

# The structural stability of protein antigens adsorbed by aluminium hydroxide in comparison to the antigens in solutions

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**Abstract.** It is believed that antigens should be adsorbed onto adjuvants in vaccines. The adsorption-modified structure of antigens is important to understand the mechanism of adjuvants and vaccine immunogenicity. The structural stability of antigens is of major importance. The changes in structure can be induced by degradation and/or increase of storage temperature. In this study the structural stability of two model antigens, bovine serum albumin (BSA) and  $\beta$ -lactoglobulin (BLG) were compared when they were adsorbed onto aluminium hydroxide and when they were in solutions using Fourier transform infrared – attenuated total reflection (FTIR-ATR) spectroscopy. The structural stability of these two proteins was studied at different temperature and during storages. The present results showed that the structure of antigens can be stabilized by adsorption onto aluminium hydroxide. Non-adsorbed protein antigens present in vaccines may facilitate the degradation of the vaccine.

Keywords: Structural stability, FTIR-ATR, antigens, aluminium

## Abbreviations

BSA, Bovine serum albumin;  
BLG,  $\beta$ -Lactoglobulin;  
FTIR, Fourier transform infrared spectroscopy;  
ATR, Attenuated total reflection;  
ADT, Accelerated degradation test.

## 1. Introduction

Aluminium-containing adjuvants are used in vaccines to adsorb antigens and enhance the immune response upon injection. Although the mechanism by which aluminium-containing adjuvants potentiate the immune response is not fully understood, it is generally accepted that the antigens should be adsorbed onto adjuvants [1]. The World Health Organization recommends that at least 80% of tetanus and

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diphtheria toxoids should be adsorbed onto aluminium-containing adjuvants [2]. Therefore, vaccines are produced by selecting adjuvants, pH, buffer and/or ionic strength to achieve maximum adsorption of the antigens [1]. Previous studies [3,4] showed conflicting results regarding to the secondary structure when antigens were adsorbed onto aluminium hydroxide adjuvants. Jones et al. [3] demonstrated by FTIR-ATR spectroscopy that protein changed its secondary structure due to adsorption and that this structural change was one of the mechanisms by which aluminium adjuvants enhances the immune response of allergens. On other hand, Dong and his co-worker [4] showed by using the same technique that no protein structural change occurred.

Therefore, further investigations of secondary structures of antigens when adsorbed to aluminium hydroxide are needed in order to elucidate, if modification of protein structure occurs. Stability of adsorption-modified protein structure is of major importance, since modifications may induce degradation of the antigens during storage. Antigen degradation could potentially pose risk to patients. The term stability, when it relates to proteins, is often used in a variety of ways, including chemical and/or physical degradation [5]. In the present study we are primarily concerned with the secondary structure of proteins. Two model antigens bound to adjuvant were investigated during heating and storage in comparison to the antigens in solutions using FTIR-ATR spectroscopy. For the storage experiments, apart from normal storage temperature, i.e. 4°C, an accelerated degradation test (ADT) was also employed, which subjected the samples to an elevated temperature, i.e. 37°C, at which denaturation should occur more rapidly than at normal temperature. It is well known that some vaccines are temperature sensitive [6]. Both high and low (freezing) temperatures can cause inactivation of vaccines. In this work, structural stability of the model antigens was in addition investigated at different heating temperatures, since temperature alterations are an important problem during the transportation and storage of vaccines, especially in developing countries where the quality of the 'cold chain' is not always optimal [7].

## 2. Materials and methods

### 2.1. Materials

Clinical reagent grade bovine serum albumin (BSA, reference L84960) and  $\beta$ -lactoglobulin (BLG, reference L3908) were purchased from ICN biomedical Inc and Sigma, respectively. Both proteins were used directly without further purification. Aluminium hydroxide was purchased from Superfos Biosector A/S. A stock aluminium hydroxide was prepared by adjusting pH to 8.0 by the addition of 1 M NaOH before use. All other chemicals were analytical grade and obtained from commercial sources without further treatment.

### 2.2. Sample preparation

A stock protein solution (20 mg/ml) was prepared by dissolving protein in Milli-Q water. Model vaccines were prepared by mixing the appropriate amount of protein stock solution and aluminium hydroxide with end-over-end rotation for 30 min to obtain the maximum protein adsorption. The final calculated aluminium concentration was 3.4 mg/ml and the final protein concentrations 4.0 mg/ml. Protein water solutions were prepared by diluting the stock solution to 4 mg/ml using Milli-Q water. Blank samples containing 3.4 mg/ml aluminium hydroxide without proteins were prepared by diluting the stock aluminium adjuvant using Milli-Q water.

