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Effect of High-Pressure Processing on Activity and Structure of Alkaline Phosphatase and Lactate Dehydrogenase in Buffer and Milk

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Changes in the activity and structure of alkaline phosphatase (ALP) and L-lactate dehydrogenase (LDH) were investigated after high pressure processing (HPP). HPP treatments (206–620 MPa for 6 and 12 min) were applied to ALP and LDH prepared in buffer, fat-free milk, and 2% fat milk. Enzyme activities were measured using enzymatic assays, and changes in structure were investigated using far-ultraviolet circular dichroism (CD) spectroscopy and dynamic light scattetering (DLS). Kinetic data indicated that the activity of ALP was not affected after 6 min of pressure treatments (206–620 MPa), regardless of the medium in which the enzyme was prepared. Increasing the processing time to 12 min did significantly reduce the activity of ALP at 620 MPa ($P < 0.001$). However, even the lowest HPP treatment of 206 MPa induced a reduction in LDH activity, and the course of reduction increased with HPP treatment until complete inactivation at 482, 515, and 620 MPa. CD data demonstrated a partial change in the secondary structure of ALP at 620 MPa, whereas the structure of LDH showed gradual denaturation after exposure at 206 MPa for 6 min, leading to a random coil structure at both 515 and 620 MPa. DLS results indicated aggregation of ALP only at HPP treatment of 206 MPa and not above and enzyme precipitation as well as aggregation at 345, 415, 482, and 515 MPa. The loss of LDH activity with increasing pressure and time treatment was due to the combined effects of denaturation and aggregation.

KEYWORDS: Alkaline phosphatase; L-lactate dehydrogenase; high-pressure processing; circular dichroism; dynamic light scattering; structure; inactivation; denaturation; aggregation

INTRODUCTION

Milk is an ideal medium for the proliferation of microorganisms that cause human disease or spoilage. Hence, the quality and safety of milk products depend on the proper inactivation of pathogenic and spoilage microorganisms present in milk. Pathogen inactivation in milk is achieved by pasteurization, which traditionally has involved thermal treatments. Although highly effective for microbial destruction, thermal treatments of milk have often been associated with reduction in aroma and loss of nutritional and sensory qualities of food products (*1–3*). Therefore, nonthermal technologies such as high-pressure processing (HPP) have received considerable attention recently because of the possibility of destroying microorganisms without significantly affecting the flavor or nutritional components of foods (*2–4*).

There is a growing interest in the use of high pressure for inactivation of indigenous milk enzymes because these enzymes serve as process indicators to assess the impact of HPP on milk (*3, 5*). Alkaline phosphatase (ALP, E.C.3.1.3.1) is a dimer with a molecular weight of about 140 kDA (*6*). The effect of HPP treatments on ALP have been extensively studied, because its inactivation temperature is slightly above that of most microorganisms likely to be found in milk (*3, 7, 8*). Inactivation of this enzyme might also indicate the absence of pathogens in pressure-treated milk (*9*). The effects of HPP on enzymes have been attributed to the fact that HPP affects hydrogen bonds and ruptures the three-dimensional configuration of proteins (*9, 10*). To provide a clear understanding of the effect of HPP on enzyme inactivation, it is important to explore a possible relationship between enzyme activity and the structure of the enzyme after HPP.

The effects of HPP on the stability of alkaline phosphatase have often been investigated using the combined effect of pressure and temperature (*4, 9*). Studies reporting exclusively the effect of HPP on ALP are very limited. Musa and

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Ramaswany (*3*) investigated the effects of HPP on the activity of ALP. They found that the enzyme was inactivated at 200, 250, 493, and 400 MPa when pressure treatments lasted 1039, 751, 250, 493, and 301 min, respectively. In their study, the time required to inactivate ALP was considered too long and led to over-processing of the milk (*4*). Other milk enzymes including γ-glutamyltransferase (GGT) and phosphohexoseisomerase have been inactivated at 600 MPa at room temperature (*9*). Likewise, lactoperoxidase was found to be pressure-resistant, because activity was only slightly affected after pressure treatments up to 600 MPa. The pressure resistance of this enzyme was greater than that of ALP and was attributed to the monomeric structure of lactoperoxidase (*11*).

L-Lactate dehydrogenase (LDH, E.C.1.1.1. 27) is a tetramer with a molecular weight of approximately 140 kDa (*12*). It is one of the most abundant enzymes in milk (*1*). LDH was designated along with ALP and γ-glutamyltransferase as a potential marker for heat inactivation of pathogens in buffalo milk (*13*). Thus, it was considered as a possible indicator of adequate pasteurization by HPP (*15*). Inactivation of LDH by HPP has been widely studied (*15–17*). However, these studies were conducted at pressures not exceeding 200 MPa. The thermal resistance exhibited by these enzymes implies that they might also exhibit resistance to HPP. It is therefore necessary to study them in a wide range of pressures to gain insights into their activity and structure following HPP. The use of structural information to investigate protein function is becoming commonplace (*18*). Relating information on an enzyme's folding, dynamics, and structure to enzyme kinetic data following HPP treatments will help to define more precisely the appropriate conditions for HPP pasteurization of milk, milk products, and food in general.

Therefore, the objectives of this research were to study the residual enzyme activity of ALP and LDH following a wide range of HPP treatments and investigate subsequent changes in secondary structure using circular dichroism spectroscopy (CD) and quaternary structure using dynamic light scattering (DLS). Possible relationships between enzyme activity and changes in enzyme structure are discussed.

MATERIALS AND METHODS

Preparation of Enzyme Solutions in Buffers and Milk. Alkaline phosphatase type VI-1 from bovine intestinal mucosa and L-lactate dehydrogenase from rabbit muscle were obtained from Sigma Aldrich, St. Louis, MO, and used to prepare 1.28 μg/mL stock solutions in 1.0 M diethanolamine buffer at pH 9.8 and 2.6 mg/mL solutions in 100 mM sodium phosphate buffer at pH 7.4, respectively. Buffers were purchased from Sigma Aldrich. St, Louis, MO. Enzymes were prepared in buffer and milk with 0 and 2% fat. Pasteurized bovine milk containing 2% fat and pasteurized fat-free milk were obtained from The Pennsylvania State University Berkey Creamery. Milks with 0 and 2% fat were tested to determine if the level of fat affected the rate of enzyme inactivation by HPP. About 438 and 1300 μg of ALP and LDH, respectively, were transferred from the stock solutions into plastic stomacher bags (VWR, West Chester, PA) containing 0.5 mL of buffer, 0% fat milk, or 2% fat milk. The bags were heat sealed using a MP-12 Impulse heat sealer (Midwest Pacific, St. Louis, MO) and placed into 4× plastic stomacher bags (VWR, West Chester, PA) containing water.

High-Pressure Treatments. Enzymes in buffer, fat-free milk, and 2% fat milk were divided into two sets. One set was pressure-treated for 6 min and the other for 12 min, at 206, 345, 415, 482, 515, and 620 MPa, at room temperature (~20–24 °C) using a 2 L pilot-scale high-pressure processing (HPP) unit (Avure Technologies, Kent, WA) and water as the pressure-transmitting fluid. After pressure was released, the bags were immediately frozen in liquid nitrogen to stop inactivation and avoid reactivation of the enzyme and were then stored at −20 °C.

