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RESEARCH

Immobilization of Bioactive Protein A from *Staphylococcus aureus* (SpA) on the Surface of *Bacillus subtilis* Spores

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Abstract Protein A from *Staphylococcus aureus* (SpA) is a 40–60 kDa cell-wall component, composed of five homologous immunoglobulin (Ig)-binding domains folded into a three-helix bundle. Each of these five domains is able to bind Igs from many different mammalian species. Recombinant SpA is widely used as a component of diagnostic kits for the detection and purification of IgGs from serum or other biological fluids. In this study, purified SpA was adsorbed and covalently linked to *Bacillus subtilis* spores. Spores are extremely stable cell forms and are considered as an attractive platform to display heterologous proteins. A sample containing about 36 μg of SpA was covalently immobilized on the surface of 4×10^{10} spores. Spore-bound SpA retained its IgG-binding activity, even after seven consecutive binding and washing steps, suggesting that it can be recycled and utilized several times. FACS analysis revealed that spores with covalently attached SpA had significantly improved fluorescence intensities when compared to those of spores with adsorbed SpA, suggesting that the covalent approach is more efficient than sole adsorption regarding protein attachment to the spore surface.

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Introduction

Display of biologically active molecules on the surface of microorganisms has become a common strategy for several biotechnological applications [1, 2]. Engineering the bacterial spore surface has attracted the interest of several scientists because of its advantages over other approaches. Spore stability, resistance, and safety are some of the clear benefits of the spore-based system that also includes the possibility to display proteins on the spore surface without the need for a membrane translocation step, which is often a limitation of other cell-based approaches [3–5]. Spore stability and resistance are in part due to the presence of a proteinaceous structure around the spore, known as the spore coat. This structure is formed of at least seventy different proteins (Cot proteins) organized into three distinct layers: an inner and an outer part and a recently discovered outermost layer, the crust [6–8].

Spore surface display can be achieved by two different methods. These include the recombinant method based on the construction of gene fusions between the DNA coding for a spore surface protein and DNA coding for the heterologous protein to be displayed, and a non-recombinant method based on the spontaneous adsorption of proteins on the spore surface. The two methods have been recently reviewed [5, 9].

Staphylococcus aureus protein A (SpA) is a 40–60 kDa protein that contains five homologous immunoglobulin (Ig)-binding domains, and a C-terminal region which is composed of a charged and a hydrophobic domain that are

necessary for the interaction and attachment of the protein to the cell-wall peptidoglycan. The Ig-binding domains (A–E) share 65–90 % amino acid sequence identity with each other [10]. SpA binds to the Fc region (crystallizable fragment) of the G class of immunoglobulins (IgG) and is widely used for antibody and Fc-tagged protein purification, immobilization of antibodies for affinity chromatography and bio-sensing [11, 12]. Affinity chromatography is a highly efficient method for obtaining antibodies in which a sorbent of immobilized immunoglobulin-binding protein(s), in particular recombinant SpA, is used for affinity purification. This method is widely used to obtain purified antibodies from cultural and ascitic fluids, and blood serum. It is also used in the immunoadsorption of antibodies and isolation of circulating immune complexes from the blood plasma [13, 14]. SpA has also been applied to couple antibodies to magnetic beads for use in both immunoassays and bacterial immuno-magnetic separation [15]. In addition, optical immunoassays have been developed by applying SpA attached to Langmuir–Blodgett films [16]. The advantage of using SpA is that its interaction with the IgG molecule does not affect the antigen-binding ability of the antibody [17].

In the present study, we displayed SpA on the surface of *Bacillus subtilis* spores by a new approach. Purified SpA was first nongenetically adsorbed onto the spore surface, and then covalently linked to the spore surface proteins. Infrared spectroscopy and flow cytometry experiments were performed to analyze the interaction between spore surface proteins and SpA. The covalent immobilization method of this study has been developed the first time to display SpA on the spore surface. It is a promising new approach for the development of a novel and cost-effective matrix to detect and purify antibodies.

Materials and Methods

Cloning of Recombinant DNA

The *spa* gene was amplified using two sets of primers as listed in the Table 1 in order to construct plasmids named pET26b-Fspa (represents the entire coding sequence) and pET26b-Tspa (represents the truncated form lacking the C-terminal hydrophobic region of *spa*). For the F-*spa*, the forward and reverse primers are corresponding to the beginning of the *spa* signal peptide and the *spa* C-terminal end, respectively. For the T-*spa*, the forward and reverse primers are corresponding to the beginning of the *spa* signal peptide and the end of the last (fifth) IgG-binding site, respectively.

Amplified DNA fragments of the expected size were double digested with the enzymes *Nde*I and *Xho*I, and ligated to the multiple cloning sites of the pET26b vector (Novagen, USA) separately (downstream of T7 promoter). By this strategy, the resulting clones contained a 6XHis-tag at the C-terminal and a stop codon from the vector located after the tag. The recombinant plasmids (pET26b-Fspa and pET26b-Tspa) were transformed into *Escherichia coli* DH5a, and then screened by colony PCR, digestion and confirmed by sequencing (MWG, Germany). They were subsequently introduced into the expression host, *E. coli* BL21 (DE3), and selected on Luria–Bertani (LB) agarose plates supplemented with kanamycin (50 mg/mL). Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of competent *E. coli* cells were carried out as described previously [18].