### 2.3. FTIR-ATR measurement

Spectra were collected using a FTIR spectrometer (PerkinElmer Spectrum One), which was mounted with a three bounces ZnSe ATR crystal (PerkinElmer). In the storage analysis, samples were stored at 4°C or 37°C between measurements. All samples were measured at room temperature. In the heating analysis experiments, the samples were divided into different vials and were heated at different temperatures (25, 37, 50, 60, 70, 80, 90°C) for 30 min, respectively; and reached to room temperature before FTIR measurement.

Total 8  $\mu\text{l}$  sample was deposited onto the ATR crystal and evaporated the solvent to make a film. The calculated amount of aluminium hydroxide for each film was 27.2  $\mu\text{g}$ ; and the calculated amount of protein was 32  $\mu\text{g}$ . Spectra were directly recorded on the film by co-adding 128 scans with a resolution of 4  $\text{cm}^{-1}$  at a scan speed 0.2  $\text{cm/s}$ . Control spectra were recorded by measuring the blank samples which contained the same components except protein under the same experimental conditions. All samples were analyzed at least in duplicate.

### 2.4. Data analysis

All FTIR spectra were analyzed using the The Unscrambler v. 9.2 software (Camo process AS, Oslo, Norway). Replicate spectra of the same sample were averaged first. For the adsorbed proteins, the spectra of a corresponding blank sample were subtracted from the spectra of the adsorbed proteins. A flat baseline from 1900 to 1740  $\text{cm}^{-1}$  was obtained for each spectrum after subtraction. A 23-point Savitsky–Golay smoothing function was applied to each spectrum from 1800  $\text{cm}^{-1}$  to 1200  $\text{cm}^{-1}$ . The second derivative spectra were calculated using an 11-point Savitsky–Golay function from 1750 to 1220  $\text{cm}^{-1}$ . The inverted second derivative spectra were obtained by multiplying by  $-1$  for convenient interpretation. Finally, the spectra were baseline corrected and mean normalized.

## 3. Results and discussion

### 3.1. Structural stability of protein antigens at different temperatures

FTIR-ATR spectroscopy was applied to monitor the secondary structure of the model antigens in solution as well as adsorbed onto aluminium hydroxide as a function of temperature. The potential changes of the secondary structure were detected in the amide I (1700–1600  $\text{cm}^{-1}$ ) and amide II (1600–1500  $\text{cm}^{-1}$ ) regions of the inverted secondary derivative spectra.

For BSA adsorbed by aluminium hydroxide, the distinguishable spectral changes occurred at or above 70°C (see Fig. 1). It showed that the  $\alpha$ -helix band around 1653  $\text{cm}^{-1}$  decreased along with an increase in  $\beta$ -sheet around 1636  $\text{cm}^{-1}$ . Another spectral difference in the amide I region observed was the intensity of the band at high wavenumber 1693  $\text{cm}^{-1}$ . This band increased as a function of temperature, especially above 70°C. In the amide II region, the intensity of bands around 1549  $\text{cm}^{-1}$  and 1533  $\text{cm}^{-1}$  decreased when the temperature increased. However, compared to the spectra of BSA in solution shown in Fig. 2, the above mentioned heat-induced spectral changes were less pronounced. BSA in solution showed significantly low thermal stability. A band resulting from intermolecular aggregation was induced around 1626 to 1622  $\text{cm}^{-1}$ , even at very low temperature. This band increased as a result of increasing temperature. Two other bands increased around 1695  $\text{cm}^{-1}$  and 1680  $\text{cm}^{-1}$  when elevated temperature. These three bands are related to intermolecular anti-parallel  $\beta$ -sheet formation [8–10].

