

Effectiveness of natural and synthetic blocking reagents and their application for detecting food allergens in enzyme-linked immunosorbent assays

Denise Huber · Judith Rudolf · Parisa Ansari ·
Brigitte Galler · Manuela Führer ·
Christoph Hasenhindl · Sabine Baumgartner

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Abstract Blocking is an important step before an enzyme-linked immunosorbent assay (ELISA) can be performed. It reduces non-specific binding to the microtiter plate to a minimum. For detecting food allergens by means of ELISA, the problem with protein blocking solutions is obvious. The blocker might interfere with the antibodies of the assay and leads to false positive results. Therefore, other blocking solutions are greatly needed. There are some alternatives like synthetic blockers or carbohydrates. Comparisons of these different blocking agents, namely proteins, carbohydrates, and synthetic blockers, were made at different reaction conditions. The incubation periods and temperatures were varied, as well as the pH. The best combinations were evaluated and compared, in respect of their blocking efficiency. The two best non-proteinaceous blockers, i.e. polyvinylalcohol and Ficoll, were subsequently applied to ELISA tests for the determination of α -casein and peanut. The study showed that Ficoll and PVA did as well as BSA in buffer solution. Therefore, they can be considered as alternative blocking reagents for ELISA, especially for the detection of food allergens.

Keywords ELISA · Blocking · PVA · Ficoll · Food allergens · α -casein

Abbreviations

Abs	Absorbance
BSA	bovine serum albumin
ELISA	enzyme-linked immunosorbent assay
HRP	horse radish peroxidase
PBS	phosphate buffered saline
PEG	polyethyleneglycol
PN	peanut
PVA	polyvinylalcohol
PVP	polyvinylpyrrolidone
TMB	tetramethylbenzidine

Introduction

Enzyme-linked immunosorbent assays are a major tool for biochemists and analysts. The ELISA offers a wide range of possibilities concerning the specific and sensitive reaction mechanism of antibody and antigen interactions. In order to ensure the specificity of the assay, the material of the microtitre plate shall not react with the other components involved in the ELISA reaction. Blocking the bottom of the microtitre plate after coating is important for the sensitivity and specificity of the following reactants, which shall bind to the coated protein only and not onto the plate. Non-specific binding is detrimental for the assay; in addition, saturation of the unoccupied sites must be achieved [1]. The most effective and common blocking solutions consist of bovine serum albumin or casein, in other words, proteins in buffer solution. Hence, non-proteinaceous blocking reagents were resorted to the study.

Tween 20, often used as detergent in washing buffers, was tested for its ability in immunoblotting (nitrocellulose

D. Huber · J. Rudolf · P. Ansari · B. Galler · M. Führer ·
C. Hasenhindl · S. Baumgartner (✉)
Christian Doppler Pilot Laboratory "Rapid Test Systems
for Allergenic Food Contaminants",
Department of Agrobiotechnology, IFA-Tulln,
Center for Analytical Chemistry,
University of Natural Resources and Applied Life Sciences Vienna,
Konrad-Lorenz Strasse 20,
3430 Tulln an der Donau, Austria
e-mail: sabine.baumgartner@boku.ac.at

membrane) and caused some artefacts, thus leading to a misinterpretation of results [2]. Therefore, it cannot be used alone as a blocking agent. Tween 20 and some other detergents were checked for their ability to influence protein coating onto polystyrene microtitre plates [3] since Tween 20 in PBS buffer was reported to prevent non-specific protein binding to polystyrene [4].

Other studies proved that the alternative synthetic blocker PVA reduced non-specific binding in enzyme-linked immunosorbent assays [5], as part of a post-coating solution and for stabilisation of the test kits itself [6, 7] as well as for serum pre-incubation of, e.g. Luminex assays [8]. The blocking capacity of PVP was demonstrated in [1] and in Western blotting by reducing the background signal without decreasing specific immunoreactivity [9]. Both PVA and PVP were used in ELISA for the detection of antibodies bound to virus-like particles. PVA reduced the amount of non-specific binding of antibodies, whereas PVP increased the sensitivity of antibody detection [10, 11]. Additionally, PEG was described as alternative blocker [1]. It was used as a block copolymer in a sandwich immunosorbent assay system with magnetic beads and turned out to be more effective than the conventional protein blocker BSA [12].

When working with food allergens, a more or less universal blocking agent would be of integral importance for assay development. Despite the efficiency of protein blockers, they are not the first choice in this case as they can possibly interfere with the analyte and the antibody. For example, defatted milk powder would not be used for the determination of any milk allergen in an ELISA. The application of BSA might be critical as well. Although, e.g. defatted milk protein and casein proved to be most effective for the blocking of the polystyrene microtitre plates [13], other possibilities must be taken into account if the allergen α -casein should be measured. Fish gelatine was described as an excellent blocker for nitrocellulose membranes for Western blot. Hence, it was also tested on polystyrene plates as protein blocker.

In respect of the specific ELISA systems, there are no general categorisations of blocking activity to refer to. The proteins have mainly been chosen after empirical testing and by convenience [13]. The alternative blockers PVA, PVP and PEG [1] were tested in our ELISA system for food allergens, in comparison with conventional protein blockers, like BSA and fish gelatine. Besides, carbohydrates, i.e. dextrans, Ficoll and trehalose, were taken into the series of tests to check their blocking efficiency and to evaluate a possible usage. In the first experiments, only the blocking agents were coated onto the plate, and their blocking efficiency was tracked by running simple standard curves on these plates. No dose-dependent signal changes were expected, which proved an efficient blocking strategy. Afterwards, the best-suited blockers were checked in two

ELISA formats with different polyclonal antibodies for the determination of α -casein and peanut for their suitability.

