Structural changes of protein antigens due to adsorption onto and release from aluminium hydroxide using FTIR–ATR

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Abstract. Structural integrity of antigens upon adsorption and release is not only important for investigating vaccine immunogenicity, but also for the epitope specificity of the resulting immune response and hence therapeutic efficacy. Moreover, the structural information is also important for understanding the mechanism of how adjuvants can enhance the immune response. However, little is known about an antigen's structure when it is adsorbed on and subsequently released from aluminium adjuvants. In this study, the structures of two protein antigens, bovine serum albumin and β-lactoglobulin, were investigated using Fourier transform infrared–attenuated total reflection (FTIR–ATR) spectroscopy. The secondary structures of both model antigens change when adsorbed to aluminium hydroxide. The structural perturbation depends on the amount of adsorbed protein. Maximal adsorption gives a more native-like structure. This may indicate that protein is adsorbed in different manners depending on the concentration. The adsorbed antigens are released using phosphate buffer pH 7.4 (PB). The recovery is approximate 80% after 40 min in the presence of PB. The recovery curves of both proteins also indicate two different adsorption modes. FTIR–ATR and circular dichroism (CD) spectroscopy yield similar results suggesting that the adsorbed antigens refold to their native-like state after release.

Keywords: Antigen, secondary structure, FTIR–ATR, aluminium hydroxide

Abbreviations

BSA	Bovine serum albumin;
BLG	β-Lactoglobulin;
FTIR	Fourier transform infrared spectroscopy;
ATR	Attenuated total reflection;
PB	Phosphate buffer solution;
CD	Circular dichroism;
BLA	Bovine α-lactalbumin.

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1. Introduction

Antigens are proteins capable of inducing specific immune responses when injected to humans. The molecular properties of antigens and the way in which these properties contribute to immune activation are central to determine the outcome of vaccination. All four levels of protein organization – primary, secondary, tertiary, and quaternary – affect its immunogenicity [1]. The structural information of the antigen is not only important for investigating its immunogenicity; it is also important in the understanding of interactions between antigen and adjuvant and the mechanism of how adjuvant can enhance the immune response. In general, proteins have a very fragile three-dimensional structure [2,3], and this structure may easily change in different environments, such as binding to membranes, at air-water interfaces, under pressure, or even during drying and storage. Protein adsorption processes are believed to cause structural changes in the protein. Previous studies [4–8] have shown that adsorption of a protein onto interfaces or solid surfaces induce structural changes in the protein. The structural change during absorption may lead to irreversible aggregate formation, prevent the ability to induce an immune response, or even cause serious side-reactions. Protein aggregation is difficult to control and hampers precise prediction of the release profile of the protein. Furthermore, protein aggregates may potentially constitute risk to patients.

Aluminium adsorbed vaccines have been safely used for many years. However, the structure of the antigen, when adsorbed to aluminium-containing adjuvants, has not been well characterized. Only a few studies [9–11] have addressed the structure of aluminium adsorbed proteins due to the lack of experimental techniques allowing structural information to be obtained directly on this complex system. Hem and White [9] demonstrated that a slight deformation of the structure of pepsin occurred when adsorbed by aluminium hydroxide using infrared spectroscopy. They regarded this as an example of ligand exchange. Jones et al. [10] studied the structural changes of model antigens as a function of temperature by FTIR–ATR spectroscopy to investigate vaccine stability. However, Dong and his co-workers [11] showed that protein antigen did not change its secondary structure due to adsorption onto aluminium adjuvants by FTIR–ATR. To our knowledge, there is no available structural information of released antigens from aluminium-containing adjuvants.

FTIR spectroscopy is a method well suited to determine the secondary structure of globular proteins. It offers many advantages over other spectroscopic techniques [12,13]. A major advantage is the lack of dependence on the physical state of the sample. The sample can either be gas, aqueous or organic solution, hydrated film, inhomogeneous suspension, or solid. FTIR is particularly suitable for the analysis of proteins adsorbed by other materials, especially when an ATR crystal is used. FTIR was applied to study the loss of secondary structure during insulin unfolding in a lipid–water interface model [14] and protein adsorption by silica, polysulfone, and other biomaterials [15–18]. The assignments of secondary structure bands of protein in a FTIR spectrum are shown in [19–24].

CD spectroscopy is a method suited to monitor the secondary and tertiary structure of proteins in solution [25]. Far-UV CD (170–250 nm) spectroscopy is a good tool to obtain the overall information of the secondary structure. However, the analyses involve complex calculations using a standard set of reference proteins as a basis for estimation and thus yield only indirect measures [26]. Moreover, CD spectroscopy is not a suitable technique for measuring the adsorbed protein in suspension because light cannot pass through such high density samples.

In this study, FTIR–ATR and CD spectroscopy were applied to investigate the structural changes of protein antigens. Model antigens were adsorbed by aluminium hydroxide and subsequently released using phosphate buffer (PB) at pH 7.4. PB was chosen because phosphate anions easily adsorb onto the

surface of aluminium hydroxide through ligand exchange displacing the antigens from the aluminium adjuvant [27]. Bovine serum albumin (BSA) and β-lactoglobulin (BLG) were used as model antigens because their structures have been well studied. They are α-helix-rich and β-sheet-rich proteins, respectively. BSA consists of 66% α-helix and BLG contains 46% β-sheet [28]. BSA is a small globular protein with a molecular weight of approximate 66.4 kDa, and pI of BSA is 4.8 [29]. BLG has a well-known structure containing one very short helix segment and eight strands of anti-parallel β-sheet, which wrap to form an anti-parallel β-barrel. BLG exists at neutral pH as a dimer in its native state. Each monomer is identical and constitutes of 162 amino acids, with one free thiol group and two disulphide bridges. The pI of BLG is 6.5 and the molecular weight is approximately 18 kDa [30,31].

2. Materials and methods

2.1. Materials

Clinical reagent grade BSA (reference L84960) and approximate 90% pure BLG (reference L3908) were purchased from ICN biomedical Inc and Sigma respectively. Both proteins were used directly without further purification. Aluminium hydroxide adjuvant was purchased from Superfos Biosector A/S (Denmark) and used without further treatment. Other chemicals were all of analytical grade and obtained from commercial sources.