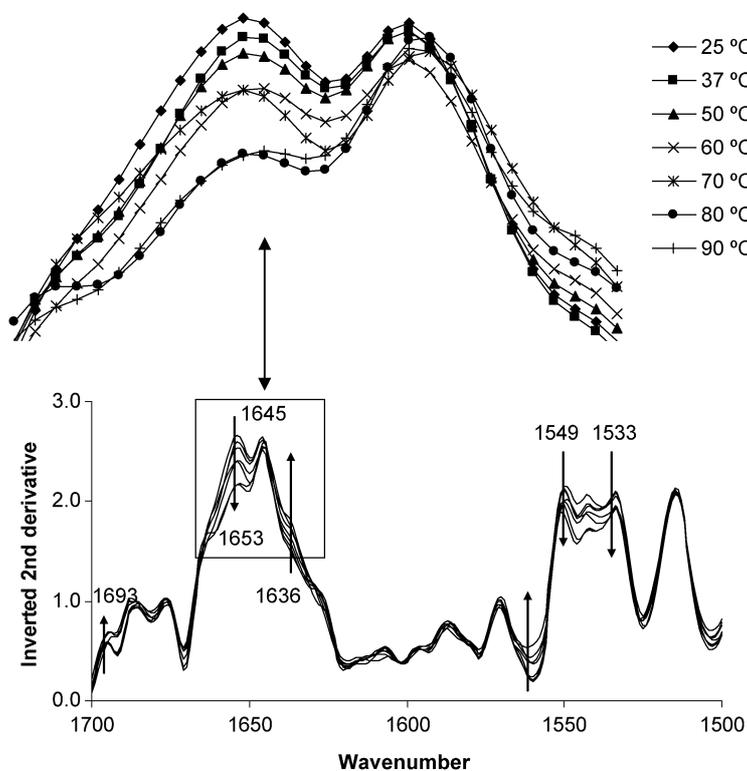


Fig. 1. Inverted 2nd derivative spectra of adsorbed BSA at different temperatures in amide I and amide II regions. BSA was adsorbed to aluminium hydroxide and incubated for 30 min before heating at different temperatures for 30 min. The spectra were collected at room temperature. The directions of arrows show the shifts in spectra of samples at increasing temperatures.

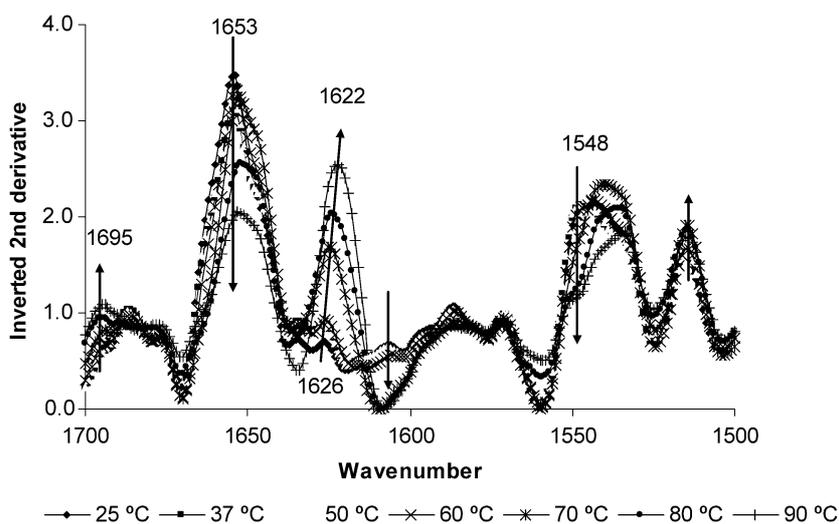


Fig. 2. Inverted 2nd derivative spectra of BSA solution at different temperatures in amide I and amide II regions. BSA solution was heated at different temperatures for 30 min, measured at room temperature. The directions of arrows show the shifts in spectra of samples at increasing temperatures.

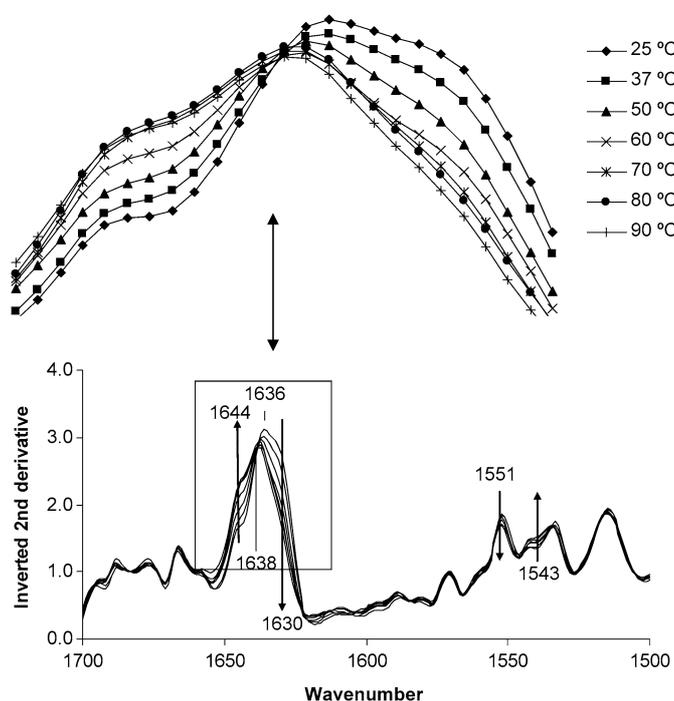


Fig. 3. Inverted 2nd derivative spectra of adsorbed BLG at different temperatures in amide I and amide II regions. BLG was adsorbed to aluminium hydroxide and incubated for 30 min before heated at different temperatures for 30 min. The spectra were collected at room temperature. The directions of arrows show the shifts in spectra of samples at increasing temperatures.

