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Effect of pulsed electric field and temperature on the secondary structure changes of β-lactoglobulin by using FTIR spectroscopy

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Abstract

The secondary structures of β-lactoglobulin in its native and denatured states when subjected to different temperatures and pulsed electric field (PEF) were evaluated by Fourier transform infrared (FTIR) spectroscopy. The denaturation of β-lactoglobulin was achieved in deuterium oxide (D_2O) solution either through heating at various temperatures or through PEF nonthermal treatment. The secondary structures of the denatured β-lactoglobulin differed according to the temperature (25 - 95^oC) of denaturation. The secondary structure of β-lactoglobulin was defined by FTIR spectroscopy after treatment with PEF. β-Lactoglobulin solution samples were treated by PEF at the electric field strengths of 20 and 30 kV cm^{-1}. The number of the electric pulses used for β-lactoglobulin ranged 0 to 200. Under all conditions (temperature and PEF), the changes in aggregation bands of β-lactoglobulin (1600 ~ 1700 cm^{-1}) were observed. Thus the electric pulses of PEF used this study did not make detectable changes in the secondary structure of the β-lactoglobulin investigated. The results provide a useful model for studying change in protein secondary structure changes under thermal and nonthermal processing conditions.

Key Words: pulsed electric field (PEF), heat treatment, secondary structure of protein, protein denaturation, FTIR spectroscopy.

Introduction

β-Lactoglobulin, which is found in the milk of several mammalian species, is one of the most abundant proteins of bovine milk whey (Dufour *et al*., 1994). The protein is made up of 162 amino acid residues and contains two disulfide groups and one free sulfhydryl group (Swaisgood, 1982). β-Lactoglobulin, which constitutes about 50% of the total protein in whey, tends to govern the behavior of the total whey protein system. Because of its dominant role, many researchers have sought to elucidate the properties of β-lactoglobulin to gain a better understanding of the properties of whey proteins in general (Nielsen *et al*., 1996).

Griffin et al. (1993) found that when β-lactoglobulin A was heated for 4 min at various temperatures, maximum aggregate size occurred when the protein solution was heated at ~ 85°C, supporting the notion that there were two different reaction mechanisms with different temperature dependences. Bovine β-lactoglobulin exists in a number of different genetic variants (Creamer & Harris, 1997), and there are commercially significant differences between the responses of milk (or whey) containing either β-lactoglobulin A or β-lactoglobulin B to heat treatment (Anema & McKenna, 1996; Boye *et al*., 1997).

The heat-induced denaturation of β-lactoglobulin is assumed generally to be a process which consists of at least two steps: a partial unfolding of the native protein and a subsequent aggregation of unfolded molecules. The factors that influence denaturation have been reviewed (Mulvihill & Kinsella, 1987). Secondary structural patterns of proteins are characterized by periodic structures such as helices, sheets and extended portions, as

well as a variety of turns, loops and disordered coils. In general, secondary structure of protein can be determined by several types of instrumental methods such as X-ray crystallography and nuclear magnetic resonance (NMR), circular dichroism (CD) and infrared (IR) spectroscopy (Kumosinski & Farrell, 1993).

A protein gives rise to many bands in infrared spectroscopy. Of these bands, the amide I band, resulting from C=O stretching vibration of the protein backbone is particularly sensitive to folding patterns in the secondary structural level. The hydrogen bonding between C=O and N-H groups of the peptide linkage determines the geometry of the polypeptide backbone which in turn determines the frequency of the IR band. Since a protein usually contains different secondary structural elements such as α-helix, β-sheet, β-turn and random coil, the amide I band is a composite band constituted of overlapping signals (Bhattacharjee *et al*., 2005). However, the band narrowing method coupled with computerized data handling enables one to resolve the bands which can be attributed to individual secondary structural elements. This also makes quantification of secondary structure possible, provided unambiguous assignments of the bands are possible (Surewicz *et al*., 1993).

β-Lactoglobulin has been studied by Casal *et al*. (1988) who reported the conformational change of β-lactoglobulin induced by change of pH and temperature. Dong *et al.* (1996) studied the subtle structural differences between β-lactoglobulin A and B forms by infrared spectroscopy. Qi *et al*. (1997) examined the possibility of the existence of a molten globule state of β-lactoglobulin at higher temperatures using an FTIR technique.