2.2. Adsorption experiments

2.2.1. Sample preparation
A protein stock solution (10 mg/ml) was prepared by dissolving protein in Milli-Q water. Working samples were prepared by mixing the appropriate amount of protein stock solution and aluminium hydroxide and subsequently diluting to 1 ml using Milli-Q water. The final calculated aluminium concentration was 3.4 mg/ml for all samples; the final protein concentrations were from 1 mg/ml to 5 mg/ml. Samples were mixed gently for 30 min to obtain the maximum protein adsorption before FTIR–ATR measurement.

2.2.2. FTIR–ATR measurement
Spectra were collected using a FTIR spectrometer (PerkinElmer Spectrum One), which was mounted with a three bounces ZnSe ATR crystal (PerkinElmer). An 8 µl sample was deposited onto the ATR crystal by evaporating the solvent to obtain a film. The calculated amount of aluminium hydroxide for a film was 27.2 µg; and the calculated value of protein was from 8 µg to 40 µg, respectively. Spectra were directly recorded on the film by co-adding 128 scans with a resolution of 4 cm^{-1} at a scan speed 0.2 cm/s. Control spectra were collected from samples containing the same components except protein and at same experimental conditions. All samples were analyzed at least in duplicate.

2.3. Release experiments

2.3.1. Sample preparation
Adsorbed protein antigens were prepared as described in Section 2.2. The final concentration of aluminium hydroxide was 3.4 mg/ml for each sample; final protein concentration was 4 mg/ml for BSA and 3 mg/ml for BLG. The samples were gently rotated at room temperature for one hour, and stored at 4°C overnight. Adsorbed proteins were released by adding PB pH 7.4 to a final concentration from 5 to 200 mM at room temperature. Samples were incubated for 40 min before FTIR–ATR analysis.

2.3.2. FTIR–ATR measurements

FTIR–ATR measurements were performed on the same instrument as described in Section 2.2. Measuring films were made in either of two ways: by evaporating the complex of protein and aluminium hydroxide; or by measuring released protein alone after centrifugation at $6800 \times g$ for 10 min. Blank samples containing the same components except protein were analyzed under the same condition. All samples were measured at least in duplicate.

2.3.3. CD measurements

Adsorbed protein was released using PB pH 7.4 at a final concentration of 150 mM for 40 min. Control samples were prepared using the same components except protein. Free protein solutions were prepared by dissolving appropriate amounts of protein in the supernatant which was obtained from control experiment.

CD measurements were carried out using an Olis spectropolarimeter (On-line instrument systems, Inc., USA), equipped with a temperature control system. Protein solutions were scanned in a 0.1 cm quartz cuvette five times at 15°C. In order to compare the CD spectra of the free protein and the released protein, protein concentrations were identical. Free protein solutions were also measured from 15°C to 90°C, with 5°C interval. At each temperature, the sample was incubated for 2 min before scanning.

2.3.4. Ultraviolet (UV) spectrophotometric measurements

After 40 minutes release, the samples were centrifuged at $6800 \times g$ for 10 min, and the supernatants were measured using an UV/VIS spectrophotometer (Lamda 800, PerkinElmer instruments) at 280 and 320 nm. The protein recovery from aluminium hydroxide was calculated from the following equation:

$$R = \frac{(A'_{280} - A'_{320})/\varepsilon - (A_{280} - A_{320})/\varepsilon}{AC} \times 100\%,$$

where R is protein recovery, ε is extinction coefficient, AC is adsorption capacity, A_{280} is the absorbance of protein at 280 nm in Milli-Q water. A'_{280} is absorbance at 280 nm in the presence of PB, likewise with A_{320} and A'_{320}.

2.4. Data analysis

2.4.1. FTIR data

All FTIR spectra were analyzed using Unscrambler Ver. 9.2 software (Camo process AS). The replicated spectra of the same sample were averaged first. The spectrum of a corresponding blank sample was subtracted from the spectrum of adsorbed proteins. A flat baseline from 1900 to 1740 cm^{-1} was obtained for each spectrum after background subtraction. A window size of 23-point Savitsky–Golay smoothing function was applied to each spectrum. The second derivative spectra were calculated using a window size of 11-point Savitsky–Golay function from 1750 to 1220 cm^{-1}. The inverted second derivative spectra were obtained by multiplying with -1 for convenient interpretation. Finally, the spectra were baseline corrected, mean normalized, transferred into Excel file and plotted.

2.4.2. CD data

Before analysis, a CD spectrum of a control sample, which contained all components except protein, was subtracted from each CD spectrum.

3. Results and discussion

3.1. Protein adsorption by aluminium hydroxide

Typical FTIR spectra of BSA and BLG adsorbed by aluminium hydroxide and recorded on films are shown in Fig. 1(A). For comparison, the spectrum of pure aluminium hydroxide is also presented in same figure. It is seen that amide I (1700–1600 cm^{-1}) and amide II (1600–1500 cm^{-1}) bands are not affected by the absorption of aluminium hydroxide enabling the aluminium hydroxide background subtraction. The subtracted spectra in the region from 1900 cm^{-1} to 1200 cm^{-1} are given in Fig. 1(B). Subtraction of background is dangerous, because over or under subtraction of the spectra may damage the final spectra. A simple and practical method is to subtract background until the region from 1900 to 1740 cm^{-1} is flat. This method works well with most proteins when adsorbed by other materials, if it is applied consistently. However, studies have also shown that the positions and intensities of the bands are maintained even with deliberate over or under subtraction of the background spectrum [32]. In this study, potential alterations of the secondary structure of model antigens are monitored in the amide I and amide II region of the final inverted 2nd derivative FTIR spectra.
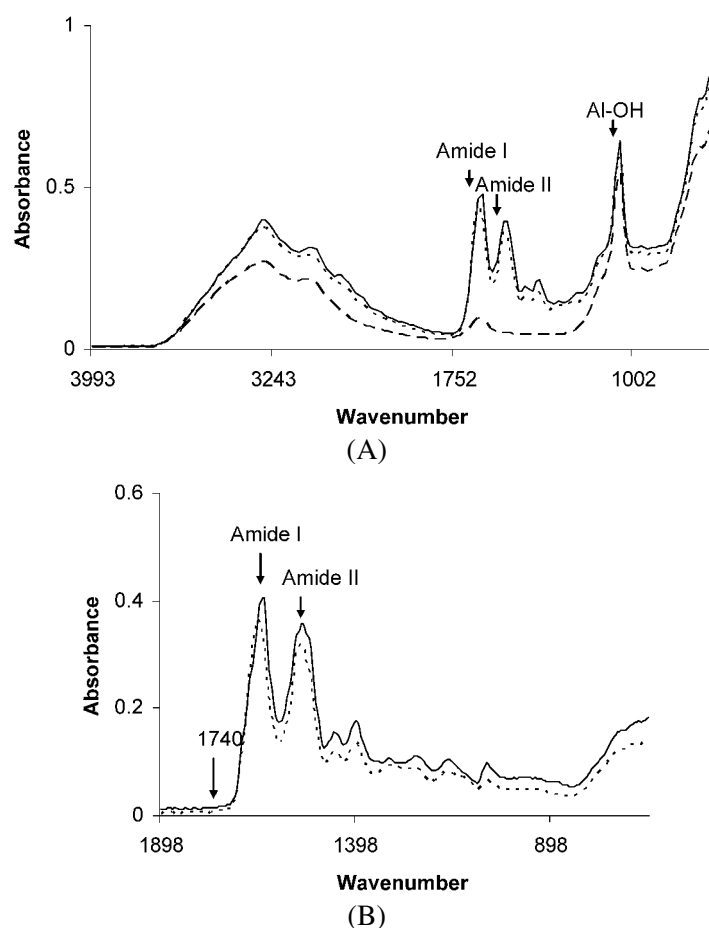