Enzymatic Assays. ALP assays were conducted as follows: a working substrate solution containing 150 mM *p*-nitrophenyl phosphate (*p*-NPP) and 0.5 mM magnesium chloride from Sigma Aldrich (St. Louis, MO) was prepared in 100 mL of 1.0 M diethanolanine buffer (pH 9.8). The pressure-treated enzyme in buffer, fat-free milk, or 2% fat milk was dissolved in 2 mL of 1.0 M diethanolanine buffer (pH 9.8) and 0.15 mL of enzyme solution was added to 3.35 mL of substrate solution. The reaction mixture contained 210 units of ALP and 15 mmol of substrate. One unit is defined as the amount of enzyme that oxidizes one micromole of *p*-NPP in 1 min. The reacting solution was stored at room temperature (25 °C), and 100 μL aliquots of the mixture were taken at time intervals of 30 min. Sample mixtures were voxtexed and acetonitrile (60 μL) HPLC grade from Sigma Aldrich (Pittsburg, PA, USA) were immediately added to stop any enzymatic reaction. After dilution (8–12 times depending on sample concentration), the samples were placed into a model Du 530 Beckman spectrophotometer (Beckman Coulter, Fullerton, CA) with a cuvette path of 1 cm and the absorbance was measured at 405 nm to determine the concentration of *p*-nitrophenol (p-NP) formed by oxidation of *p*-nitrophenyl phosphate. The blank solution used for measurements was similar to the reaction mixture except that it contained no enzyme. Absorbances of five different concentrations of *p*-NP from Sigma Aldrich (St. Louis, MO) were measured spectrophotometrically and used to construct a standard curve that was used to determine the concentration of *p*-NP in each sample. Each data point was the average of three measurements. The oxidation of *p*-nitrophenyl to *p*-nitrophenol with formation of inorganic phosphate (pi) in the presence of alkaline phosphatase proceeds according to the following equation:

$$p\text{-nitrophenlyphosphate} + H_2O \xrightarrow{\text{ALP}} p\text{-nitrophenol} + \text{pi} \quad (1)$$

In the presence of L-lactate dehydrogenase, pyruvate was transformed to L-lactate, whereas β-NADH was oxidized to β-NAD$^+$. The enzymatic reaction can be described as:

$$\text{pyruvate} + \beta\text{-NADH} \xrightarrow{\text{LDH}} L\text{-lactate} + \beta\text{-NAD}^+ \quad (2)$$

The change in the concentration of β-NADH was measured spectrophotometrically at 340 nm. A working substrate solution containing 0.11 mM β-nicotinamide reduced form (β-NADH), 620 mM sodium pyruvate solution and 10 g/L BSA obtained from Sigma (St. Louis, MO, USA) were prepared in 100 mM PBS pH 7.4. Fifteen milliliters of pressure-treated LDH in buffer, fat-free milk, or 2% fat milk was dissolved in 100 mL of 100 mM buffer, 200 μL of which was added to 0.3 mL of substrate solution. In this assay, β-NADH was the measurable substrate. One unit of LDH was defined as the amount of LDH required to oxidize 1 μmol of β-NADH in one minute. The reacting solution was stored at room temperature (25 °C) and vortexed at time intervals of 10 min; 100 μL of the mixture was sampled and assayed. Acetonitrile (60 μl) was added immediately after sampling to stop the enzymatic reaction. After dilution, the sample was placed into a model Du 530 Beckman spectrophotometer, and the absorbance was measured at 340 nm. To construct a standard curve, we prepared five different concentrations of β-NADH and measured their absorbances spectrophotometrically at 340 nm. Each data point of the standard curve was the average of three measurements. The standard curve was used to calculate the concentration of remaining β-NADH in the samples. The activity of ALP and LDH were determined by measuring reaction rate constants of *p*-NP formation and substrate (β-NADH) disappearance, respectively, using a first-order kinetic model as described by Ludikhuyze (*6*) and Pandey and Ramaswany (*19*).

Circular Dichroism Analysis. Circular dichroisnm (CD) spectra of pressure-treated ALP and LDH were recorded with a Jasco 810 spectrophotopolarimeter (Wayne Kottkamp, Easton, MD) equipped with a Peltier thermostatic system under constant nitrogen flux at 25 °C using a 0.2 cm quartz cuvette. Scannings were performed at wavelengths

between 190 and 250 nm, at a rate of 20 nm per/min. The enzyme concentration was 0.1 mg/mL and spectra were measured in 100 mM PBS, pH 7.4, instead of diethanolamine buffer to avoid interference with the strong near-UV signal from the amine bond in diethanolamine. The composition of the secondary structure of pressure-treated enzymes was determined using the K2d algorithm (*20, 21*).

Circular dichroism measures the differential adsorption of left- and right-handed circular polarized light. It is a form of spectroscopy used to determine the optical isomerism and secondary structure of molecules. At a given wavelength

$$\Delta A = A_L - A_R \tag{3}$$

where ΔA is the difference between absorbance of left circularly polarized (LCP) and right circularly polarized (RCP) light. This equation is also expressed by applying Beer's law, as

$$\Delta A = (\varepsilon_L - \varepsilon_R)Cl \tag{4}$$

where ε_L and ε_R are the molar extinction coefficients for RCP and LCP light, C is the molar concentration, and l is the path length in centimeters (cm). The molar circular dichroism that corresponds to the circular dichroism of the substance is expressed as

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R \tag{5}$$

ΔA and the molar ellipticity, $[\theta]$, are readily interconverted using the equation

$$[\theta] = 32982\Delta\varepsilon \tag{6}$$

Dynamic Light Scattering. DLS measurements were performed using a Viscotek 802 DLS (Houston, TX) equipped with a class 3B 60mW laser diode with a wavelength of 830 nm and model 802 DLS Omnisize 2.0 software. One milliliter of 0.2 mg/mL pressure-treated ALP and LDH in Tris-HCl, pH 8.5, and PBS, pH 7.4, buffers, respectively, was centrifuged for 5 min at 15 000 g using a table-top microcentrifuge (Beckman, Coulter, Fullerton, CA), and filtered prior to DLS measurements. Tris-HCl buffer was chosen for DLS measurements instead of diethanolanine buffer to avoid interference between the amine groups in diethanolanine and the enzyme that could affect measurement of protein content. A volume of 100 μL of supernatant was transferred to a clean Eppendorf polypropylene tube (VWR, International, Chester, PA) and filtered through a Whatman syringe filter of pore size 1 μm (VWR International, Chester, PA). Fifteen microliters of the filtrate was transferred into a 1 cm path quartz cuvette and placed into the DLS cell for measurement. Ten measurements were made for each sample and the data were analyzed using the model 802 DLS Omnisize 2.0 software. DLS measures the autocorrelation function $g^1(\tau)$, which is related to the size distribution of particles in solution (*18*).

$$g^{(1)}(\tau) = \int_0^\infty m^2 P(q,\Gamma) G(\Gamma) e^{-\Gamma\tau} d\Gamma, \tag{7}$$

where $G(\Gamma)$ is the normalized number distribution function for the decay constants Γ, m_Γ the particle scattering factor, $\Gamma = q^2 D_T$, the decay constant, $q = (4\pi n/(\lambda \sin(\theta/2))$, θ the angle between the incident and scattering beam, n the refractive index of the medium, and D_T the translational diffusion coefficient related to the hydrodynamic radius R_h by

$$D_T = \frac{k_B T}{6\pi\eta R_h} \tag{8}$$

where k_B is the Boltzmann's constant, T the temperature, and η the viscosity of the sample.