Nonthermal food processing methods are of great interest in the food industry, which recognizes their potential to replace or complement the traditional thermal processing methods. Pulsed electric fields (PEF) technology has been applied in food processing. It involves the application of pulses of high voltage to liquid or semi-solid foods placed between two electrodes. While it is well demonstrated that pulsed electric fields of adequate voltage efficiently inactivate many kinds of microorganisms (Wouters & Smelt, 1997; Barsotti & Cheftel, 1999; Wouters *et al*., 1999), much less is known concerning the effects of pulsed electric fields on proteins and other food constituents (De Jong & Van Heesch 1998; Barsotti & Cheftel, 1999; Fernandez-Diaz *et al*., 2000; Li *et al*., 2005). Studies on enzyme inactivation by pulsed electric fields of enzyme solutions or liquid foods reveal varying results, perhaps partly due to differences in electric systems and pulse types (Vega-Mercado *et al*., 1995; Barbosa-Cánovas *et al*., 1996, 1998; Ho et al., 1997; Barsotti & Cheftel, 1999, Yang *et al*., 2004). Fernandez-Diaz *et al*. (2000) investigated the effect of PEF on ovalbumin solutions and dialyzed egg white and indicated that the electric pulses of high-field strength (~31.5 kV cm^{-1}) did not cause notable modifications in ovalbumin and dialyzed egg white. Recently, Li *et al*. (2005) investigated the effect of PEF on the stability and secondary structure of bovine immunoglobulin G and concluded that the PEF treatments ranging from 0 to 41.1 kV cm^{-1}, for 0 to 91.4 μs did not cause detectable changes in either bovine IgG secondary structure or immunoactivity.

This present study deals with the effects of pulsed electric fields and temperature on the structure changes of β-lactoglobulin. In this work, we describe FTIR spectroscopy studies on the structural changes of β-lactoglobulin during PEF nonthermal method and heat treatment.

Materials and Methods

Materials

β-Lactoglobulin was purchased from Sigma (St. Louis, MO, USA) and was used without further purification. Deuterium oxide (D_2O) (product 15, 188-2, minimum 99.9 atom %D) was purchased from Sigma (St. Louis, MO, USA).

Sample Preparation

The β-lactoglobulin solution was prepared by dissolving the powder at 5% (w/v) in deuterium oxide (D_2O). D_2O was used for making the buffers in which the protein was dispersed because of its greater transparency in the region of interest (1600-1700 cm^{-1}). It should be mentioned that the preparation and storage history of the β-lactoglobulin sample from Sigma was not known. Changes may occur in the secondary structural characteristics of the β-lactoglobulin variants during their isolation and purification.

Heat treatment

The β-lactoglobulin sample (5% w/v β-lactoglobulin in D_2O) was stored at a small content, then was measured the pD with the Accumet pH meter and the temperature with the thermocouple. The heat treatment was set up by FTIR from 25-95^{o}C. The temperature of the sample was regulated by placing the cell in a thermostatic holder employing an Omega temperature controller (Omega Engineering, Laval, QC, Canada). The temperature was

increased in 5°C increments in 10 minutes and the cell was allowed to equilibrate for 10 min prior to data acquisition. The reported temperatures were accurate to within ± 0.5°C.

Pulsed electrical field treatment

Electrical treatment of the protein solution was made with high electric field pulse generator, the circuit of which is shown in Figure 1. A variable autotransformer AT (Powerstat Type 3PN116C 0-140 V) was used to supply the circuit. The input voltage was regulated by autotransformer AT to obtain a pulse frequency of 1 Hz. The voltage was then transformed by the high voltage transformer - T (model 62159A - Apotex Inc., Weston, ON, Canada) and rectified by diode D. The capacitor C was charged through the limited resistor R. The charge capacitor voltage and the initial treatment voltage supplying the treatment chamber were the same and depended on the distance between the spheres of discharger, ℓ_{sph}. The discharger was made from stainless steel spheres of 15 mm diameter. The break voltage V_0 for this diameter can be calculated by the following equation (Armyanov et al., 2001):

$$V_0 = 4.85\ \ell_{sph}^{0.75}$$

The sample was placed in a treatment chamber specially designed to hold approximately 2.0 ml and was subjected to electrical treatment as shown in the Figure 2. The well of the chamber was equipped with stainless steel electrodes (as shown at 1 and 2 in Figure 2) and the walls (3) were made of Teflon material. The distance between the two electrodes (gap) was 5.0 mm (shown at 4) which was filled with the sample. Four screws

(5) were used to keep the electrodes tight and prevent them of moving due to the high pressure obtained during high voltage treatment.

The treatment chamber was filled with the β-lactoglobulin sample (5% w/v β-lactoglobulin in D_2O) and it was exposed to 0, 40, 80, 120, 160, and 200 pulses at an electric field strength 20 and 30 kV cm^{-1} with the capacitor's capacitance of discharge set at 20 or 60 nF. The treatment chamber was washed and dried before putting in the new sample for PEF treatment. The pulse width was determined by measuring the current that passed through the sample using a current monitor (Model 411, Pearson Electronics, Inc., CA, USA) connected to a digital oscilloscope (model 54621A, Agilent Technologies, Inc., CA). The temperature and pD (pH meter corrected for deuterium isotope effect) of the samples were measured before and after the treatment using a thermocouple and pH meter (Accumet 25, Fisher Scientific Inc., NH), respectively. The initial temperature of the sample was about 17^{o}C. The pD was measured with the Accumet pH meter and the value was corrected according to pD = pH + 0.4 (Lefèvre & Subirade, 2001).

