



Pulsed electric field (PEF)-induced aggregation between lysozyme, ovalbumin and ovotransferrin in multi-protein system



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ABSTRACT

The aggregation of multi-proteins is of great interest in food processing and a good understanding of the formation of aggregates during PEF processing is needed for the application of the process to pasteurize protein-based foods. The aggregates formation of a multi-protein system (containing ovalbumin, ovotransferrin and lysozyme) was studied through turbidity, size exclusion chromatography and SDS-PAGE patterns for interaction studies and binding forces. Results from size exclusion chromatography indicated that there was no soluble aggregates formed during PEF processing. The existence of lysozyme was important to form insoluble aggregates in the chosen ovalbumin solution. The results of SDS-PAGE patterns indicated that lysozyme was prone to precipitate, and was relatively the higher component of aggregates. Citric acid could be effective in inhibiting lysozyme from interacting with other proteins during PEF processing. Blocking the free sulphhydryl by *N*-ethylmaleimide (NEM) did not affect aggregation inhibition.

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1. Introduction

Pulsed electric fields (PEF) is a very promising non-thermal technology for preservation of liquid foods due to reduced heating effects. It preserves the colour, flavour and nutrients of products better than traditional pasteurization methods (Elez-Martínez, Sobrino-López, Soliva-Fortuny, & Martín-Belloso, 2012, chap. 4). This technology is widely employed in pump-able foods or semi-fluid foods (Pereira & Vicente, 2010). Current PEF research focuses on microorganism inactivation, endogenous enzymes inactivation, product quality and shelf life of protein-based foods and fruit juices (Kazmierska, Rudzinska, Jarosz, Dobrzanski, & Trziszka, 2012; Rieger, Noci, Cronin, Morgan, & Lyng, 2008; Rivas, Rodrigo, Martínez, Barbosa-Cánovas, & Rodrigo, 2006; Saldana, Puértolas, Monfort, Raso, & Alvarez, 2011; Sepulveda, Góngora-Nieto, Guerrero, & Barbosa-Cánovas, 2009; Shamsi, Versteeg, Sherkat, & Wan, 2008). With growing understanding of PEF technology and improvement of equipment, it is also increasingly being used in bioactive compounds' extraction and decontamination (Gusbeth, Frey, Volkmann, Schwartz, & Bluhm, 2009; Loginova, Vorobiev, Bals, & Lebovka, 2011; Luengo, Álvarez, & Raso, 2013; Puértolas, López, Saldaña, Álvarez, & Raso, 2010). This technology is used

mostly at pilot-scale, but it is also advancing towards industrial scale applications (López, Puértolas, Condón, Raso, & Alvarez, 2009; Puértolas et al., 2010).

Unlike fruit and vegetable juices, the protective effects of food components such as proteins and lipids make severe PEF conditions a requirement for sufficient microbial reduction in protein-based foods (Martín-Belloso et al., 1997; Monfort, Gayán, Condón, Raso, & Álvarez, 2011; Sampedro, Rivas, Rodrigo, Martínez, & Rodrigo, 2006). Most PEF studies focus on the alteration of protein conformation and inactivation of endogenous enzymes under PEF treatment. However, the mechanism of such effects has not yet been elucidated (Li, Chen, Liu, & Chen, 2008; Shamsi, Versteeg, Sherkat, & Wan, 2008; Zhao & Yang, 2009). Severe PEF conditions cause protein unfolding which ultimately results in aggregate formation through weakly covalent and non-covalent bonds. Perez and Pilosof (2004) analysed egg white proteins by electrophoresis and found that aggregates were formed by covalent bonds. They proposed that during PEF processing, protein molecules polarized first, and then hydrophobic amino acids or sulphhydryl groups were exposed as the conformation of protein changed. Lastly, if the electric pulse energy is high enough, aggregates could form hydrophobic or covalent bonds (Perez & Pilosof, 2004). However, no further studies have been carried out on the binding types of aggregates. Similar conclusions were presented by Li, Chen, and Mo (2007) and Zhao et al. (2009). However,

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protein-based foods such as liquid eggs and milk usually contain a variety of proteins, which may display different aggregation characteristics due to the interactions among them. Currently, there is no investigation on the effects of protein composition and formation of protein aggregates under PEF treatments.