The concentration of enzyme in the filtrate was measured using the Biorad Protein Assay Reagent concentrate, using bovine serum albumin (BSA) as a protein standard. Aliquots of the filtrates (20 μL) were taken and their absorbances were measured at 505 nm using a model Du 530 Beckman Spectrophotometer. The concentration of protein in the filtrate was calculated using a standard curve made of known concentrations of BSA.

Statistical Analysis. Origin software (Origin 7.5 software, Origin Laboratory) was used to assess the means and standard deviations. Minitab Software (Minitab, State College, PA) was used for analysis of variance and differences between treatment means. Tukey's test was used to analyze differences between treatments pairs, with a 95% confidence limit.

RESULTS AND DISCUSSION

Enzyme Activity. Figure 1 shows first-order plots of ALP activity determined after 6 and 12 min HPP in buffer, fat-free milk, and 2% fat milk. The rate constant k of formation of p-NP was 6.3×10^{-3} min^{-1} for non-pressure-treated (untreated) ALP in buffer. When the enzyme was subjected to various pressures from 206 to 620 MPa, reaction rate constants were practically the same ($P > 0.1$). In fat-free and 2% fat milk, k values for β-NADH oxidation by untreated ALP were 6.4×10^{-3} and 6.3×10^{-3} min^{-1}, respectively. Similar to ALP in buffer, k did not change significantly when submitted to higher HPP ($P > 0.1$). Furthermore, at each pressure treatment, k values for ALP in buffer, fat-free milk, and 2% fat milk were not significantly different ($p > 0.05$), indicating that changing the suspending medium did not affect the activity of ALP. When ALP was treated for 12 min, k was 6.3×10^{-3}, 6.3×10^{-3}, and 6.8×10^{-3} min^{-1} in buffer, fat-free milk, and 2% fat milk, respectively. Increasing pressure treatments did not make any significant difference in the rate constants ($P > 0.09$) except at 620 MPa, where k values for ALP in buffer, fat-free milk, and 2% fat milk were decreased to 3.2×10^{-3}, 2.6×10^{-3}, and 4.2×10^{-3} min^{-1}, respectively ($p < 0.005$). This suggested that ALP was resistant to pressures below 515 MPa and a combination of time (12 min) and pressures as high as 620 MPa was necessary to induce change in activity. However, ALP did not entirely lose its activity at 515 and 620 MPa (**Figure 1**). Our results agree with those of Mussa and Ramaswamy (*3*), who reported no inactivation of ALP at 200–482 MPa, and with Rademacher et al. (*10*), who reported that 99% inactivation of ALP was achieved when the enzyme was subjected to HPP at 600 MPa.

The activity of LDH in buffer, fat-free milk, and 2% fat milk expressed as the changes in the reaction rate constant was measured using a first-order kinetic model after 6 and 12 min of HPP. Non-pressure-treated (untreated) LDH was used as the control. The slope of the plots of the logarithm of the concentration of β-NADH versus time was taken as reaction rate constant k (**Figure 2**). Rate constants of LDH treated in buffer (PBS), fat-free milk, and 2% milk are shown in **Table 1**. The k values of untreated LDH in PBS, fat-free milk, and 2% milk were 1.9×10^{-2}, 1.8×10^{-2}, and 2×10^{-2} min^{-1}, respectively. When LDH was subjected to HPP of 206 MPa, k dropped to 1.0×10^{-2} min^{-1} in all three processing media and continued decreasing as HPP increased. Upon HPP of 415 MPa, k values were 9×10^{-3}, 8.6×10^{-3}, and 9×10^{-3} min^{-1}, in PBS, fat-free milk, and 2% fat milk, respectively ($p < 0.005$). Increasing HPP up to 620 MPa did not induce further reduction of k, which remained between 4.7×10^{-3} and 5.2×10^{-3} min^{-1}. After 12 min of HPP treatement, k values of LDH were 1.8×10^{-2}, 1.9×10^{-2}, and 1.9×10^{-2} min^{-1}, in PBS, fat-free milk, and 2% fat milk, respectively, and decreased as pressure increased in a manner similar to the decrease observed for 6 min (**Figure 2**). In the 415–620 MPa range, k did not change significantly in all media ($p > 0.05$,) suggesting that complete enzyme inactivation had occurred in this pressure range. In contrast, a significant decrease in the rate constant of oxidation of β-NADH by LDH was observed at 206 MPa and at higher-pressure treatments. These results revealed that in the