FTIR Measurements

FTIR spectra of the β-lactoglobulin solutions having undergone PEF treatment and heat treatment were recorded with an 8210 Nicolet FTIR spectrometer equipped with a deuterated triglycine sulfate detector (Boye *et al*., 1995). A total of 512 scans were averaged at 4 cm^{-1} resolution. Wavenumber accuracy was within (0.01 cm^{-1}). The spectrometer was purged with dry air from a Balston dryer (Balston, Haverhill, MA, USA). The samples were held in an infrared cell with CaF_2 windows separated by 50 μm

polyethylene terephthalate film spacers. Deconvolution of the observed spectra was performed using the Nicolet FTIR Software, Omnic 6.0 (Thermo Electron Inc., Bellefonte, PA, USA). The deconvolution of the infrared spectra was done by using Fourier self-deconvolved (FSD) and the bandwidth used for deconvolution was 20 cm^{-1} with the enhancement set at 2.4. All FTIR experiments were done in duplicate.

Results and Discussion

Effect of temperatures on the secondary structure changes of β-lactoglobulin

The Fourier self-deconvolution infrared spectra of β-lactoglobulin at pD 7.6 as a function of temperature from 25 to 95^{o}C during the heating steps are shown in Figure 3. Heat treatment significantly altered the secondary structure of β-lactoglobulin when the temperature was greater than 70°C. The secondary structure changes of the β-lactoglobulin took place gradually, but a critical temperature apparently exists. The FTIR spectra of β-lactoglobulin at 25^{o}C showed five bands at 1692 (β-sheet), 1678 (turn), 1648 (α-helix), 1634 (anti-parallel β-sheet) and 1623 cm^{-1} (β-sheet). When the temperature was set at 65^{o}C, the 1692 and 1623 cm^{-1} bands were disappeared and a new band appeared at 1684 cm^{-1}, the band at 1678 cm^{-1} shifted to 1674 cm^{-1} and the band at 1648 cm^{-1} shifted to 1644 cm^{-1} and the band at 1634 cm^{-1} shifted to 1631 cm^{-1}. The intensity of the band 1684 cm^{-1} increased with increasing temperature while the band at 1678 cm^{-1} disappeared after the temperature was higher than 70^{o}C. As the temperature was higher than 75^{o}C, the new band 1616 cm^{-1} appeared and a marked increase in the intensity of the band at 1616 cm^{-1} was observed accompanied by an apparent increase in the 1684 cm^{-1} band. Upon denaturation of β-

lactoglobulin, the major changes observed in the protein secondary structure were a decrease in the intensity of the band 1648 cm^{-1} which had been attributed to an α-helical structure (Casal *et al*., 1988; Susi & Byler, 1988; Boye, 1995); a substantial decrease in the intensity of the band 1634 cm^{-1} attributed to anti-parallel β-sheet structures (Susi & Byler, 1988). The increase in the intensity of these bands suggests an increase in side-chain vibration formation with increasing temperature. The 1678, 1648 and 1634 cm^{-1} bands shifted by 2-5 cm^{-1} to lower wavenumbers as the temperature were increased from 25 to 65^{o}C. Such shifting of the amide I bands to lower wavenumbers may be attributed to a decrease in the strength of the C=O bond stretching vibration resulting from an increase in hydrogen bonding (Krimm & Bandekar, 1986). On aggregation of β-lactoglobulin, the major changes observed were an increase in the intensity of the bands at 1684 and 1616 cm^{-1} attributed to intermolecular hydrogen-bonded β-sheet structures (Ismail *et al*., 1992; Boye, 1995).

The Fourier self-deconvolution spectra of β-lactoglobulin at pD 7.6 as a function of temperature from 95 to 25^{o}C during the cooling steps are shown in Figure 4. As temperature decreased from 95 to 25°C, no major conformational changes were observed except the amide I bands at wavenumbers 1640 and 1616 cm^{-1} have shifted to lower wavenumbers by about 2-5 cm^{-1}. On cooling the heated β-lactoglobulin solution, the spectra of β-lactoglobulin kept almost the same profile, which suggested that β-lactoglobulin remained in its aggregated form and that aggregation was an irreversible process. This study confirmed the findings of Bhattacharjee & Das (2000) who showed that

β-lactoglobulin heated beyond 70^{o}C underwent an irreversible change in secondary structure during subsequent cooling.

Effect of PEF on the secondary structure changes of β -lactoglobulin

The energy applied to the sample during PEF treatment depends on the voltage capacity of discharged capacitor. This investigation used pulses with energies 1 and 2.25 J with a pulse width of 1 μs, as well as pulses with energies 3 and 6.75 J and a pulse width of 3 μs. The sample temperature before treatment was 17^{o}C and after treatment the temperature differed according to circuit parameters and number of pulses. Sample temperature within 1 min from the last pulse never exceeded 29^{o}C.