These three aggregation-induced bands were also observed in other heat-denatured protein aggregates [11–13]. In the amide I region, the main  $\alpha$ -helix band at  $1653\text{ cm}^{-1}$  shifted to  $1652\text{ cm}^{-1}$  and the intensity decreased dramatically with increasing temperature. In other words, BSA in solution loses  $\alpha$ -helix structure and gains intermolecular  $\beta$ -sheet structure at high temperature.

The spectral difference between adsorbed BSA and BSA solution revealed that a modified structure was induced by adsorbing onto aluminium hydroxide, which agreed with previous study [3]. The less changeable spectra of adsorbed BSA indicated that adsorption-modified structure increased the protein thermal stability, which prevented intermolecular aggregating of protein during the heating process.

In the case of BLG, the spectra of adsorbed protein at different temperature were shown in Fig. 3, which were also significantly different from that of the protein in solution, seeing Fig. 4. The bands of adsorbed protein were located at wavenumbers that were not similar to the corresponding components of BLG in solution, which indicated that the secondary structure of adsorbed BLG changed as compared to native protein. As the temperature increased, the band of the adsorbed BLG around  $1630\text{ cm}^{-1}$  lost intensity in Fig. 3, but there was a gain in the random structure as seen around  $1644\text{ cm}^{-1}$ . The main broad  $\beta$ -sheet band shifted from  $1636\text{ cm}^{-1}$  to  $1638\text{ cm}^{-1}$ .

For BLG solution, the spectra at different temperatures were presented in Fig. 4. The main  $\beta$ -sheet band appeared at  $1628\text{ cm}^{-1}$  and the band broadened with increasing temperature. It can be seen that the band at  $1694\text{ cm}^{-1}$  increased at high temperatures (80,  $90^\circ\text{C}$ ), but no peak was induced around  $1623\text{ cm}^{-1}$ . The association state of BLG results from equilibrium between its monomeric and dimeric forms. Dimeric BLG is characterized by having two components around  $1632\text{ cm}^{-1}$  and  $1623\text{ cm}^{-1}$ , while monomeric BLG reveals only one component near  $1630\text{ cm}^{-1}$  [14]. Therefore, in the current ex-

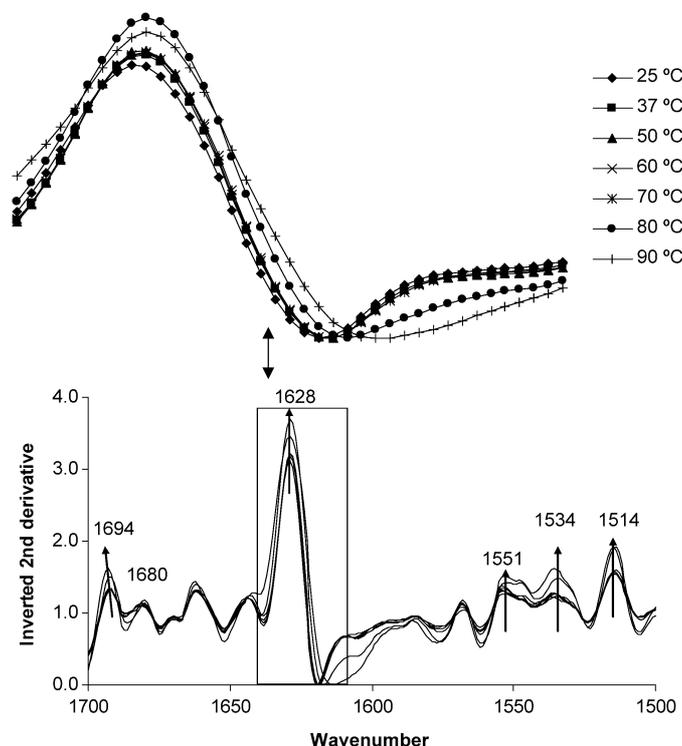


Fig. 4. Inverted 2nd derivative spectra of BLG solution at different temperature in amide I and amide II regions. BLG solution was heated at different temperatures for 30 min but measured at room temperature. The directions of arrows show the shifts in spectra of samples at increasing temperatures.

