Direct Biosensor Immunoassays for the Detection of Nonmilk Proteins in Milk Powder

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The low prices of some nonmilk proteins make them attractive as potential adulterants in dairy products. An optical biosensor (BIACORE 3000) was used to develop a direct and combined biosensor immunoassay (BIA) for the simultaneous detection of soy, pea, and soluble wheat proteins in milk powders. Affinity-purified polyclonal antibodies raised against the three protein sources were immobilized in different flow channels (Fcs) on the biosensor chip (CM5). Dissolved milk powders were injected (20 μL injections at 20 μL min^{-1}) through the serially connected Fcs, and the antibody-bound plant proteins were detected directly. The total run time between samples, including a regeneration step with 5 μL of 10 mM HCl, was 5 min. The limits of detection in milk powder were below 0.1% of plant protein in the total milk protein content. The antibodies also recognized some proteins from other plant sources, which made this BIA even more suitable as a broad screening assay for nonmilk proteins.

Keywords: *Plant proteins; soy; pea; soluble wheat proteins; milk powder; direct biosensor immunoassay; surface plasmon resonance; BIACORE*

INTRODUCTION

The compositional standards for most milk products require that they contain no other proteins than milk proteins, unless declared. The low prices of some nonmilk proteins make them attractive as potential adulterants in milk powders and other dairy products. Soy protein is probably the nonmilk protein most commonly used in milk replacers such as simulated yogurts, coffee whiteners, and frozen desserts, and it is likely to be a major potential adulterant. Several preparations of soy proteins are commercially available, such as soy flour (42−52% of protein), soy protein concentrates (62−69% of protein), soy isolates (82−87% of protein), and soy hydrolysates (∼20% of protein). Other possible sources of nonmilk proteins include plant proteins such as wheat gluten, maize, pea, bean, rice, and potato proteins, soluble cereal protein hydrolysates, and even gelatin, blood plasma, egg, or fish proteins. In addition, cheap wheat proteins, mainly in the form of hydrolysates, can be found as a byproduct of the production of glucose and fructose from starch. For the detection of plant proteins in dairy products, several analytical techniques can be applied.

Of the polyacrylamide gel electrophoresis (PAGE) methods, sodium dodecyl sulfate (SDS)-PAGE is the most common technique. Down to 5% of processed soy milk could be detected in pasteurized skimmed milk by four distinct soy characteristic bands in SDS-PAGE (*1*). By using an immunoblotting step after the electrophoretic separation, as little as 25 ng of soy protein per lane was detected in milk replacers (*2*). The detection limit of SDS-PAGE was lowered to detect 0.06% of soy protein in total protein of melted cheese by introducing a selective sample treatment removing soluble casein from insoluble soy protein (*3*).

Capillary zone electrophoresis (CZE) has the potential to obtain rapid separations with high plate numbers together with easy quantification of the peaks. CZE was applied to analyze soy and milk proteins that could potentially be detected on the basis of their different CE patterns (*4*).

Compared with electrophoresis, the main advantages of immunochemical methods are the high specificity, sensitivity, and large sample throughput. The AOAC adopted an enzyme-linked immunosorbent assay (ELISA) procedure for the detection of soy protein in meat products, which proved to be semiquantitative in a collaborative study (*5*). A modified immunoassay for the detection of soy milk in pasteurized skimmed bovine milk was also described (*6*). ELISA and immunoblotting procedures were developed for the specific determination of SDS-denatured glycinin A (*7*). This protein exists in all of the soy variants described so far, and the antigenicity of the denatured protein is independent of food-processing conditions (*7*). Highly specific monoclonal antibodies have been produced against soybean glycinin and have been used as molecular probes to investigate its structure, subunit composition, and structural modifications induced by technological treatments (*8*). Monoclonal antibodies have also been developed against various gluten protein fractions (*9*, *10*). An ELISA format and several monoclonal antibody combinations were developed to determine wheat gliadins in unheated gluten-free food for celiac patients (*11*). Other applications include varietal identification in cereals

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10.1021/jf010440p CCC: $20.00
Published on Web 10/05/2001

and quantification of baking-quality related proteins in gluten. Similarly, highly active antisera against several isolated zeins and maize glutelin have also been obtained (*12*).

