

2020

The Effect of Atmospheric Cold Plasma Treatment on the Antigenic Properties of Bovine Milk Casein and Whey Proteins

Sing Wei Ng
University College Dublin

Peng Lu
University College Dublin

Aleksandra Rulikowska
Technological University Dublin, aleksandra.rulikowska@tudublin.ie

See next page for additional authors

Follow this and additional works at: <https://arrow.tudublin.ie/schfsehart>

 Part of the [Nanotechnology Commons](#)

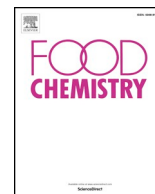
Recommended Citation

Ng, S.W., Lu, P. & Rulikowska, A. (2020). The Effect of Atmospheric Cold Plasma Treatment on the Antigenic Properties of Bovine Milk Casein and Whey Proteins. *Food Chemistry*, vol. 342, no. 128283. doi:10.1016/j.foodchem.2020.128283

This Article is brought to you for free and open access by the School of Food Science and Environmental Health at ARROW@TU Dublin. It has been accepted for inclusion in Articles by an authorized administrator of ARROW@TU Dublin. For more information, please contact arrow.admin@tudublin.ie, aisling.coyne@tudublin.ie, vera.kilshaw@tudublin.ie.

Authors

Sing Wei Ng, Peng Lu, Aleksandra Rulikowska, Daniela Boehm, Graham O'Neill, and Paula Bourke



The effect of atmospheric cold plasma treatment on the antigenic properties of bovine milk casein and whey proteins

Sing Wei Ng^{a,b}, Peng Lu^a, Aleksandra Rulikowska^b, Daniela Boehm^b, Graham O'Neill^b, Paula Bourke^{a,b,c,*}

^a Plasma Research Group, School of Biosystems and Food Engineering, University College Dublin, Dublin 4, Ireland

^b Environmental Sustainability and Health Institute, Technological University Dublin, Dublin 7, Ireland

^c Institute for Global Food Security, School of Biological Sciences, Queens University Belfast, Northern Ireland, United Kingdom

ARTICLE INFO

Keywords:

Milk
Atmospheric cold plasma
Spark discharge
Glow discharge
Allergen
Casein
Whey

ABSTRACT

Casein, β -lactoglobulin and α -lactalbumin are major milk protein allergens. In the present study, the structural modifications and antigenic response of these bovine milk allergens as induced by non-thermal treatment by atmospheric cold plasma were investigated. Spark discharge (SD) and glow discharge (GD), as previously characterized cold plasma systems, were used for protein treatments. Casein, β -lactoglobulin and α -lactalbumin were analyzed before and after plasma treatment using SDS-PAGE, FTIR, UPLC-MS/MS and ELISA. SDS-PAGE results revealed a reduction in the casein and α -lactalbumin intensity bands after SD or GD treatments; however, the β -lactoglobulin intensity band remained unchanged. FTIR studies revealed alterations in protein secondary structure induced by plasma, particularly contents of β -sheet and β -turn. The UPLC-MS/MS results showed that the amino acid compositions decreased after plasma treatments. ELISA of casein and α -lactalbumin showed a decrease in antigenicity post plasma treatment, whereas ELISA of β -lactoglobulin showed an increase in antigenicity. The study indicates that atmospheric cold plasma can be tailored to mitigate the risk of bovine milk allergens in the dairy processing and ingredients sectors.

1. Introduction

Milk is one of the fourteen major allergenic foods identified in Annex II of Regulation (EU) No 1169/2011. Milk allergy occurring through ingestion, inhalation and skin contact makes it an important public and industrial health concern (Quirantes Sierra, Lara Jiménez, & Skodova, 2017). Milk allergy is caused by an adverse immune response to dairy-based allergens. The antigenic determinants of allergens are epitopes, which are categorized into linear and conformational epitopes depending on the structure (Bannon, 2004; Davis & Williams, 1998). The major allergens in bovine milk are caseins and whey proteins (Tammineedi & Choudhary, 2014). Casein is a heat-stable milk specific protein that encompasses approximately 80% of the total milk protein, comprising four main subtypes: α S1-, α S2-, β -, and κ -caseins. α -casein, constituting approximately 50% of the total caseins, is a major allergen, causing severe allergic reactions and contains both conformational and sequential IgE epitopes (Tsabouri, Douros, & N Priftis, 2014). Heat labile whey proteins, comprise 20% of the total milk protein, consisting of β -lactoglobulin and α -lactalbumin. β -lactoglobulin occurs naturally in the form of a 36 kDa dimer and each subunit possesses two disulfide

bridges and one free cysteine (McKenzie, Ralston, & Shaw, 1972). β -lactoglobulin is considered the most important allergen in whey protein because it is normally absent in human breast milk (Savilahti & Kuitunen, 1992). α -lactalbumin is a globular protein with four disulfide bridges and molecular weight of 14.4 kDa. Bovine α -lactalbumin has extensive amino acid sequence homology with human α -lactalbumin (Wal, 1998). However, despite the high similarity, bovine α -lactalbumin is considered as a major bovine milk allergen.

Food processing is primarily carried out to retain or enhance the safety, quality, sensory attributes and shelf-life of foods. Food processing approaches can modify the protein structure by chemical and physical reactions, such as denaturation, Maillard reaction or aggregation (Bloom et al., 2014; Davis & Williams, 1998). This may lead to the inactivation or destruction of the epitope structure or the formation of new epitopes, which can result in changes to allergenic properties of proteins (Tsabouri et al., 2014). There is a demand for both thermal and non-thermal technologies to mitigate food allergy by modifying epitopes. Heat treatment is the conventional processing technique used in dairy industry, and can considerably reduce allergenicity by altering antigenic epitopes of some of the heat labile

* Corresponding author at: Plasma Research Group, School of Biosystems and Food Engineering, University College Dublin, Dublin 4, Ireland.
E-mail address: paula.bourke@ucd.ie (P. Bourke).

<https://doi.org/10.1016/j.foodchem.2020.128283>

Received 31 March 2020; Received in revised form 29 September 2020; Accepted 29 September 2020

Available online 02 October 2020

0308-8146/© 2020 Elsevier Ltd. All rights reserved.

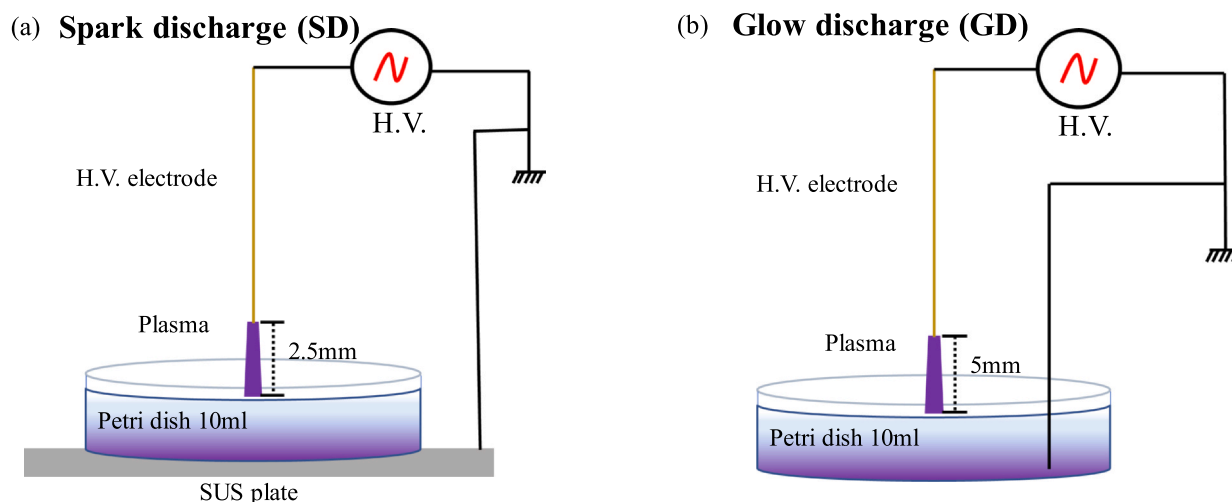


Fig. 1. Schematic of air discharges ((a) SD and (b) GD) above liquids.

proteins and thereby changing their IgE recognition (Sharma, Kumar, Betzel, & Singh, 2001). However, some studies reported that bovine milk proteins retained their antigenicity and allergenicity even when subjected to severe heat treatments (Bu, Lu, Zheng, & Luo, 2009).