Materials and methods

Reagents and instrumentation

High-binding 96-well microtitre plates from Greiner Bio One (Kremsmuenster, Austria) were chosen for the assay. For washing the microtitre plate, Tecan 96PW™ from Tecan Austria GmbH (Groedig, Austria) was employed. The 12-channel ELISA reader Sunrise™ from Tecan Austria GmbH was used with Magellan5 software.

The antibodies of rabbit and chicken (IgY) were prepared in house. IgY preparation was followed according to the protocol as described in [14]. The labelled antibodies anti-rabbit-IgG-HRP and anti-chicken-IgG-HRP were obtained from Sigma Aldrich (Vienna, Austria), as well as Ficoll ($M_w \sim 400$ kDa), polyvinylpyrrolidone ($M_w 40\text{--}50$ kDa), bovine serum albumin (Fraction V, $\geq 96\%$), fish gelatine (gelatine from cold water fish skin) and polyvinylalcohol ($M_w 13\text{--}23$ kDa). D(+)-Trehalose ($>99\%$ purity) was purchased from Roth (Karlsruhe, Germany), polyethyleneglycol ($M_w \sim 20$ kDa) from BioChemika Fluka AG (Buchs, Switzerland) and two different dextrans ($M_w 40$ and $2,000$ kDa) from Pharmacia Biotech (Vienna, Austria). The salts for phosphate buffer, citric acid and sulphuric acid were bought from Merck (Vienna, Austria). Potassium sorbic acid, polyoxyethylene-(20)-sorbitan monolaurate (Tween 20), sodium azide, 3,3',5,5'-tetramethylbendizidine (TMB), hydrogen peroxide and dimethyl sulphoxide were purchased from Sigma Aldrich (Vienna, Austria). Water was purified by reverse osmosis before use. Samples used as blank matrices were cookies "Eierbiskotten" from Manner (Vienna, Austria) and soy milk "Alpro Soya" from Tirolmilch (Wörgl, Austria). The extraction buffer was obtained from an available Ridascreen test kit from R-Biopharm (Darmstadt, Germany). The cellulose acetate filters with glassfiber prefilters and a pore size of $0.45 \mu\text{m}$ were purchased from Sartorius Stedim Biotech (Aubagne Cedex, France).

Stock solutions and buffers

Coating buffer (pH 9.6) was made using 12 mM sodium carbonate, 38 mM sodium hydrogencarbonate and 0.01% sodium azide. A stock of 0.2 M PBS buffer containing 0.36 M NaCl, pH 7.5 was used for all phosphate buffer dilutions. Blocking solutions were varied in the assays: To dissolve 1% of blocking reagent, either 10 mM phosphate buffer containing 18 mM sodium chloride (pH 7.6) or coating buffer (pH 9.6) were used. Phosphate buffer (10 mM), containing 18 mM sodium chloride, was also

applied as washing buffer, adding 0.1% Tween 20. The assay buffer consisted of 50 mM phosphate buffer and 0.1% Tween 20. For the substrate solution, 12.5 mL substrate buffer pH 4.0, containing 0.2 M citric acid and 0.01% potassium salt of sorbic acid, was mixed with 2.5 μ L hydrogen peroxide (30% w/v) and 100 μ L TMB stock solution. TMB stock solution included 1.25% (w/v) tetramethylbenzidine dissolved in 5 mL dimethyl sulphoxide, then adding 25 mL methanol. H_2SO_4 (1 M) was used as stop solution. The extraction buffer for blank matrices was diluted 1:20 with deionised water.

Assay set-up for comparison of blocking reagents

Coating and blocking of the microtitre plate

Plates were coated with blocking solutions of 1% BSA, fish gelatine, PVA, PEG, Ficoll, dextran 40, dextran 2000, 3% PVP and 5% trehalose in coating buffer at pH 9.6 or in PBS buffer at pH 7.6. Blocking solution (300 μ L) was filled in each well, and the sealed plate was incubated at 4 °C overnight, at room temperature for 2 h or at 37 °C for 2 h. Afterwards, it was washed three times with washing buffer containing Tween 20.

Assay procedure

α -Casein standards (100 μ L; dilutions with assay buffer from 0 to 130 $\mu\text{g mL}^{-1}$) was filled into each cavity and shaken at room temperature for 1 h. The plate was washed again three times with washing buffer containing Tween 20. Rabbit-anti- α -casein (100 μ L; diluted 1:10,000 with assay buffer) was added and shaken at room temperature for 1 h. After washing the plate three times with washing buffer containing Tween 20, 100 μ L of anti-rabbit-HRP antibody (diluted 1:50,000 with assay buffer) was put into each well and shaken at room temperature for an hour. After the final procedure of triple washing with washing buffer containing Tween 20, 100 μ L of substrate solution was filled into each cavity and shaken at room temperature for 30 min protected from light. Eventually, 100 μ L stop solution was added, and the absorbance was measured at 450 nm with an ELISA reader.

Indirect sandwich immunoassay for α -casein and peanut

Coating of microtitre plate

Anti- α -casein antibody was coated onto the microtitre plate for the sandwich ELISA: IgY-anti- α -casein or rabbit-anti- α -casein was diluted with coating buffer up to a final concentration of 1 $\mu\text{g mL}^{-1}$. The sandwich was performed subject to the coated antibody used. The respective diluted

antibody (100 μ L) was coated onto the high-binding microtitre plate and incubated at 4 °C overnight. The washing step after coating was carried out three times with phosphate buffer without Tween 20.

For the peanut sandwich assay, anti-peanut antibody was coated. On the one hand, rabbit-anti-peanut was diluted with coating buffer up to a final concentration of 100 ng mL^{-1} , and on the other hand, IgY-anti-peanut antibody was diluted with coating buffer up to a final concentration of 2 $\mu\text{g mL}^{-1}$. The following coating procedure was the same as mentioned above for α -casein.