Due to the difficulties of studying complicated multi-protein systems in detail, it was preferential to examine model systems comprising a few major proteins. A multi-protein system (including ovalbumin, ovotransferrin and lysozyme, Ova 1), pure ovalbumin (Ova 2) and lysozyme solution were selected for study of protein interactions under PEF treatments. Although the self-aggregation, and structure changes of ovalbumin and lysozyme under PEF treatments have received much attention, the interaction between main egg white proteins such as ovalbumin, ovotransferrin and lysozyme during PEF processing has not been reported. Therefore, in the present study, different compositions of ovalbumin solutions were used to determine the effects of aggregates formation upon protein interaction. The mechanism of the PEF-induced aggregation of lysozyme with other proteins is also reported and a possible way to suppress aggregation presented.

2. Materials and methods

2.1. Materials

Multi-ovalbumin (Ova 1) was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Ovalbumin (A5503, Ova 2), lysozyme (62970) and *N*-ethylmaleimide (NEM, E3876) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Main components in Ova 1 were ovalbumin (46.57 kD, relative amount was 87.2%), ovotransferrin (76.27 kD, relative amount was 8.12%) and lysozyme (14.56 kD, relative amount was 4.6%). Compared to Ova 1, Ova 2 was purer (Fig. 1).

2.2. Preparation of different composition ovalbumin solutions

All the ovalbumin samples (2 mg/mL) were prepared in ultrapure water (pH 7.2), and the electrical conductivity was adjusted to 2000 $\mu\text{S}/\text{cm}$ by adding NaCl (0.1096 g NaCl to 100 mL). Electrical conductivity was determined with a conductivity meter (EL30, Mettler-Toledo International, Inc, Switzerland). For the preparation

of citric acid samples, Ova 1 solution (2 mg/mL, ultrapure water) was used as stock solution. Citric acid solutions of different concentrations were made. The pH and conductivity were determined separately.

2.3. PEF devices and treatments

A bench-scale continuous PEF system (OSU-4L, Ohio State University, Columbus, OH, USA) with square-wave pulses was used. As described in our previous study (Zhao & Yang, 2009), the PEF apparatus has six co-field flow tubular chambers with a 2.92 mm electrode gap and 2.3 mm inner diameter. The chambers are grouped in three pairs and connected with stainless steel tubing. After going through each pair of chambers, the sample was cooled by passing through a stainless steel tube, which was submerged in a heat exchange water bath (Fisher Scientific Inc., Pittsburgh, PA, USA) at 4 °C. The pre- and post-PEF exposure temperatures at the inlet and outlet of the treatment chamber were measured by using a K type thermocouple (OMEGA, Stamford, CT, USA). Samples (100 mL) were exposed to a constant electric field intensity of 25 kV/cm for 200, 400, 600 and 800 μs , corresponding to treatment energies of 681.3, 1362.6, 2043.9 and 2726.3 kJ/L, respectively. In this study, the pulse repetition rate and pulse width were set at 100 Hz and 2 μs , respectively. The highest temperature achieved in all tests was 18 °C (18 °C represented the highest temperature detected by the K type thermocouple near the outlet of the sixth chamber). Finally, the control sample was also pumped through the PEF chambers at 18 °C. Depending on the protein solutions treated, clear solutions or cloudy suspensions of protein aggregates were obtained.

2.4. Measurement of turbidity

Turbidity is the cloudiness or haziness of a fluid caused by individual particles (totally suspended or dissolved solids). It is inversely proportional to the transmittance (Zoubida Akkouché, Lyes Aissat, & Khodir Madani, 2012). After PEF treatments, the samples were left for 20 min at room temperature, and the transmittance ($T\%$) was measured at 600 nm using a UV-Vis spectrophotometer (UV-1800 UV-Vis Spectrophotometer, SHIMADZU Co., Kyoto, Japan). All the samples were treated and analysed in triplicate. They were shaken well before being measured, using deionised water (pH 7.2, 2000 $\mu\text{S}/\text{cm}$) as a standard ($T\% = 1$). Turbidity was calculated as percentage of transmittance ($T\%$) using the following equation:

$$\text{Turbidity} = -\ln(T\%) \quad (1)$$

where $T\%$ is the record transmittance of protein solutions. A turbidity of 0% corresponds to a totally clear solution.