Recently, atmospheric cold plasma has emerged as a promising non-thermal technology for addressing risks associated with foods (Bourke, Ziuzina, Boehm, Cullen, & Keener, 2018). Plasma, known as the fourth state of matter, can be generated using electrical energy which is applied with the help of electrodes on neutral carrier gas (Pankaj & Keener, 2017). When air is used as the inducer gas the production of reactive oxygen (like $O\cdot$, $\cdot OH$, $HO_2\cdot$, OH^- , O_2^- , O^- , O_2^+ , O^+ , O_3 and H_2O_2) and reactive nitrogen (such as $N\cdot$, N_2^* , N^* , N_2^+ , N^+ , NO and $NO\cdot$) species occurs along with ultraviolet light, shock waves and cavitation (Sarangapani et al., 2017), and these may interact with the compounds present in the food, further changing their properties (Ramazzina et al., 2016). Several authors have reported cold plasma induced the structural modification of protein (Attri et al., 2015; Chauvin, Judee, Yousfi, Vicendo, & Merbahi, 2017; Segat, Misra, Cullen, & Innocente, 2015; Takai, Kitano, Kuwabara, & Shiraki, 2012). Tamineedi et al. (2013) reported that the nonthermal atmospheric plasma treatment they applied on milk proteins has no significant change in antigenicity. The authors treated the samples using indirect plasma, resulting in no significant reduction in antigenicity. A recent study on in-package cold plasma treatment on peanut demonstrates a significant reduction of antigenicity of peanut allergen Ara h1 (Venkataratnam, Sarangapani, Cahill, & Ryan, 2019). The author found modifications in the secondary structure induced by plasma reactive species. Moreover, a recent attempt on treating king prawn using cold plasma showed up to 17.6% reduction of IgE recognition to tropomyosin (Ekezie, Sun, & Cheng, 2019). Therefore, the objective of this study was to elucidate the effects of cold plasma on allergenicity of bovine casein, β -lactoglobulin and α -lactalbumin.

Previously, we demonstrated that spark discharge (SD) and glow discharge (GD), as a characterized and tunable cold plasma, generate different amounts and patterns of reactive species (Lu, Boehm, Bourke, & Cullen, 2017; Lu, Boehm, Cullen, & Bourke, 2018). SD generates more reactive oxygen species (ROS) than reactive nitrogen species (RNS), and these lead to production of H_2O_2 in the water. GD generates more RNS than ROS, and these lead to production of NO_2^- in the water. As the uniqueness of our tunable plasma setup allows control of output chemistry, the objective of the current study is to reveal a better understanding of the potential interaction of two different direct plasma discharges with casein, β -lactoglobulin and α -lactalbumin, and track the proteomic profile of caseins and whey proteins before and after plasma treatments.

2. Materials and methods

2.1. Materials

Casein from bovine milk (powder, technical grade) was purchased from Sigma-Aldrich, Dublin, Ireland. Whey proteins were purchased from Fonterra in New Zealand. All chemicals used in this study were purchased from Sigma-Aldrich, Dublin, Ireland, unless otherwise stated. The commercial ELISA kits of caseins and β -lactoglobulin were purchased from RBiopharm $\text{\textcircled{R}}$ IDASCREEN, Darmstadt, Germany. The commercial ELISA kits of α -lactalbumin were purchased from ELISAGENIE, United Kingdom. A molecular weight marker (ab116029) was purchased from Abcam, United Kingdom.

2.2. Preparation of protein samples

Casein was dissolved in phosphate buffered saline (PBS) with 0.01 M phosphate buffer, 2.7 mM potassium chloride and 0.137 M sodium chloride, pH 7.4, and adjusted to a final concentration of 2 mg/mL. Whey protein was dissolved in the same buffer and adjusted to a final concentration of 2 mg/mL. The protein was restored by dialysis with PBS after plasma treatment to bring the pH back to neutral and remove the reactive species in the treated solution. The protein concentration was determined using Bradford reagent before and after plasma treatment and restoration, due to the water loss and precipitation.

2.3. Cold plasma treatment

A tunable plasma device based on two types of electrical discharges: spark discharge (SD) and glow discharge (GD) previously described in Lu et al (2017, 2018) was employed. The discharge configurations are presented in Fig. 1. Both setups comprise a high voltage (HV) half bridge resonant inverter circuit; PVM500 plasma driver (Information Unlimited Inc., USA) with maximum output of 20 kV with a variable frequency of 20–70 kHz as a power supply and Simran THG5000UD 5000 W step up-step down transformer. The system was composed of a tungsten-copper needle which served as the HV electrode and was fixed perpendicular to the liquid surface. The distance between the HV needle tip and the liquid surface was adjusted to 2.5 or 5 mm in all experiments. For each treatment of the liquid sample, a volume of 10 mL liquid sample was pipetted into a polystyrene petri dish with inner diameter of 55 mm, which corresponded to a liquid depth of approximately 4.2 mm. The ground electrode connections of both setups differ. For SD setup, the polystyrene petri dish was placed on a

stainless-steel plate which was connected to the ground, shown in Fig. 1(a). In GD setup, a thin stainless-steel ground electrode was submerged into the liquid sample contained in the petri dish, shown in Fig. 1(b). The system was operated at atmospheric pressure using atmospheric air as working gas. There was a modification to the treatment procedure described in Lu et al. (2017). For SD plasma treatment, the distance of HV between electrode tip and sample surface was 2.5 mm due to the stability of plasma discharge. However, for GD treatment, the distance was 5 mm. SD treatment was performed at a voltage of 8 kV and a frequency of 25 kHz (Fig. S1(a)). GD treatment was performed at a voltage of 5 kV and a frequency of 25 kHz (Fig. S1(b)). The plasma treatment times were 0, 10, 20 and 30 min for both discharge configurations. Control and treated samples were stored at $-20\text{ }^{\circ}\text{C}$ prior to chemical analyses.

2.3.1. Plasma diagnostic

Applied voltage and discharge current were monitored by a Tektronix P6015A HV probe and a Pearson 4100 0.5–1.0 W wideband current monitor. The voltage probe and current probe were connected to the high-impedance inputs of a DSO-X 2014A digital oscilloscope, InfiniiVision 2000 X-Series with 100 MHz bandwidth and 2-G sample/s sampling rate (Agilent Technologies, Ireland).

2.3.2. Physico-chemical analysis

The pH was measured by an Orion pH meter (model 420A, Thermo Electron Corporation, USA). The temperature of the samples was measured by infrared thermometer (Radionics Ltd., Dublin, Ireland) immediately after the plasma treatment. The treatment temperature was kept below $45\text{ }^{\circ}\text{C}$.

2.4. SDS-PAGE analysis

The SDS-PAGE analysis was performed using 4–12% hand-cast gels. Protein concentrations of all samples were adjusted to 1 mg/mL by mixing with loading buffer (2.5% of sodium dodecyl sulphate (SDS), 10 mM of Tris-HCl, 1 mM of EDTA and 0.002% of bromophenol blue) with or without 5% of β -mercaptoethanol. β -mercaptoethanol was used as reducing agent to break down the disulfide bonds in proteins. All samples were boiled for 5 min at $100\text{ }^{\circ}\text{C}$ before electrophoresis. A 10 μL sample and marker were added to the wells in the gel. The gels were run at 200 V for 1 h and stained with Coomassie brilliant blue overnight. The gel was de-stained three times using 10% acetic acid solution washes over a period of 24 h. Gel imaging was performed using Syngene G Box Chemi XRQ for visible analysis.

