity did not exceed 1 W/cm^2.

3.3. Molecular weight of whey proteins

Molecular weight of whey proteins in all gels were obtained with ImageJ software, where curves were plotted pixel intensity of protein bands. The share of each component is derived from the pixel intensity of protein bands that is displayed as area below the surface peak.

From the untreated whey protein gels (Figs. 1 and 2) one can clearly see the protein bands corresponding to polypeptides. From standard Anamed, a curve is obtained which shows the share of individual standards, i.e. a certain molecular weight of proteins. Fig. 6 shows the curve obtained from pixel intensity of protein bands from the gel of untreated WPI. Share of molecular weight of 14,200 Da, is corresponding to a share of 23.4%, and this corresponds to the molecule of α-lactalbumin. Then, share of molecular weight of 18,300 Da, the proportion is 45.8%, and the corresponding β-lactoglobulin. The remainder, corresponding to molecular masses of 5590 Da, with a share of 5.6%, 66,400 Da, with a share of 6.4%, and this corresponds to molecule bovine serum albumin (BSA). Then, molecular mass of 92,642 Da, with a share of 8.1% and 166,425 Da, with a share of 10.5%. The latter refers mainly to the immunoglobulin of mole masses greater than 100 kDa. There is the visible presence of the trimers and tetramers of whey protein, molecular mass >60 kDa. Fig. 2 shows the gel of untreated whey protein concentrate. The corresponding protein bands are shown by the intensity of pixels as curve (Fig. 9). Share of a molecular weight of 14,200 Da, is corresponding to a share of 16.4%, and this corresponds to the molecule of α-lactalbumin. Then, share of molecular weight of 18,300 Da, the proportion is 24.2%, and the corresponding β-lactoglobulin. The remainder, is corresponding to molecular masses of 5647 Da, with a share of 11.3%, 20,980 Da, with a share of 19.8%, 42,138 Da, with a share of 14.3%, 66,400 Da, with a share of 5.4% (this corresponds to molecule serum albumin (BSA)) and 118,054 Da, with a share of 5.8%. The latter refers mainly to the immunoglobulin of mole masses greater than 100 kDa.

Figs. 3–5 show whey protein gels after treatment by ultrasound of 20 kHz and 40 kHz. Also, the protein bands of standards Anamed and BioLabs. After treatment with probe of 20 kHz there was a significant ($p < 0.05$) decrease in molecular weight and protein fractionation (Figs. 7 and 10). Longer treatment shows no splitting and tendency to larger proteins. Ultrasonic bath treatment with 40 kHz ultrasound also showed a significant changes ($p < 0.05$) in

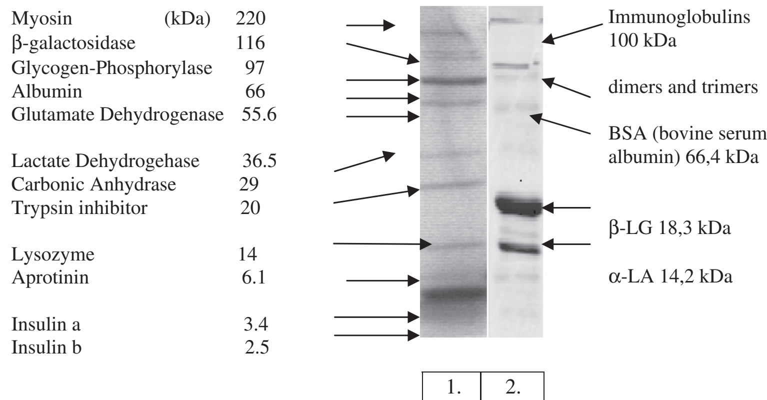

Fig. 1. SDS–PAGE gel with protein bands of untreated, no ultrasound A (line 2-right) whey protein isolate (WPI) protein sample, and bands of molecular marker (ANAMED-line 1-left).