Protein Expression, Purification, and Western Blotting

The transformed *E. coli* cells were cultured at 37 °C in 5 mL of fresh LB medium containing 50 mg/mL of kanamycin for 12 h, and then diluted and subcultured in 100 ml of fresh LB medium until an optical density of 0.9 (OD600) was achieved. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added at a final concentration of 0.2 mM, and the cultures were kept at 30 °C for about 6 h under aerobic conditions. Expressed F-SpA and T-SpA were visualized on a Coomassie Blue-stained gel. Recombinant proteins were purified from crude bacterial lysate by affinity chromatography using Ni-NTA Agarose (Qiagen, USA) according to the manufacturer's instructions [19]. Ni-NTA Agarose is an affinity chromatography matrix for purifying recombinant proteins carrying a 6XHis-tag. Histidine residues in the 6XHis-tag bind to the vacant positions in the immobilized nickel ions with high specificity and affinity. Briefly, 250 µL of the affinity matrices were added to the 10 mL of bacterial cell lysate and let them bind for 2 h at 4 °C. Unbound and non-

Table 1 List of oligonucleotides

Name	Sequence (5′–3′)	Restriction site
PAF4 (Forward primer for T-SpA)	GGGGCATATGAAAAAGAAAAACATTTATTC	N*de*I
PAR4 (Reverse primer for T-SpA)	GGGGCTCGAGTTTTGGTGCTTGAGCATCGT	X*ho*I
PAF6 (Forward primer for F-SpA)	GGGGCATATGAAAAAGAAAAACATTTATTC	N*de*I
PAR6 (Reverse primer for F-SpA)	GGGGCTCGAGTAGTTCGCGACGACGTCC	X*ho*I

specific proteins were removed by several washing steps using the following buffers. Elution can be achieved under non-denaturing conditions using elution buffer mentioned below.

[lysis buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 1 % Tween 20 (Adjust pH to 8.0 using NaOH.), Wash buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 0.05 % Tween 20 (Adjust pH to 8.0 using NaOH.), Elution buffer: 50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.05 % Tween 20 (Adjust pH to 8.0 using NaOH.)] [19].

For Western blot analysis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, USA) and treated with a rabbit anti-goat IgG antibody conjugated to horseradish peroxidase (HRP) (Roche, USA) at a 1:2000 dilution. The blot was developed with hydrogen peroxide, and 4-chloronaphthol as substrate. It should be noted that in all experiments regarding expressed proteins, equal amount of proteins were used. The quantity of the purified SpA was determined by the Bradford method.

Preparation and Purification of *B. subtilis* Spores

The *B. subtilis* 168 *trp*C2 is an auxotrophic laboratory strain which requires tryptophan for growth [20]. The *B. subtilis* 168 *trp*C2 was induced to sporulate by the exhaustion method in the Difco-Sporulation Medium (DSM) [21]. The spores were harvested by centrifugation at 10,000×*g*, 20 min, 4 °C. The pellet were treated with a solution containing $MgSO_4$ (2.5 μg/mL), lysozyme (200 μg/mL), DNase I (2 μg/mL) for 30 min at 37 °C. Each batch of spores was then heat-treated (68 °C, 45 min) to ensure killing of all vegetative cells and inactivating enzymes. After repeated centrifugation and washing steps in distilled water, the purified spores were suspended in double distilled water (pH 7) and stored in aliquots (1×10^{11} spore/mL) at −20 °C until use [21, 22]. Spore purity was checked under the microscope, and the number of viable spores was determined by the plate count method.

Spore Adsorption and Covalent Linking

Kyte and Doolittle online program (www.expasy.org/protscale/pscale/Hphob.Doolittle.html) was used to predict different hydrophobic plots of F-SpA and T-SpA proteins [23]. In order to compare the immobilization efficiencies of these two proteins and investigate the role of the potentially hydrophobic region on SpA immobilization, the same concentrations of the two forms of SpA were adsorbed on the spore surface in citrate buffer (0.1 M citric acid monohydrate [5.9 mL] and 0.1 M trisodium citrate, dihydrate [4.1 mL], pH 4). The amount of SpA adsorbed on the spore surface was measured by fluorescent spectroscopy (see below).

For covalent immobilization of the protein onto the spore surface, glutaraldehyde was used as a linker reagent to activate the functional groups present on the spore surface. Spores (4×10^{10} spores) were incubated with 1 mL of 0.5 % (v/v) glutaraldehyde under mild stirring for 2 h at room temperature. After two washes with 1 mL of deionized water, the reaction mixture was centrifuged and the resulting pellet was mixed with different amounts of protein in 50 mM phosphate buffer (pH 7.5) for 2 h at room temperature. Proteins were stabilized by reduction of imine bonds with sodium borohydride (1 mg/mL $NaBH_4$). $NaBH_4$ (powder) was dissolved in distilled water as a stock solution, and then was added to the previously treated spores (treated with glutaraldehyde and then protein A) at the final concentration of 1 mg/mL and after incubation at RT for 1 h, the spores were harvested by centrifugation (5000 rpm, 10 min) and washed three times with 50 mM phosphate buffer (pH 7.5). Finally, a high salt solution (1 M NaCl) was added to the SpA-conjugated spore to remove non-covalently adsorbed proteins and assure that all the attached SpA proteins have been attached covalently. The resulting spores with covalent immobilized SpA on their surface were then used for the IgG-binding assay.

FTIR Analysis of Functional Groups on the Spore Surface

The samples of free untreated spores (SP), spores treated with glutaraldehyde (SP-G), and spores treated with glutaraldehyde plus protein A bound to their surface (SP-G-SpA) were first lyophilized, and then ground into the fine particles using a mortar and pestle. Each sample was then mixed with potassium bromide (1 mg of spores in 100 mg of KBr), compressed into a disk with a thickness of 0.25 mm and stabilized under controlled relative humidity prior to acquiring the FTIR spectra (Bruker-Vector22, Inc. Billerica, MA, USA).