2.5. Secondary structural analysis

Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy was performed on untreated and treated proteins to determine conformational changes in the secondary structure before and after plasma treatments. A Nicolet iS7 Fourier FTIR spectrometer coupled with iD7 ATR (Thermo Fisher Scientific Inc., USA) was employed under ambient conditions as per the method described in Liu et al. (2009) with a slight modification. In brief, the solid protein was prepared by dialyzing untreated and treated protein solution against distilled water and then freeze dried. The solid protein was pressed into a small pellet between two aluminum foils to ensure good contact with ATR crystal. Each FTIR spectrum was recorded from 4000 to 400 cm^{-1} with 64 scans and a 4 cm^{-1} resolution. The secondary structural analysis was estimated by conducting the second derivative of the amide I peak in the wavenumbers ranging from $1580\text{ to }1720\text{ cm}^{-1}$ for deconvolution analysis with the aid of OMNIC software. The deconvoluted spectrum was curve-fitted with Gaussian band shapes. The resultant peaks were assigned to different secondary structures using guideline of Zhang, Deng, and Zhao (2017). The measurements were repeated 6 times, and the values were averaged to minimise baseline

effects.

2.6. Amino acid profile analysis

The protein solution samples were placed into a sealed glass tubes and hydrolyzed with 6 M HCl at $110\text{ }^{\circ}\text{C}$ for 24 h (Rafiq et al., 2016). The samples were transferred to a vial for further amino acid analysis. Tryptophan could not be detected due to it is acid-lability. Amino acid analysis was performed following the method from Kennedy and Bivens (2017). Ultra-high-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis was performed using Agilent 6470 triple quadrupole mass spectrometer system, equipped with a G7104A Agilent 1290 Infinity II Flexible Pump, a G7167B Agilent 1290 Multisampler, a G7116B Agilent 1290 Infinity II Multicolumn Thermostat and Agilent Jet Stream Electrospray ionization source (Agilent Technologies, Ireland). Poroshell 120 A Hydrophilic Interaction Chromatography (HILIC)-Z column ($2.1 \times 100\text{ mm}$, $2.7\text{ }\mu\text{m}$ particles) was used. The UPLC-MS/MS system was operated at a flow rate of 0.8 mL/min using 20 mM ammonium formate in water (eluent A) and 20 mM ammonium formate in acetonitrile (eluent B). The following gradient of eluent B was applied: 0 min: 100% B; 0–10 min: ramped to 70% B; 10–11 min ramped to 100% B and held for 5 min. With column equilibrium, the run time is 16 min. The column temperature was maintained at $30\text{ }^{\circ}\text{C}$. The injection volume was 0.25 μL . The triple quadrupole instrument was operated in multiple reaction monitoring (MRM) mode with Electrospray Ionization (ESI) positive. In the case of cystine; a cysteine dimer, the precursor ion, product ion, fragmentor and collision energy used were 241 m/z , 151.9 m/z , 75 V and 12 V, respectively. The amino acid standard curve was made using known concentrations of amino acid standard (0, 31.25, 62.5, 125, 187.5, 250, 312.5, 625 and 1250 $\mu\text{mol/L}$) and used to convert the recorded response into amino acid concentrations.

2.7. Antigenicity analysis

The antigenicity of casein, β -lactoglobulin and α -lactalbumin samples were determined using commercial ELISA kits (RBIopharm IDASCREEN Fast Casein, RBIopharm IDASCREEN β -lactoglobulin and ELISAGENIE bovine α -lactalbumin ELISA kits, respectively). The detailed procedures were performed according to the manufacturer's instructions. The standard curve of known protein concentration was prepared by diluting the specific protein prepared by the manufacturer. The standard curve was used to convert absorbance into antigenicity of specific protein concentrations. The percentage of antigenicity of the protein was calculated as (treated concentration/untreated concentration) $\times 100$.

2.8. Statistical analysis

Statistical analysis was performed using Prism 8.0 (GraphPad Software Inc., La Jolla, USA) and the results were analyzed by analysis of variance (ANOVA) with the Tukey's honest significant difference (HSD) test at $\alpha = 0.05$. All values are mean \pm standard deviation (SD) of triplicate measurements from two independent experiments ($n = 6$).

3. Results and discussion

3.1. Characteristics of SD and GD plasma treatment

Two types of discharges above the liquids were observed through an oscilloscope coupled with HV probe and current probe. The applied voltage and current waveforms differentiate between SD and GD, as can be observed in Fig. S1. SD was ignited at the positive polarity half period of the applied voltage shown in Fig. S1(a), while GD was ignited at the negative polarity half period of the applied voltage shown in Fig. S1(b). SD predominantly generates reactive oxygen species while GD

predominantly generates reactive nitrogen species (Lu et al., 2017, 2018). Each plasma treatment was observed to reduce the liquid volume of casein and whey solutions (Table S2). The volume decreased with increasing treatment time. This moisture loss was due to increase in temperature (up to 43.3 °C) which led to evaporation of water molecules. Approximately 6.2 mL was collected after 30 min of plasma treatment from 10 mL of initial sample. Moreover, the protein concentrations were increased after plasma treatment (Table S2). Hence, it was necessary to recover the protein concentration of treated samples as per control (2 mg/mL) before further analysis. In addition, the phosphate precipitated during GD treatment.

Applying SD or GD treatments decreased the pH value of the protein solutions after 10 min. The pH value was reduced from 7 and 7.3 (untreated casein and whey solutions, respectively) to a minimum of 4.15 and 4.87 after 30 min SD or GD treatment time, respectively. The decrease of pH value could be attributed to nitrogen oxides produced in the plasma interacting with liquids and producing nitric and nitrous acids (Tsoukou, Bourke, & Boehm, 2018).

3.2. SDS-PAGE analysis

SD and GD treated caseins were analyzed using SDS-PAGE under non-reducing and reducing conditions for their electrophoretic profiles, shown in Fig. 2(a) and (b), respectively. Casein is known as the major allergen in milk. The band of native casein occurred around the protein marker of 25–35 kDa under non-reducing and reducing conditions Fig. 2(a) and (b). As shown in Fig. 2(a), the band intensity of casein was slightly decreased with SD treatment time for both non-reducing and reducing conditions. Observing the control in lane 1 and 5 at the top of the gel in Fig. 2(a), the buffer soluble aggregate disappeared after reducing the disulfide bond with β -mercaptoethanol as a reducing agent. However, in the presence of reducing agent, the soluble aggregates

were found after SD treatment in gel compared to control. This could be explained by a change in solubility as a result of SD treatment, which is induced by the new formation of higher molecular weight aggregates via the formation of disulfide bonds, hydrophobic and electrostatic interactions and inter protein crosslinking (Tammineedi et al., 2013). As shown in Fig. 2(b), the band intensity of casein declined dramatically with increasing GD treatment time in both conditions. Observing the untreated and GD treated casein in non-reducing conditions, no protein band was found in lane 4 of the gel (Fig. 2(b)). The lost protein band in lane 4 could be attributed to the formation of new, buffer insoluble aggregates via the generation of disulfide bonds, hydrophobic and electrostatic interactions and intra/inter protein crosslinking. After 10 min and 20 min GD treatment, we can observe the soluble aggregate in reducing conditions, however, after 30 min GD treatment, we can see very little soluble aggregate in reducing condition (Fig. 2(b)). This is attributed to insolubilization of protein after extended GD treatment. In the presence of reducing conditions (Fig. 2(b), lane 8) the existence of soluble aggregate was attributed to the formation of disulfide bonds by comparison with the GD treated casein (30 min) in non-reducing and reducing conditions, lanes 4 and 8, respectively. This emphasizes the greater changes in the protein profile of casein induced by GD than by SD treatment.