Fig. 1. Typical FTIR spectra of protein before (A) and after (B) subtraction of aluminium hydroxide: absorbed BSA (dotted line), adsorbed BLG (solid line), and aluminium hydroxide (dashed line) (A); BSA (dotted line) and BLG (solid line) (B).
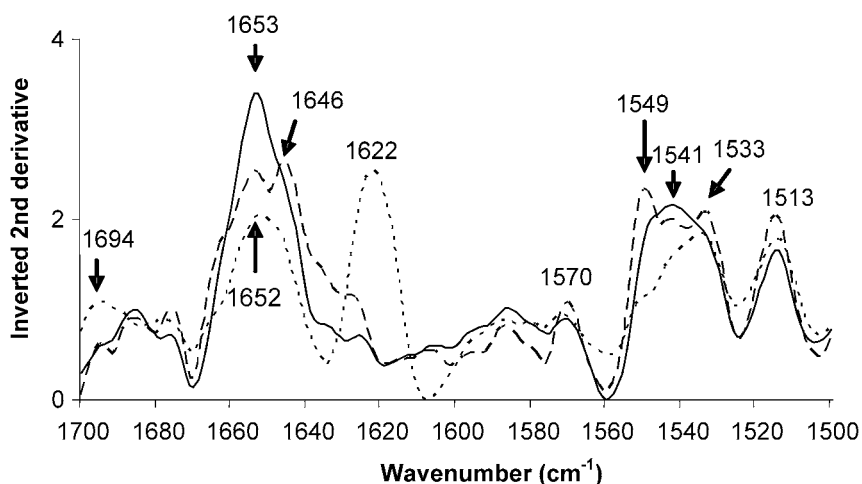
Fig. 2. Inverted 2nd derivative spectra of BSA in amide I and amide II region: free BSA (solid line), adsorbed BSA (dashed line) and aggregated BSA (dotted line).

The inverted 2nd derivative FTIR spectra of free BSA, adsorbed BSA and aggregated BSA are given in Fig. 2. Except when specifically mentioned, in this paper free protein means pure protein in solution; adsorbed protein means protein adsorbed onto aluminium hydroxide; and the aggregated protein is protein that has been preheated at 90°C for 30 min. In Fig. 2 there are band-broadening and the introduction of new peak for adsorbed BSA in amide I and amide II regions. A new peak at 1646 cm^{-1} is introduced and can be assigned to random structure. A band at 1653 cm^{-1} decreases in intensity, which ascribes to α-helix. In other words, adsorbed BSA loses its α-helix but gains random structure in amide I range. In amide II region, two new bands are induced at 1549 cm^{-1} and 1533 cm^{-1}. The band around 1570 cm^{-1} and 1513 cm^{-1} increases in intensity due to adsorption, respectively. The significant difference in the spectra of adsorbed BSA and free BSA indicates that the secondary structure of BSA changes when adsorbed onto aluminium hydroxide.

In order to compare the structural difference of adsorbed protein and the aggregated denatured protein, BSA was preheated at 90°C for 30 min and the spectrum was recorded under identical experimental condition. The final inverted 2nd derivative spectrum of aggregated BSA is also shown in Fig. 2. For aggregated BSA, there are two new bands around 1622 cm^{-1} and 1694 cm^{-1} in the amide I region. The band at 1622 cm^{-1} is extremely strong. These two bands relate to intermolecular anti-parallel β-sheet formation. The result agrees with other heat-denatured protein aggregates [33–35]. In the amide II range, aggregated BSA loses intensities at 1549 cm^{-1} and 1541 cm^{-1} compared to adsorbed and free BSA. These significant differences indicate that the adsorption-induced secondary structural change is not the same as observed for protein aggregation.

We have also studied protein adsorption capacity by aluminium-containing adjuvants. Aluminium hydroxide can adsorb different amounts of protein depending on the protein. Adsorption of different amounts of protein may not result in same structural changes. To elucidate the absorption level effect, we investigated the structures at different protein to aluminium hydroxide ratios.