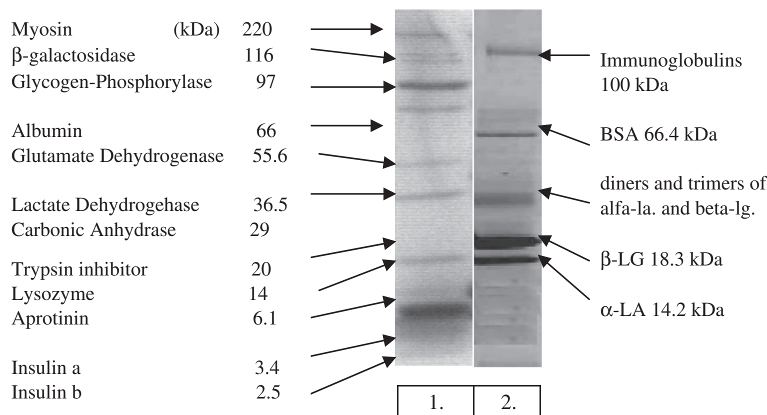

Fig. 2. SDS–PAGE gel with protein bands of untreated, no ultrasound A (line 2-right) whey protein concentrate (WPC) protein sample, and bands of molecular marker (ANAMED-line 1-left).

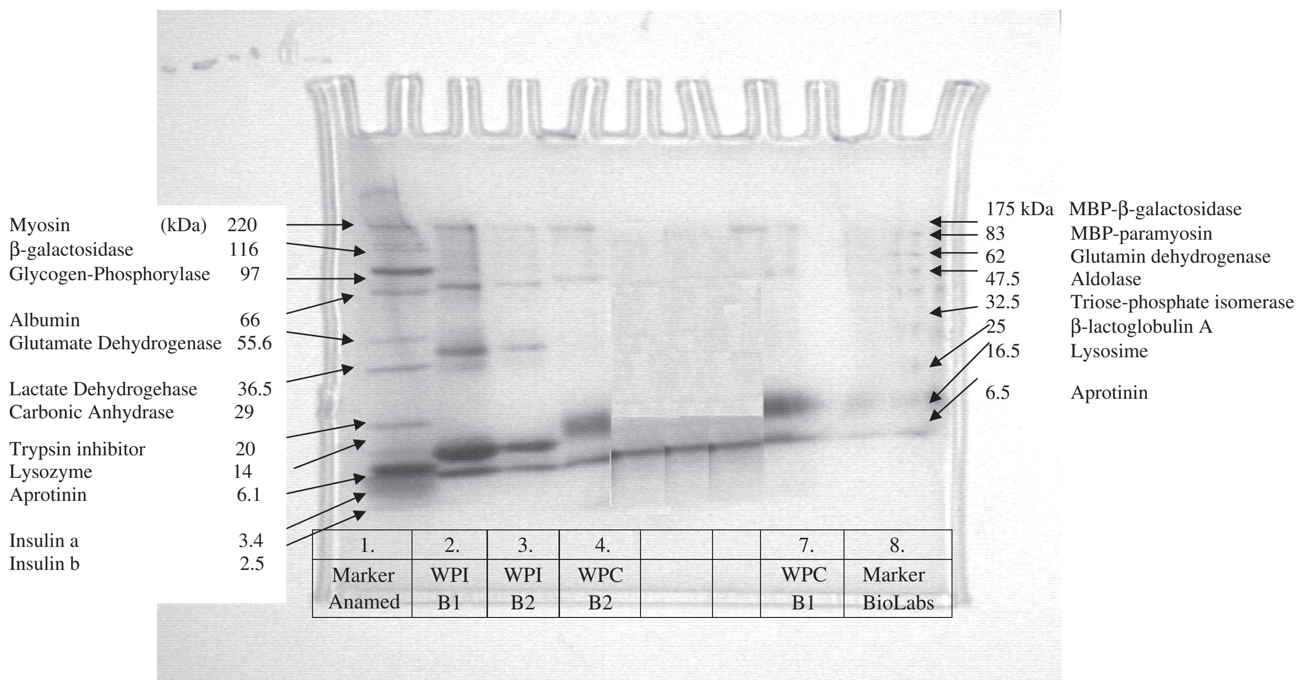

Fig. 3. SDS–PAGE gel with protein bands of ultrasound treated whey protein isolate (WPI) and whey protein concentrates (WPC) and bands of molecular marker ANAMED (line 1) and BioLabs (line 8); 20 kHz probe – 15 min (WPI B1-line 2); 20 kHz probe – 30 min (WPI B2-line3); 20 kHz probe– 15 min (WPC B2-line 4); 20 kHz probe– 30 min (WPC B1-line 7).