SDS-PAGE profiles of SD and GD treated whey proteins under non-reducing and reducing conditions are shown in Fig. 2(c) and (d). Whey proteins, as major allergens in milk, are composed of β -lactoglobulin and α -lactalbumin. As shown in Fig. 2, the band at 17 kDa corresponds to β -lactoglobulin; and the band at 11 kDa corresponds to α -lactalbumin. The band located approximately at 35 kDa is the dimer of β -lactoglobulin. Applying SD, the results in Fig. 2(c) showed that the band intensity of native and dimer β -lactoglobulin was largely unchanged even at the maximum treatment duration of 30 min. However, the band of native and dimer β -lactoglobulin became more smeared and broader.

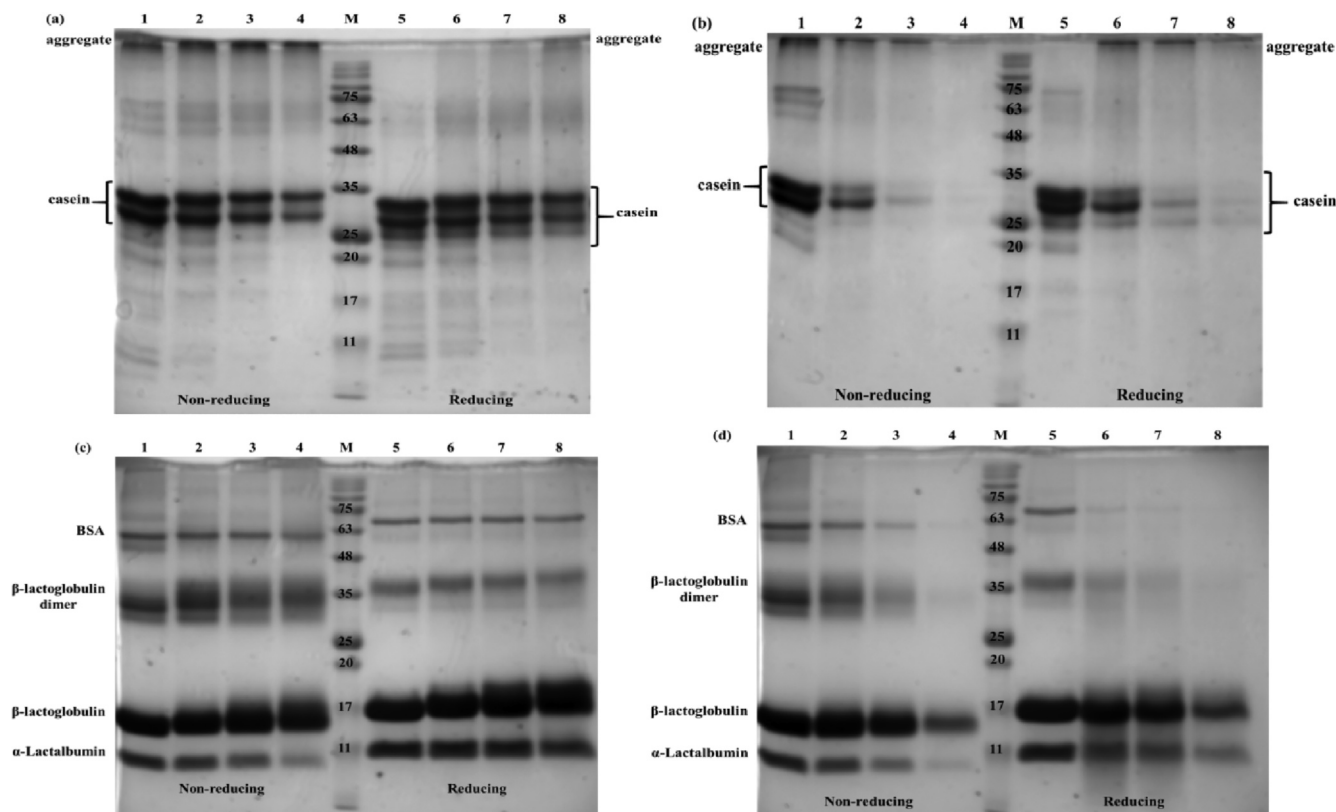


Fig. 2. SDS-PAGE profiles of untreated; (a) SD and (b) GD treated caseins; (c) SD and (d) GD treated whey proteins subjected to different exposure times. M: the standard protein marker. Lane 1–4: control and samples treated for 10, 20, 30 min, respectively under non-reducing condition. Lane 5–8: control and samples treated for 10, 20, 30 min, respectively under reducing condition.

This could indicate some SD induced structural modification of β -lactoglobulin, resulting in changes to the molecular weight of β -lactoglobulin. Zhou et al. (2016) reported the modification of amino acids by reactions induced by plasma, such as nitration, oxidation, hydroxylation, dehydrogenation, sulfonation and dimerization. These reactions can modify the protein structure and cause changes in the molecular weight of proteins. The band intensity of bovine serum albumin (BSA) remained unchanged, except for a slight decrease in band intensity following 30 min of SD treatment. Similar trends were recorded for the band intensity of α -lactalbumin in the presence of reducing agent. However, the α -lactalbumin band density faded upon prolonged exposure to SD treatment in the absence of reducing agent. This could be explained by the new generation of disulfide bonds during SD exposure.

Employing GD, Fig. 2(d) shows notable changes in the profile of both β -lactoglobulin and α -lactalbumin when compared to control. The native and dimer of β -lactoglobulin bands and BSA bands gradually decreased with increased GD treatment time. Similar to GD treated α -lactalbumin, its band intensity diminished after a treatment time of 30 min in non-reducing condition. Moreover, the protein bands in lanes 6 and 7 in Fig. 2(d) were smeared, indicating that the structure of whey proteins could be modified by GD treatment. As shown in Fig. 2(c) and (d), little or no soluble aggregate was found under reducing condition at the top of the gel. Presumably, the formation of insoluble aggregates, particularly in the case of Fig. 2(d), were induced by the plasma treatment which led to disappearance of protein bands in SDS-PAGE, as only soluble proteins can be detected in the electrophoretic profile. Analysis of whey proteins demonstrated a higher notable change in protein profile of whey treated by GD than by SD.

Atmospheric cold plasma offers a source of reactive oxygen and nitrogen species along with UV irradiation, which have potential to modify protein structures (Ramazzina et al., 2016). A previous study on the effect of a plasma jet system using the noble gas argon as the working gas on major milk allergens α -casein, β -lactoglobulin and α -lactalbumin showed no significant change in SDS-PAGE band intensity compared to control (Tammineedi et al., 2013). However, the samples were not directly exposed to the plasma discharge or afterglow, unlike the current work, where samples were directly exposed to the tunable discharge.

3.3. Secondary structural analysis

The FTIR spectra of casein and whey proteins were used to define the extent of secondary structural changes induced by cold plasma. The deconvoluted amide I (1600–1700 cm^{-1}) region is widely used in the analysis of protein secondary structure. The resultant peaks at $1620 \pm 20 \text{ cm}^{-1}$, $1645 \pm 5 \text{ cm}^{-1}$, $1654 \pm 4 \text{ cm}^{-1}$, and $1680 \pm 20 \text{ cm}^{-1}$ corresponded to β -sheets, random coils, α -helices, and β -turns, respectively, in the fitting procedure (Hu, Zheng, Liu, Deng, & Zhao, 2016).

As shown in Table 1, the FTIR analysis of the amide I band showed SD and GD treatment changed the secondary structural components of casein, particularly β -sheets and β -turns. The β -sheet content of both SD and GD treated casein decreased. The β -turn content of SD treated casein increased slightly ($p < 0.05$) after 30 min treatment, while the β -turn content of GD treated casein sharply increased. The α -helix content of both SD and GD-treated caseins increased slightly ($p < 0.05$) compared to control. However, the random coil content of SD treated and GD treated caseins was largely unaffected. These data suggested that the proteins were unfolded or were only folded partially by plasma exposure.

The FTIR analysis of the deconvoluted amide I band illustrated the secondary structural components of whey proteins, particularly β -sheets and β -turns, considerably changed after SD or GD treatment (Table 1). The β -sheet content of SD treated whey sharply increased; while the β -sheet content of GD treated whey drastically decreased with increased treatment time. The β -turn content of SD treated whey

decreased, while the β -turn content of GD treated whey increased. SD and GD treatment showed an opposite effect on the fractions of β -sheet and β -turn of whey. The α -helix content of SD-treated and GD-treated whey slightly decreased ($p < 0.05$) compared to control. However, similarly to casein, the random coil content of SD treated and GD treated whey was largely unaffected.