perimental conditions, BLG seemed to be primarily in the monomeric form. This probably resulted from low protein concentration [15,16]. Infrared spectra of BLG varied significantly in the low concentration range [17]. The spectra were characterized by one band between 1640 and 1623  $\text{cm}^{-1}$  at low concentrations, whereas two bands around 1634 and 1623  $\text{cm}^{-1}$  were present at high concentrations. High temperatures [17] and low ionic strength [18] may also result in the BLG spectrum having one FTIR band between 1640 and 1623  $\text{cm}^{-1}$  instead of two bands around 1634 and 1623  $\text{cm}^{-1}$ .

A small band around 1680  $\text{cm}^{-1}$  was induced for BLG solution at high temperature, i.e. 80 and 90°C. It can also be attributed to the formation of intermolecular  $\beta$ -sheets that result from the self-aggregation of the proteins [19]. The intensity of this band was weak probably because the spectra were recorded at room temperature. The decreased temperature may facilitate refolding of the BLG structure. In amide I region the intensity decreased around 1610–1600  $\text{cm}^{-1}$ . It is usually not assigned to any secondary structural elements, which makes understanding the increasing intensity rather difficult. In the amide II region, the intensity of bands around 1551, 1534 and 1514  $\text{cm}^{-1}$  increased significantly when elevated temperatures.

No intermolecular aggregation was observed for adsorbed protein at high temperature, i.e. above 70°C, while protein in solution aggregated under similar conditions. The results suggested that the adsorption onto aluminium hydroxide can prevent intermolecular aggregation of the antigen when it was exposed to high temperature. However, the structure of adsorbed protein also changed slightly at low temperature, i.e. 37°C. Jones and co-workers [3] demonstrated that the transition of aluminium hydroxide adsorbed BSA started at approximately 35°C using differential scanning calorimetry. This temperature was lower

than the temperature which caused transition of BSA in solution. Thus, it was concluded that proteins adsorbed to aluminium-containing adjuvant show significantly reduced thermal stability compared to the protein in solution. However, they observed the temperatures at which the protein started to destabilize, and made no conclusion concerning the high temperature effects. The difference of the FTIR measurements in these two studies was that Jones et al. [3] measured the slurry after centrifugation compared to evaporated films which were analyzed in this work. Another difference is Jones et al. [3] measured the sample at the denaturing temperature in comparison to room temperature in this work. The buffer difference in vaccine preparation may also play an important role in the structural stability of BSA [20]. The thermal effect on the stability of adsorbed BSA and BLG is not the same. This result agrees with the study of Jones et al. [3] showing that the stability of ovalbumin was less affected by adsorption than BSA.

### 3.2. Structural stability during the storage at two temperatures

The structural stability of adsorbed antigen is important especially when the vaccine is stored for long periods prior to use. It is well known that vaccines lose their potency during storage, especially at high temperature [21]. The mechanism by which vaccine loses potency is not clear. The structural stability may influence the immunogenicity of the vaccine. FTIR-ATR spectroscopy was applied here to monitor the structural changes of protein antigens during the storage in the amide I and the amide II region.

The alteration of the secondary structure of two model antigens during 4 months of storage was studied in this work. The second derivative FTIR spectra of adsorbed BSA which was stored both at 37°C and 4°C did not change much (see Fig. 5). Similar spectra indicated that the structure of BSA was