Five amounts of BSA, from 8 μg to 40 μg with 8 μg interval, were adsorbed by same amount of aluminium hydroxide (27.2 μg), and measured under identical experimental condition. The inverted 2nd derivative spectra are given in Fig. 3. The spectra were significantly different in the amide I and amide II regions due to the intensity changes and peak shifts. For low ratio of adsorbed BSA, e.g. 8 μg, bands in
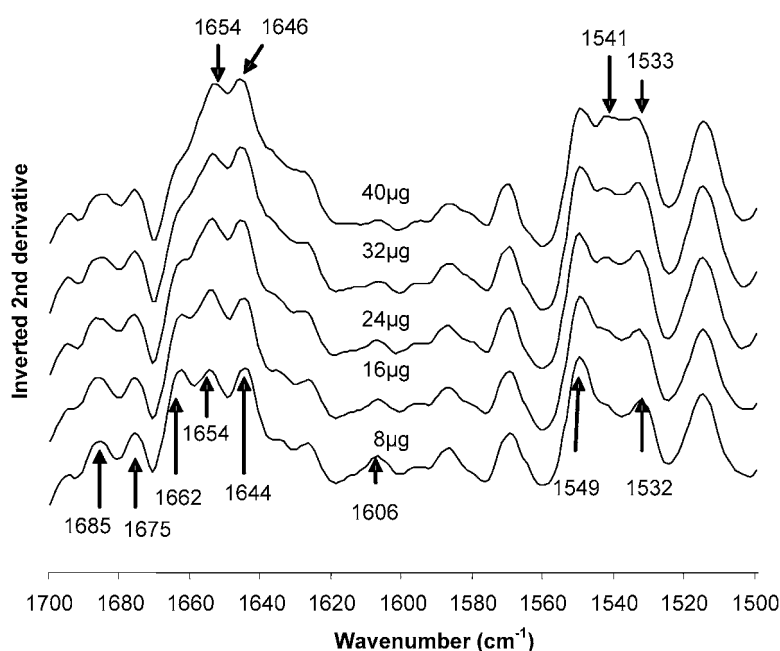
Fig. 3. Inverted 2nd derivative spectra of different amount of BSA adsorbed by same amount of aluminium hydroxide in amide I and amide II region. The spectra have been shifted vertically for case of interpretation.
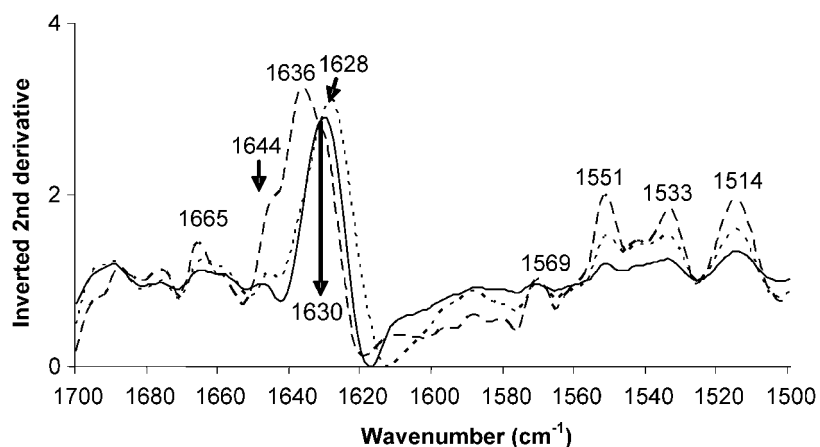

Fig. 4. Inverted 2nd derivative spectra of BLG in amide I and amide II region: free BLG (solid line), adsorbed BLG (dashed line) and aggregated BLG (dotted line).

amide I region increase in intensity around 1685, 1675 and 1662 cm^{-1}. On the other hand, in the spectra at a high ratio of adsorbed BSA, e.g. 40 μg, bands increase around 1654 and 1646 cm^{-1}, which are the α-helix and random structure. In the amide II region, at a high ratio of adsorbed protein there is a loss in intensities at 1549 and 1532 cm^{-1}, but the band at 1541 cm^{-1} increase. The band at 1532 cm^{-1} shifts to 1533 cm^{-1} at a low ratio of adsorbed BSA. Therefore, the structure of adsorbed protein depends on how much protein is adsorbed by the aluminium adjuvant. A high ratio of adsorbed BSA gives a native-like structure.

In the case of BLG, the experiments were performed using the same experimental conditions as for BSA. Figure 4 presents the inverted 2nd derivative spectra of free BLG, adsorbed BLG and aggregated

BLG. The spectral difference between adsorbed BLG and free BLG are due to peak shifts and band broadening in the amide I region, and intensity changes in the amide II region. The broadest band widens and shifts from 1630 to 1636 cm^{-1} for adsorbed BLG, which can be ascribed to β-sheet. A shoulder peak is introduced at 1644 cm^{-1}, which can be assigned to random structure. Another difference in the amide I range is the intensity of the band at 1665 cm^{-1}; adsorbed BLG has a stronger absorption. In the amide II region, the intensities of three peaks increase considerably around 1551, 1533 and 1514 cm^{-1} for adsorbed BLG. These significant spectral alterations indicated that the secondary structure of BLG is dramatically modified when adsorbed by aluminium hydroxide, revealing a strong effect of the aluminium adjuvant on BLG. The result is in excellent agreement with another study, in which BLG rearranged when adsorbed to dimyristoylphosphatidylglycerol (DMPG) [36].

In order to compare the adsorption-modified structure and the heat-induced structure, heat-induced aggregation of BLG was also investigated. Figure 4 presents the spectrum of aggregated BLG. It has been preheated at 90°C for 30 min and measured under identical experimental conditions. The spectrum of aggregated BLG differs from free BLG by band-broadening and band shift from 1630 to 1628 cm^{-1} in amide I region. In contrast, this band of adsorbed BLG shifts to higher wavenumber, 1636 cm^{-1}. In the amide II range, the intensities of three bands around 1551, 1533 and 1514 cm^{-1} increase for both aggregated BLG and adsorbed BLG. Surprisingly, adsorbed BLG has stronger absorption for these three bands than aggregated BLG. This is an indication that BLG is not extensively unfolded at this heat process or that the unfolded structure refold when retuning to room temperature. However, the structure is dramatically modified by adsorption onto aluminium hydroxide.