Applications for the simultaneous detection of several plant proteins in milk products were not described with regard to any of these methods. In the present study, the application of an automated four-channel optical biosensor (BIACORE 3000) was tested as a possible tool for the simultaneous detection of nonmilk proteins in milk products. The detection principle of this biosensor is based on surface plasmon resonance (SPR), which has been described previously (*13*). The major advantages of this system are that the detection can be performed without labeling of the reagents, results can be obtained within minutes, four assays can be run simultaneously as a result of the application of the four-channel mode, and the automation of the total procedure, which renders this system suitable for high-throughput screening. SPR-based biosensor applications in food control are described for the detection of drug residues in milk (*14*, *15*) and pig bile (*16*, *17*). For these low molecular weight drug residues, the competitive inhibition immunoassay format was applied.

For the detection of the plant proteins, we have selected the direct biosensor immunoassay (BIA) format in which antibodies are immobilized on the sensor surface and the binding of plant proteins to the immobilized antibodies is detected directly (without labeling). Commercially available preparations of vegetal proteins [soy isolate, pea isolate, and soluble wheat protein (SWP)] were selected as potential adulterants. The aim was to detect at least 1% of the plant proteins in the total protein content (~37%) of milk powders. Lower levels of adulteration are not of commercial interest.

MATERIALS AND METHODS

Instruments and Reagents. The BIACORE 3000, sensor chips (CM5), HBS-EP buffer [pH 7.4, consisting of 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 150 mM sodium chloride, 3 mM EDTA, 0.005% (v/v) surfactant polysorbate 20] and an amine coupling kit [containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), and 1 M ethanolamine hydrochloride−NaOH (pH 8.5)] were supplied by BIACORE AB (Uppsala, Sweden). The fast protein liquid chromatography (FPLC) system, HiTrap protein G columns (1 mL), and CNBr-Sepharose were supplied by Amersham Pharmacia Biotech AB (Uppsala, Sweden). Affinity-purified total rabbit IgG (10 mg/mL) was obtained from Lampire (Pipersville, PA). Centricon centrifugal filter devices (YM-10, 2 mL) were obtained from Millipore (Bedford, MA). Bovine serum albumin (BSA) and sodium caseinate were obtained from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Whey protein concentrate (Bipro) was supplied by Davisco Food International. The BCA protein assay was supplied by Pierce (Rockford, IL).

Preparation of Plant Protein Extracts. Commercially available plant protein products were used. Supro 500 E soy protein isolate (I) was obtained from Anvisa (Madrid, Spain), and the Europroducts 595 soy protein isolate (II) was obtained from Europroducts (Milan, Italy). The pea protein isolate (Pisane HD) is produced by Cosucra SA (Fontenoy, Belgium), and the soluble wheat protein (SWP100) was from the Amylum group in Belgium. SWP 100 is an enzymatically modified and deamidated wheat protein product, which is widely used as an emulsifier.

For the extraction of proteins, 1 g of the plant product was mixed with 20 mL of a solution containing 50 mM tris(hydroxymethyl)aminomethane (Tris) and 200 mM NaCl (pH 8.0). After mixing (magnetic stirrer) for 30 min and centrifugation (5 min at 5000*g*), the supernatants were filtered (0.45 μm). The protein contents of the filtrates were determined by the BCA protein assay, with BSA as a standard. The concentrations of protein in the filtrates of soy, pea, and SWP were 4, 7.5, and 18 mg mL^{-1}, respectively.

Immunization of Rabbits. Polyclonal antibodies were raised in rabbits against one of the soy protein isolates (Supro 500E), the pea protein isolate (Pisane HD), and SWP100.

The immunizations were performed at the Laboratory of Hormonology (Marloie, Belgium). Two rabbits (New Zealand White SPF) were immunized with the particular protein extracts. Injections were performed subcutaneously according to the laboratory standard protocol: first injection at day 0, second injection at day 14, third injection at day 28, and thereafter one injection every 4 weeks. The first injection consisted of 0.5 mg of protein in 0.1 mL mixed with 0.4 mL of a solution of 0.9% (m/v) NaCl and 0.5 mL of Freund's complete adjuvant. In all subsequent injections, the complete adjuvant was replaced by Freund's incomplete adjuvant. The first bleeding was performed at day 0 before the first injection (preimmune serum) and the second bleeding at day 38. The following bleedings were performed every 10 days after each injection. After collection, the blood samples were placed in vacutainer tubes at room temperature for 24 h. Thereafter, the blood was centrifuged, and the collected serum was stored at −80 °C until further use. The sera obtained from the sixth bleeding were used in this study.