Extraction of Spore Surface Proteins

Surface proteins were extracted from a suspension of approximately 4×10^{10} spores by incubation in decoating extraction buffer (50 mM Tris–HCl [pH 8.0], 1 % (w/v) sodium dodecyl sulfate [SDS], 8 M urea, 50 mM dithiothreitol, 10 mM EDTA) for 90 min at 37 °C [24]. The spores were pelleted by centrifugation, and the supernatant containing the extracted proteins was analyzed by SDS-PAGE and Western blotting.

Determination of the Immobilized Protein Yield by Fluorescence Spectroscopic Method

The binding saturation of SpA was measured by adding increasing amounts of the protein to a suspension of 4×10^{10} spores. Immobilized protein yield is the ratio of the protein immobilized on the spore surface to the amount of introduced protein, and was calculated according to the following equation [25]:

$$\text{SpA immobilization yield } (\%) = (\text{amount of immobilized SpA} / \text{amount of introduced SpA}) \times 100.$$

In the spectroscopic method used in this study, after determination of the maximum excitation wavelength of the pure protein A, its emission was measured using several defined SpA concentrations at that specific wavelength. The calibration curve was then drawn. Now the emission of protein in primary loaded solution, supernatants, and washing solutions was determined and converted to concentration using standard curve. In this way, we can be almost certain that the concentration of protein A is calculated and thus enhance precision. Having the protein concentration of primary loaded, supernatant non-bounded protein A and also detached protein A from the spore surface during the washing process, the concentration of attached protein can be calculated. The amount of SpA immobilized onto the spore surface was determined by subtracting the amount of unbound protein from the initial loaded protein.

IgG-Binding Assay

The ability of SP-G-SpA to bind IgG was analyzed by the binding assay using rabbit serum. Free spores and SP-G-SpA were washed with 1 ml of starting buffer [100 mM Tris–HCl, (pH 8)]. The pH of rabbit serum was adjusted to 7.5–8 with 1 M Tris (pH 8) and added to the washed spores and incubated for 1 h. Thereafter, the spores were washed with 100 mM Tris–HCl (pH 8) and 10 mM Tris–HCl (pH 8), respectively. Bound IgGs were eluted using a solution of 100 mM glycine (pH 3) containing 1 M KCl. Spores were then washed with starting buffer and stored at 4 °C for reuse. Eluted fractions were analyzed on SDS-PAGE and quantified by ELISA.

Protein A Coating-ELISA (PAC-ELISA) Test

ELISA was used to measure the amount of IgG eluted from the spore-based immunoadsorbents. To increase the efficiency of coating antibody onto the 96-well plates, the Protein A coating-ELISA (PAC-ELISA) method was used, but with minor modifications [26]. Briefly, SpA was diluted in 100 mM carbonate buffer [3.03 g Na_2CO_3 and 6.0 g $NaHCO_3$ in 1 L of distilled water (pH 9.6)] and added to plates at a final concentration of 10 µg/mL. IgG eluted from the free spores and the spore-based immunoadsorbents (SP-G-SpA) were added to different wells. Serial dilutions of the rabbit antibody were subsequently added as standards. After blocking with 1 % (w/v) Bovine serum Albumin (BSA) and a final washing step, an HRP-conjugated anti-rabbit antibody at 1:2000 dilution was added to each well, and the development of color reaction was carried out by adding ABTS (2,2′-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) substrate.

Reusability Test

IgG purification from rabbit serum and its quantification was used for reusability analysis of the SP-G-SpA. At the end of each cycle of reusing, the SP-G-SpA was removed from the reaction and washed three times with 10 mM Tris buffer (pH 8), after which a sample of new serum was added to suspension of SP-G-SpA to start a new cycle.

Fluorescence-Activated Cell Sorting (FACS) Analysis

In surface display technology, it is necessary and useful to know the number of cells that express the protein of interest on their surface and also the amount of the expressed protein. The fluorescence-activated cell sorting (FACS) is designed to show that. Spores which T-SpA was attached to their surface covalently, spores with adsorbed T-SpA on their surface as well as free spores were washed three times with phosphate-buffered saline (PBS). The washed spores were resuspended in 1 mL PBS solution containing rabbit antibody conjugated to FITC (1:1000) and incubated for 1 h on ice. Spores were washed three times with PBS again, resuspended in 500 µL of PBS. The fluorescent signal located on the surface of the free and SpA-conjugated spores were analyzed using a fluorescence-based flow cytometry device (FACSCalibur, BD, USA). The Cell Quest ver. 1.0 software was used for data analysis.

Results

Cloning, Expression, and Purification of the Full-Length (F-SpA) and the Truncated Form of SpA (T-SpA)

T-SpA and F-SpA were expressed in *E. coli* cells and purified in order to be displayed on spore surface by adsorption or covalent methods. For this purpose, DNA

fragments of 930 and 1560 bp in length, encoding a truncated (T-*spa*) and a full-length (F-*spa*) version of the *spa* gene from *S. aureus,* were amplified by PCR and cloned into expression vectors, as described in Materials and Methods.