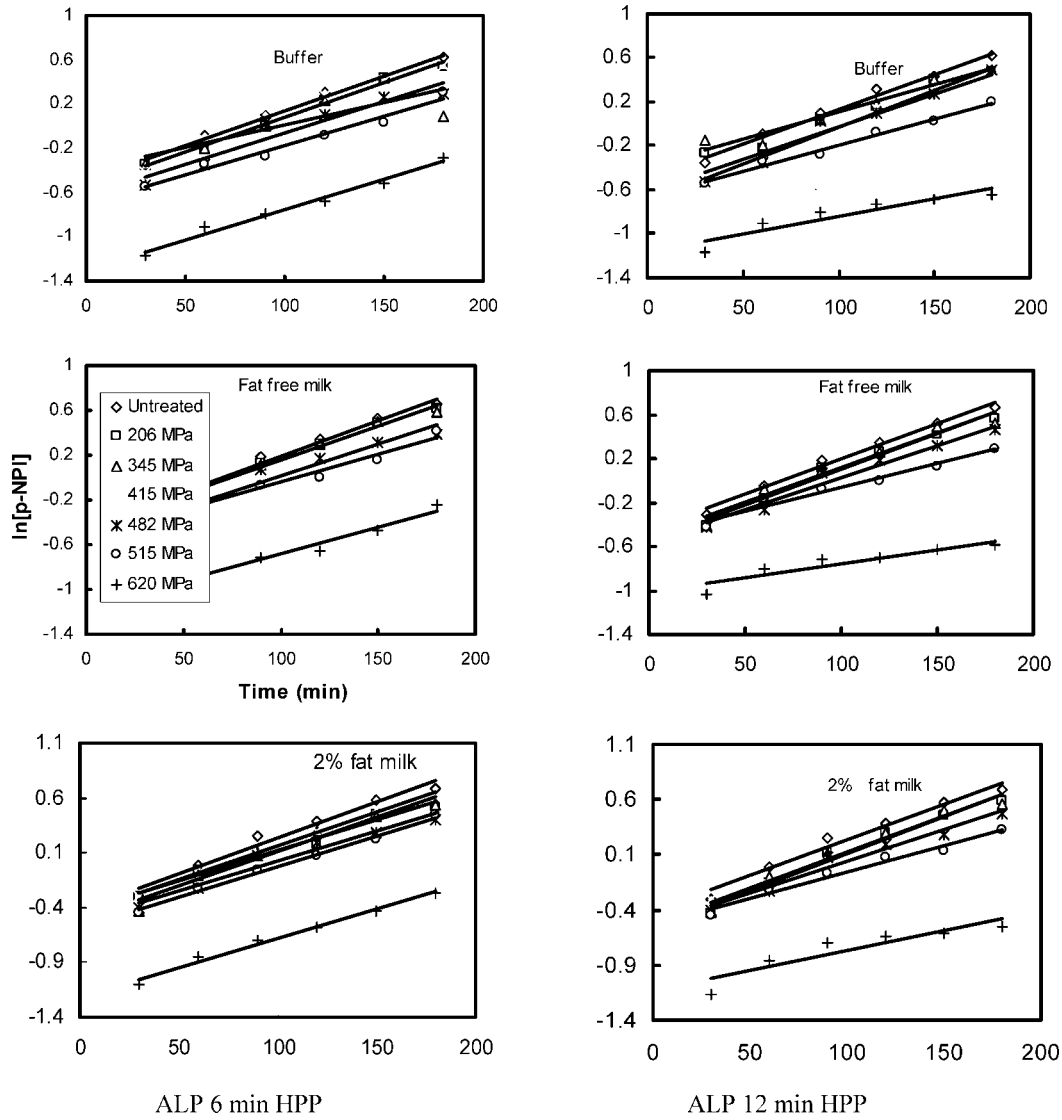

Figure 1. Activity of ALP in buffer, fat-free milk, and 2% fat milk, measured after 6 and 12 min at 206, 345, 415, 482, 515, and 620 MPa using a first-order kinetic model.

pressure range of 206–415 MPa, the activity of LDH was gradually affected and total inactivation occurred at 482 MPa and above. When LDH was treated for 12 min, the activity change at 206 and 345 MPa was similar to treatment at 206–482 MPa for 6 min treatment. The rate constants of inactivation of LDH in buffer, fat-free milk, and 2% milk were not significantly different, regardless of the suspending media used ($P > 0.05$). Our results did not agreed with those of Seyderhelm et al. (*22*), who claimed that bovine milk has a protective effect on enzyme inactivation by HPP. Furthermore, the difference in fat composition (2%) in the milk did not have any significant effect on ALP or LDH activity ($p > 0.05$).

Circular Dichroism. Changes in the secondary structure of ALP and LDH after HPP were studied using the far-ultraviolet CD spectrum in the 190–250 nm range. In general, protein activation is observed as a shift downward of the CD spectrum (*23–26*), whereas inactivation shifts the CD spectrum upward (*28–30*). A denatured protein is thus observed as an upward shift in ellipticity. **Figure 3a–d** show CD spectra of ALP and LDH treated at various pressures.

The CD spectrum of untreated enzyme ALP (**Figure 3a** 1) is typical of a blend of α-helix, *β*-sheets, and unordered structure as described by Brahms et al. (*30*), having a negative ellipticity in the 205–240 nm region with two peaks at 210 and 222 nm, respectively, and a positive ellipticity in the 190–210 nm region. After 6 min of HPP, similar CD spectra were obtained at 206, 345, 415, 482, 515, and 620 MPa, suggesting that no major change in ellipticity occurred as a result of HPP. When ALP was treated for 12 min, CD spectra were similar to those obtained after 6 min of HPP except at 620 MPa, where an increase in ellipticity of 4.9 deg cm^2 was observed (**Figure 3b**). This change in ellipticity indicates a modification in the composition of the secondary structure of ALP (*26*). However, the overall CD spectra of ALP did not suggest a complete denaturation of the enzyme. **Figure 3c** shows the CD spectra of untreated LDH (*1*) as well as LDH treated during 6 min at 206, 345, 415, 482, 515, and 620 MPa (2–6). The spectrum of untreated LDH has characteristics of α-helix/*β*-sheet/unordered structure, with a negative ellipticity in the 205–240 nm region and showed two peaks at 208 and 224 nm and one major peak at 195 nm. The ellipticity of the spectrum increased with HPP, indicating a loss of the native structure of the enzyme. When LDH was subjected to 515 and 620 MPa, the shift upward of the ellipticity was almost total, synonymous to loss of the secondary structure of the enzyme. Increasing the treatment time to 12 min (**Figure 3d**) produced similar effects. This indicated that the secondary structure of LDH was gradually lost as LDH was subjected to higher-pressure treatments.

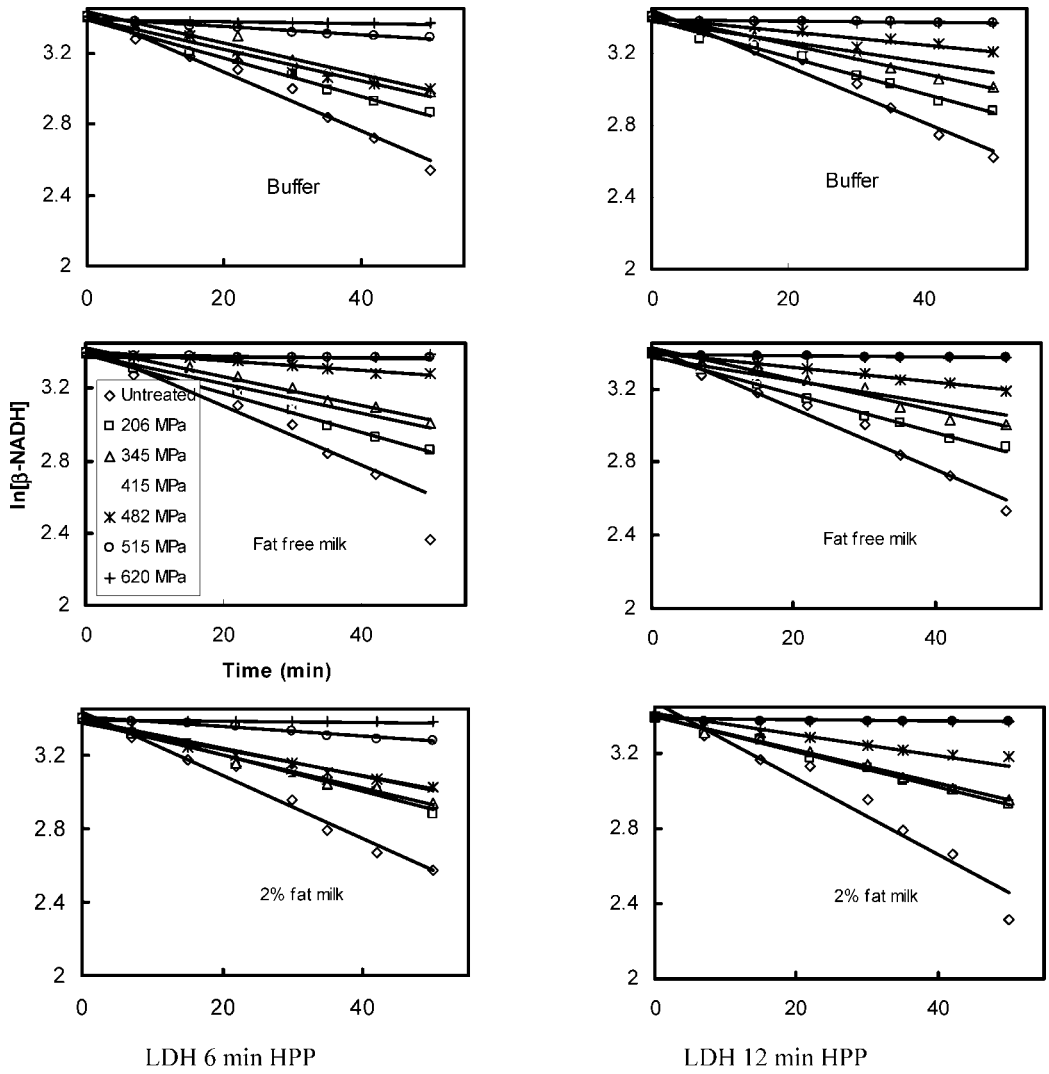

Figure 2. Activity of LDH in buffer, fat-free milk, and 2% fat milk, measured after 6 and 12 min at 206, 345, 415, 482, 515, and 620 MPa using a first-order kinetic model.