2.5. Size exclusion chromatography

A Protein-Pak 125 Column (10 μm , 7.8 $\times$ 300 mm, WAT084601) was used to characterize the egg white proteins aggregation by PEF and heat process. The buffer was 50 mM phosphate buffered saline at pH 6.8, containing 0.15 M NaCl. Then 10 μL samples of each solution were injected, eluted at a flow rate of 0.6 mL/min and detected at 280 nm.

2.6. Gel electrophoresis and binding forces of aggregates

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out at a constant voltage (80 V) in a Mini-Protein Tetra system with PowerPac Basic Power Supply (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The sample preparation and experiments were carried out according to Zhao et al.

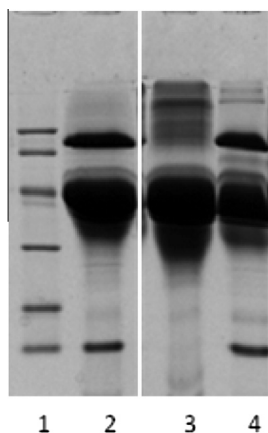


Fig. 1. SDS–PAGE pattern of different ovalbumin solutions. Lane 1, molecular weight standard markers (a-lactalbumin, 14.4 kD; trypsin inhibitor, 20.1 kD; carbonic anhydrase, 31.0 kD; ovalbumin, 43.0 kD; bovine serum albumin, 66.2 kD; phosphorylase B, 97.4 kD); lane 2, ovalbumin ($\geq 90\%$ by agarose gel electrophoresis, Ova 1); lane 3, ovalbumin ($\geq 98\%$ by agarose gel electrophoresis, Ova 2); lane 4, egg white.

(2009). After PEF treatment, the solution was centrifuged at 10,000g at 4 °C for 10 min to get supernatant and insoluble aggregates. Supernatants were added to loading buffers for SDS–PAGE. Aggregates were washed thrice with distilled water and then centrifuged. Binding forces of aggregates were determined as described by Zhao et al. (2009). Aggregates were dissolved in 0.6 M NaCl (solution A), 0.6 M NaCl plus 2% SDS (solution B), 0.6 M NaCl plus 8 M urea (solution C), and 0.6 M NaCl and 8 M urea plus 2% 2-mercaptoethanol (solution D). Solution A rupture electrostatic bonds, while solution B cleave electrostatic and hydrogen bonds. As solution B, solution C also break hydrophobic bonds. Solution D cleave the above mentioned bonds and disulphides bonds. Each solution was well mixed with a vortex mixer and then centrifuged. The supernatant fractions were analysed by SDS–PAGE. The images of gels were scanned with GEL-Doc 2000 gel documentation system (BIO-RAD GelDoc 2000, Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the band intensities were analysed by Image Lab (3.0).

3. Results and discussion

3.1. Changes in the turbidity of PEF-treated samples

In this study, turbidity was used to directly characterize the aggregates formed during PEF treatment. As shown in Fig. 2A, PEF-dependant formation of aggregates occurred upon PEF treatment of different ovalbumin solutions. Other researches undertakings have also observed the formation of aggregates of protein such as whey protein (Sui, Roginski, Williams, Versteeg, & Wan, 2011), soy bean protein isolate (Li, Chen, & Mo, 2007) and enzymes

(Zhao & Yang, 2009). The formation of these aggregates depends on the variety of protein, concentration of protein solution and conditions of PEF treatment. In the present research, through changes in solution turbidity, it was found that aggregate formation depended on the constituent of the protein solution. Ova 2, a relatively pure solution, did not flocculate during treatment. However, under PEF treatment, the turbidity of Ova 1 solution decreased with treatment time. At the beginning of the process, there were no visible particles present in the solution and the solution was transparent. The difference between Ova 1 and Ova 2 protein solutions was that lysozyme (relative amount was 4.6%) and ovotransferrin (relative amount was 8.12%) were present. However, no coagulation occurred during the same treatment of lysozyme (Fig. 2B). On the other hand, adding lysozyme (0.05 mg/mL) to Ova 2 (2 mg/mL) solution caused turbidity to increase sharply (Fig. 2C). These results indicate that lysozyme could be the reason for aggregate formation during PEF treatment.

3.2. Composition and binding type of PEF-induced aggregates of Ova 1 solution

Size exclusion chromatography data for PEF treated Ova 1 (Fig. 3A) and Ova 2 (Fig. 3B) solutions show major peaks of ovalbumin at elution time 12.31 min, indicating that no soluble aggregates were formed during processing.

The SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was implanted to access the composition and binding type of PEF-induced aggregation of Ova 1 and Ova 2 solutions. From the SDS–PAGE pattern in the absence and presence of 2-mercaptoethanol, there were no changes in the bands of Ova 2 (Fig. 4A

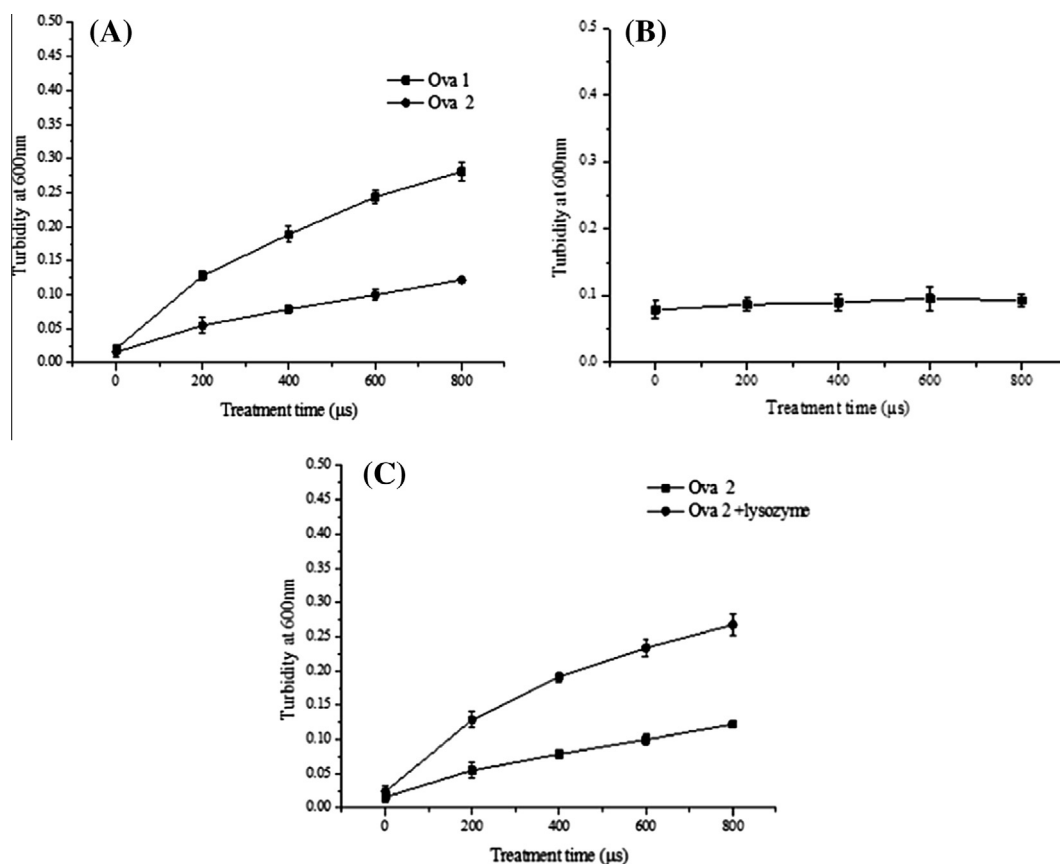


Fig. 2. Effects of PEF treatment time (25 kV/cm, 0–800 μs) on turbidity of different ovalbumin solution (A), lysozyme solution (B) and lysozyme (0.05 mg/mL) mixed Ova 2 (2 mg/mL) (C) solution.