For an electric field strength E = 20.0 kV cm^{-1}, capacitor C= 60 nF the temperatures varied 17 to 26^{o}C, depending on the number of pulses. At an electric field strength E = 20.0 kV cm^{-1}, capacitor C= 20 nF, the temperatures varied 17 to 19^{o}C. Similar results were obtained at a higher electric field strength E = 30.0 kV cm^{-1}, capacitor C = 60 nF where the temperature changed from 17 to 28^{o}C and an electric field strength E = 30.0 kV cm^{-1}, capacitor C = 20 nF where the temperature changed from 17 to 22^{o}C. All of these showed that the high voltage PEF treatment increased the sample temperature, but in this case the higher temperature was not reflected to the β-lactoglobulin denaturation. Compared to the heat treatment of β-lactoglobulin, the FTIR spectra of β-lactoglobulin showed almost no change, confirming that the secondary structure of β-lactoglobulin had suffered no changes when the temperature was between 17 to 28^{o}C with PEF treatment.

The Fourier self-deconvolution infrared spectra of β-lactoglobulin with PEF treatments (electric field strength E = 20.0 kV cm^{-1}, capacitor C= 60 nF and different pulse numbers of 0 to 200) are shown in Figure 5. The FTIR spectra of β-lactoglobulin with no PEF treatment showed five bands at 1692 (β-sheet), 1678 (turn), 1648 (α-helix), 1634 (anti-parallel β-sheet) and 1623 cm^{-1}(β-sheet). These bands had almost no shifts as the pulse number was increased from 0 to 200. That is to say that the spectra of β -lactoglobulin had no detectable change with PEF treatment. The shape of the FTIR spectra of the PEF-treated samples were the same as that acquired from the non-treated PEF sample. PEF treatments, ranging from 0 to 20 kV cm^{-1}, demonstrated advantages in maintaining β-lactoglobulin intact.

The Fourier self-deconvolution infrared spectra of β-lactoglobulin with PEF treatments (electric field strength E = 20.0 kV cm^{-1}, capacitor C= 20 nF and different pulse numbers 0 to 200) are shown in Figure 6. The FTIR spectra of β-lactoglobulin with no PEF treatment showed the same five bands as the previous case (C = 60 nF; Figure 5). Again, these bands had no shifts as the pulse number was increased from 0 to 200. No detectable changes in the FTIR spectra were observed between the control sample and the PEF-treated β-lactoglobulin samples, implying that the PEF-treated β-lactoglobulin samples maintained the same secondary structure characteristics as that of the nontreated β-lactoglobulin sample.

The Fourier self-deconvolution infrared spectra of β-lactoglobulin with PEF treatments (electric field strength E = 30.0 kV cm^{-1}, capacitor C= 60 nF and different pulse numbers 0

to 200) are shown in Figure 7. The temperatures of β-lactoglobulin shifted from 17 to 28^{o}C with different pulse numbers with the greater electric field strength. The FTIR spectra of β-lactoglobulin with no PEF treatment showed the same five bands, and again no shifts were observed as the pulse numbers were increased from 0 to 200 indicating that there was no significant difference in the secondary structure of β-lactoglobulin before and after PEF treatments.

Conclusion

In this study, the secondary structure of β-lactoglobulin was found to behave differently when subjected to different temperatures and PEF treatment. With rising temperatures, the spectra of β-lactoglobulin were altered at temperatures exceeding 65^{o}C, and especially at temperatures above 75^{o}C. But when the temperature was decreased from 95 to 25^{o}C, the spectra of β-lactoglobulin showed almost no change and the β-lactoglobulin remained in its aggregated form and that aggregation was an irreversible process. But no detectable changes in the secondary structure of β-lactoglobulin were found to occur with the PEF treatments ranging from 0 to 30 kV cm^{-1} and from 0 to 200 pulses, indicating that nonthermal processing methods could maintain the secondary structure of β-lactoglobulin and allow the β-lactoglobulin keep its native physicochemical properties. Further studies will be required to determine the nature of molecular interactions involved in the aggregation of β-lactoglobulin under different PEF parameters, different temperatures and different protein concentrations.

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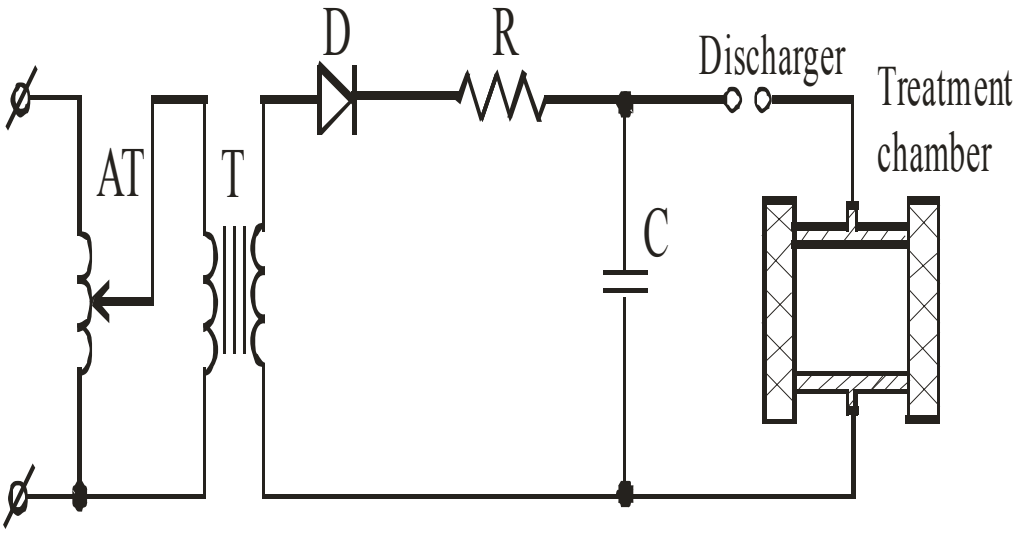