Table 1. Reaction Rate Constants of ALP and LDH after 6 and 12 min HPP in Buffer, Fa-Free Milk, and 2% Fat Milk (rate constants expressed in min^{-1} were calculated using the enzyme assay data for ALP and LDH)

	alkaline phosphatase						L-lactate dehydrogenase					
	6 min			12 min			6 min			12 min		
pressure (MPa)	buffer	fat-free milk	2% fat milk	buffer	fat-free milk	2% fat milk	buffer	fat-free milk	2% fat milk	buffer	fat-free milk	2% fat
0	6.3×10^{-3}	6.4×10^{-3}	6.3×10^{-3}	6.3×10^{-3}	6.3×10^{-3}	6.8×10^{-3}	1.9×10^{-2}	1.8×10^{-2}	2.0×10^{-2}	1.8×10^{-2}	1.9×10^{-2}	1.9×10^{-2}
206	6.3×10^{-3}	6.0×10^{-3}	6.2×10^{-3}	6.4×10^{-3}	6.2×10^{-3}	6.6×10^{-3}	1.0×10^{-2}	1.0×10^{-2}	1.0×10^{-2}	1.1×10^{-2}	1.0×10^{-2}	9.5×10^{-3}
345	6.8×10^{-3}	6.1×10^{-3}	5.6×10^{-3}	6.2×10^{-3}	6.2×10^{-3}	6.4×10^{-3}	9.0×10^{-3}	8.0×10^{-3}	9.0×10^{-3}	8.2×10^{-3}	8.5×10^{-3}	9.0×10^{-3}
415	5.7×10^{-3}	6.0×10^{-3}	5.4×10^{-3}	5.9×10^{-3}	6.5×10^{-3}	5.7×10^{-3}	9.0×10^{-3}	7.9×10^{-3}	9.0×10^{-2}	5.8×10^{-3}	6.5×10^{-3}	5.6×10^{-2}
482	5.3×10^{-3}	5.7×10^{-3}	5.6×10^{-3}	6.7×10^{-3}	6.0×10^{-3}	6.0×10^{-3}	5.2×10^{-3}	5.0×10^{-3}	6.7×10^{-2}	4.0×10^{-3}	4.3×10^{-3}	4.0×10^{-2}
515	5.3×10^{-3}	5.0×10^{-3}	5.3×10^{-3}	5.8×10^{-3}	5.5×10^{-3}	5.5×10^{-3}	5.0×10^{-3}	5.0×10^{-3}	4.7×10^{-3}	4.0×10^{-3}	4.0×10^{-3}	4.0×10^{-3}
620	5.5×10^{-3}	4.8×10^{-3}	5.3×10^{-3}	3.2×10^{-3}	2.6×10^{-3}	4.2×10^{-3}	5.0×10^{-3}	5.0×10^{-3}	4.1×10^{-3}	5.1×10^{-3}	4.4×10^{-3}	5.0×10^{-3}

In summary, CD results revealed that the secondary structure of ALP was only affected after 6 min at 620 MPa, but not at pressures below this threshold. When the same treatments were applied for 12 min, HPP effects were also only significant at 620 MPa. In contrast, the secondary structure of LDH was affected by 206 MPa and gradually lost as pressure was increased. The loss of the secondary structure observed in this study following pressure treatments is in agreement with the finding of Kinsho et al. (*28*) in their investigation of the effects of HPP treatment on carboxypeptidase and with Chapleau et al. (*30*) in their study on the effect of HPP on myofibrilar protein structure. The composition of the secondary structure of ALP and LDH obtained using the K2.d program provided an estimation of the percentage of α-helix, β-sheet, and others, including turn and coil, and are shown in **Table 2**. The structure of untreated ALP was composed of 24.7% α-helix, 14.2% β-sheet, and 61% others (turn and coil). Yan et al. (*25*) reported a similar secondary structure composition for this enzyme. This composition changed very little as pressure treatment increased. In the 0–620 MPa range, α-helix composition decreased only from 24.7 to 22.3%, whereas the β-sheet