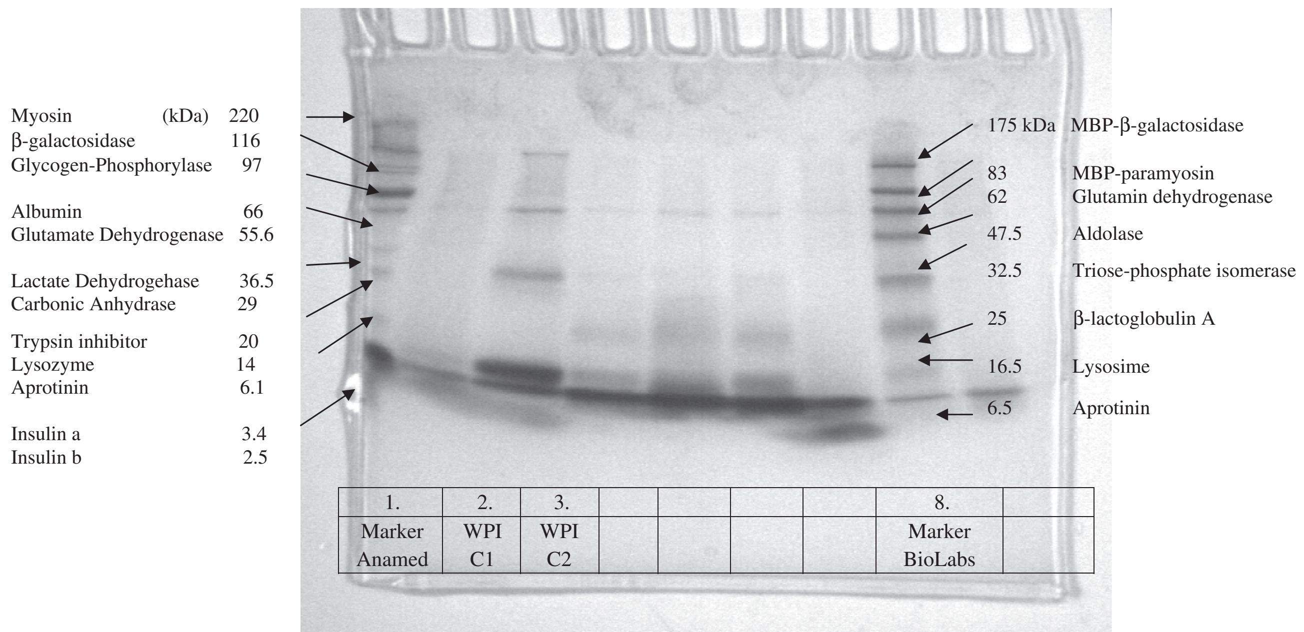

Fig. 4. SDS–PAGE gel with protein bands of ultrasound treated whey protein isolate (WPI) and bands of molecular marker ANAMED (line 1) and BioLabs (line 8); 40 kHz bath – 15 min (WPI C1-line 2); 40 kHz bath – 30 min (WPI C2-line 3).