Blocking of the microtitre plate

Three hundred microlitres of 1% PVA in 10 mM PBS buffer or 1% Ficoll in coating buffer was used for each well of the microtitre plate as blocking reagent for the sandwich assay. Blocking with PVA was performed at 4 °C overnight, blocking with Ficoll at room temperature for 2 h. Subsequently, the plates were washed three times with washing buffer containing Tween 20.

Sandwich assay procedure

α -Casein standards (100 μ L; dilutions with assay buffer from 0 to 200 $\mu\text{g mL}^{-1}$) were filled into each cavity and shaken at room temperature for 1 h. The plate was washed three times with washing buffer containing Tween 20. Secondary antibody (100 μ L) was added and shaken at room temperature for 1 h. For IgY-anti- α -casein coating, rabbit anti- α -casein (diluted 1:10,000 with assay buffer) was applied as secondary antibody. For rabbit-anti- α -casein coating, IgY-anti- α -casein (diluted 1:1,000 with assay buffer) was employed. After the triple washing procedure with washing buffer containing Tween 20, 100 μ L of anti-rabbit-HRP antibody (diluted 1:50,000 with assay buffer) or 100 μ L of anti-IgY-HRP (diluted 1:30,000 with assay buffer) was put into the corresponding well and shaken at room temperature for an hour. The addition of substrate and stop solution was performed as for the blocking assays. The absorbance was measured at 450 nm with an ELISA reader. Four-parameter equations were formed to describe the standard curves.

For the peanut sandwich assay, 100 μ L of peanut standard (dilutions with assay buffer from 0 to 400 $\mu\text{g mL}^{-1}$) was filled in each well. The assay was performed as described for α -casein, but for rabbit-anti-peanut coating, the secondary antibody was IgY-anti-peanut (diluted 1:1,000 with assay buffer), and for IgY-anti-peanut coating, the secondary antibody was rabbit-anti-peanut (diluted 1:10,000 with assay buffer). The enzyme-labelled antibody was either anti-IgY-HRP (diluted 1:30,000 with assay buffer) or anti-rabbit-HRP (diluted 1:50,000 with assay buffer).

Indirect competitive immunoassay for α -casein and peanut

Extraction procedure of blank matrices

Five grams solid sample (or 5 mL liquid sample) was extracted with 50 mL (or 45 mL) preheated extraction buffer at 60 °C for 15 min. Subsequently, the samples were centrifuged at 9,500 rpm for 10 min. The supernatant was filtered through cellulose acetate filters. The extracted matrices were treated like buffers and diluted in the same way to receive matrix standard curves.

Coating of the microtitre plate

For the competitive ELISA, α -casein was coated with 500 ng mL⁻¹, diluted in coating buffer. α -Casein solution (100 μ L) was put into each cavity and incubated at 4 °C overnight. Washing was performed three times with phosphate buffer without Tween 20.

For the peanut assay, the procedure was the same as mentioned above. Peanut standard was coated in a final concentration of 500 ng mL⁻¹, diluted in coating buffer. This standard (100 μ L) was put into each cavity and incubated at 4 °C overnight.

Blocking of the microtitre plate

Blocking and washing were performed as described for the sandwich ELISA for Ficoll and PVA. BSA (1%) was dissolved in coating buffer (pH 9.6) and used at room temperature for 2 h.

Assay procedure

α -Casein standard (75 μ L; dilutions with assay buffer from 0 to 5,000 μ g mL⁻¹) was filled into each cavity. IgY-anti- α -casein (25 μ L; diluted 1:1 000 with assay buffer) or rabbit-anti- α -casein (diluted 1:10,000) was added immediately afterwards and shaken at room temperature for 1 h. After triple washing with washing buffer containing Tween 20, 100 μ L of anti-IgY-HRP antibody (diluted 1:30,000 with assay buffer) or anti-rabbit-HRP (diluted 1:50,000 with assay buffer) was put into each well and shaken at room temperature for 1 h. The addition of substrate and stop solution was performed as for the sandwich ELISA. Four-parameter equations were formed to describe the standard curves.

For the competitive peanut assay, 75 μ L of peanut standard (dilutions with assay buffer from 0 to 5,000 μ g mL⁻¹) was filled into each cavity. IgY-anti-peanut antibody (25 μ L; diluted 1:1,000 with assay buffer) or rabbit-anti-peanut (diluted 1:10,000 with assay buffer) was added immediately and shaken at room temperature for 1 h. The

rest of the assay was performed as described for the sandwich ELISA.

Results and discussion

Comparison of different blocker efficiencies

Three main groups of blocking reagents were tested: proteins, synthetic reagents and carbohydrates. At first, the blocking reagents were used for blocking the plate without any other coating substance at three different temperatures and times (37 °C/2 h, room temperature/2 h and 4 °C/overnight). Additionally, two different buffers at pH 7.6 and pH 9.6 were applied. The standard curves that formed resulted from the application of α -casein and the corresponding polyclonal antibody, which was detected with a second HRP-labelled antibody. Two protein blockers, BSA and fish gelatine, and three synthetic blockers, PVA, PVP and PEG, were used, respectively. Furthermore, carbohydrates with high molecular weight were used to determine their blocking capacity and efficiency.

Blank values, preferably at absorbances around 0.1 or below, indicate an optimal blocking reagent. Higher values are an indication of non-specific binding of the standard on the microtitre plate, thus signalling insufficient blocking efficiency of the blocking reagents used. Table 1 lists the minimal, maximal absorbances, obtained by Magellan software⁵, and calculated differences in absorbances (Δ abs) for all the blockers used at the different reaction conditions. Δ abs values show the behaviour of the curve with regard to rising standard concentrations.