Figure 1. Electrical circuit for high voltage treatment

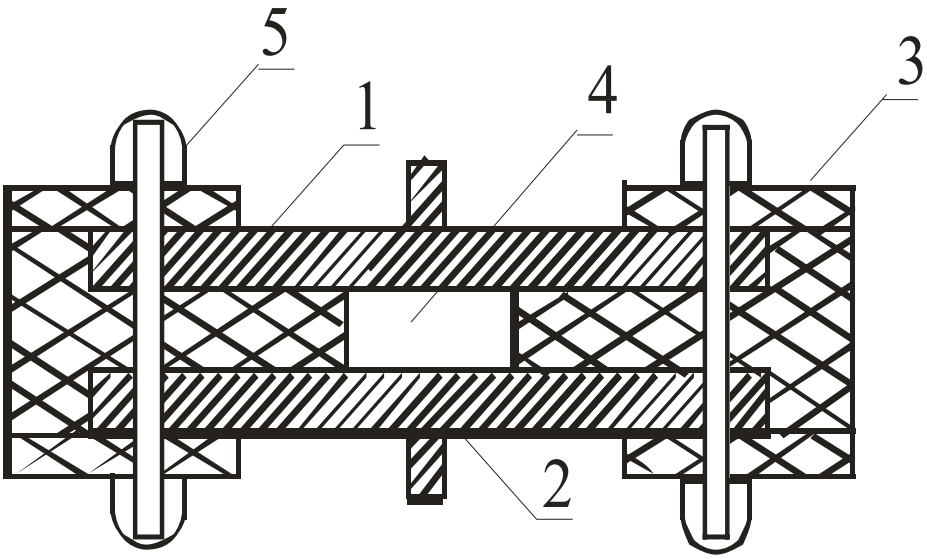

Figure 2. Pulsed electric field treatment chamber

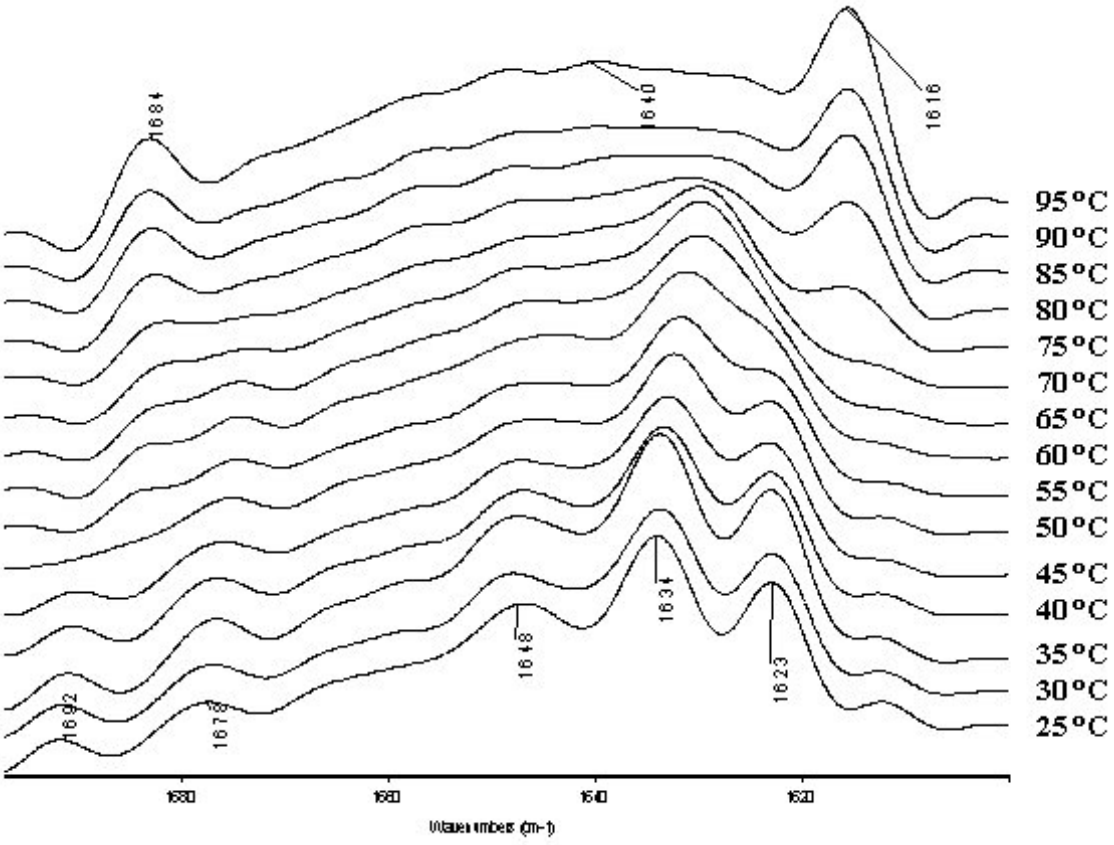

Figure 3. The Fourier self-deconvolution (FSD) spectra of the β-lactoglobulin at temperatures during the heating steps