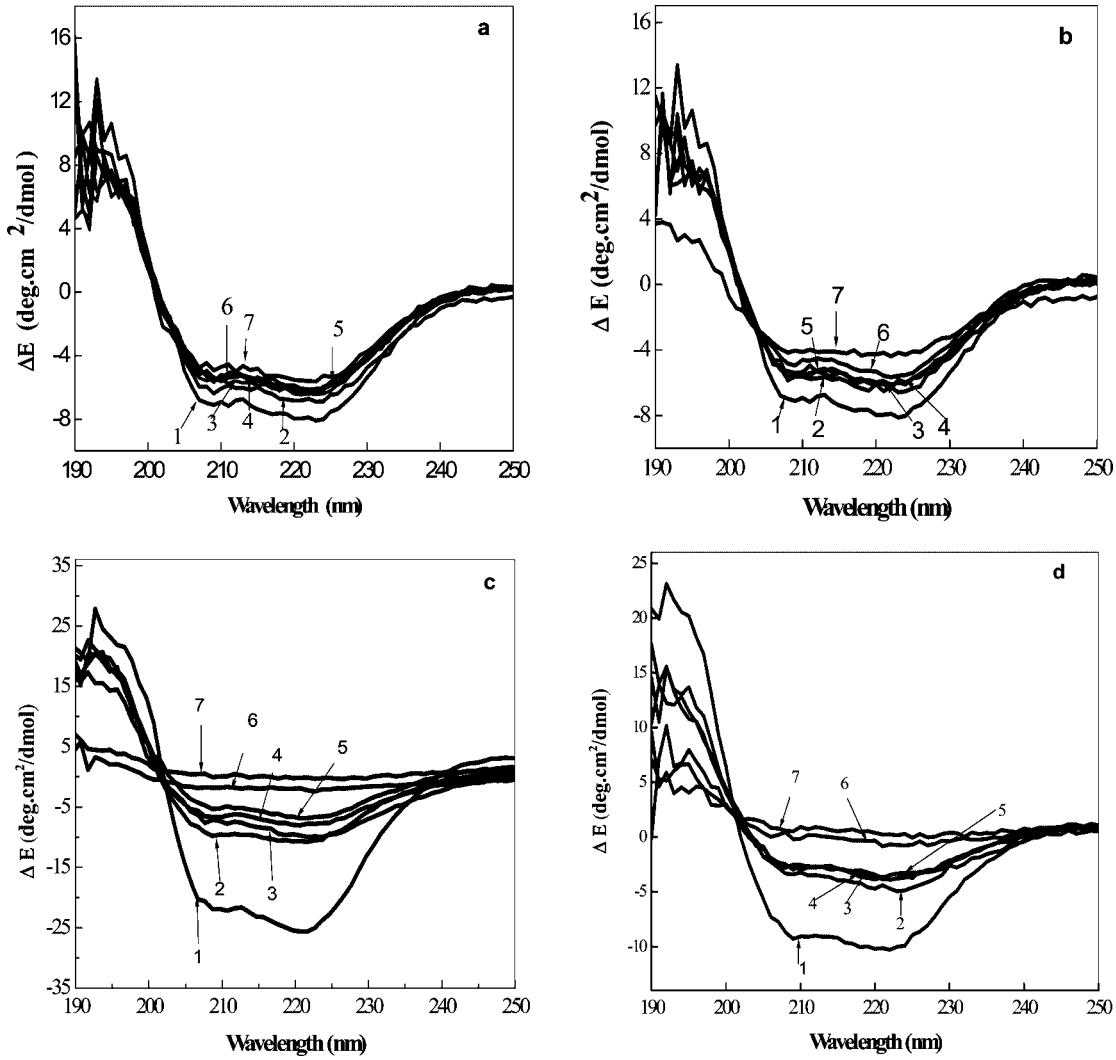

Figure 3. CD spectroscopy of ALP and LDH. (**a**, **b**) CD spectra of ALP after 6 and 12 min HPP, respectively. (**c**, **d**) CD spectra of LDH after 6 and 12 min HPP, respectively. Numbers 1–7 denote spectra of untreated enzyme and treated enzyme at 206, 345, 415, 482, 515, and 620 MPa, respectively.

Table 2. Secondary Structure of ALP and LDH Measured from Circular Dichroism Spectra; Data are Expressed as Percentages of Total Composition Structure[a]

	allcaline phosphatase						L-lactic dehydrogenase					
pressure	6 min			12 min			6 min			12 min		
(MPa)	α -helix	β-sheet	other	α-helix	β-sheet	other	α-helix	β-sheet	other	α-helix	β-sheet	other
0	24.7 a	14.2 a	61.1 a	24.6 a	14.2 a	61.2 a	39.2 a	22.4 a	38.4 a	37.6 a	24.4 a	37.5 a
206	23.5 a	14.7 a	61.7 a	23.3 a	14.5 a	62.2 a	28.5 b	25.2 a	46.3 b	25.3 b	29.5 b	45.1 a
345	23.2 a	14.6 a	62.2 a	23.1 a	14.6 a	62.7 a	26.3 b	27.1 a	46.1 b	21.2 b	32.0 b	46.8 a
415	23.1 a	14.7 a	62.3 a	23.1 a	14.7 a	62.3 a	25.0 b	28.4 a	46.3 b	19.4 b	35.2 b	46.2 a
482	23.1 a	14.5 a	62.3 a	22.9 a	14.9 a	62.2 a	24.5 b	28.6 a	46.9 b	19.0 b	35.4 b	45.0 a
515	22.8 a	14.8 a	62.9 a	18.6 b	16.2 a	65.1 a	09.6 c	13.3 b	77.1 b	03.0 c	0.76 c	96.2 c
620	22.3 a	15.1 a	62.6 a	11.6 c	21.4 b	67.0 a	04.8 d	07.7 c	94.4 b	01.6 a	0.34 a	98.1 d

[a] Identical letters next to values within a column indicate that they are not significantly different.

fraction and others increased only from 14.2 to 15.1% and 62.6 to 71.1%, respectively.

These results indicate that the secondary structure of ALP was preserved after 6 min of HPP, which suggests that at these processing conditions no change in the secondary structure of ALP was observed except in the 515–620 MPa pressure range where the α-helix concentration dropped from 18.6 to 11.6%, leading to a subsequent increase in β-sheet and unordered fraction. The secondary structure of the untreated LDH contained 39.2, 22.4, and 38.4% of α-helix, β-sheet, and others, respectively. Such a composition was consistent with the secondary structure of LDH reported by Brahms et al. (*30*). As HPP treatment increased, the α-helix content decreased from 22.4 to 4.8%, whereas the β-sheet fraction first increased from 38.4 to 46.3 % at 345 MPa, and the contents of others increased from 46.3 to 77. 1%. After 12 min of HPP in the 0 to 206 MPa range, a more severe reduction occurred from 37.7 to 1.6% in the α-helix composition; meanwhile, the β-helix fraction increased from 24.4% to a maximum of 35.4% at 482 MPa, followed by a

Table 3. Pearson Correlation Coefficients (*r*) of Reaction Rate Constants with Components of the Secondary Structure of ALP and LDH at State Pressures for 6 min[a]

	ALP		LDH	
	r	*p*	*r*	*p*
α-helix	0.772	0.042	0.828	0.021
β-sheet	−0.674	0.097	0.304	0.507
others	−0.804	0.022	−0.628	0.131

[a] *p* is the probability of getting a value for the test statistic that is as extreme as or more than the observed value.

drastic reduction to 0.76 and 0.34% at 515 and 620 MPa, respectively. This led to an increase in the composition of other structures from 37.5 to 98.1%. LDH lost most of its α-helix and *β*-sheet components to the detriment of other unordered structures. The increased in ellipticity observed in this study as a consequence of HPP is in accordance with Sun et al. (*4*) and Brahms et al. (*30*) and may be ascribed to the occurrence of changes in water molecules surrounding proteins in the proteins hydration shell that gives the enzymes a molten-globule-like structure. The moderate change observed in the secondary structure of ALP in contrast to the severe changes in the LDH structure were reflected in the CD spectra, as well as enzyme activity, and suggested a possible link between pressure inactivation of the LDH due to loss of the secondary structure in this enzyme. The Pearson correlation coefficient (r) between the reaction rate constant