The full-length and truncated forms of SpA were overproduced in *E. coli* and affinity purified as described in the previous section. Purified proteins of 34 and 57 kDa, corresponding to the truncated and full-length versions of SpA (T-SpA and F-SpA) were visualized by Western blot analysis using HRP-conjugated rabbit anti-goat IgG (Fig. 1). As SpA can bind to the Fc region of IgG, one-step Western blot analysis using any conjugated IgG can usually be used to detect protein A. We used HRP-conjugated rabbit anti-goat IgG to detect this protein. Lane 1 in both panel a and b, show negative control which is the uninduced total lysate of the same bacteria transformed with the same plasmid and were grown in similar conditions but were not induced with IPTG. As can be seen antibody bound to the truncated and full-length SpA but failed to recognize the negative control.

Adsorption of the Full-Length (F-SpA) and the Truncated Form of SpA (T-SpA) onto the Spore Surface

To determine whether an immunoadsorbent can be developed by immobilizing the *S. aureus* protein A onto the spore surface and compare the efficiency of this method to the one using the covalent attachment, two recombinant forms of SpA were adsorbed onto the spore surface. We also extended this study by determining whether the presence of a potentially hydrophobic region in the protein structure could affect the efficiency of the adsorption and compared the binding efficiency of both forms of SpA onto the spore surface.

Both purified proteins (10, 20, 40, and 60 μg) were adsorbed on the spores surface (4.0×10^{10}) as previously described [22], and protein immobilization yield was determined from the calculation described in the "Materials and Methods" section. The binding saturation of both forms of SpA was measured by adsorbing increasing amounts of the proteins to a suspension of 4×10^{10} spores. The results showed that both forms of SpA were immobilized onto the spore surface but with different efficiencies. Immobilization yield was higher in the case of F-SpA than T-SpA. The maximum immobilization yield was 38.5 and 34 % for F-SpA and T-SpA, respectively. These results indicated that, compared to T-SpA, F-SpA bound more efficiently onto the spore surface using adsorption method (Fig. 2). Based on *T* test statistical analysis, the difference between the immobilization yield of F-SpA and T-SpA was significant in all concentrations (20, 40, and 60 μg/4×10^{10} spore) except for the concentration of 10 μg/4×10^{10} spore (where $p < 0.05$).

Covalent Linking of Adsorbed Proteins

As the adsorption is a weak interaction and the adsorbed protein can be detached easily from the surface, we used a modified method of the covalent attachment previously developed by Gashtasbi et al. [27] in order to have a more stable immunoadsorbent and also to check the impact of different surface interactions.

In the covalent method, the hydrophobic and electrostatic interactions do not play an important role. Our data also proved that contrary to adsorption method, presence of a potentially hydrophobic region does not affect the immobilization yield in the covalent method (data not

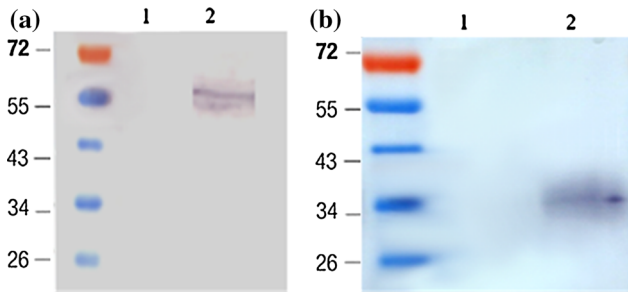

Fig. 1 Western blotting analysis for confirmation of F-SpA and T-SpA. **a** *Lane 1* un-induced total lysate of the same bacteria transformed with the same plasmid as a negative control; *lane 2* F-SpA protein; **b** *lane 1* un-induced total lysate of the same bacteria transformed with the same plasmid as a negative control, *lane 2* T-SpA protein. The SpA proteins were visualized with a rabbit anti-goat IgG antibody conjugated to HRP at 1:2000 dilution, followed by staining with hydrogen peroxide, and 4-chloronaphthol as substrate

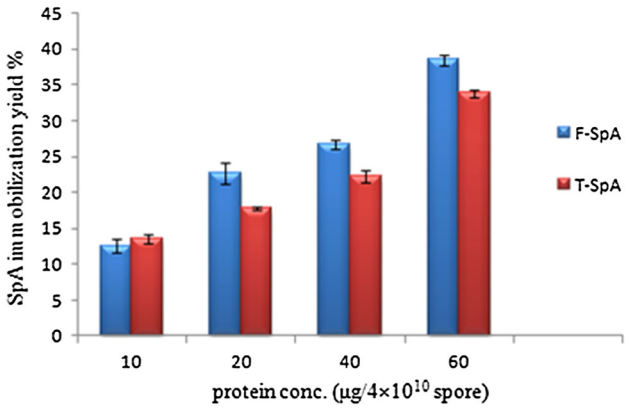

Fig. 2 Comparison between F-SpA and T-SpA immobilization yields on the spore surface. Increase in F-SpA immobilization was significant at all concentrations except 10 μg/4×10^{10} spores ($p < 0.05$) using *T*-test statistical analysis

shown). As in this type of cross-linking method, glutaraldehyde activate the functional groups on the surface and links these groups to the free amino groups of SpA, we decided to use the shorter form of the protein for all other experiment throughout this study.

Covalently linked T-SpA was extracted from the spore surface by the decoating treatment, as indicated in "Materials and Methods" section and proved by Western blotting (Fig. 3). The extracted proteins from the free and SpA-conjugated spore were run on a 12 % SDS-PAGE and electroblotted onto the PVDF membrane. The membrane was then incubated with the rabbit anti-goat IgG antibody conjugated to HRP and then visualized. SpA was just detected and visualized from the spore coat extract of the SP-G-SpA and not from the free spores. No SpA protein was extracted and detached from the spore surface after treatment with 1 M NaCl (data not shown) which shows that the SpA proteins were covalently attached to the surface not by adsorption (Table 2).