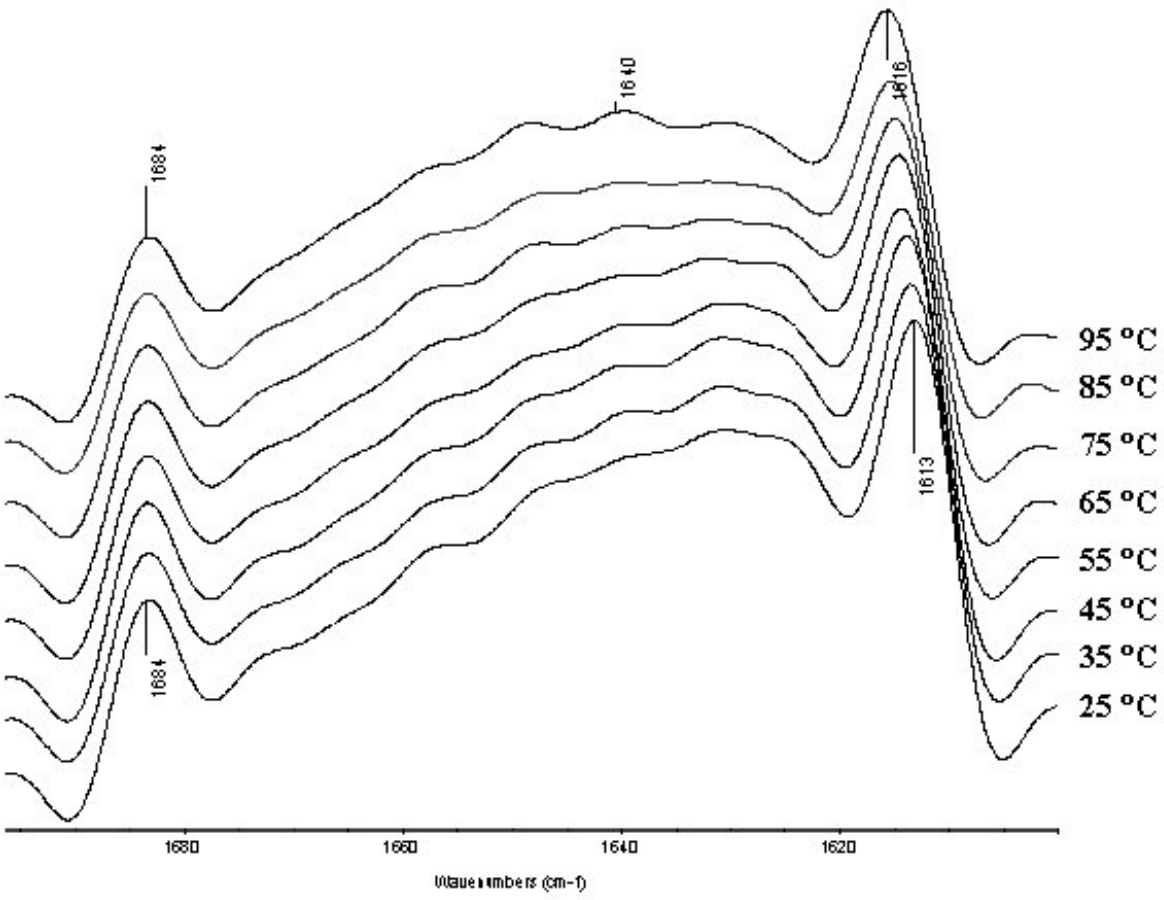

Figure 4. The Fourier self-deconvolution (FSD) spectra of the β-lactoglobulin at temperatures during the cooling steps