Five concentrations of BLG, from 8 to 40 μg with an 8 μg interval, were adsorbed by 27.2 μg aluminium hydroxide, respectively. FTIR spectra were collected at identical conditions. The inverted 2nd derivative spectra are shown in Fig. 5. Spectral differences are mainly due to band shifts and intensity
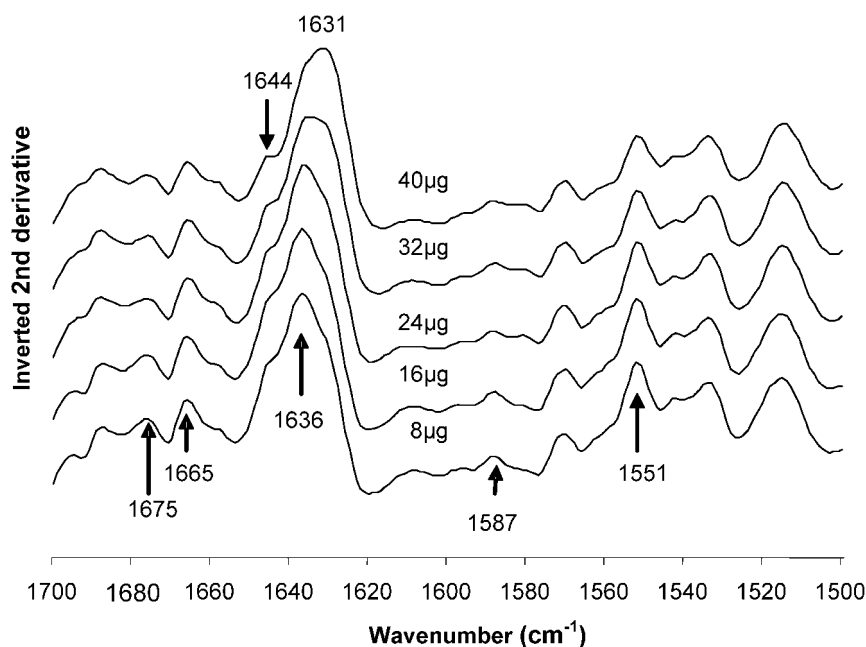

Fig. 5. Inverted 2nd derivative spectra of different amount of BLG adsorbed by same amount of aluminium hydroxide in amide I and amide II region. The spectra have been shifted vertically for case of interpretation.

changes. The β-sheet band shifted from 1636 cm^{-1} to 1631 cm^{-1} according to increasing protein adsorption, e.g. from 8 µg to 40 µg. The band at 1644 cm^{-1}, which is random structure, loses intensity at a high ratio of adsorbed BLG, e.g. 40 µg. This phenomenon is different from was observed with adsorbed BSA.

Two bands at 1675 and 1665 cm^{-1} have stronger absorption at a low ratio adsorbed of BLG. In the amide II range, a low ratio of adsorbed BLG has a stronger absorption at 1551 cm^{-1}. Therefore, the structures differ as a function of amount of adsorbed BLG. The more BLG adsorbed, the more native-like the structure is. Interference from free BLG in the adsorbed sample cannot be excluded in this case, as BLG can not be totally adsorbed by aluminium hydroxide even at very low concentrations. Free protein may affect the final spectra, especially for high amount of protein adsorption such as 40 µg.

Protein antigen adsorption is believed to result from electrostatic attraction between protein and aluminium adjuvant. When antigen is adsorbed onto the aluminium surface, the resulting intermolecular electrostatic interaction may weaken the intramolecular forces that stabilize the protein native structure. It may be the reason of adsorption-induced structural changes in the antigens.

The adsorption manner depends on the amount of adsorbed protein, which is in agreement with other studies [37,38]. Urano et al. [37] demonstrated that BSA was adsorbed on the Al$_2$O$_3$ surface by formation of a monomolecular layer. A model of BSA adsorption was proposed according to which protein was adsorbed in two different configurations depending on the amount of adsorbed protein. Nevertheless, Urano et al. [37] using potentiometric titration could not conclude that protein structure changed due to adsorption. It is probably because the conformation of adsorbed protein depends on the properties of the aluminium hydroxide surface [39,40]. In this study, two model proteins are adsorbed by aluminium hydroxide resulting in different conformations. Small amounts of protein are strongly adsorbed by aluminium hydroxide. The protein molecules form a monomolecular layer on aluminium hydroxide and secondary structure becomes more denatured. On the other hand, the structural perturbation of the protein becomes smaller when the degree of banding decreases. A high ratio of adsorbed protein is weakly adsorbed via few linking points. The protein molecules are oriented "perpendicular" onto the aluminium hydroxide, which results in a native-like structure. However, structural changes of the proteins when adsorbed on the ATR crystal cannot be excluded. Previous study showed that the changes in protein structure may occur upon adsorption onto the ATR surface [41].

The mechanism of how aluminium adjuvants can enhance the immune response is still poorly understood. Jones et al. [10] proposed that protein structure became destabilized upon adsorption by aluminium adjuvants. These physical changes of adsorbed antigens made them more susceptible to proteolytic processing by the immune system, resulting in an enhanced antigen presentation. According to this hypothesis, vaccines with low ratio of protein adsorption should induce stronger or similar immune response as compared to high ratio adsorption, since it has a more destabilized structure. The denatured protein may be pharmaceutically inactive for treating purposes, and denaturation may be the reason why causing immunogenicity, which was regarded as side effect in other fields [42,43].