To evaluate the amount of T-SpA linked to 4×10^{10} spores in phosphate buffer (pH 7.5), we used a fluorescent spectroscopy measurement approach to be more precise. In this method, different excitation wavelengths were evaluated, and the maximum excitation and emission wavelengths were obtained at 230 and 312 nm, respectively. By increasing the concentration of the pure T-SpA, the fluorescence intensity of the SP-G-SpA increased markedly and was maximal when 60 μg of SpA was used with 4×10^{10} spores (Table 3). Thereafter, it showed an initial decrease in protein immobilization, which then became constant, which could show saturation of surface with the attached SpA as depicted in Fig. 4.

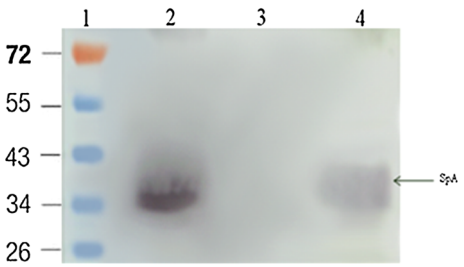

Fig. 3 Western blotting analysis to confirm the immobilization of SpA on the surface of *B. subtilis* spores. Attached proteins to the spore coat from free spores and SP-G-SpA spores were extracted and separated on 12 % polyacrylamide gels, transferred to PVDF membranes, and reacted with a rabbit anti-goat IgG antibody conjugated to HRP at a 1:2000 dilution and then visualized. *Lanes* are loaded as follows; *Lane 1* protein size marker; *lane 2* purified recombinant SpA (T-SpA) to verify the Western blot procedure; *lane 3* free spore coat and *lane 4* spore coat from SP-G-SpA

FTIR Analysis

The covalent attachment and the mode of interaction of T-SpA molecules on the surface were also confirmed by A Fourier transform infrared (FTIR) spectroscopic approach which is shown in Fig. 5. Using this method, the T-SpA was covalently linked to the functional groups present on the spore surface using glutaraldehyde, as described in "Materials and Methods" section. Consequently, FTIR spectra of SP, SP-G, and SP-G-SpA were obtained. The main absorption peaks were: 3299.85 cm^{-1} (N–H stretching), 2962.62 cm^{-1} (–CH stretch), 1654.56 cm^{-1} (carboxylic groups), 1541.35 cm^{-1} (carboxylic groups), 1444.59 cm^{-1} (CH_3 bending vibration), 1390.90 cm^{-1} (C–C stretch [in–ring]), and 1082.78 cm^{-1} (O–H alcohols [primary and secondary] and aliphatic ethers), which were similarly present in all three samples. Some differences were observed in the 4000–500 cm^{-1}region of the FTIR spectra. As indicated in Table 2, the FTIR spectrum of SP-G when compared to that of SP, revealed that the N–H stretching at 3299.85 cm^{-1} shifted to 3308.62 cm^{-1}, the –CH stretch at 2962.62 cm^{-1} shifted to 2931.55 cm^{-1}, C–C stretch (in-ring) at 1390.90 cm^{-1} shifted to 1386.08 cm^{-1}, and O–H alcohols at 1082.78 cm^{-1} shifted to 1077.88 cm^{-1}. Such peak shifts indicated the formation of new bonds between the aldehyde groups of glutaraldehyde and the amine groups of the spore surface proteins. Meanwhile, differences between the SP-G and SP-G-SpA spectra were more significant. The N–H stretching at 3308.62 cm^{-1}, and the C=O bending (out-of-plane) at 589.75 cm^{-1}shifted to 3313.53 and 533.09 cm^{-1}, respectively. Furthermore, the C–N stretch at 1160.02 cm^{-1} and the N–H bending (out-of-plane) at 860.63 cm^{-1}, were observed in the FTIR spectrum of SP-G-SpA, but not in those of of SP and SP-G (Table 2; Fig. 5) which was an indicator of the covalent linkage of SpA to the spore surface via glutaraldehyde. The different spectral peaks observed, demonstrated that the spore is a heterofunctional matrix with different functional groups on its surface that are able to interact with the groups present on a protein surface under different conditions. Based on spore surface characteristics (anion-rich hydrophobic surfaces [22] and functional groups), the protein can be immobilized by different methods of the adsorption and the covalent attachment [28, 29].

IgG-Binding Activity of Immobilized SpA and Reusability

One of the most important points in protein immobilization methods is that the immobilized protein remains functional and retains its activity. SpA can bind to the Fc region of different subclasses of IgG through its five binding

Table 2 The FTIR spectral characteristics of spores before and after treatment with glutaraldehyde and protein A attachment

Wave number range (cm^{-1})	Spore (SP)	Spore + glutaraldehyde (SP-G)	Spore + glutaraldehyde + protein (SP-G-SPA)	Assignment
3100–3500	3299.85	3308.62	3313.53	N–H stretching
2700–2950	2962.62	2931.55	2933.09	–CH stretching
1670–1500	1654.56	1655.93	1657.77	Carboxylic groups
1670–1500	1541.35	1540.19	1542.21	Carboxylic groups
1490–1350	1444.59	1445.23	1445.24	–CH bending vibrations
	1390.90	1386.08	1387.24	C–C stretch (in-ring)
	–	–	1160.2	C–N stretch
1350–1000	1082.78	1077.88	1077.76	O–H alcohols (primary and secondary) and aliphatic ethers
640–800	–	–	860.63	NH bending (Out-of-plane)
537–606	559.23	589.75	533.09	C=O bending (Out-of-plane)