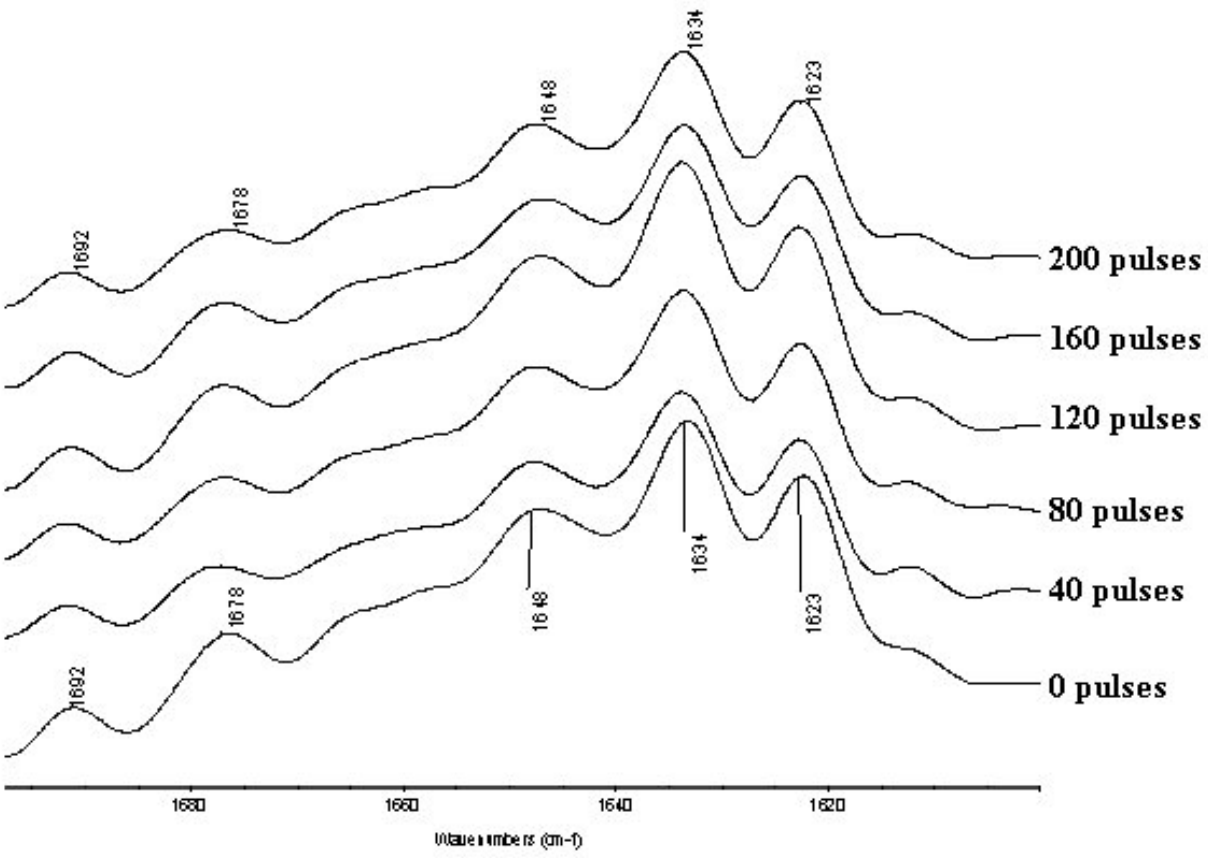

Figure 5. The Fourier self-deconvolution (FSD) spectra of the β-lactoglobulin at electric field strength E = 20.0 kV cm^{-1}, capacitor C= 60 nF)