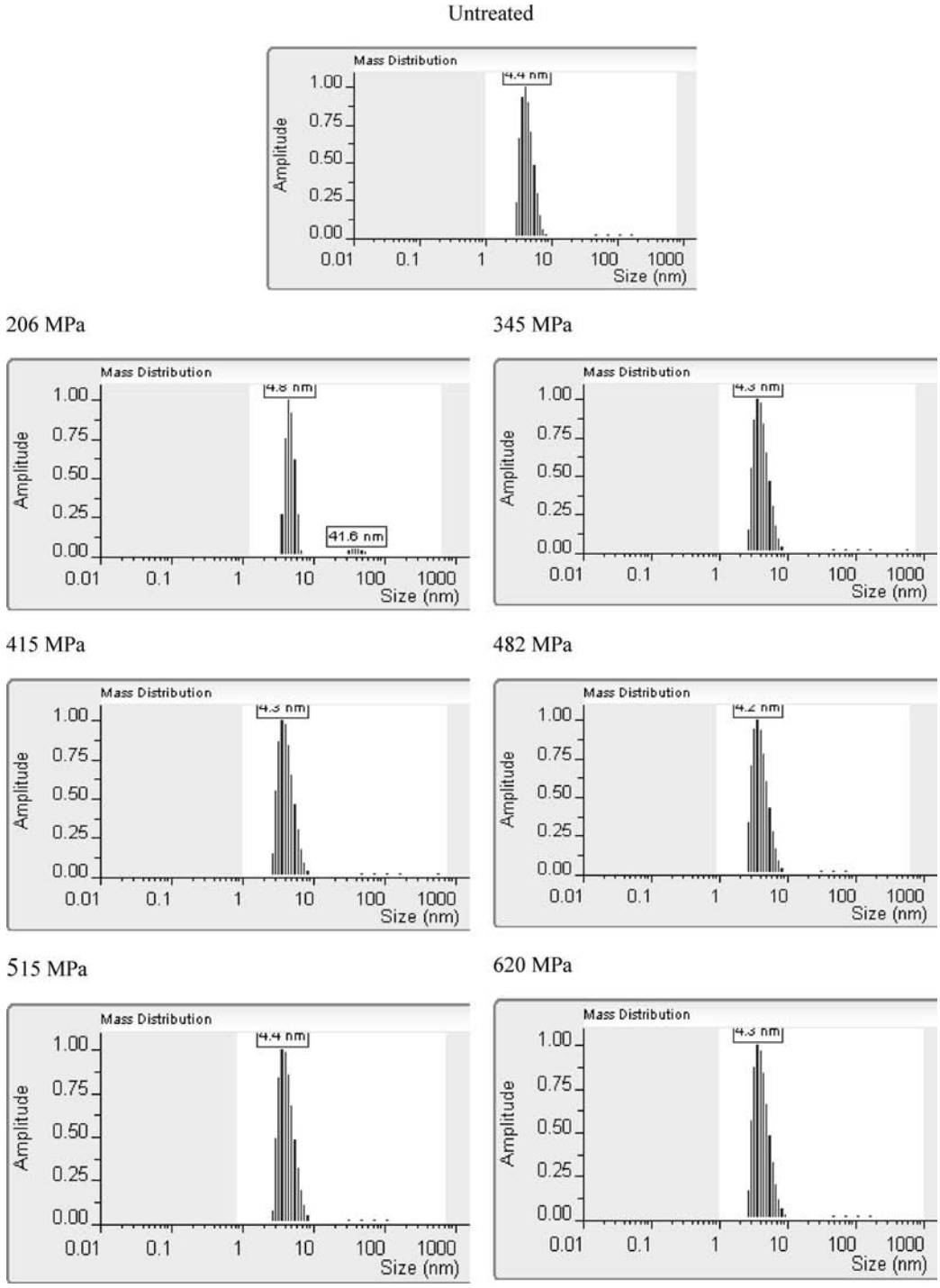

Figure 4. Mass distribution histogram of ALP obtained from DLS measurements after 6 min held at 206, 345, 415, 482, 515, and 620 MPa.

Table 4. Characteristics of the Quaternary Structure Observed from DLS Analysis of Untreated and Treated ALP and LDH at 206, 345, 415, 482, 515, and 620 MPa for 6 min

pressure (MPa)	distribution	Rh (nm)	MW (kDA)	RSD %	peak area (%)
		ALP			
untreated	monomodal	4.4	143.1	21.9	100
206	bimodal	4.5	142.1	22.5	99.5
		37.6	17429.9	25.5	0.5
345	monomodal	4.3	145.1	25.1	100
415	monomodal	4.3	141.5	16	100
482	monomodal	4.2	141.5	26.3	100
515	monomodal	4.3	144.4	33.1	100
620	monomodal	4.3	143.2	26.7	100
		LDH			
untreated	monomodal	4.4	145.1	19.4	100
206	bimodal	4.5	149.2	27.9	12.3
		39.8	21173.9	31.3	87.7
345	bimodal	4.5	148.4	16.7	99
		39.9	1220.8	30	1
415	bimodal	4.2	145.5	16.6	99.3
		37.4	11224.4	20	0.7
482	bimodal	4.1	91.5	17.3	98.9
		31.2	11224.4	20.3	1.1
515	bimodal	4.3	143.1	21.9	98.8
		33.4	13134.9	29.3	1.2
620	monomodal	4.4	146.8	25.6	100

k and each fraction of the secondary structure of ALP and LDH are presented in **Table 3**. The Pearson correlation coefficient *r* between *k* and the α-helix, *β*-sheet, and other fractions of ALP after treatment for 6 min at 206–620 MPa were 0.772, −0.674, and −0.804, respectively. These values indicate a poor correlation between secondary structure and enzyme activity. The poor correlation could be explained by the fact that HPP did not induce changes when samples were treated for 6 min. However, For LDH, *r* values between *k* and α-helix, *β*-sheet, and other fractions of LDH after 6 min at 206–620 MPa were 0.888, 0.304, and −0, 628, respectively. This suggests a strong correlation between LDH activity and α-helix component; however, good correlation was also found between LDH activity and its *β*-sheet, and other fractions. The relationship between the α-helix component and the activity supports the idea that the secondary structure plays a crucial role in enzyme activity.

Dynamic Light Scattering. ALP samples did not precipitate and appeared stable after all HPP treatments. In contrast, extensive precipitation was visually observed in LDH samples at pressures of 482 MPa and higher. The amount of precipitation increased with increasing pressure; however, the precipitates were not visible after centrifugation and filtration. The mass distribution of ALP obtained from DLS is shown in **Figure 4**. The hydrodynamic radius (R_h) is the radius of enzyme in solution, and the relative standard (RSD) or polydispersity is indicative of the distribution in the peak or subpeak; the peak area refers to the proportion of each particle in the sample. The mass distribution of untreated samples is characterized by the presence of a single component of hydrodynamic radius (R_h) 4.4 with a molecular weight (MW) of 143 kDA and 100% peak area (**Table 4**). These characteristics suggest that this component corresponded to ALP.

At 206 MPa, the enzyme solution showed a bimodal distribution as illustrated by the occurrence of two peaks in the mass distribution histogram (**Figure 4**). The R_h and the molecular weight (MW) of this component were 4.46 nm and 142.14 kDa, respectively, which were consistent with native ALP, whereas the second peak with R_h 36.7 nm and MW 17429.9 was characteristic of an aggregate of ALP. However, this aggregate represented only 0.55% of the protein in solution, indicating that 99.5% of ALP in filtrates remained in its initial state. At 345–620 MPa, the distribution of the enzyme was monomodal and no aggregation was detected. The absence of aggregation at higher pressures suggested that aggregation observed in ALP at 206 MPa was reversible and at pressures above 206 MPa, aggregates dissociated. In general, when the percentage of polydispersity (%Pd) of a solution is below 15%, the solution is likely to crystallize. A solution having polydispersity below 30% has a moderate polydispersity. When the Pd% is higher than 30%, the solution is highly polydispersed and therefore the quaternary structure of biomolecules in this solution can be analyzed (*31*). In this study, the %Pd of ALP was below 30% except at 515 MPa, where it was 33.1%. This suggests the pressure-treated samples are relatively stable and likely to crystallize except at 515 MPa, where the likehood of crystallization is reduced possibly because of low enzyme concentration or hazing in the enzyme solution (*31, 32*).