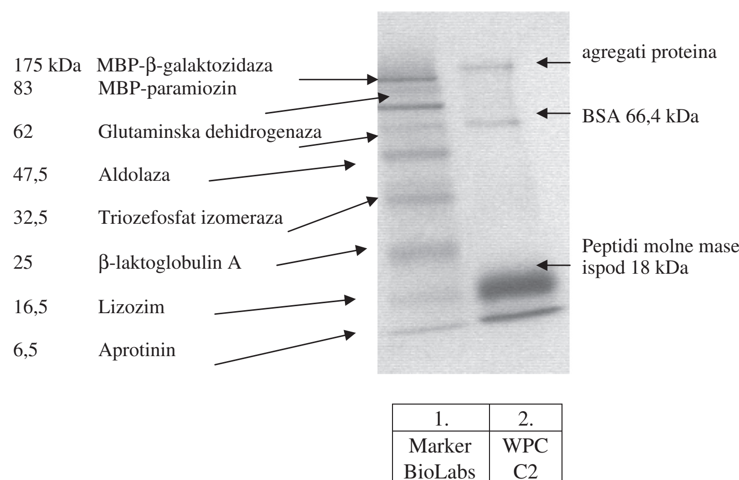

Fig. 5. SDS–PAGE gel with protein bands of ultrasound treated whey protein concentrate (WPC) and bands of molecular marker BioLabs (line 1); 40 kHz bath – 30 min (WPC C2-line 2).

the composition of the molecular weight of protein fractions (Figs. 8 and 10), and this is to a greater extent in all samples than in the treatment with probe. More effective is the treatment for 15 min (in terms of molecular weight reduction). Longer treatment shows no splitting and larger protein chains, except for samples of whey protein concentrate, where the treatment with bath was even more effective than the treatment with probe of 20 kHz. Prolonged treatment (for 30 min, intensity was 1 W/cm^2) of WPI with ultrasonic bath of 40 kHz encourages the formation of aggregates of molecules.

Fig. 7 shows the curves from corresponding protein bands from gel of treated WPI samples with ultrasonic probe of 20 kHz after 15 (B1) and 30 (B2) min. In comparison to untreated whey protein isolate (Fig. 6), 20 kHz ultrasound treatments resulted in changes in the composition of the molecular weight of protein fractions. Share of molecular masses after treatment of 20 kHz ultrasound for 15 min (B1) is as follows: molecular mass of 5156 Da, corresponding to a share of 17%, the molecular mass of 6981 Da, the share was 41.5%; molecular mass of 29,351 Da, with a share of 20.4%; molecular mass of 72,243 Da, corresponding to a share of 9.4%, and 176,822 Da of molecular mass, with a share of 11.6%. It can be seen that there has been change and shift towards to an area of about 7000 Da. Protein chains of high molecular masses in the range of 14,000–70,000 Da were most exposed to ultrasound. This can be explained by the fact, that these chains are more accessible and easier to reach and to their cleavage. Zisu et al. (2011) sonicated the aqueous solutions of reconstituted whey protein concentrate (WPC) and isolate (WPI) powders at 20 kHz in a batch process for 1–60 min. Sonication at 20 kHz increased the clarity of WPC solutions largely due to the reduction in the size of the suspended insoluble aggregates. Shares in molecular masses after treatment with 20 kHz ultrasound for 30 min (B2) is as follows: molecular mass of 5590 Da, corresponding to a share of 26.7%; for the molecular weight of 7723 Da, the share was 45.1%;

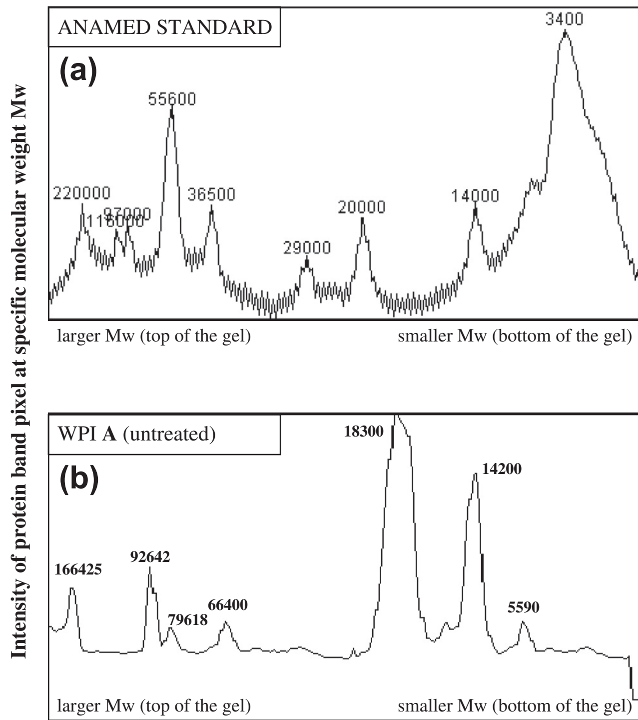

Fig. 6. Pixel intensity (shown as graph) of whey protein isolate (WPI) protein band of SDS–PAGE gel at specific molecular weight (Mw) of ANAMED standard (a) and untreated (A) protein (b).