BSA and fish gelatine were expected to be very reliable protein blockers. However, in reality, even 1% BSA in coating buffer (pH 9.6), as common blocker, yielded absorbances up to 0.2, but there was no trend visible between blank and highest used standard concentration (130 ppb), which showed that BSA was very effective (Δ abs < 0.1, Fig. 1) in all conditions. Fish gelatine behaved a bit differently. At pH 7.6, incubated at room temperature and 4 °C, higher Δ abs of 0.254 and 0.317 were detected, which corresponded to a visible rise in the standard curve. A comparison of the blocking efficiency of BSA with fish gelatine revealed that the latter could also be used as alternative protein blocking reagent at pH 9.6 at all incubation conditions; for pH 7.6, only the incubation at 37 °C for 2 h showed sufficient efficiency (Fig. 1). There was no enhancing trend evident for increasing standard concentrations. In general, 1% fish gelatine reached higher abs_{min} values than BSA, around 0.4 (Table 1), which has to be taken into account during the assay development.

Polyvinylalcohol, polyvinylpyrrolidone and polyethyleneglycol are the three synthetic blocking reagents

Table 1 Minimal and maximal absorbances are listed for various blocking reagents, tested at different conditions (measured at 450 nm)

Incubation temperature and period		37°C 2h			Room temperature 2h			4°C overnight		
	Buffer solution	abs _{min}	abs _{max}	Δabs	abs _{min}	abs _{max}	Δabs	abs _{min}	abs _{max}	Δabs
Proteins										
1% BSA	A	0.183	0.230	0.047	0.160	0.248	0.087	0.193	0.284	0.091
	B	0.182	0.207	0.025	0.158	0.182	0.024	0.239	0.201	0.038
1% fish gelatine	A	0.379	0.470	0.092	0.306	0.560	0.254	0.417	0.734	0.317
	B	0.304	0.367	0.063	0.306	0.342	0.036	0.350	0.386	0.036
Synthetic blockers										
1% PVA	A	0.104	0.787	0.682	0.133	0.491	0.358	0.067	0.240	0.173
	B	0.107	0.509	0.402	0.116	0.736	0.620	0.060	0.728	0.668
3% PVP	A	0.128	1.709	1.580	0.128	2.099	1.971	0.130	1.637	1.507
	B	0.155	1.698	1.543	0.154	1.653	1.499	0.129	1.587	1.458
1% PEG	A	0.116	1.311	1.196	0.081	1.465	1.384	0.091	1.240	1.148
	B	0.139	1.457	1.318	0.127	1.544	1.416	0.101	1.165	1.064
Carbohydrates										
1% dextran 40	A	0.134	2.266	2.132	0.157	2.604	2.447	0.133	1.665	1.532
	B	0.146	2.132	1.986	0.143	1.761	1.619	0.161	1.009	0.848
1% dextran 2000	A	0.140	1.515	1.375	0.125	1.547	1.422	0.156	0.828	0.672
	B	0.115	0.516	0.401	0.110	0.537	0.427	0.127	0.406	0.279
1% Ficoll	A	0.141	0.473	0.332	0.135	0.314	0.178	0.102	0.331	0.229
	B	0.204	0.182	0.023	0.130	0.184	0.054	0.123	0.209	0.086
5% trehalose	A	0.221	2.318	2.097	0.183	1.820	1.638	0.331	1.634	1.303
	B	0.163	2.164	2.000	0.135	1.629	1.493	0.268	1.667	1.399

Differences (Δabs) were calculated by subtraction of abs_{min} from abs_{max}. Each four-parameter curve was formed by Magellan software, based on the measurement of samples in triplicate. Abs_{min} and abs_{max} are parameters from this four-parameter curve. Buffer A=10 mM PBS buffer, pH 7.6; buffer B=carbonate buffer, pH 9.6

employed. As it can be seen from the Δabs values, summarised in Fig. 1, only 1% PVA at pH 7.6 incubated at 4 °C overnight showed sufficient blocking capacity. In prior studies, PVP was considered an alternative synthetic blocker, but neither 3% PVP in coating buffer nor in PBS buffer showed any blocking effects. Curves were obtained, ranging in the absorbance from 0.1 to 2.1. The same effect was shown for 1% polyethyleneglycol in coating and PBS buffer. The results were standard curves ranging in the absorbance from around 0.1 to 1.6. Yet, these results are not satisfactory. PVP and PEG can therefore not be recommended as alternative blocking agents. Only 1% PVA in PBS buffer incubated at 4 °C overnight achieved an absorbance maximum of 0.2. The Δabs reached only 0.173 (Fig. 1) in this case, indicating a comparable blocking capacity to BSA blocking. Hence, 1% PVA could be taken into account for the blocking in ELISA assays at pH 7.6. Higher amounts of PVA were not subject of these experiments because of the increasing insolubility at higher concentrations.

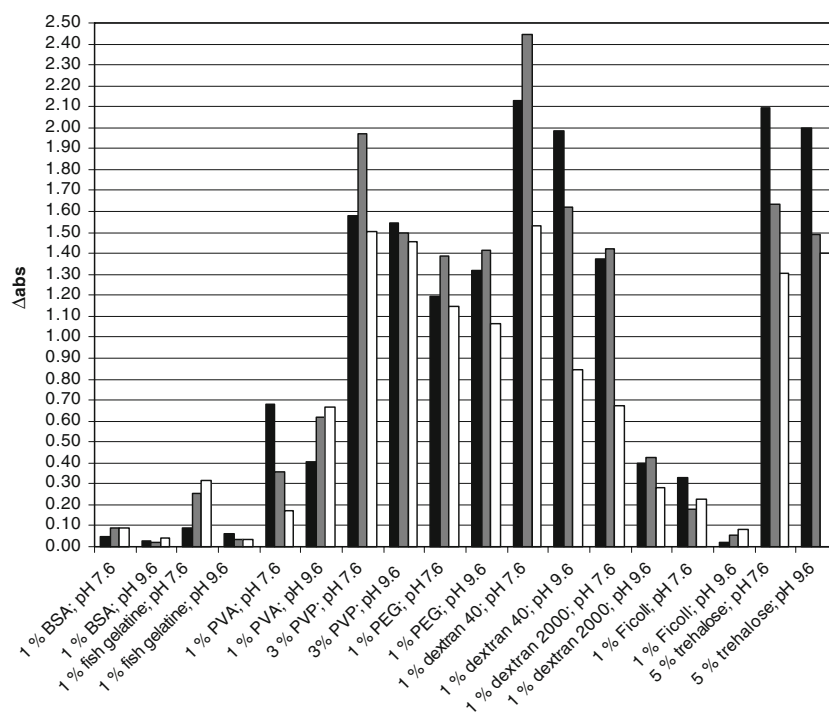
Finally, carbohydrates were tested as alternative blocking agents because of the great amount of hydroxyl groups available for the interaction with the activated surface of the