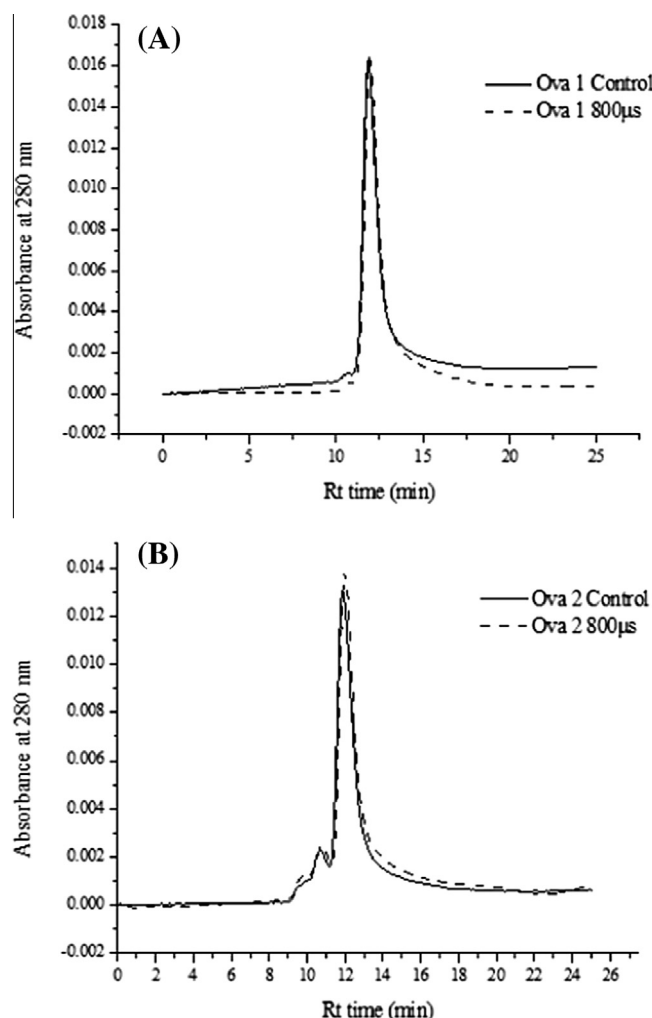


Fig. 3. Effects of PEF treatment (25 kV/cm, 800 μs) on the size exclusion chromatograms of Ova 1 (A) and Ova 2 (B).

and B, lane 4 and 5) solution at 25 kV/cm for 800 μs. This suggests that no soluble aggregate formed or could be sustained by dissociation of SDS and 2-mercaptoethanol.

In contrast, the PEF treatment induced band changes which were observed from the soluble fraction of Ova 1. Relative to the content of lysozyme in Ova 1 before treatment (Fig. 4B, lane 1), it (Fig. 4B, lane 2) decreased to 74% after treatment. The relatively contents of ovotransferrin and ovalbumin after treatment only decreased by 5% and 7%, respectively. During treatment, it was noticed that along with the formation of invisible aggregates, the turbidity of Ova 1 increased. In order to study the composition and binding type of the aggregates from PEF treatment, various protein-denaturing reagent solutions were applied to dissolve the aggregates. The aggregates partially dissolved in the A (0.6 M NaCl), B (0.6 M NaCl plus 2% SDS), and C (0.6 M NaCl plus 8 M urea) and totally dissolved in D (0.6 M NaCl plus 8 M urea and 2% mercaptoethanol). These results showed that non-covalent bonds such as electrostatic interactions, hydrogen bonds and hydrophobic interactions played an important role in formation of aggregates during treatment. The SDS-PAGE patterns showed that lysozyme was the main component in the NaCl, SDS and urea solution. More bands were observed in the presence of 2-mercaptoethanol, including lysozyme (relative content was 34.4%), ovalbumin (relative content was 36.2%), ovotransferrin (relative content was 24.1%) and other high molecular bands, suggesting that disulphide bonds also play an important role in the formation of aggregates during PEF treatment. The turbidity changes (Fig. 2) of samples with different compositions of ovalbumin, lysozyme and Ova 2 plus lysozyme showed that the existence of lysozyme in ovalbumin solution could be the reason for the formation of aggregates during PEF processing.

3.3. Inhibition of protein aggregation during PEF processes

Ovalbumin is a protein which belongs to the serpin family, although it lacks any protease inhibitory activity. It exists widely in avian egg-whites (Huntington & Stein, 2001). The crystal structure of ovalbumin was determined by Stein, Leslie, Finch, and Carrell (1991), and it comprised of 385 residues (McReynolds et al., 1978; Nisbet, Saundry, Moir, Fothergill, & Fothergill, 1981). Its molecular weight was 44.5 kD and isoelectric point (pI) was 4.5. It is the only egg white protein containing free sulphydryl groups (Mine, 1995). Ovotransferrin contained 686 amino acid residues and had a molecular weight of 77.7 kD and pI of 6.1. It accounts for 12–13% of the hen egg white protein (Wu & Acero-Lopez, 2012). Lysozyme contains 129 amino acid residues and they have been studied widely. It has a molecular weight of 14.3 kD and