Table 3 Protein concentrations in different fractions measured by spectroscopy fluorescence method

Initial conc. of protein (μg/mL)	Fluorescence intensities of supernatant	Conc. of protein in supernatant (μg/mL)	Fluorescence intensities of wash solutions (1 and 2)	Conc. of protein in wash solutions (μg/mL)	Conc. of immobilized protein	SpA immobilization yield (%)
10	117.26	4.14	102.99	1.83	4.03	40.3
20	140.29	7.87	108.43	2.71	9.42	47.1
40	169.33	12.57	117.19	4.13	23.30	58.25
60	209.16	19.02	118.37	4.32	36.66	61.1
80	306.06	34.71	149.37	9.34	33.95	44.93
100	373.16	45.57	155.43	10.32	44.11	44.11

The concentration of the immobilized protein was determined by the difference between the content of the initial protein concentrations and those determined in the supernatant and pooled washing solutions

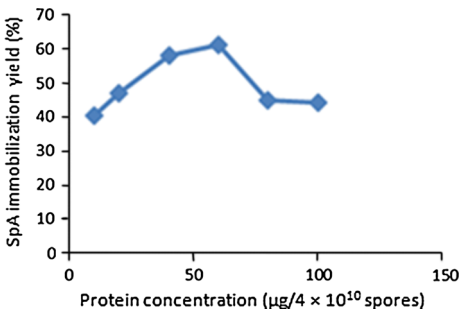

Fig. 4 Determination of the SpA immobilization yield on the spore surface using gradual increasing of loaded protein in covalent attachment method. By gradual increasing concentration of the pure SpA added to the spores, the immobilization yield increased markedly and was maximal when 60 μg of SpA was used with 4×10^{10} spores. The maximum immobilization yield in this situation is 61.1 %

domains and can be used for purification and detection of these antibodies [14, 30]. In this study, IgG from the rabbit serum was used to evaluate the activity of spore-immobilized T-SpA. Spores were mixed with rabbit serum, washed, and collected by centrifugation as described in "Materials and Methods" section. Bound IgG was then eluted and analyzed by SDS-PAGE using a 12 % (w/v) polyacrylamide gel. The presence of proteins corresponding to the IgG heavy (approximately 50 kDa) and light (approximately 25 kDa) chains (Fig. 6) indicated that IgGs were bound to the SpA-conjugated spore surface.

The efficiency of spore-attached T-SpA in binding to IgG was also assessed by a PAC-ELISA approach mentioned in the Materials and Methods section. Based on our data, 40 μg SpA/4×10^{10} spores was able to pull down about 180 μg/mL of IgG (Fig. 7).

The main object of immobilization of proteins on a solid surface is that the immobilized protein can be retrieved easily from the reaction solution, and the developed matrix can be recycled such that it can be used many times. This is an advantageous in industry and their application is increasing continuously. Therefore, the expenses regarding

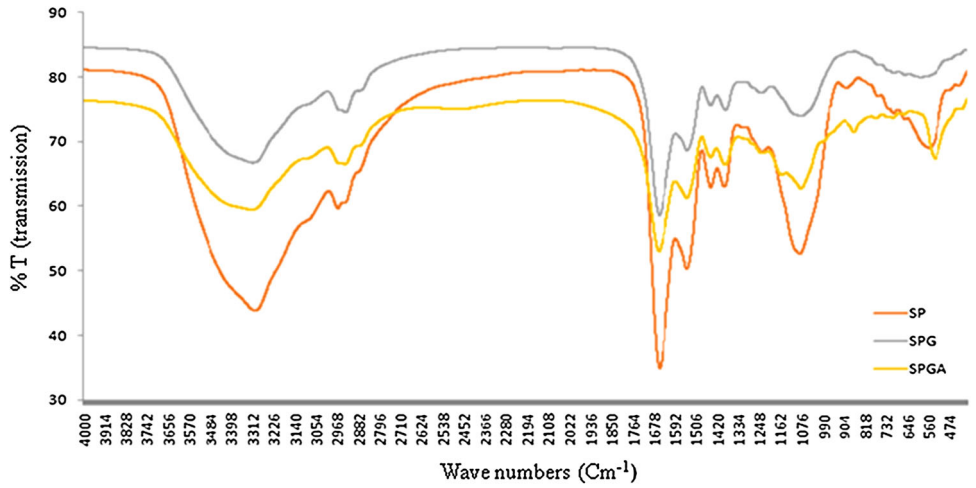

Fig. 5 FTIR analysis of free spores (SP), spore + glutaraldehyde (SP-G) and spore + glutaraldehyde + protein (SP-G-SpA)

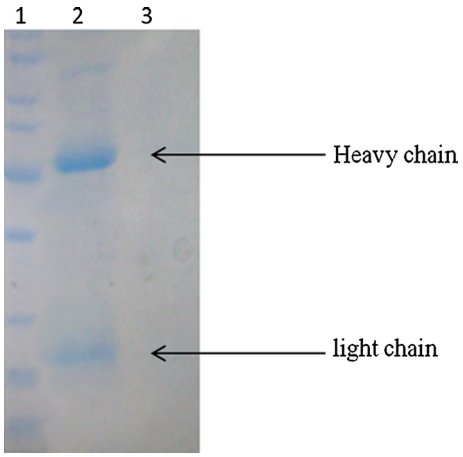

Fig. 6 SDS-PAGE analysis of the antibody fractions during IgG-binding assay of different spore-based immunoadsorbent. The bound IgG were eluted with 100 mM glycine (pH 3) plus 1 M KCl solution after binding to sp-G-SpA. The presence of proteins corresponding to the IgG *heavy and light chains* indicated that IgG could specifically bind to SpA immobilized onto the spore surface. *Lanes* are loaded as follows: *lane 1* protein size marker; *lane 2* antibody heavy and light chains bound to and eluted from the surface of the sp-G-SpA; *lane 3* No antibody was bound to and detected from free spores as negative controls