3.2. Release of protein from aluminium hydroxide

PB is capable of replacing pre-adsorbed BSA and BLG from aluminium hydroxide. The protein recovery (amount of released proteins/adsorbed protein) at different PB concentration is shown Fig. 6. Protein release from aluminium hydroxide is quite fast; recovery is approximately 80% within 40 min both for BSA and BLG. These high values indicate that PB at pH 7.4 is a good candidate for replacement of protein from aluminium hydroxide. Some surfactants have also been used for protein release
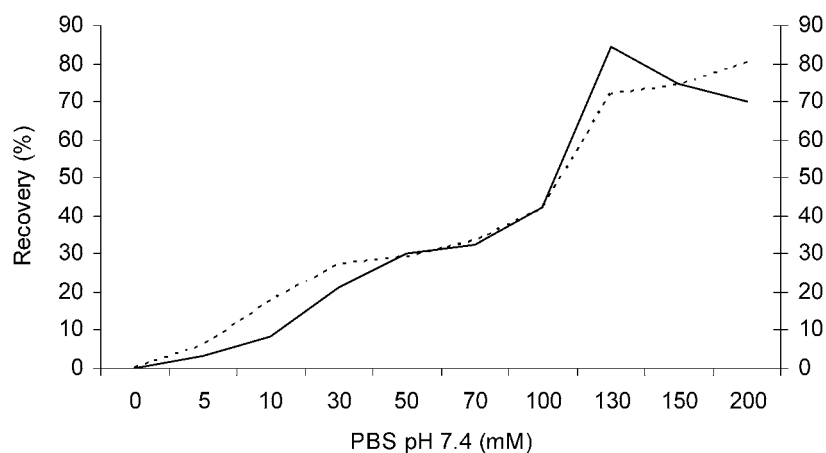
Fig. 6. Recovery of proteins that are first adsorbed by aluminium hydroxide and subsequently released using pH 7.4 PB at different concentrations: BSA (solid line), BLG (dotted line). The incubation time in the presence of PB is 40 min. The recovery was calculated by the released amount/adsorbed protein.
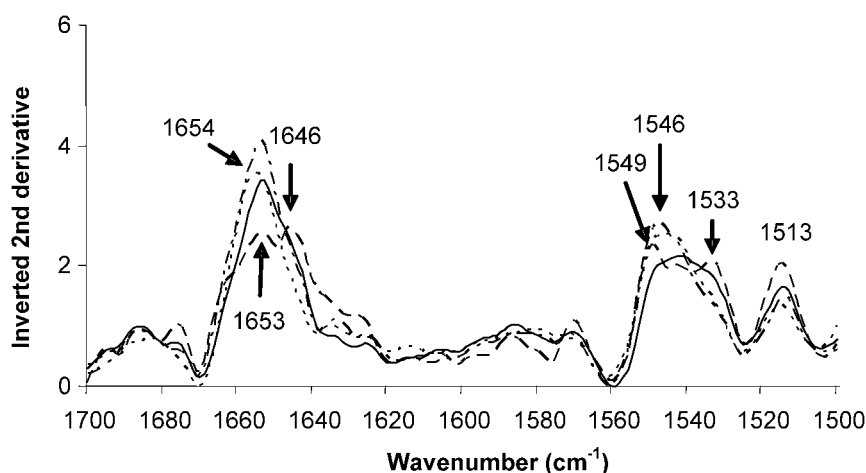

Fig. 7. Inverted 2nd derivative spectra of BSA in amide I and amide II region: free BSA (solid line), adsorbed BSA (dashed line), released BSA, the complex of protein and aluminium hydroxide (dotted line), and released BSA, protein alone after centrifugation (dash-dotted line).

from aluminium adjuvant [44,45]. The recovery of protein is proportional to the concentration of buffer, especially for BLG. Both for BSA and BLG, adsorbed protein can not be totally released from aluminium hydroxide. Incomplete release may associate with aggregation. The aggregated protein may not be released from aluminium hydroxide. Protein recoveries do not increase significantly even when increasing the final PB concentration from 50 mM to 100 mM. The curves have two plateaus, which may indicate that proteins have different adsorption conformations on the aluminium hydroxide.

The structure of released protein was measured by FTIR spectroscopy in two ways, either using the complex of protein and aluminium hydroxide or using only the released protein without adjuvant after centrifugation. Figure 7 shows the inverted 2nd derivative spectra of released BSA in the amide I and the amide II regions. For comparison, the adsorbed BSA and free BSA are also shown in the same figure. In the amide I range, released BSA loses random structure at 1646 cm^{-1} and regains α-helix

structure around $1654\,\mathrm{cm^{-1}}$. In the amide II range, band at $1546\,\mathrm{cm^{-1}}$ shifts from $1549\,\mathrm{cm^{-1}}$ comparing to adsorbed BSA, and a band is lost at $1533\,\mathrm{cm^{-1}}$. The spectrum of released BSA is similar to the spectrum of free BSA. This may indicate that released BSA again regains the native structure. This is in agreement with the study of Engel et al. [45]. They showed that released bovine α-lactalbumin (BLA) from polystyrene nanospheres had native spectroscopic properties. The structure of adsorbed BLA refolded to its native-like state upon release from aluminium hydroxide. There is a small spectral difference between released BSA when measured in complex of the released protein and aluminium and when measured on the released protein alone. This can be explained by the former sample having 20% adsorbed BSA, which cannot be released.

The refolding procedure was studied by investigating the release using different final PB concentrations, from 5 mM to 200 mM. The spectra were recorded on the complex sample of protein and aluminium hydroxide. For low PB concentrations, the sample has more adsorbed protein, whereas, there is more released protein in samples containing high PB concentrations. Figure 8 presents the inverted 2nd derivative spectra of released BSA. It is seen that in amide I range, band at $1646\,\mathrm{cm^{-1}}$ is diminished, and the band at $1653\,\mathrm{cm^{-1}}$ becomes stronger with increasing PB concentration. Random structure is lost and the amount of α-helix increases. In amide II region, bands at 1569, 1549, 1533 and $1514\,\mathrm{cm^{-1}}$ lose
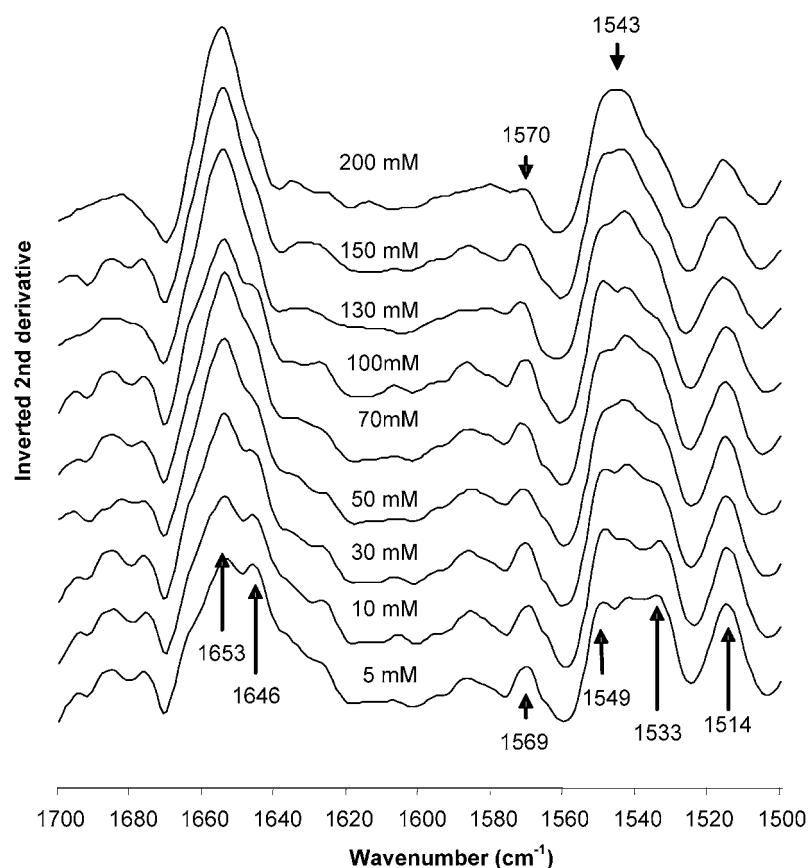