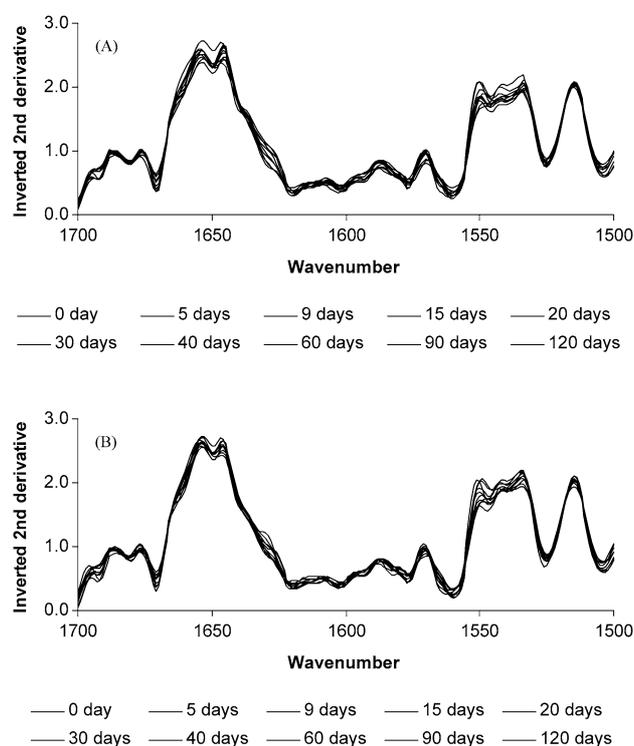


Fig. 5. Inverted 2nd derivative spectra of adsorbed BSA during 120 days storage in amide I and amide II regions. BSA was adsorbed to aluminium hydroxide and stored at 37°C (A) and 4°C (B) respectively.

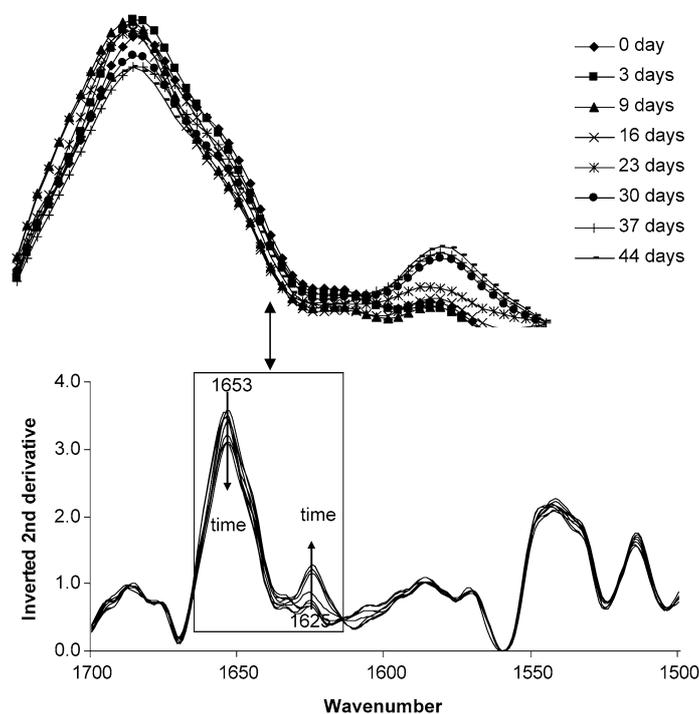


Fig. 6. Inverted 2nd derivative spectra of BSA solution after 44 days storage at 37°C in amide I and amide II regions.

stable, when adsorbed to aluminium hydroxide. Slight spectral differences did occur, but the changes were irregular and the spectral variability may be caused by experiment-to-experiment variation and inconsistent measurement of the temperature.

The stability of BSA in solution when stored at 37°C and 4°C were also investigated. The secondary structure of BSA in solution changed dramatically in 44 days when stored at 37°C (Fig. 6) and white sedimentation was observed within 65 days. A new peak was induced at 1625 cm<sup>-1</sup> after 16 days storage. The intensity of the peak increases as a function of time. The band is due to the intermolecular anti-parallel  $\beta$ -sheet formation. On the other hand, the  $\alpha$ -helix band at 1653 cm<sup>-1</sup> decreased with increasing storage time. Thus, for BSA in solution,  $\alpha$ -helix was reduced accompanied by an increase in anti-parallel  $\beta$ -sheet explained by intermolecular aggregation. BSA in solution was stable when it is stored at 4°C. The spectrum changed slightly after 65 days of storage (data not shown).

In the case of BLG, the secondary derivative FTIR spectra of adsorbed BLG stored at 37°C and 4°C were given in Fig. 7 and Fig. 8, respectively. The  $\beta$ -sheet band around 1636 cm<sup>-1</sup> shifted to higher wavenumbers, and the intensity decreased as a function of time. The change was more pronounced in the early stage of the storage, i.e. first three weeks. On the other hand, the bands from 1606 to 1587 cm<sup>-1</sup> increased with storage. In the amide II region, bands around 1551 and 1534 cm<sup>-1</sup> increased. The results agreed with another study [21], in which it demonstrated that adsorption of BLG to an oil–water interface lead to time-dependent changes in the protein structure. The dramatic changes of adsorbed BLG may result from free protein in the samples. BLG (4 mg/ml) cannot be totally adsorbed by 3.4 mg/ml aluminium hydroxide. Adsorbed BLG was released from aluminium hydroxide during storage, while the amount of adsorbed BSA remained constant (data not shown). Moreover, if 8 mg/ml BSA was added to 3.4 mg/ml aluminium hydroxide it would give more non-adsorbed BSA in the system and the sam-