The SD and GD cold plasma processes significantly increased β -turn content and decreased β -sheet content, revealing that the protein structural modifications were dominated by partial unfolding. The β -turn structure is considered a product of protein unfolding of any higher order structures, whereas the anti-parallel β -sheet structure could be formed in aggregated protein molecules (Zhao et al., 2013). Cold plasma discharges of various configurations, can provide RONS in gaseous or liquid form; these can then modify the amino acid side chains, leading to the disruption of non-covalent bonds and alteration in secondary structures (Ekezie et al., 2019). Such rearrangement, destruction and disruption in protein secondary structures due to protein folding/unfolding can ultimately lead to modification of antigen epitopes and attenuation of their antigenicity (Zhu, Wang, Chen, & Zhou, 2018).

3.4. Amino acid profile analysis

Primary protein structure can be changed by modifying amino acid profiles, therefore the amino acid compositions of untreated and treated caseins by SD and GD treatments were assessed (Table 2). The total amino acid contents of SD and GD treated casein declined with increasing treatment time. The concentrations of all the amino acids decreased with increasing SD or GD treatment time. The total amino acid contents of SD and GD treated whey also decreased with increased treatment time (Table 3). Alanine, arginine, cystine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine contents decreased significantly, while aspartic acid, glycine and leucine contents increased with SD and GD treatments of whey proteins. However, glutamic acid content of whey proteins was unchanged after SD or GD treatments.

Sulfur-containing amino acids, such as cystine and methionine, which have been described as particularly susceptible to reactive oxygen species (ROS) (Takai et al., 2014), showed significant decrease ($p < 0.05$) after 10 min SD and GD treatment of casein and whey proteins. The tertiary structures of casein and whey proteins may have collapsed due to the reduction of cystine content after SD or GD treatment. Aromatic amino acids, such as tyrosine and phenylalanine, quantitatively declined using either SD or GD treatments of casein and whey proteins. Five-membered ring amino acids, such as histidine and proline, also declined post-treatment. The structures of sulfur-containing, aromatic and five-membered ring amino acids were reported as modified by hydroxylation, dehydrogenation, nitration and dimerization induced by atmospheric-pressure air microplasma (Zhou et al., 2016). Leucine, as a hydrophobic, aliphatic amino acid, showed a slight increase after SD or GD treatment of whey proteins, in accordance with results from Zhou et al (2016). Glutamic acid and aspartic acid, as acidic, hydrophilic amino acids, were largely unaffected by plasma treatments, as these amino acids are reported as less susceptible to ROS (Pal et al., 2016).

3.5. Antigenicity analysis

ELISA was performed to quantify the change in the antigenicity of casein, α -lactalbumin and β -lactoglobulin treated with SD and GD plasma treatment. The antigenicity of casein after SD and GD treatment is presented in Fig. 3(a) and (b), respectively. The antigenicity of SD and GD treated caseins declined was aligned with increasing treatment time. The antigenicity of caseins treated for 30 min with SD or GD treatments were reduced by 49.9% and 91.1% respectively, by comparison with untreated control. GD treatment was more effective for

Table 1
Secondary structures of SD and GD treated caseins and whey proteins at different treatment time (10, 20, 30 min).

Samples	Secondary structure (%)			
	α -helix	β -sheet	β -turn	random
<i>Casein</i>				
Control	18.80 \pm 0.26 ^a	45.44 \pm 0.21 ^a	23.17 \pm 0.25 ^a	12.6 \pm 0.21 ^a
SD10	19.24 \pm 0.29 ^b	44.6 \pm 0.24 ^b	23.56 \pm 0.37 ^a	12.6 \pm 0.17 ^a
SD20	19.46 \pm 0.1 ^{bc}	44.06 \pm 0.25 ^c	23.60 \pm 0.19 ^a	12.88 \pm 0.09 ^{ab}
SD30	19.68 \pm 0.15 ^c	43.59 \pm 0.25 ^d	23.65 \pm 0.17 ^b	13.08 \pm 0.14 ^b
GD10	19.06 \pm 0.21 ^{ad}	42.16 \pm 0.27 ^{ef}	26.35 \pm 0.28 ^c	12.42 \pm 0.14 ^a
GD20	19.36 \pm 0.15 ^{de}	41.74 \pm 0.32 ^f	26.59 \pm 0.33 ^{cd}	12.31 \pm 0.17 ^{ac}
GD30	19.71 \pm 0.24 ^e	37.99 \pm 0.34 ^g	30.33 \pm 0.53 ^e	11.97 \pm 0.24 ^c
<i>Whey</i>				
Control	18.89 \pm 0.16 ^a	43.04 \pm 0.13 ^a	24.77 \pm 0.24 ^a	13.3 \pm 0.26 ^a
SD10	18.52 \pm 0.29 ^a	47.03 \pm 0.27 ^b	21.74 \pm 0.15 ^{bc}	12.71 \pm 0.16 ^{ab}
SD20	17.41 \pm 0.26 ^{bc}	48.64 \pm 0.38 ^{cd}	21.5 \pm 0.3 ^{bc}	12.45 \pm 0.29 ^{bc}
SD30	17.27 \pm 0.18 ^c	48.91 \pm 0.39 ^d	21.48 \pm 0.34 ^b	12.35 \pm 0.13 ^c
GD10	18.75 \pm 0.26 ^{ad}	39.71 \pm 0.21 ^e	28.01 \pm 0.15 ^d	13.52 \pm 0.06 ^a
GD20	18.37 \pm 0.16 ^d	40.45 \pm 0.38 ^f	27.54 \pm 0.3 ^e	13.63 \pm 0.11 ^a
GD30	17.85 \pm 0.39 ^e	41.29 \pm 0.33 ^g	27.17 \pm 0.15 ^f	13.69 \pm 0.21 ^a

*Values are expressed as mean \pm SD. Values with different letters in the same column are significantly different ($p < 0.05$).

Table 2
Amino acid profile in untreated and treated caseins by SD and GD at different treatment time (10, 20, 30 min) (g amino acid/100 g protein).

Amino acids	Casein control	SD10	SD20	SD30	GD10	GD20	GD30
Alanine	2.38 ^a	1.85 ^b	0.93 ^c	1.58 ^b	2.45 ^a	2.35 ^a	1.95 ^b
Arginine	3.31 ^a	2.85 ^b	0.78 ^c	2.31 ^d	3.22 ^a	2.89 ^b	2.16 ^d
Aspartic acid	7.25 ^a	5.54 ^b	4.56 ^c	5.83 ^b	7.52 ^a	8.59 ^d	7.90 ^c
Cystine	0.29 ^a	0.07 ^b	0.04 ^b	0.06 ^b	0.07 ^b	0.03 ^b	0.01 ^b
Glutamic acid	24.65 ^a	23.35 ^a	21.99 ^b	17.38 ^c	24.45 ^a	24.40 ^a	19.02 ^c
Glycine	1.42 ^a	1.28 ^b	1.25 ^b	1.29 ^a	1.51 ^a	1.48 ^a	1.22 ^b
Histidine	3.33 ^a	2.42 ^b	0.69 ^c	1.88 ^d	3.05 ^a	2.42 ^b	1.69 ^d
Isoleucine	4.51 ^a	3.43 ^b	2.86 ^c	3.02 ^c	4.61 ^a	4.53 ^a	3.57 ^b
Leucine	4.76 ^a	4.08 ^b	2.19 ^c	3.86 ^d	4.92 ^a	5.18 ^c	3.59 ^d
Lysine	5.08 ^a	4.07 ^b	0.84 ^c	3.06 ^d	4.81 ^a	3.99 ^b	1.93 ^c
Methionine	0.23 ^a	0.09 ^b	0.04 ^b	0.05 ^b	0.07 ^b	0.03 ^b	0.02 ^b
Phenylalanine	4.25 ^a	3.72 ^b	2.84 ^c	2.02 ^d	4.31 ^a	3.25 ^b	0.83 ^c
Proline	9.77 ^a	6.52 ^b	4.97 ^c	4.60 ^d	9.06 ^c	7.16 ^f	6.19 ^g
Serine	5.53 ^a	4.16 ^b	2.36 ^c	1.76 ^d	3.16 ^c	1.97 ^f	1.21 ^g
Threonine	3.76 ^a	2.49 ^b	0.89 ^c	0.94 ^c	1.60 ^d	0.61 ^e	0.33 ^f
Tyrosine	6.27 ^a	2.06 ^b	0.02 ^c	0.04 ^c	2.16 ^b	0.05 ^c	0.04 ^c
Valine	4.85 ^a	4.20 ^b	3.52 ^c	3.03 ^d	4.85 ^a	4.67 ^a	3.52 ^c
Total	91.64 ^a	72.17 ^b	50.79 ^c	52.70 ^c	81.81 ^d	73.60 ^b	55.18 ^e