molecular mass of 31,123 Da, with a share of 9.7%; molecular mass of 72,242 Da, corresponding to a share of 5.2%, and 173,286 Da molecular mass, with a share of 3.7%. It can be seen that there has shift towards an area of about 8000 Da. However, we can see that the longer ultrasound treatment caused cleavage of larger and larger chains, i.e. chains of larger molecular weight where their share was reduced to approx. 9%. Ultrasound is splitting molecules by moving from the centre of the molecule (Grönroos et al., 2004, 2008). In this ultrasonic experiment the ultrasonic intensity was 43–48 W/cm^2 that is higher power input that in case of ultrasound bath.

Fig. 8 shows the curves from corresponding protein bands from gel of treated WPI with ultrasonic bath of 40 kHz after 15 (C1) and 30 (C2) min. In comparison with untreated whey protein isolate bath treatment resulted in changes in the composition of the molecular weight of protein fractions. Shares in molecular weight after treatment with ultrasonic bath of 40 kHz for 15 min is as follows: molecular mass of 4661 Da, corresponding to a share of 87.6%, while the proportion of molecular mass of approx. 66,000 Da to about 11.3%. It can be seen that there has been much greater changes in intensity and greater strength even in relation to the treatment with probe. There has been a shift towards inching of small mass protein fractions of about 5000 Da. Ultrasonic bath of 40 kHz has a higher frequency but lower power. These treatments are more prevalent with the effect of turbulence and shear stress within the liquid medium, and there was a major collision of molecules in which there was a major split of proteins. Also, here is 40,000 cycles of wave propagation, while the probe has 20,000, which is the definition of frequency. Hence more speed and cycle, which causes shear and subjected collisions in the medium. Here, almost complete reduction in protein fractions of larger molecular weight was noticed, and there was almost uniform distribution. Shares in molecular weight after treatment with ultrasonic bath of 40 kHz for 30 min (C2) is as follows: molecular mass of 3886 Da, corresponding to a share of 25.2%; the molecular weight of 5156 Da, the proportion is 41.4%; molecular mass of