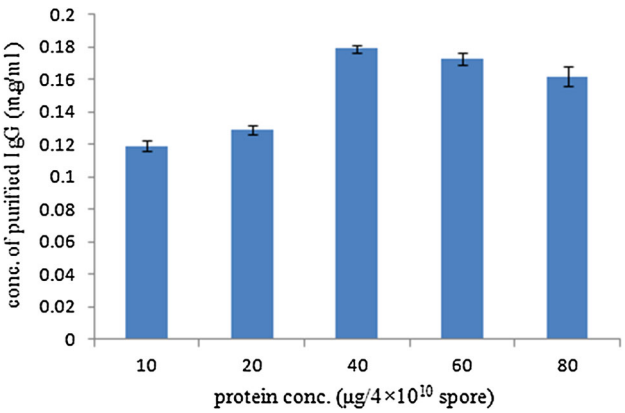

Fig. 7 The efficiency of spore-attached T-SpA in binding to IgG assessed by a PAC-ELISA approach. The maximum efficiency was about 180 µg/mL of IgG for 40 µg of SpA/4 × 10^{10} spores

protein expression, purification, and immobilization can be decreased. Meanwhile, covalent attachment of proteins on a solid surface confers mechanical advantages because the target protein becomes much robust and less prone to damage during handling.

After elution of the bound IgG from the SpA-conjugated spore on their surfaces, they were re-used to bind the IgG in the rabbit serum several times. The amount of antibody bound to the spore-immobilized SpA was evaluated for each round of IgG binding/elution, and the activity was compared with the initial activity, which was assumed as 100 %. The residual activity of the immobilized protein A was found to decrease after multiple uses. After the 7th cycle, the remaining IgG-binding activity was approximately 60 % of that of the initial activity (Fig. 8).

Covalent Linking is More Efficient than Adsorption in Immobilizing SpA

Comparison of immobilization efficiency of the T-SpA onto the spore surface by the adsorption and the covalent approach was evaluated by the FACS method.

The ability of spore-immobilized SpA to bind FITC-conjugated rabbit IgG, allowed us to use a flow cytometry approach to compare the efficiency of these two methods in SpA attachment on the spore surface. As shown in Fig. 9d, the percentage of the positive fluorescence spores, gated by panel M1, was used for measurement of the SpA immobilization efficiency on the surface of spore. The FACS

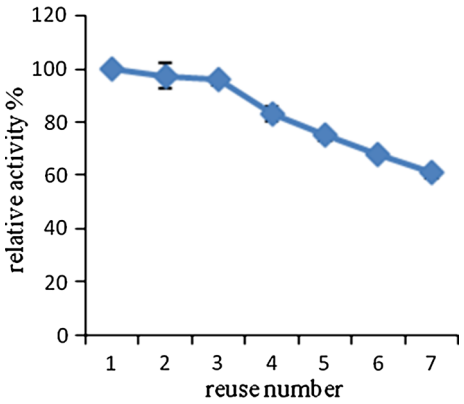

Fig. 8 Determination of the reusability of the SpA immobilized by covalent method on the spore surface

analysis showed that spores with covalently attached SpA had a significantly higher fluorescence intensity than that of spores coated with SpA using the adsorption method (95.67 and 26.99 %, respectively) (Fig. 9), indicating that glutaraldehyde is an efficient crosslinker for SpA display. In this study, two negative controls including, free spores without staining (Fig. 9a) and free spores stained with rabbit IgG conjugated with FITC (Fig. 9b) were used. Flow cytometry analysis revealed that the spores with covalently attached SpA showed significantly improved fluorescence intensity compared to the negative controls (Fig. 9d).

Discussion

In this study, the SpA from *S. aureus* was displayed on the surface of *B. subtilis* spores by spontaneous adsorption and by a new approach in which adsorbed SpA was covalently linked to the proteins present on the spore surface. Although Gashtasbi et al. immobilized the alpha-amylase enzyme on the spore surface by the covalent method using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N hydroxysulfosuccinimide (NHS), here, glutaraldehyde was used as an efficient and low-cost crosslinker to immobilize SpA on the spore surface [31].

After several washing steps, the amount of SpA displayed on the spores was higher with the covalent linking method than by spontaneous adsorption.

It is generally hypothesized that hydrophobic and electrostatic interactions are two major forces for protein adsorption to the negatively charged surface of the spore [22, 32–36]. To investigate the effects of the potentially hydrophobic region of the target protein (SpA) on spore surface immobilization via the adsorption method, we used the full-length SpA (F-SpA), and the truncated SpA (T-SpA) lacking the potentially hydrophobic region. Our results showed that the amount of F-SpA attached to the surface was significantly greater than that of T-SpA. Since, the difference between these two forms is in the presence of a potentially hydrophobic region in the F-SpA, and the spore surface is also highly hydrophobic, then it could be assumed that the hydrophobic interactions might have a positive role in the adsorption of SpA on the spore surface. However, more different proteins are needed to be analyzed in order to achieve a conclusive result.