* Values with different letters in a row are significantly different ($p < 0.05$).

allergenicity reduction of casein than SD treatment.

The antigenicity of α -lactalbumin after SD and GD treatment is presented in Fig. 3(c) and (d), respectively. Similarly, α -lactalbumin antigenicity was reduced by 49.5% by SD treatment and 45.5% by GD treatment for 30 min. Apart from the plasma treatment, the heat induced by the cold plasma process may decrease the α -lactalbumin antigenicity. Xu et al. (2015) reported that the α -lactalbumin antigenicity was significantly decreased after heat treatment of 65 °C for 30 min. The antigenicity of β -lactoglobulin after SD and GD treatment is presented in Fig. 3(e) and (f), respectively. In contrast to other proteins, there was a notable increase in antigenicity of β -lactoglobulin observed after SD or GD treatments. β -lactoglobulin antigenicity increased by approximately 2.5 to 3 times compared to control after SD or GD treatment for 10 min.

The reduction in antibody binding potential could be attributed to modification of conformational structure and release of hydrophobic or hydrophilic residues by the cold plasma treatments, which permit re-orientation or destruction of antigenic epitopes. Conversely as observed with β -lactoglobulin, an increase in IgE binding potential could be due to the formation of new epitopes (Sathe, Liu, & Zaffran, 2016). The antibody binding epitopes on casein were characterized by a high

Table 3
Amino acid profile in untreated and treated whey proteins by SD and GD at different treatment time (10, 20, 30 min) (g amino acid/100 g protein).

Amino acids	Whey control	SD10	SD20	SD30	GD10	GD20	GD30
Alanine	4.84 ^a	4.58 ^b	3.73 ^c	3.93 ^c	4.87 ^a	4.69 ^b	4.20 ^c
Arginine	2.27 ^a	2.14 ^a	2.00 ^{ab}	1.92 ^b	2.18 ^a	2.33 ^a	1.90 ^b
Aspartic acid	11.32 ^a	12.32 ^b	12.39 ^b	12.69 ^c	13.37 ^d	11.78 ^{ab}	12.22 ^b
Cystine	2.86 ^a	0.09 ^b	0.00 ^c	0.00 ^c	0.19 ^d	0.00 ^c	0.00 ^c
Glutamic acid	17.14 ^a	17.46 ^b	17.02 ^a	16.63 ^c	17.52 ^b	17.62 ^b	17.19 ^a
Glycine	1.38 ^a	1.42 ^a	1.40 ^a	1.76 ^a	1.69 ^a	1.53 ^a	1.75 ^a
Histidine	2.11 ^a	1.91 ^a	1.66 ^b	1.44 ^b	2.07 ^a	1.53 ^b	1.22 ^c
Isoleucine	6.43 ^a	6.26 ^a	5.14 ^b	4.89 ^b	6.25 ^a	5.34 ^b	4.62 ^c
Leucine	7.13 ^a	8.17 ^b	9.35 ^c	7.97 ^b	7.22 ^a	7.76 ^{ab}	7.94 ^b
Lysine	10.08 ^a	9.51 ^b	8.01 ^c	7.23 ^d	9.70 ^b	7.24 ^d	5.91 ^e
Methionine	2.25 ^a	0.13 ^b	0.06 ^c	0.05 ^c	0.21 ^c	0.26 ^c	0.15 ^b
Phenylalanine	2.92 ^a	2.80 ^a	2.41 ^b	2.25 ^c	2.93 ^a	2.87 ^a	2.33 ^{bc}
Proline	3.94 ^a	3.27 ^b	2.84 ^c	2.72 ^c	3.39 ^b	2.92 ^c	2.58 ^d
Serine	3.47 ^a	2.82 ^b	2.14 ^c	2.08 ^c	3.09 ^b	2.65 ^b	1.71 ^d
Threonine	4.73 ^a	3.85 ^b	2.56 ^c	2.21 ^c	4.48 ^a	3.03 ^d	1.34 ^e
Tyrosine	3.17 ^a	0.14 ^b	0.05 ^c	0.05 ^c	0.10 ^c	0.08 ^c	0.04 ^c
Valine	5.11 ^a	4.55 ^b	4.07 ^c	3.94 ^c	5.04 ^a	4.90 ^a	3.78 ^c
Total	91.15 ^a	81.38 ^b	74.81 ^c	71.75 ^d	84.29 ^b	76.54 ^c	68.86 ^d

*Values with different letters in a row are significantly different ($p < 0.05$).

content of alanine, glutamic acid, leucine, lysine, proline, serine and valine (Busse, Järvinen, Vila, Bayer, & Sampson, 2002; Chatchatee, Järvinen, Bardina, Bayer, & Sampson, 2001; Chatchatee, Järvinen, et al., 2001). Combining the results of amino acid profile analysis and antigenicity analysis, the decrease in these amino acid contents induced by both plasma treatments led to the decrease in casein antigenicity. Asparagine, isoleucine, lysine and valine were the major compositions of antibody binding epitopes on α -lactalbumin; while aspartic acid, glutamic acid and leucine were the major compositions of antibody binding epitopes on β -lactoglobulin (Järvinen, Chatchatee, Bardina, Bayer, & Sampson, 2001). The decrease in these corresponding amino acid contents induced by plasma treatment led to the decrease in α -lactalbumin antigenicity; whilst the increase in these corresponding amino acid contents by plasma treatment led to the increase in β -lactoglobulin antigenicity. Reactive species induced by cold plasma can modify amino acids, which are sensitive to oxidation, hydroxylation and nitration, presumably resulting in amino acid reconciliation or disruption of binding sites for the antibodies investigated in this study.

Although there are multiple mechanisms affecting food allergen reactivity, the underlying concept is the modification of the conformational as well as linear epitopes by cold plasma. For example,