Fig. 8. Inverted 2nd derivative spectra of released BSA in amide I and amide II region: adsorbed protein has been released using pH 7.4 PB at different concentrations (from 5 mM to 200 mM) for 40 min respectively. The spectra are recorded on the complex of protein and aluminium hydroxide. The spectra have been shifted vertically for case of interpretation.
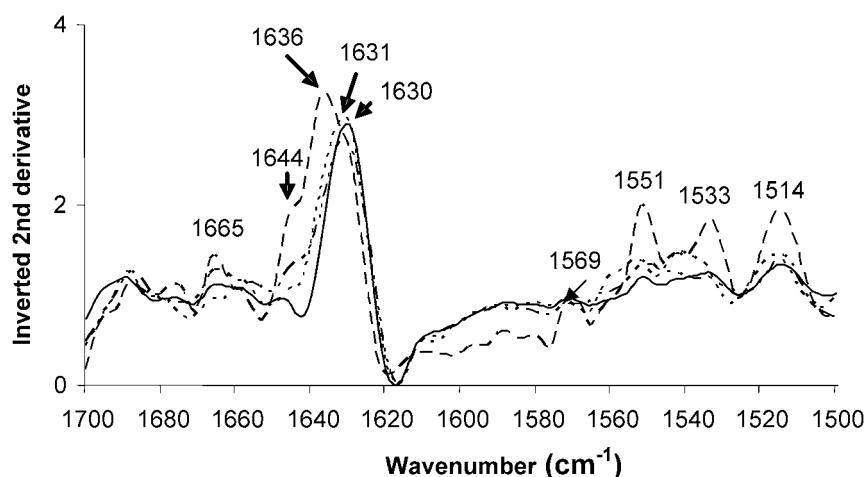
Fig. 9. Inverted 2nd derivative spectra of BLG in amide I and amide II region: free BLG (solid line), adsorbed BLG (dashed line), released BLG, the complex of protein and aluminium hydroxide (dotted line), and released BLG, protein alone after centrifugation (dash-dotted line).

intensity, and the band at 1543 cm^{-1} increase in intensity. These structural alterations make the spectrum more native-like following the increase of PB concentration.

The inverted 2nd derivative FTIR spectra of free BLG, adsorbed BLG, and released BLG are given in Fig. 9. In the amide I region, β-sheet band shifts from 1636 cm^{-1} to 1631 cm^{-1} for released BLG as compared to adsorbed BLG, and thus the spectrum of released BLG more closely resembles that of free BLG. In the amide II range, bands at 1569, 1551, 1533 and 1514 cm^{-1} for released BLG are similar to free BLG. This indicates that released BLG regain its native-like structure. Figure 10 shows the inverted 2nd derivative spectra of BLG which are released using different PB concentrations, from 5 mM to 200 mM. The spectra were also measured on the complex of protein and aluminium hydroxide. It is seen that β-sheet band at 1636 cm^{-1} shifts to 1631 cm^{-1}, and band at 1541 cm^{-1} becomes stronger. A few bands 1665, 1644, 1569, 1551, 1533 and 1514 cm^{-1} decrease intensity as a function of PB concentration. These changes make the spectrum become more native-like since there is more released protein in the sample.

The structural information from the CD spectra is in agreement with the FTIR results. The CD spectra of released BSA and free BSA as well as released BLG and free BLG are given in Fig. 11. The almost identical spectra illustrate that released BSA has similar structure with free protein. The released BLG and free BLG show similar spectra except lower ellipticity between 194–206 nm. This may indicate that released BLG cannot totally refold to its native state. In addition, CD experiments confirm the results obtained by FTIR. To compare the CD spectra of denatured and native BSA and BLG, free proteins were also measured at different temperatures; see Fig. 11(C) and (D). The significant difference of the CD spectra between high and low temperature indicates that proteins are in their native state before measurements.

4. Conclusion

FTIR–ATR spectroscopy provides direct information on the secondary structural changes of the model antigens when first adsorbed to aluminium hydroxide and following release. It is shown that the sec-
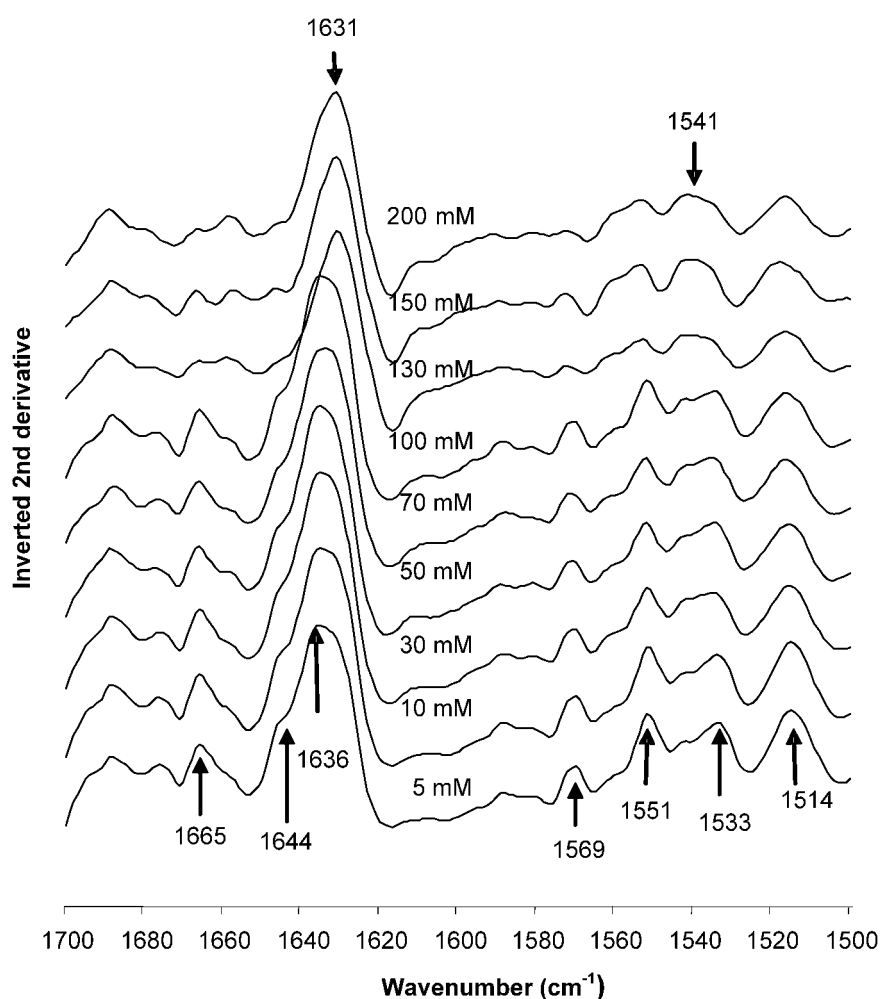
Fig. 10. Inverted 2nd derivative spectra of released BLG in amide I and amide II region: adsorbed protein has been released using pH 7.4 PB at different concentrations (from 5 mM to 200 mM) for 40 min respectively. The spectra are recorded on the complex of protein and aluminium hydroxide. The spectra have been shifted vertically for case of interpretation.