Figure 5 shows the mass distribution histogram of DLS measurements of LDH samples after HPP treatments and the characteristics of the samples are shown in **Table 4**. Before HPP treatments, LDH is stable in the buffer as indicated by the presence of a single component of R_h 4.4 and MW 149.2 kDa attributable to native LDH. From 206 to 515 MPa, the enzyme exhibited a bimodal distribution with two components, one of which was attributable to LDH in its native state on the basis of the molecular weight, with the other components being an aggregate. The sizes of the aggregates varied with pressure and the R_h ranged from 31.2 to 39.7 nm, with MW between 1220.77 and 21173 kDa. Such an aggregate would have involved as many as 150 molecules that assembled to form a massive cluster of molecules. To estimate the magnitude of LDH aggregation, it is necessary to take into account the formation of larger aggregates, which are eventually removed through filtration. Table 5 shows the concentrations of ALP and LDH remaining after centrifugation and filtration. The concentration of ALP changed only from 0.20 to 0.17 mg/mL in the 206–482 MPa range, which corresponded to about 15% protein loss. Increasing pressure treatments up to 620 MPa induced a further reduction in the concentration of the enzyme to 45% of the initial concentration. However, the concentration of LDH was gradually reduced from 0.3 and 0.1% as pressure increased from 515 to 620 MPa, respectively. This reduction of the LDH concentration is attributable to denaturation as well as aggregation, as suggested by the DLS results. The relative stability in the concentration of ALP is consistent with the stability in the activity and the secondary structure of this enzyme on the basis of the results of CD spectroscopy analysis. On the other hand, the disappearance of LDH upon HPP treatments as result of denaturation and aggregation is in accordance with the drastic loss of activity and change in the secondary structure of this enzyme.

ALP owes its stability to its relative resistance to aggregation and the stability of its secondary structure. The results obtained on LDH agree with those of Chapleau et al. (*29*), who demonstrated that pressures above 300 MPa induced denaturation of myofibrillar proteins by destabilizing the secondary structure and causing irreversible aggregation.

Conclusions. In this study, we have shown that ALP exhibited resistance to inactivation and its secondary and quaternary structure were preserved after 6 min at 206–620 MPa. The study also

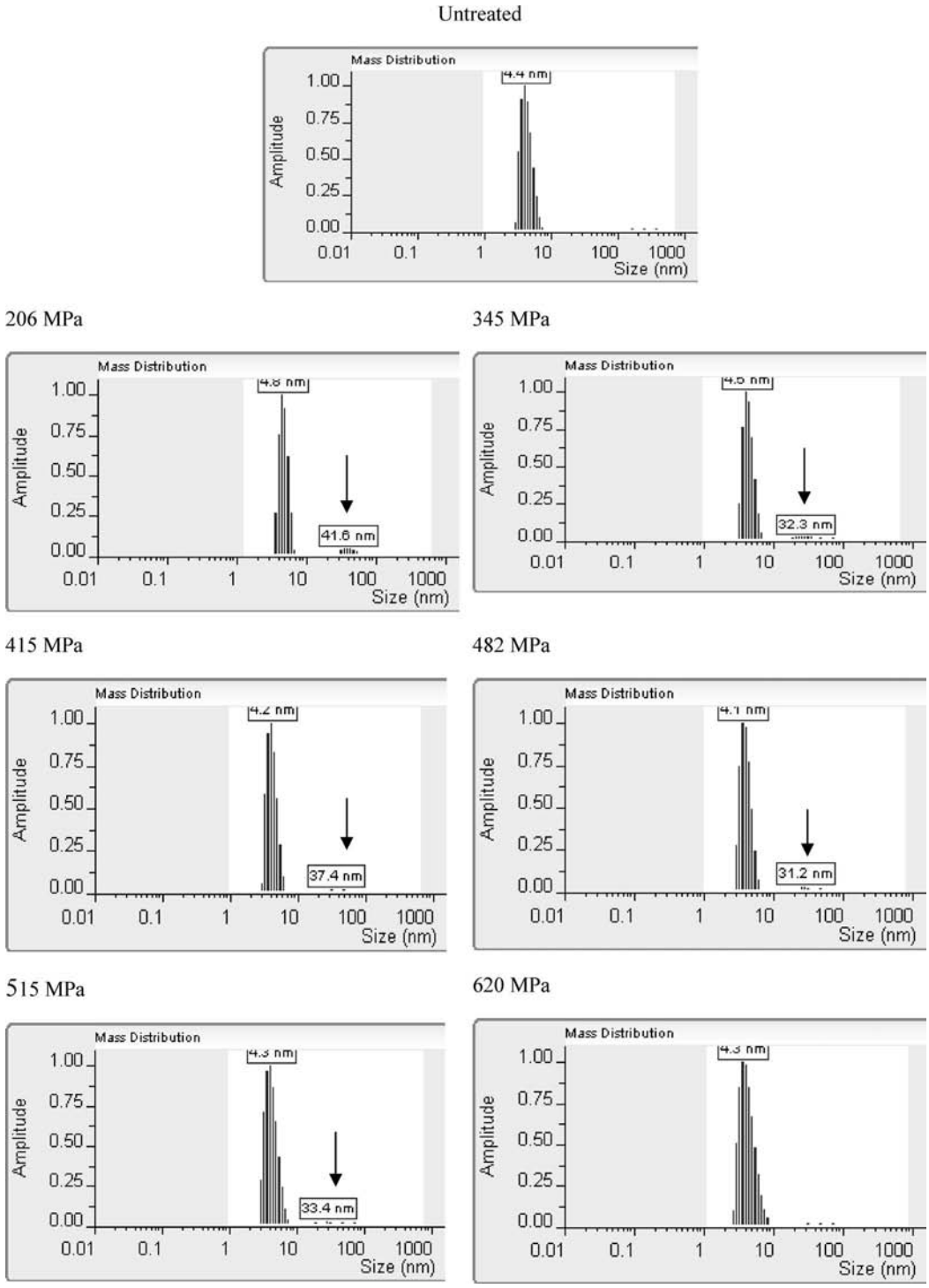

Figure 5. Mass distribution histogram of LDH obtained from DLS measurements after 6 min hold at 206, 345, 415, 482, 515, and 620 MPa. The arrows represent formation of aggregates.

revealed that increasing HPP from 6 to 12 min at 620 MPa reduced ALP activity without causing complete inactivation of the enzyme. Alkaline phosphatase likely owes its pressure resistance to the stability of its secondary structure, demonstrated by the preservation of its α-helix content and the limited amount of aggregates formed upon various pressure treatments. In contrast, LDH underwent extensive loss of activity, which was in accordance with the alteration of its secondary structure and the concomitant aggregation observed via DLS. This study has demonstrated that HPP can inactivate an enzyme by altering the stability of its secondary structure and inducing aggregation of enzyme molecules. In turns, aggregation impacts enzyme activity by reducing the concentration of active enzyme in the reaction medium. Future studies might investigate LDH crystal formation following HPP. The information obtained from this study is essential to understanding the mechanism(s) of HPP inactivation of enzyme activity. Such information is necessary to understand the inactivation of both microorganisms and enzymes that affect the safety and quality of foods processed using high pressure.

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