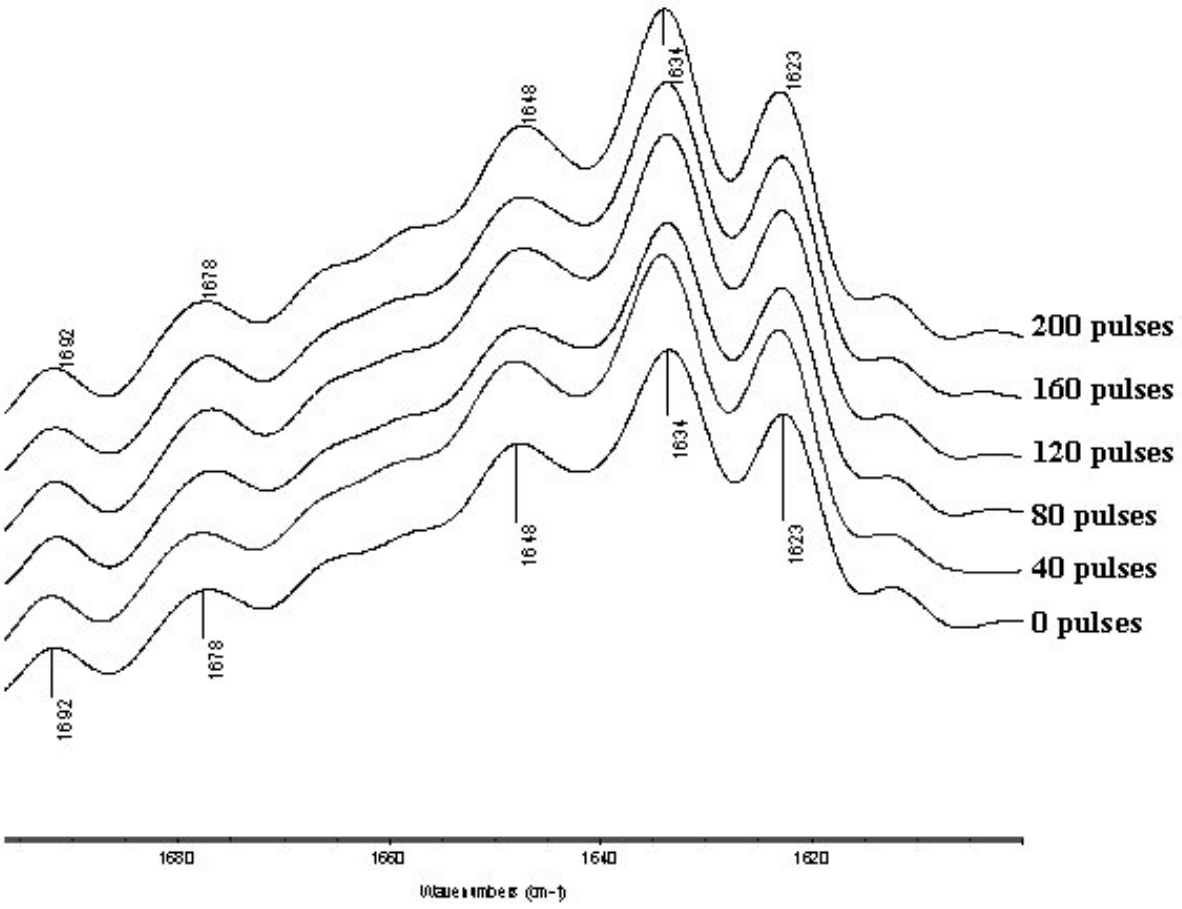

Figure 6. The Fourier self-deconvolution (FSD) spectra of the β-lactoglobulin at electric field strength E = 20.0 kV cm^{-1}, capacitor C= 20 nF)

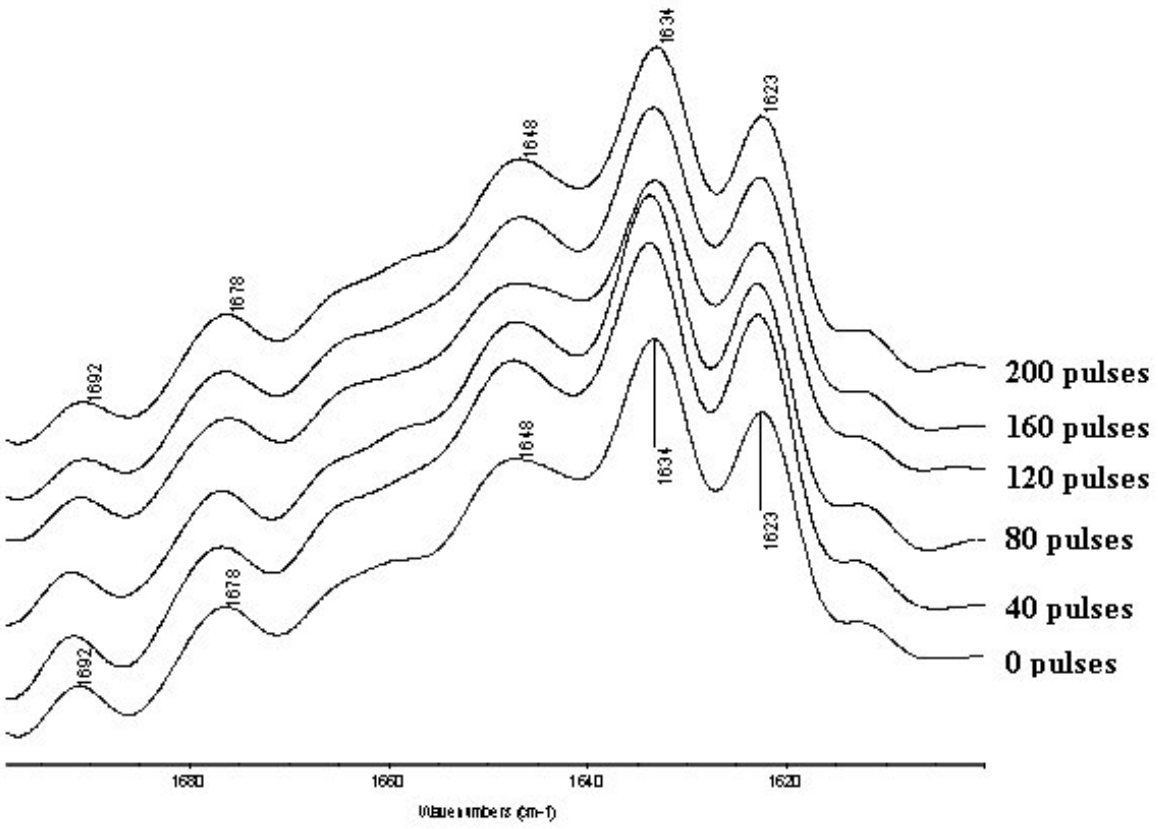

Figure 7. The Fourier self-deconvolved (FSD) spectra of the β-lactoglobulin at electric field strength E = 30.0 kV cm^{-1}, capacitor C= 60 nF)