ondary structures of BSA and BLG changes when adsorbed by aluminium hydroxide. The adsorption-induced structural changes are dependent on how much protein is adsorbed onto aluminium hydroxide. Maximal adsorption gives a more native-like structure than low protein adsorption.

PB replaces pre-adsorbed antigen from aluminium hydroxide. The recovery of released antigen is approximate 80% after 40 min in the presence of PB. FTIR–ATR and CD experiments show that the released antigens generally refold to their native state. Overall, the method has been shown to be relevant in development of new vaccines.
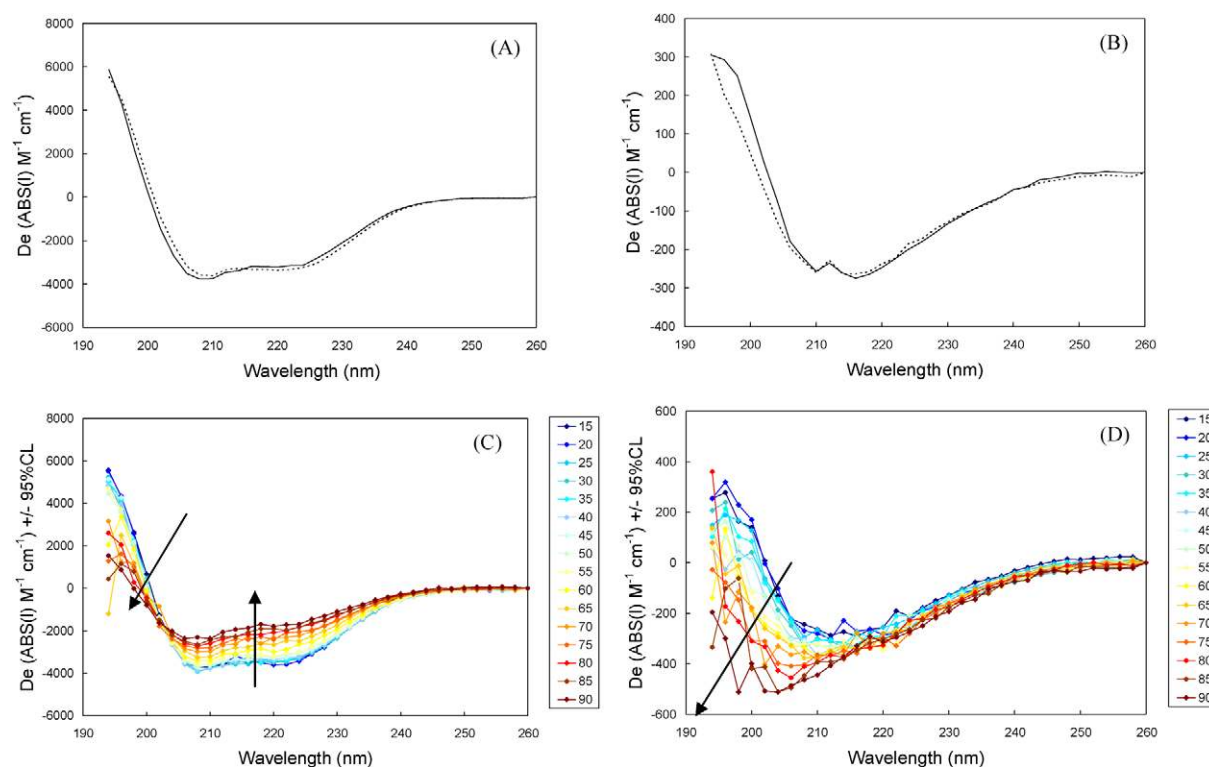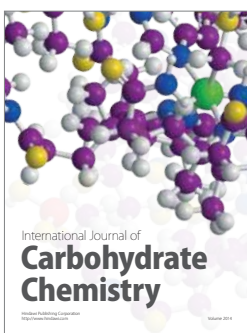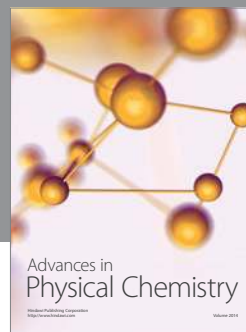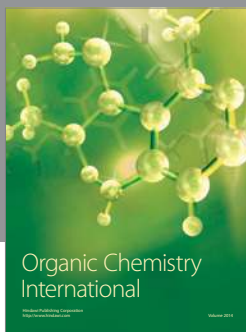
Acknowledgement

Fig. 11. CD spectra BSA (A, C) and BLG (B, D): free protein (solid line) and released protein (dotted line); C and D show CD spectra of BSA and BLG at different temperatures respectively. The arrows indicate the direction of increasing temperature.

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