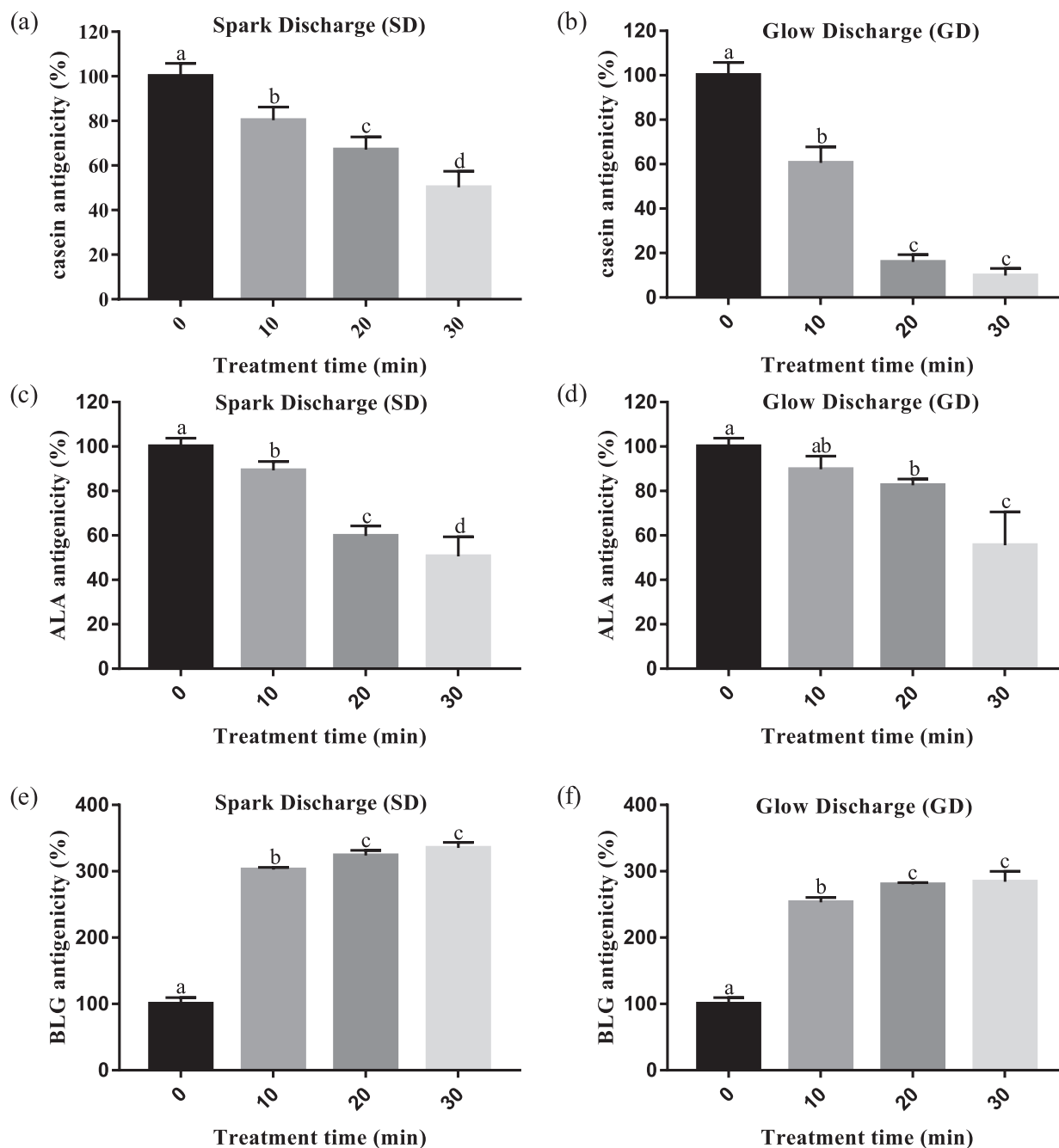


Fig. 3. Sandwich ELISA response of casein subjected to different (a) SD and (b) GD treatment time against antigenicity (%). Sandwich ELISA response of α -lactalbumin (ALA) subjected to different (c) SD and (d) GD treatment time against antigenicity (%). Competitive ELISA response of β -lactoglobulin (BLG) subjected to different (e) SD and (f) GD treatment time against antigenicity (%). *Different superscript letters indicate significantly different ($p < 0.05$) compared with the other.

conformational epitopes can be changed by plasma treatment-induced protein aggregation or crosslinking of proteins as result of a loss of protein solubility, whereas sequence epitopes can be affected by plasma treatment-induced protein fragmentation. The protein aggregation induced by cold plasma could be partly responsible for reducing the food allergen reactivity by rendering the epitopes unavailable. In the case of ingesting a food directly treated in this way, there may be a theoretical risk that such aggregates could still pose a risk if the hidden epitope was re-exposed by digestion.

4. Conclusions

The current study investigated the effect of tunable atmospheric cold plasma treatment on milk proteins (casein, β -lactoglobulin and α -lactalbumin) structure and antigenicity. The results clearly showed that direct cold plasma treatment was effective for attenuation of casein and α -lactalbumin antigenicity as assessed by ELISA. However, β -lactoglobulin antigenicity increased by direct cold plasma treatment, of either SD or GD. Additionally, cold plasma treatments resulted in changes in the content of secondary structures and changes in the amino acid compositions of milk proteins. These modifications in secondary and primary structures may collectively affect the antibody binding

capacity of milk proteins. Thus, cold plasma poses a promising alternative approach to reduce milk derived allergenicity in dairy processing. However, the precise mechanisms through which cold plasma affects antigenicity require further elaboration. There was a strong relationship between either Spark or Glow plasma process duration and efficacy for allergenicity attenuation, which may also be affected by concentration. The results achieved point to application for reducing the allergenicity of food allergen residues in food processing environments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by a research grant from Science Foundation Ireland (SFI) under the Grant Number SFI/16/BBSRC/3391 and the BBSRC under the Grant Reference BB/P008496/1.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2020.128283>.