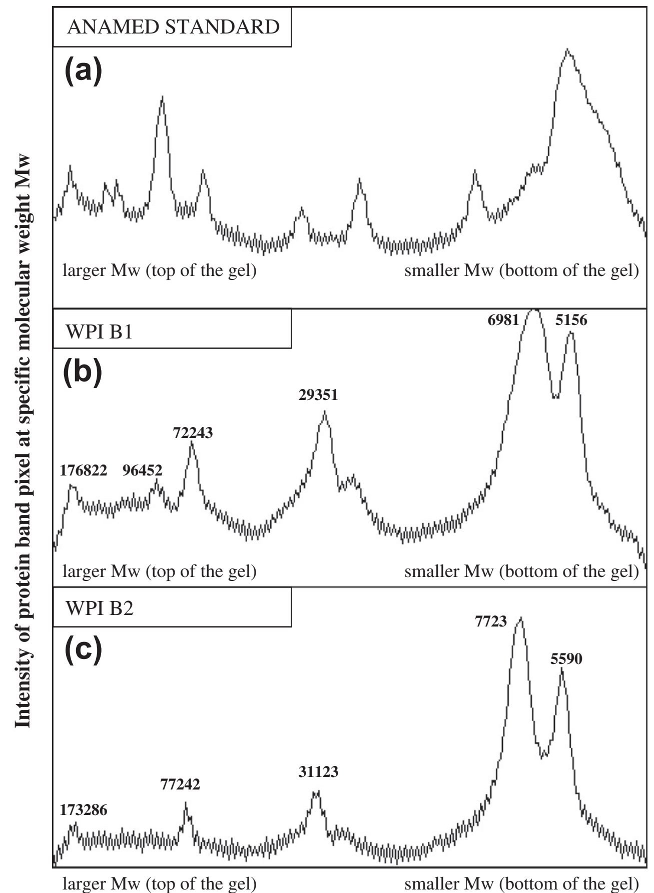

Fig. 7. Pixel intensity (shown as graph) of whey protein isolate (WPI) protein band of SDS–PAGE gel at specific molecular weight (Mw) of ANAMED standard (a) and ultrasound treated whey protein isolates (WPI) with 20 kHz probe for 15 min (B1) (b) and 20 kHz probe for 30 min (B2) (c).

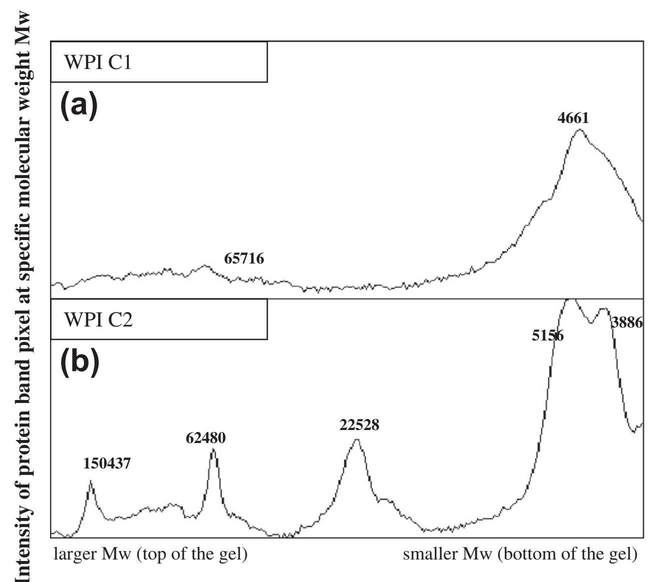

Fig. 8. Pixel intensity (shown as graph) of whey protein isolate (WPI) protein band of SDS–PAGE gel at specific molecular weight (Mw) of ultrasound treated whey protein isolates (WPI) with 40 kHz bath for 15 min (C1) (a) and 40 kHz bath for 30 min (C2) (b).

22,528 Da, with a share of 14.3%; molecular mass of 62,480 Da, corresponding to a share of 6.1%, and 150,437 Da molecular mass,

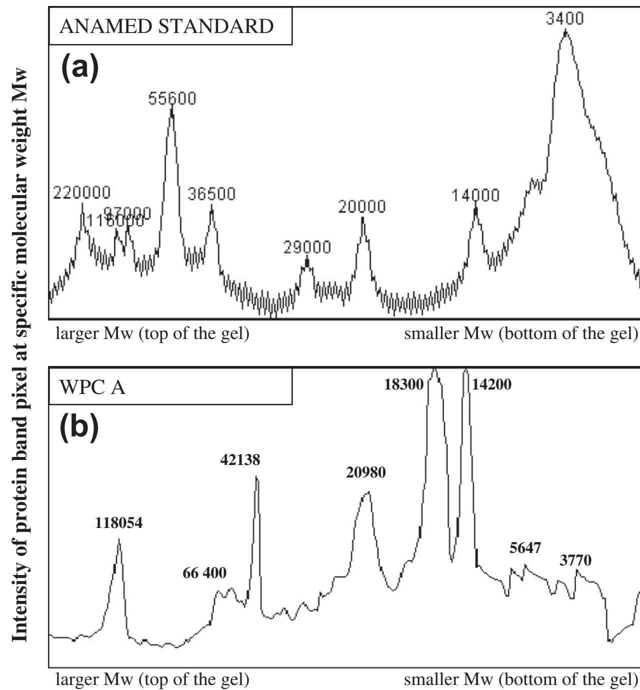

Fig. 9. Pixel intensity (shown as graph) of whey protein concentrate (WPC) protein band of SDS–PAGE gel at specific molecular weight (Mw) of ANAMED standard (a) and untreated (A) protein (b).