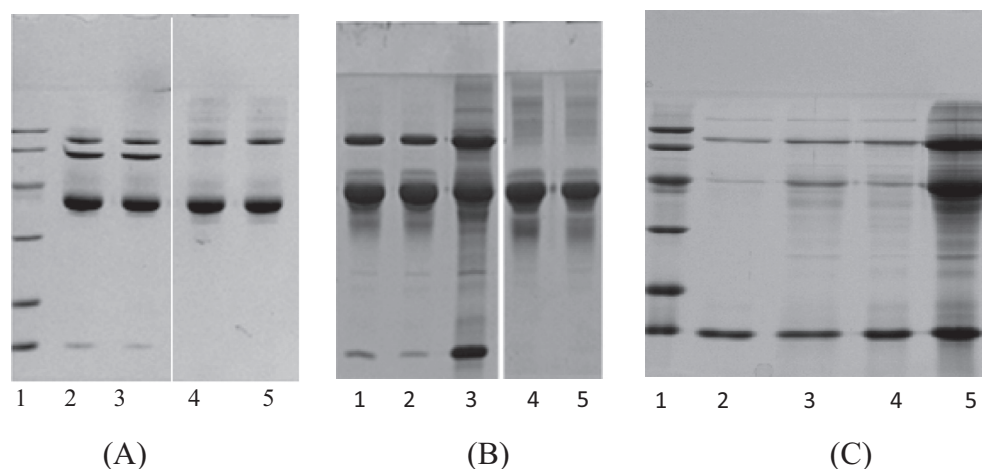


Fig. 4. SDS-PAGE patterns of ovalbumin solutions under PEF treatment (25 kV/cm, 800 μs) with (B and C) or without (A) 2-mercaptoethanol. (A) Lane 1, marker; lane 2 and 3, Ova 1 before and after PEF treatment; lane 4 and 5, Ova 2 before and after PEF treatment; (B) lane 1 and 2, Ova 1 before and after PEF treatment; lane 3, precipitate of Ova 1 from PEF treatment; lane 4 and 5, Ova 2 before and after PEF treatment; (C) the solubilisation of aggregates was performed using 0.6 M NaCl (solution A, lane 2), 0.6 M NaCl plus 2% SDS (solution B, lane 3), 0.6 M NaCl plus 8 M urea (solution C, lane 4), and 0.6 M NaCl and 8 M urea plus 2% 2-mercaptoethanol (solution D, lane 5).

pl of 10.7 (Johnson, 1998; Mine, 1995). Many previous studies have focused on the changes of microstructure of a single protein (Fernandez-Diaz, Barsotti, Dumay, & Cheftel, 2000; Yang, Li, & Zhang, 2004; Zhao & Yang, 2008). Protein-rich liquid foods are rich in heterogeneous components. Currently, there is no report about interaction of the proteins with opposite charges during PEF processing.

In the present study, it was realized that the ovalbumin system remained stable when lysozyme was absent. When lysozyme was present, it interacted with ovalbumin and ovotransferrin, and formed non-covalent or covalent bonds. Based on this finding, *N*-ethylmaleimide (NEM) and citric acid were selected to try and prevent the formation of insoluble aggregates during processing. NEM has always been used in protein solutions to block free sulphhydryls (Zhu et al., 2013), thereby preventing interactions by disulphide bonds during PEF processing. Fig. 5A shows that (0.01%) NEM had no effect on prevention of aggregate formation.

However, with increment in citric acid concentration, the pH of Ova 1 solution decreased (Table 1). When the citric acid concentration was 0.20%, and pH 2.82, no obvious change in turbidity of protein solutions occurred during processing (Fig. 5B). Gongora-Nieto et al. (2003) reported that citric acid (0.50%) could reduce energy consumption, improve the treatment effect and protect product quality (Gongora-Nieto, Pedrow, Swanson, & Barbosa-Canovas, 2003). However they did not mention that citric acid influenced

Table 1

Different contents of citric acid in Ova 1 solution (2 mg/mL).