microtitre plate. The same property is valid for synthetic reagents: They have nothing in common with the proteins to be detected; consequently, no interference with the food allergens is expected in the ELISA. The following carbohydrates were tested: Ficoll, dextran 40, dextran 2000 and trehalose. All carbohydrates were used as 1% solutions except for trehalose, which was a 5% solution dissolved in coating buffer or PBS buffer.

Ficoll is a polysaccharide made of saccharose cross-linked with epichlorohydrin to form a highly branched, high molecular weight and hydrophilic polysaccharide, which behaves neutrally and shows good solubility in aqueous solutions. The size of the Ficoll used was at about 400 kDa.

Dextran is a complex branched glucan synthesised by lactic acid bacteria. Different molecular weights are available. For this study, dextrans of an average size of 40 and 2,000 kDa were used. Trehalose was applied as stabilising agent for plate storage in the dry state due to its high water retention capability. As disaccharide, it contains two α-1,1-glycosidic linked glucose molecules. Trehalose was not able to cover the empty sites on the microtitre plate, as expected. Trehalose (5%) was dissolved in coating and PBS buffer, but

Fig. 1 Summarised data of all Δabs values (450 nm) of Table 1. Tested conditions: 37 °C 2 h (black bars), room temperature 2 h (grey bars), 4 °C overnight (white bars) for each blocking reagent at pH 7.6 (10 mM PBS buffer) and at pH 9.6 (coating buffer)



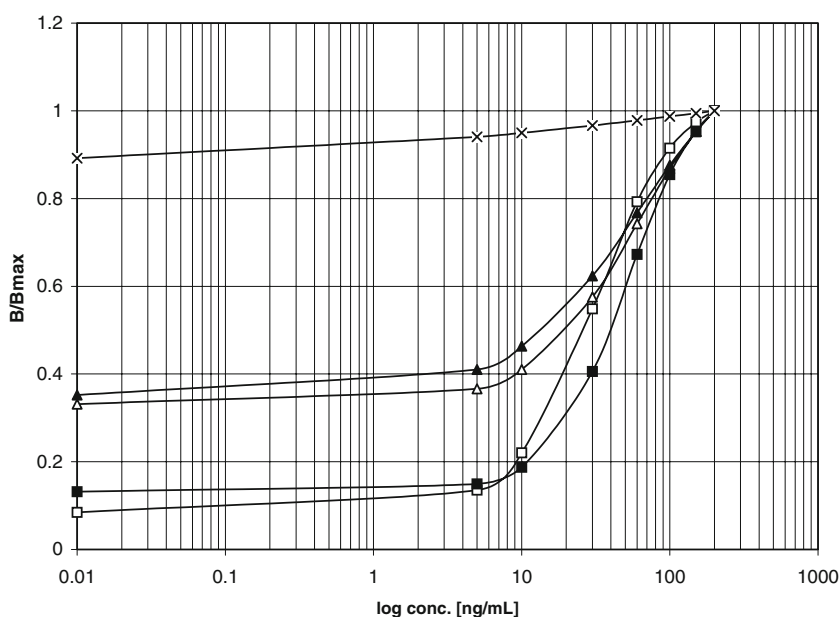
no blocking effects were observed, as shown in the resulting Δabs from 1.3 to 2.1 (Table 1). Standard curves were obtained within a range in absorbance from 0.2 to 2.0. Dextran 40 (1%) was dissolved in coating and PBS buffer, but the blocking results were poor. Dextran 2000 (1%) did better in both buffer solutions, but there was still a visible trend with increasing standard concentrations. Blocking with 1% Ficoll was efficient for all incubation parameters used at pH 9.6 ($\Delta\text{abs} < 0.1$), but not at pH 7.6, where Δabs up to 0.33 was measured (Fig. 1).

Based on these findings, Ficoll and PVA were further investigated for their suitability as alternative “synthetic” blockers for the detection of α -casein and peanut in the sandwich and the competitive ELISA format.

Ficoll and PVA blocking for α -casein indirect sandwich and competitive ELISA format

Chicken anti- α -casein and rabbit anti- α -casein antibodies were coated and blocked with 1% Ficoll in coating buffer

Fig. 2 α -Casein sandwich ELISA: Blocking was performed once with PVA at pH 7.6 and once with Ficoll at pH 9.6. Two sandwich formats were tested: coating $1 \mu\text{g mL}^{-1}$ rabbit-anti- α -casein with PVA blocking (filled triangles), coating $1 \mu\text{g mL}^{-1}$ rabbit-anti- α -casein with Ficoll blocking (empty triangles), coating $1 \mu\text{g mL}^{-1}$ IgY-anti- α -casein with PVA blocking (filled squares), coating $1 \mu\text{g mL}^{-1}$ IgY-anti- α -casein with Ficoll blocking (empty squares). The assays were performed without coating antibody before to check the blocking efficiency too. PVA blocking in the sandwich format with IgY-anti- α -casein coating is shown (multiplication signs)