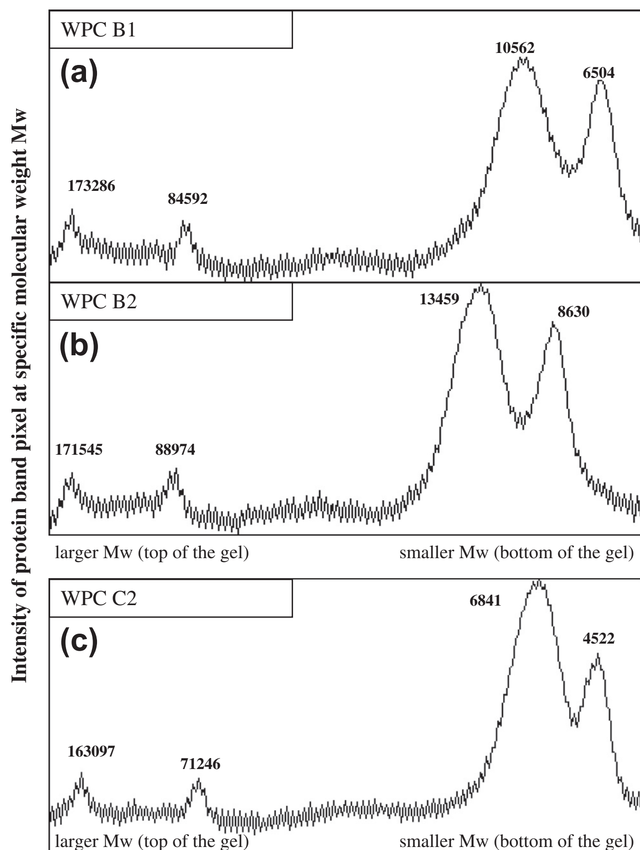

Fig. 10. Pixel intensity (shown as graph) of whey protein concentrate (WPC) protein band of SDS–PAGE gel at specific molecular weight (Mw) of ultrasound treated whey protein concentrates (WPC) with 20 kHz probe for 15 min (B1) (a); 20 kHz probe for 30 min (B2) (b) and 40 kHz bath for 30 min (C2) (c).

with a share of 2.4%. Here has been much cleavage of protein chains into smaller molecular masses. Prolonged ultrasonic treatment can have effects that stimulate the formation of aggregates of molecules (Mason, 1998). It is possible that there was an amino acid residue linking intermolecularinteractions, such as hydrogen bonding, free-SH groups, protein–protein interactions and that newly created group by the action of ultrasound came to the surface. It can be seen that there has been change and shift towards an area of about 5000 Da.

Molecular changes occurring during protein hydrolysis may result in modified techno functional behaviour of the hydrolysates compared to the intact protein such as altered solubility, viscosity, sensory properties and foam properties (Panyam and Kilara, 1996; Nielsen, 1997; Caessens et al., 1999). Ultrasound caused similar, proteins were hydrolysed to peptides which have been shown to have health promoting properties (lower blood pressure, gastrointestinal balance, etc.) (Zhu and Damodaran, 1994). Ultrasound treatment could be used in preparing of low molecular weight peptides and to formulate food products.