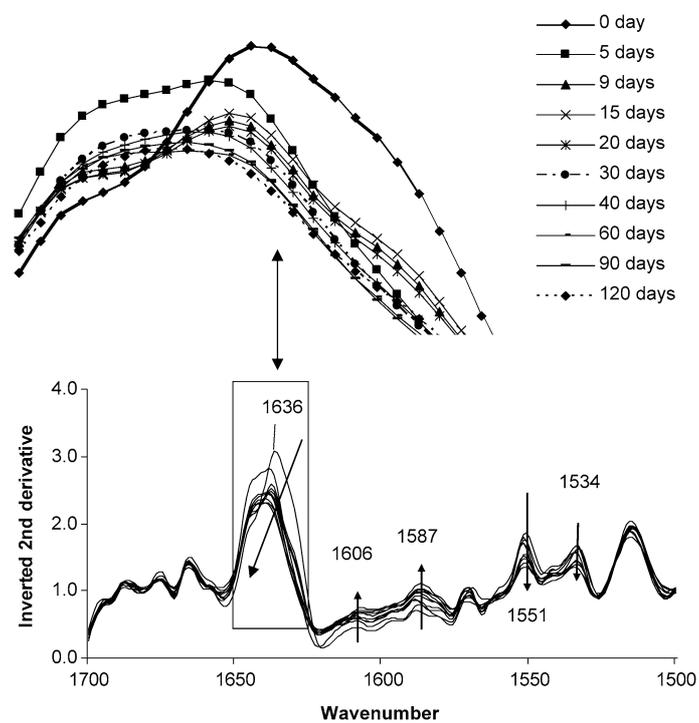


Fig. 7. Inverted 2nd derivative spectra of adsorbed BLG after 120 days storage at 37°C in amide I and amide II regions.

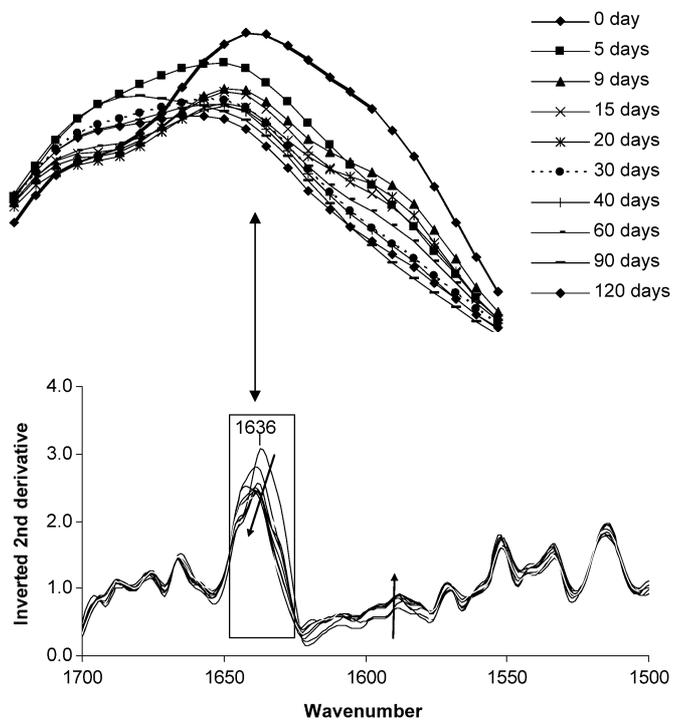


Fig. 8. Inverted 2nd derivative spectra of adsorbed BLG after 120 days storage at 4°C in amide I and amide II regions.