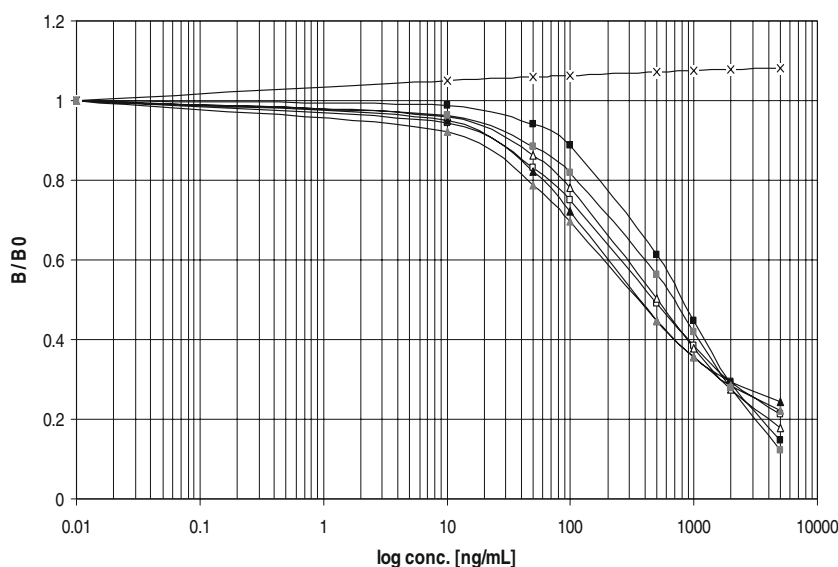


Fig. 3 α -Casein competitive ELISA: The assay was performed with rabbit-anti- α -casein and IgY-anti- α -casein. α -Casein (500 ng mL^{-1}) was coated on the microtitre plate and blocked with PVA at pH 7.6, BSA at pH 9.6 or Ficoll at pH 9.6. Rabbit-anti- α -casein with PVA blocking (black triangles), rabbit-anti- α -casein with BSA blocking (grey triangles) and rabbit-anti- α -casein with Ficoll blocking (white triangles)

were diluted 1:10,000. IgY-anti- α -casein with PVA blocking (black squares), IgY-anti- α -casein with BSA blocking (grey squares) and IgY-anti- α -casein with Ficoll blocking (white squares) were diluted 1:1,000. The assays were performed without coating antibody before to check the blocking efficiency as well. PVA blocking in the sandwich format with rabbit-anti- α -casein coating is shown (multiplication signs)

for 2 h at room temperature or with 1% PVA in PBS buffer at 4°C overnight. Four-parameter equations were formed with the measured absorbances by Magellan5 software. The measurements were all performed in triplicate. B/B_0 and $B/B_{\max}$ were calculated. The results for the sandwich assay are shown in Fig. 2. Each assay could be directly compared at the two different blocking conditions and they perfectly matched. There was a difference between the two formats, depending on the antibody used for coating and for detection of α -casein. The curves for rabbit-anti- α -casein coating started with higher blank values and showed a much lower increase in absorbance than the curves obtained with IgY-anti- α -casein coating.

The same assays without coated antibody showed good blocking effects in both assay formats for the corresponding blocking solutions. Hence, only one blocking curve is shown in Fig. 2. No curve in sigmoid shape was achieved, indicating the good inhibition by the blocking solution.

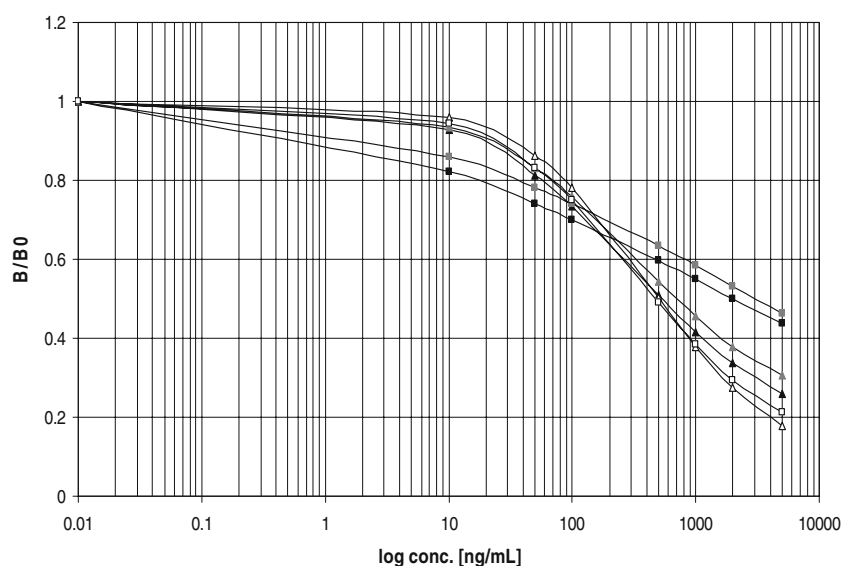
The same assumption was made for the competitive ELISAs shown in Fig. 3. The results were calculated in the same way as for the sandwich ELISA. The B/B_0 values were received from the four-parameter curve from Magellan5 software. Again, only one curve is shown for the blocking capacity of the two blocking reagents. Two aspects in Fig. 3 are worth considering: The standard curves for rabbit-anti- α -casein showed the same behaviour no matter if blocked with BSA, Ficoll or PVA. The blocking solutions were equally suitable. The same was shown for IgY-anti- α -casein, but the curve which was blocked with PVA showed a steeper slope

than the one blocked with Ficoll. The standard curve obtained with BSA blocking can be found in between these two curves. Hence, the efficiencies of PVA and Ficoll blocking were proven.