Citric content (%)	pH	Electrical conductivity ($\mu\text{S}/\text{cm}$)
0.20	2.82	2000
0.10	3.09	1980
0.08	3.19	2120
0.05	3.39	2132
0.03	3.77	2080
0.01	4.23	2000
0.005	4.66	2095
0	7.20	2000

the interactions of proteins during PEF processing. The results indicated that electrostatic interactions, hydrogen bonds and hydrophobic interactions played important roles in the formation of aggregates during PEF processing. Because pH could affect the charge of proteins, it is reasonable to speculate that proteins with the same charge were more stable than were oppositely charge. These results suggest that during PEF processing, electrostatic interactions are very important for the formation of aggregates from oppositely charged proteins. This also coincides with the mechanism of PEF treatment which involves the application of different voltages at the treatment chamber, changing the microbial membrane voltage, and then achieving the purpose of pasteurization. PEF may accelerate protein movement depending on its charge, thus creating more opportunities for proteins to interact with each other. When proteins in the solution have opposite charges, they are precipitated during PEF processing. However, aggregates formation can effectively be prevented by controlling the pH of the solution.

4. Conclusion

No obvious aggregates were formed in ovalbumin and lysozyme solutions at an electric field strength of 25 kV/cm for 800 μs . However, when lysozyme (0.05%) was present in ovalbumin (0.2%) solution, insoluble protein aggregation occurred due to electrostatic interactions and disulphide bonds. SDS-PAGE patterns showed that lysozyme (34.4%), ovalbumin (36.2%) and ovotransferrin (24.1%) existed in the aggregates formed in Ova 1 solution (the content of ovalbumin was 87.2%) after PEF treatment. Adding NEM (0.01%) to block free sulphhydryl did not prevent the formation of insoluble aggregates. However, addition of citric acid (0.20%) could effectively inhibit lysozyme from interacting with other proteins during PEF processing. Therefore, our results provide useful information for the application of PEF on pasteurization of liquid egg white. Mechanism of the inhibition of protein aggregation under PEF treatments by citric acid requires further study. It is also important to consider the effects of citric acid on the conformation and functional properties of proteins.

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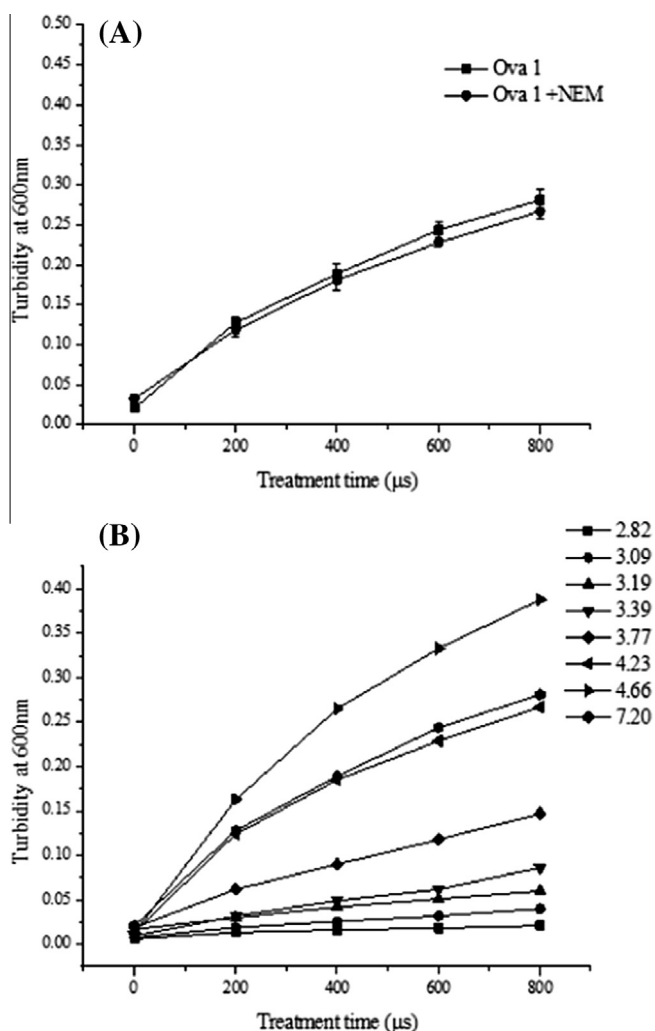


Fig. 5. Effects of different content of NEM (A) and citric acid (B) on Ova 1 solution under PEF treatment (25 kV/cm).

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