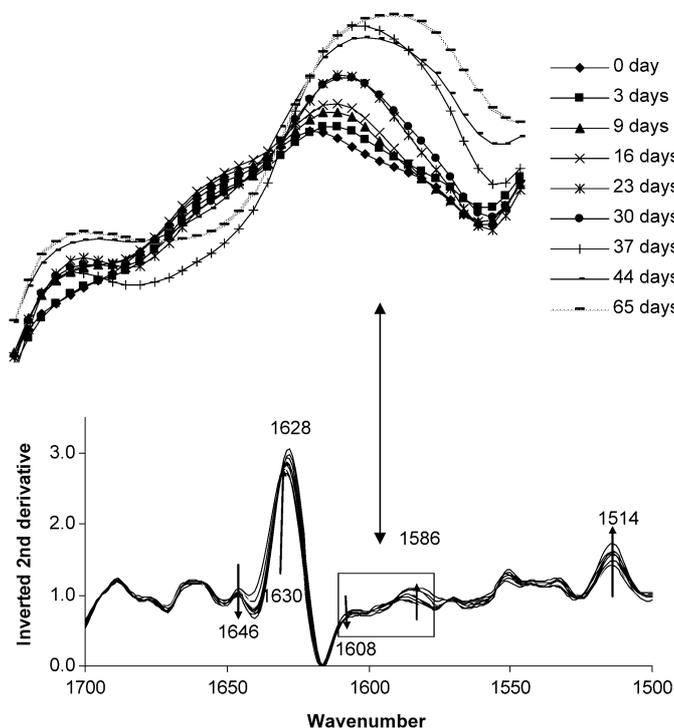


Fig. 9. Inverted 2nd derivative spectra of BLG solution after 65 days storage at 37°C in amide I and amide II regions.

ple aggregates rapidly. The data did not allow for an accurate estimation of structural analysis due to the aggregation after 4 weeks at 37°C. Therefore, non-adsorbed antigens in vaccines may facilitate the deterioration of the vaccine. Furthermore, the alteration of pH from 7.6 to 7.0 during storage may also contribute a little to the structural change of adsorbed BLG.

For BLG in solution stored at 37°C and 4°C, the FTIR spectra were shown in Figs 9 and 10, respectively. The  $\beta$ -sheet band at 1630  $\text{cm}^{-1}$  increased and it shifted to lower wavenumber around 1628  $\text{cm}^{-1}$  for BLG solution with increasing storage time at 37°C. It indicated that the molecules of BLG aggregate due to intermolecular interaction. If BLG solution was stored at 4°C, the  $\beta$ -sheet band at 1630  $\text{cm}^{-1}$  lost intensity as function of storage time. However, a band around 1545  $\text{cm}^{-1}$  increased, which can be described as random structure. In other words, BLG lost  $\beta$ -sheet but gained random structure when stored at low temperature. When BLG solution was stored at 37°C, the variability of the spectra around 1586  $\text{cm}^{-1}$  was evident. The intensity of this band increased as function of time.

#### 4. Conclusion

This preliminary study showed that the structure of an antigen can be stabilized by adsorption to aluminium hydroxide during exposure to high temperature and/or during storage. The phenomenon is obvious for BSA when it is stored at 37°C. In the case of BLG, due to the low protein concentration and non-adsorbed protein influence, it is not pronounced. Non-adsorbed protein antigens present in vaccines may facilitate the degradation of the vaccine. It requires further experiments to confirm this hypoth-

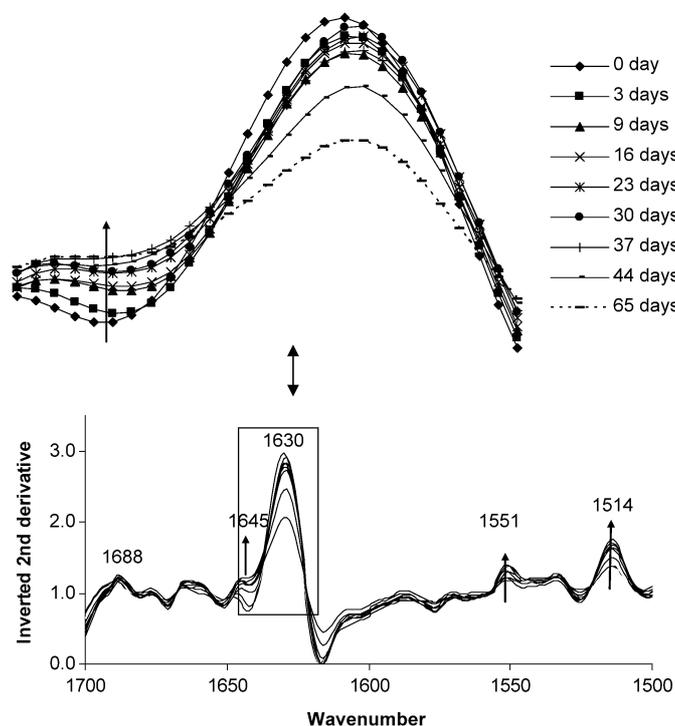


Fig. 10. Inverted 2nd derivative spectra of BLG solution after 65 days storage at 4°C in amide I and amide II regions.

esis. As expected, at elevated temperature, i.e. 37°C, two proteins no matter adsorbed by aluminium hydroxide or not degrade more rapidly than at the normal storage temperature, 4°C.

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