By comparing the two types of polyclonal antibodies according to their different origin, different sensitivities can be shown with the competitive ELISA format (Fig. 3). The rabbit antibody was diluted ten times higher than the chicken antibody. However, the antibodies seemed to be equally sensitive within the appropriate dilutions in the competitive ELISA format. The curves were all very close and had a similar shape. In particular, the standard curves of both antibodies, which were blocked with Ficoll, were nearly identical.

To find out the behaviour of the synthetic and carbohydrate blocker in food matrices, one solid and one liquid sample were tested in the indirect competitive format (Figs. 4 and 5). Figure 4 shows the results of buffer and matrix standard curves blocked with Ficoll at pH 9.6. The rabbit antibody gave the same standard curves, not influenced by the different matrices used. In contrast, the chicken antibody was influenced by the cookies and the soy milk, resulting in a different shape of the curve compared to the standard curve recorded in buffer solution. Although PVA blocking had proved satisfactory results in buffer solution, Fig. 5 demonstrates high matrix effects for both antibodies used. For using PVA as blocking reagent in ELISA development, additional studies are necessary to overcome matrix effects as shown in Fig. 5.

Fig. 4 α -Casein competitive ELISA: The assay was performed with rabbit-anti- α -casein and IgY-anti- α -casein. α -Casein (500 ng mL^{-1}) was coated on the microtitre plate and blocked with Ficoll at pH 9.6. Rabbit-anti- α -casein in buffer (white triangles), rabbit-anti- α -casein in cookie matrix (black triangles) and rabbit-anti- α -casein in soy milk (grey triangles) were diluted 1:10,000. IgY-anti- α -casein in buffer (white squares), IgY-anti- α -casein in cookie matrix (black squares) and IgY-anti- α -casein in soy milk (grey squares) were diluted 1:1,000



Ficoll and PVA blocking for peanut indirect sandwich and competitive ELISA format

Chicken and rabbit antibodies were coated onto the microtitre plate and blocked with 1% Ficoll in coating buffer for 2 h at room temperature and 1% PVA in 10 mM PBS buffer at 4 °C overnight. The measurements were performed in triplicate, and Magellan5 software formed a four-parameter curve out of these values, which was then applied for calculating B/B_0 and $B/B_{\max}$ values for each concentration measured. These data can be seen in Fig. 6 for the sandwich ELISA for peanut. The curves can be compared in respect of the blocking solution and of the antibodies. All curves were very close and showed the same behaviour. Both

Ficoll and PVA proved to be effective blockers and yielded results that perfectly matched.

The assays were performed without coating antibodies before as well. One outcome is shown in Fig. 6 because they did not differ in behaviour. It was a straight line close to 1 for $B/B_{\max}$, lacking non-specific binding.

The competitive ELISA assays were equally performed with chicken and rabbit antibodies and blocked with 1% Ficoll in coating buffer for 2 h at room temperature and with 1% PVA in 10 mM PBS buffer at 4 °C overnight. The calculation of the curves was done as described for the sandwich ELISA format. Figure 7 shows the results, which are comparable for both blocking solutions. Parallel standard curves were obtained with the same slope and slightly different IC_{50} values for

Fig. 5 α -Casein competitive ELISA: The assay was performed with rabbit-anti- α -casein and IgY-anti- α -casein. α -Casein (500 ng mL^{-1}) was coated on the microtitre plate and blocked with PVA at pH 7.6. Rabbit-anti- α -casein in buffer (white triangles), rabbit-anti- α -casein in cookie matrix (black triangles) and rabbit-anti- α -casein in soy milk (grey triangles) were diluted 1:10,000. IgY-anti- α -casein in buffer (white squares), IgY-anti- α -casein in cookie matrix (black squares) and IgY-anti- α -casein in soy milk (grey squares) were diluted 1:1,000

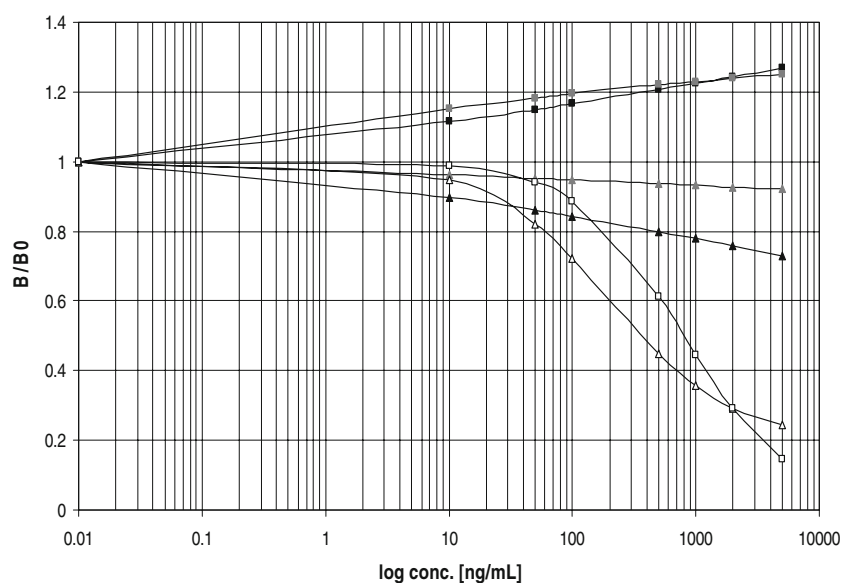
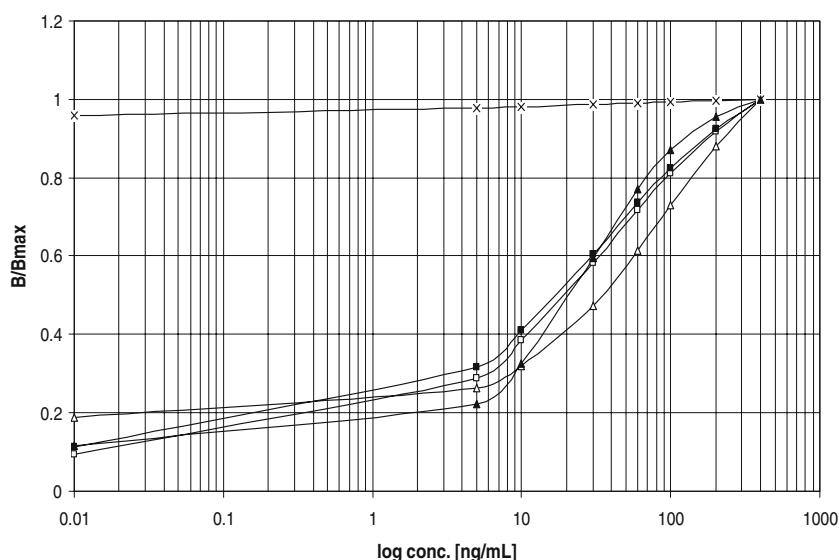