References

- Attri, P., Kumar, N., Park, J. H., Yadav, D. K., Choi, S., Uhm, H. S., ... Lee, W. (2015). Influence of reactive species on the modification of biomolecules generated from the soft plasma. *Scientific Reports*, 5, 8221. <https://doi.org/10.1038/srep08221>.
- Bannon, G. A. (2004). What makes a food protein an allergen? *Current Allergy and Asthma Reports*, 4(1), 43–46.
- Bloom, K. A., Huang, F. R., Bencharitwong, R., Bardina, L., Ross, A., Sampson, H. A., & Nowak-Wegrzyn, A. (2014). Effect of heat treatment on milk and egg proteins allergenicity. *Pediatric Allergy and Immunology*, 25(8), 740–746. <https://doi.org/10.1111/pai.12283>.
- Bourke, P., Ziuzina, D., Boehm, D., Cullen, P. J., & Keener, K. (2018). The potential of cold plasma for safe and sustainable food production. *Trends in Biotechnology*, 36(6), 615–626. <https://doi.org/10.1016/j.tibtech.2017.11.001>.
- Bu, G., Lu, J., Zheng, Z., & Luo, Y. (2009). Influence of Maillard reaction conditions on the antigenicity of bovine α -lactalbumin using response surface methodology. *Journal of the Science of Food and Agriculture*, 89(14), 2428–2434.
- Busse, P. J., Järvinen, K.-M., Vila, L., Beyer, K., & Sampson, H. A. (2002). Identification of sequential IgE-binding epitopes on bovine α s2-casein in cow's milk allergic patients. *International Archives of Allergy and Immunology*, 129(1), 93–96.
- Chatchatee, P., Järvinen, K.-M., Bardina, L., Beyer, K., & Sampson, H. (2001). Identification of IgE- and IgG-binding epitopes on alpha(s1)-casein: Differences in patients with persistent and transient cow's milk allergy. *The Journal of Allergy and Clinical Immunology*, 107(2), 379–383. <https://doi.org/10.1067/mai.2001.112372>.
- Chatchatee, P., Järvinen, K. M., Bardina, L., Vila, L., Beyer, K., & Sampson, H. (2001). Identification of IgE and IgG binding epitopes on β - and κ -casein in cow's milk allergic patients. *Clinical and Experimental Allergy*, 31(8), 1256–1262.
- Chauvin, J., Judee, F., Yousfi, M., Vicendo, P., & Merbahi, N. (2017). Analysis of reactive oxygen and nitrogen species generated in three liquid media by low temperature helium plasma jet. *Scientific Reports*, 7(1), 4562. <https://doi.org/10.1038/s41598-017-04650-4>.
- Davis, P., & Williams, S. (1998). Protein modification by thermal processing. *Allergy*, 53, 102–105.
- Ekezie, F.-G.-C., Sun, D.-W., & Cheng, J.-H. (2019). Altering the IgE binding capacity of king prawn (*Litopenaeus Vannamei*) tropomyosin through conformational changes induced by cold argon-plasma jet. *Food Chemistry*, 300, Article 125143.
- Hu, G., Zheng, Y., Liu, Z., Deng, Y., & Zhao, Y. (2016). Structure and IgE-binding properties of alpha-casein treated by high hydrostatic pressure, UV-C, and far-IR radiations. *Food Chemistry*, 204, 46–55. <https://doi.org/10.1016/j.foodchem.2016.02.113>.
- Järvinen, K.-M., Chatchatee, P., Bardina, L., Beyer, K., & Sampson, H. A. (2001). IgE and IgG binding epitopes on α -lactalbumin and β -lactoglobulin in cow's milk allergy. *International Archives of Allergy and Immunology*, 126(2), 111–118.
- Kennedy, A., & Bivens, A. (2017). Methods for the Analysis of Underivatized Amino Acids by LC/MS. from https://www.agilent.com/cs/library/applications/5991-8582EN_HILIC_Underivatized_Amino_Acid_application.pdf.
- Liu, G., Li, J., Shi, K., Wang, S., Chen, J., Liu, Y., & Huang, Q. (2009). Composition, secondary structure, and self-assembly of oat protein isolate. *Journal of Agriculture and Food Chemistry*, 57(11), 4552–4558.
- Lu, P., Boehm, D., Bourke, P., & Cullen, P. J. (2017). Achieving reactive species specificity within plasma-activated water through selective generation using air spark and glow discharges. *Plasma Processes and Polymers*, 14(8), 1600207. <https://doi.org/10.1002/ppap.201600207>.
- Lu, P., Boehm, D., Cullen, P., & Bourke, P. (2018). Controlled cytotoxicity of plasma treated water formulated by open-air hybrid mode discharge. *Applied Physics Letters*, 110(26), Article 264102.
- McKenzie, H., Ralston, G., & Shaw, D. (1972). Location of sulfhydryl and disulfide groups in bovine. beta.-lactoglobulins and effects of urea. *Biochemistry*, 11(24), 4539–4547.
- Pal, P., Kaur, P., Singh, N., Kaur, A., Misra, N., Tiwari, B. K., ... Virdi, A. S. (2016). Effect of nonthermal plasma on physico-chemical, amino acid composition, pasting and protein characteristics of short and long grain rice flour. *Food Research International*, 81, 50–57.
- Pankaj, S. K., & Keener, K. M. (2017). Cold plasma: Background, applications and current trends. *Current Opinion in Food Science*, 16, 49–52.
- Quirantes Sierra, B., Lara Jiménez, A., & Skodova, M. (2017). Sensitization to cow's milk protein in a dairy worker. *Occupational Medicine*, 67(7), 579–580.
- Rafiq, S., Huma, N., Pasha, I., Sameen, A., Mukhtar, O., & Khan, M. I. (2016). Chemical composition, nitrogen fractions and amino acids profile of milk from different animal species. *Asian Australasian Journal of Animal Sciences*, 29(7), 1022–1028. <https://doi.org/10.5713/ajas.15.0452>.
- Ramazina, I., Tappi, S., Rocculi, P., Sacchetti, G., Berardinelli, A., Marseglia, A., & Rizzi, F. (2016). Effect of cold plasma treatment on the functional properties of fresh-cut apples. *Journal of Agriculture and Food Chemistry*, 64(42), 8010–8018.
- Sarangapani, C., Danaher, M., Tiwari, B., Lu, P., Bourke, P., & Cullen, P. (2017). Efficacy and mechanistic insights into endocrine disruptor degradation using atmospheric air plasma. *Chemical Engineering Journal*, 326, 700–714.
- Sathe, S. K., Liu, C., & Zaffran, V. D. (2016). Food allergy. *Annual review of Food Science and Technology*, 7, 191–220.
- Savilahti, E., & Kuitunen, M. (1992). Allergenicity of cow milk proteins. *The Journal of Pediatrics*, 121(5), S12–S20.
- Segat, A., Misra, N. N., Cullen, P. J., & Innocente, N. (2015). Atmospheric pressure cold plasma (ACP) treatment of whey protein isolate model solution. *Innovative Food Science & Emerging Technologies*, 29, 247–254. <https://doi.org/10.1016/j.ifset.2015.03.014>.
- Sharma, S., Kumar, P., Betzel, C., & Singh, T. P. (2001). Structure and function of proteins involved in milk allergies. *Journal of Chromatography B: Biomedical Sciences and Applications*, 756(1–2), 183–187.
- Takai, E., Kitamura, T., Kuwabara, J., Ikawa, S., Yoshizawa, S., Shiraki, K., ... Kitano, K. (2014). Chemical modification of amino acids by atmospheric-pressure cold plasma in aqueous solution. *Journal of Physics. D. Applied Physics*, 47(28), Article 285403. <https://doi.org/10.1088/0022-3727/47/28/285403>.
- Takai, E., Kitano, K., Kuwabara, J., & Shiraki, K. (2012). Protein Inactivation by Low-temperature Atmospheric Pressure Plasma in Aqueous Solution. *Plasma Processes and Polymers*, 9(1), 77–82. <https://doi.org/10.1002/ppap.201100063>.
- Tamineedi, C. V. R. K., & Choudhary, R. (2014). Recent advances in processing for reducing dairy and food allergenicity. *International Journal of Food Science and Nutrition Engineering*, 4, 36–42. <https://doi.org/10.5923/j.food.20140402.02>.
- Tamineedi, C. V. R. K., Choudhary, R., Perez-Alvarado, G. C., & Watson, D. G. (2013). Determining the effect of UV-C, high intensity ultrasound and nonthermal atmospheric plasma treatments on reducing the allergenicity of α -casein and whey proteins. *LWT – Food Science and Technology*, 54(1), 35–41. <https://doi.org/10.1016/j.lwt.2013.05.020>.
- Tsabori, S., Dourou, K., & N Priftis, K. (2014). Cow's milk allergenicity. *Endocrine, Metabolic & Immune Disorders-Drug Targets (Formerly Current Drug Targets-Immune, Endocrine & Metabolic Disorders)*, 14(1), 16–26.
- Tsoukou, E., Bourke, P., & Boehm, D. (2018). Understanding the differences between antimicrobial and cytotoxic properties of plasma activated liquids. *Plasma Medicine*, 8(3), <https://doi.org/10.1615/PlasmaMed.2018028261>.
- Venkataratnam, H., Sarangapani, C., Cahill, O., & Ryan, C. B. (2019). Effect of cold plasma treatment on the antigenicity of peanut allergen Ara h 1. *Innovative Food Science & Emerging Technologies*, 52, 368–375. <https://doi.org/10.1016/j.ifset.2019.02.001>.
- Wal, J. M. (1998). Cow's milk allergens. *Allergy*, 53(11), 1013–1022.
- Xu, Q., Shi, J., Yao, M., Jiang, M., & Luo, Y. (2015). Effects of heat treatment on the antigenicity of four milk proteins in milk protein concentrates. *Food and Agricultural Immunology*, 27(3), 401–413. <https://doi.org/10.1080/09540105.2015.1117059>.
- Zhang, Y., Deng, Y., & Zhao, Y. (2017). Structure-based modelling of hemocyanin allergenicity in squid and its response to high hydrostatic pressure. *Scientific Reports*, 7, 40021. <https://doi.org/10.1038/srep40021>.
- Zhao, Q., Xiong, H., Selomulya, C., Chen, X. D., Huang, S., Ruan, X., ... Sun, W. (2013). Effects of spray drying and freeze drying on the properties of protein isolate from rice dreg protein. *Food and Bioprocess Technology*, 6(7), 1759–1769.
- Zhou, R., Zhou, R., Zhuang, J., Zong, Z., Zhang, X., Liu, D., ... Ostrikov, K. (2016). Interaction of Atmospheric-Pressure Air Microplasmas with Amino Acids as Fundamental Processes in Aqueous Solution. *PLoS ONE*, 11(5), Article e0155584. <https://doi.org/10.1371/journal.pone.0155584>.
- Zhu, Y., Wang, W., Chen, T., & Zhou, G. (2018). The effect of ultra-high pressure and heat on the allergenicity and structural properties of bovine serum albumin. *Biomedical Research* (0970-938X).