Fig. 10 shows the curves from corresponding protein bands from gel of treated whey protein concentrate with ultrasonic probe of 20 kHz after 15 (B1) and 30 (B2) min, and an ultrasonic bath of 40 kHz for 30 min (C2). In comparison with untreated whey protein concentrate (Fig. 9) this treatment has resulted in changes in the composition of the molecular weight of protein fractions. Shares in molecular masses after treatment with 20 kHz ultrasound for 15 min (B1) is as follows: molecular mass of 6504 Da, corresponding to a share of 34.5%; the molecular weight of 10,562 Da, the share was 62.1%; molecular mass of 84,592 Da, with a share of 2.9%, and 173,286 Da molecular mass, with a share of 3.3%. It can be seen that there has been change and shift towards an area of about 10,000 Da, which is not much a shift towards smaller molecular masses like for WPI. This is explained by the different composition of protein powders, where the presence of whey proteins, fats and disaccharides showing the protective effect of the protein molecule as well as in pressure treatments (Dumay et al., 1994). However, one can see better splitting of large molecular fractions of proteins weight of approx. 7%, which was not the case for WPI. Shares in molecular masses after treatment of 20 kHz ultrasound for 30 (B2) min is as follows: molecular mass of 8630 Da, corresponding to a share of 29.5%; the molecular weight of 13,459 Da, the proportion is 59.1%; molecular mass of 88,974 Da, with a share of 2.9%, and 171,545 Da molecular mass, with a share of 2.8%. It can be seen that there has been change and shift towards an area of about 13,000 Da. Here one can see that longer ultrasound treatment did not cause chain scission and then a similar treatment of 15 min. Fig. 10 shows the corresponding protein bands of treated whey protein concentrate with ultrasonic bath of 40 kHz after 30 (C2) min. In comparison with untreated whey protein concentrate, ultrasound treatment has resulted in changes in the composition of the molecular weight of protein fractions. Share in molecular weight after treatment with ultrasonic bath of 40 kHz for 30 min is as follows: molecular mass of 4522 Da, is corresponding in share of 20.3%; the molecular weight of 6841 Da was 69.7%, the molecular mass of 71,246 Da was 3.2%, while the proportion of molecular a mass of 163,097 Da, 4%. It can be seen that there has been change, even in relation to the treatment with probe of much greater intensity and greater strength. There has been a shift towards very small molecular masses of protein fractions from about 7000 Da, nearly 70%. Ultrasonic bath of 40 kHz has a higher frequency but lower power. With this treatment there is more prevalent effect of turbulence and shear stress within the liquid medium, and there was a major collision of molecules in which there was a major split. Also this is due of using lower ultrasound intensity which was less than 1 W/cm^2.- The mechanism, by which the degradation of macromolecules was

carried by ultrasound, has not yet been sufficiently clarified. However it is considered that the hydrodynamic forces have primary importance. Hydrodynamic forces arise as a result of increased friction force between the ultrasound and accelerated solvent molecules, smaller and larger more mobile, macromolecules (Grönroos et al., 2004, 2008). Hydrodynamic forces can arise due to the high pressure that is associated with the collapse of cavitation bubbles.

The largest proportion of protein fractions after various treatments was: whey protein isolates (WPI): 20 kHz/15 min – In 7000 Da, the share of 41.5%, 20 kHz/30 min – In 8000 Da, the share of 45.1%, 40 kHz/15 min – In 5000 Da, the share of 87.6%, 40 kHz for 30 min – In 5000 Da, the share of 41.4%. Whey protein concentrates (WPC): 20 kHz/15–10000 Da, the proportion of 62.1%, and 30 min of 13000 Da, the share of 59.1% and an ultrasonic bath of 40 kHz/30 min – In 7000 Da the share of 69.7%.

4. Conclusions

In this study the effect of ultrasound treatment on particle size distribution and molecular weight were studied. Ultrasound caused significant changes in particle size and molecular weight of whey proteins. The results of particle size distribution have shown that, after treatment with an ultrasonic probe of 20 kHz, ultrasound caused a decrease in particle size, narrowed their distribution, and significantly increased the specific free surface in all samples. After treatment with ultrasonic bath of 40 kHz, there was a significant reduction in the size of particles, but not to the extent as treatment probe. Ultrasound-induced structural changes in proteins are associated with partial cleavage of intermolecular hydrophobic interactions, rather than peptide or disulphide bonds. After treatment with probe of 20 kHz there was a significant decrease in molecular weight and protein fractionation. Ultrasonic bath treatment with 40 kHz ultrasound also showed significant changes in the composition of the molecular weight of protein fractions. Prolonged treatment of WPI with ultrasonic bath of 40 kHz encourages the formation of aggregates of molecules.

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