Fig. 6 Peanut sandwich ELISA: Blocking was performed once with PVA at pH 7.6 and once with Ficoll at pH 9.6. Two sandwich formats were tested: coating $0.1 \mu\text{g mL}^{-1}$ rabbit-anti-peanut with PVA blocking (*filled triangles*), coating $0.1 \mu\text{g mL}^{-1}$ rabbit-anti-peanut with Ficoll blocking (*empty triangles*), coating $2 \mu\text{g mL}^{-1}$ IgY-anti- α -peanut with PVA blocking (*filled squares*), coating $2 \mu\text{g mL}^{-1}$ IgY-anti-peanut with Ficoll blocking (*empty squares*). The assays were also performed without coating antibody before to check the blocking efficiency. PVA blocking in the sandwich format with IgY-anti-peanut coating is shown here (*multiplication signs*)



rabbit-anti-peanut antibody. For IgY-anti-peanut antibody, the IC_{50} values differ by a factor of 3, but the behaviour of the curve is similar. The blocking solutions were both effective.

The assays prepared without coating peanut did not show OD values above 0.2. Therefore, the blocking efficiency of Ficoll and PVA was established once again.

Conclusion

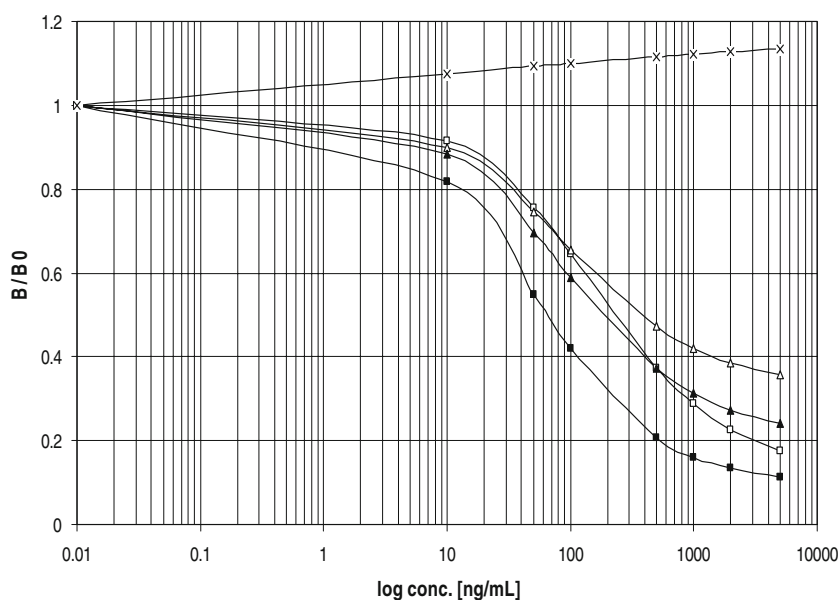
This study showed that non-specific binding on polystyrene microtitre plates during immunosorbent assays can be reduced by various blocking solutions. The most effective blockers remain the proteins, e.g. BSA and fish gelatine. However, for food allergens, alternatives to proteins were

required. PVA, PVP and PEG were tested, representing synthetic blockers, but only PVA was capable of inhibiting non-specific binding in buffer systems. Furthermore, carbohydrates were taken into account owing to their high molecular weight and their hydroxyl groups. Dextran 40, dextran 2000, Ficoll and trehalose were considered to represent a range of different carbohydrates. Only Ficoll showed the desired blocking efficiency.

Based on these findings, two feasible blockers other than proteins remained with their specific reaction conditions: 1% Ficoll at pH 9.6 for 2 h at room temperature, 1% PVA at pH 7.6 at 4 °C overnight (only in buffer systems).

The application of Ficoll and PVA for immunosorbent assays for food allergens was shown in two examples: α -casein and peanut detection. For each food allergen,

Fig. 7 Peanut competitive ELISA: The assay was performed with rabbit-anti-peanut and IgY-anti-peanut. Peanut (500 ng mL^{-1}) was coated on the microtitre plate and blocked with PVA at pH 7.6 or Ficoll at pH 9.6. Rabbit-anti-peanut with PVA blocking (*filled triangles*) and rabbit-anti-peanut with Ficoll blocking (*empty triangles*) were diluted 1:10,000. IgY-anti-peanut with PVA blocking (*filled squares*) and IgY-anti-peanut with Ficoll blocking (*filled squares*) were diluted 1:1,000. Again, the assays were run without coating antibody before to check the blocking efficiency. PVA blocking in the sandwich format with rabbit- α -casein coating is shown (*multiplication signs*)



sandwich and competitive formats were performed. Both Ficoll and PVA proved suitable for the different ELISA formats in buffer systems. PVA was not capable of dealing with the matrix; in contrast, Ficoll gave comparable results in the buffer and the matrix.

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