Activation of the spore surface functional groups by glutaraldehyde was checked using FTIR analysis. The rise of absorbance in the wave numbers regions 537–606 cm^{-1} for the activated spores indicated that a reaction with glutaraldehyde had occurred and imine groups were formed. Meanwhile, decreasing the height of this peak after incubation with SpA shows that the aldehyde groups were

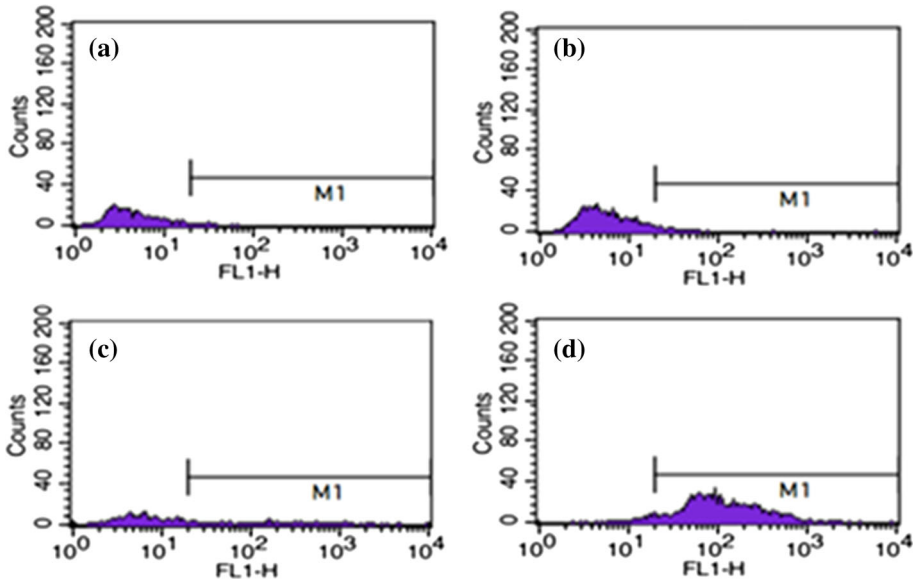

Fig. 9 FACS histograms of **a** Free spores without staining; **b** free spores stained with FITC-labeled rabbit antibody; **c** spores with SpA immobilized on their surface via adsorption stained with FITC-labeled rabbit antibody; **d** spores with SpA immobilized on their surface via covalent attachment by glutaraldehyde stained with FITC-labeled rabbit antibody. M1 shows a gate to detect only the cells that fall in positive area; all the cells falling outside this gate are ignored

involved in the reaction. These analyses also demonstrated that the amine groups on the surface of SpA had reacted with the aldehyde groups of glutaraldehyde, leading to the formation of imine groups. The appearance of two new peaks [C–N stretch at 1160.02 cm^{-1} and N–H bending (out-of-plane) at 860.63 cm^{-1}] in the spectrum of the SP-G-SpA sample can also be attributed to the attachment of protein A to the activated spores.

Our results showed that the best condition for SpA immobilization was obtained using 60 μg of SpA/4 × 10^{10} spores in phosphate buffer (pH 7.5). However, the best concentration for maximum SpA activity was determined as 40 μg SpA/4 × 10^{10} spores in phosphate buffer (pH 7.5). As it was mentioned before, although less protein was immobilized onto the spore surface under this condition, but the SpA activity was higher, which could be due to less steric hindrance. We can hypothesize that the steric hindrance between IgGs that bind to protein A on the spore surface could influence the SpA binding capacity with both inter-protein A steric hindrance (between immobilized IgGs) and intra-protein A hindrance (between the various binding domains within a single ligand). Theoretically, five antibodies could bind to each molecule of SpA, as it contains five IgG-binding domains. However, all five domains of SpA may not be simultaneously available for binding. When an antibody molecule binds to one of these domains, it can sterically block the access of another antibody molecule to the adjacent domains [37]. Furthermore, at 40 μg of SpA/4 × 10^{10} spores, the purity of the eluted IgG was greater than at other concentrations, because more specific molecules (IgG rather than albumin) could bind to protein A in the ELISA plate.

To the best of our knowledge, there is no report of displaying protein A on biological surfaces, except for the work conducted by Steidler et al. which used the Pap Pili as a carrier to display only one domain of SpA on the *E. coli* surface [38]. In contrast to that study in which just one domain of SpA were displayed, in our study, both full-length and truncated forms of SpA containing 5 IgG-binding domains were displayed on the spore surface. Another advantage of the present system is the very high stability of the *B. subtilis* spores in tolerating possible harsh industrial conditions, when compared to that of the *E. coli* cells [6, 39].

Furthermore, spores displaying SpA on their surfaces are more cost-effective than the commercially available polymeric matrices like Sepharose or agarose used for displaying protein A.

In conclusion, compared to the conventional immobilization matrices, the many advantages of spores are attributed to simplicity, low costs, high production rates, and ease of handling [6]. Here, we showed that hydrophobic interactions can be regarded as important in surface adsorption of SpA to the spore surface. Furthermore, covalent attachment of heterologous proteins could be a good alternative for surface adsorption, since a higher load of protein can be attached by using this approach. All in all, the mentioned advantages of covalent spore surface display highlight the potential applications of immobilized SpA for the further development of immunological and biochemical research. However, it should also be stated that this is a model study that evaluates the potential application of glutaraldehyde in activating spore surfaces that can then be used as matrices.

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