Isolation of Total IgG. The rabbit IgGs were isolated from crude serum samples by ammonium sulfate precipitation (*18*), followed by affinity chromatography using a HiTrap Protein G column in accordance with the manufacturer's instruction manual.

Isolation of Specific IgG. Affinity columns were prepared by coupling soy protein, pea protein, and SWP to CNBr-activated Sepharose in accordance with the manufacturer's instruction manual. The amounts of coupled protein for soy, pea, and SWP were 2.5, 3.5, and 4.7 mg mL^{-1} of gel, respectively. The crude antisera (3−4.5 mL) were five times diluted with phosphate-buffered saline (PBS), and the mixtures were transferred to the affinity columns. The columns were washed with PBS (10 mL), and the bound antibodies were eluted with five fractions of 1 mL of a 0.1 M HCl solution. The pH of the eluted fractions was adjusted (between 6.5 and 8.0) by the addition of 0.1 mL of a 0.67 M phosphate buffer and 0.1 mL of a 1 M NaOH solution. The fractions were combined, and a buffer exchange to PBS was performed by means of a Centriprep (10 kDa). The protein concentration of the final retentate was determined by the BCA protein assay. The retentates were stored at −20 °C until further use.

Biosensor Chip Preparation. The antibodies were immobilized on the sensor surface of a CM5 sensor chip by the use of the amine coupling kit and the Surface Preparation Wizard as present in the BIACORE 3000 control software. The biosensor surface was activated by injecting (35 μL at a flow rate of 5 μL min^{-1}) a mixture of EDC and NHS (1:1, v/v) into one of the four flow channels. Then the antibodies, diluted (0.1 mg mL^{-1}) in coupling buffer (10 mM sodium acetate; pH 4.5) were injected and attached to the carboxymethylated dextran surface via primary amine groups. After coupling, active groups were blocked with ethanolamine (1 M).

Sample Materials. Soy protein isolate, pea protein isolate, and soluble wheat protein (SWP100) were used to prepare calibration standards in nonheated freeze-dried milk powder and to prepare adulterated milk samples, which were heat treated (pasteurized and UHT) prior to spray-drying.

Preparation of Calibration Standards. Skimmed milk powder (NILAC; 160 g) was added to 1.60 L of water of 40 °C and stirred for 2 h. To facilitate freeze-drying, the calibration standards were prepared with extra added milk proteins. Whey protein concentrate (23 g) and sodium caseinate (93 g) were dissolved in 1.60 L of water at ~10 °C using mechanical stirring and ultrasonic treatment. The pH was adjusted to 7.8 by adding 4 M NaOH. After both solutions (pH 7.1) had been mixed and the protein content measured, the plant protein

was added to 1.2 kg of solution (5.62 g of soy, 5.3 g of pea, and 5.99 g of SWP100, respectively), resulting in ~8% of plant protein added to the protein of the reconstituted milk. To avoid settling out of the plant proteins, the soy and pea standard solutions were homogenized twice at 500 bar at 45 °C. The efficiency of homogenization was checked by means of microscopy. The homogenized solutions did not show any precipitated matter after a few days at 4 °C. Dilutions were made on the basis of weight and subsequently freeze-dried. The freeze-dried powder was extremely hygroscopic, making weighing of small amounts difficult. To obtain a product with lesser hygroscopic properties, and thus easier to handle, the product was equilibrated with air having ~50% humidity. Spread in a layer ~1 cm thick, the powder was equilibrated in ~3 h (checked by weighing), resulting in a moisture content of ~10%.

For the soy calibration standards, the average total protein content was 57.4 ± 0.5% and the concentrations of soy (percent of soy protein in the milk protein content) were 8.48, 4.24, 3.18, 2.12, 1.06, 0.53, and 0%, respectively. For the pea calibration standards, the average total protein content was 57.0 ± 0.7% and the concentrations of pea (percent of pea protein in the milk protein content) were 7.11, 3.69, 1.87, 0.94, 0.48, and 0%. For the SWP calibration standards, the average total protein content was 57.2 ± 0.6% and the concentrations of SWP (percent of SWP protein in the milk protein content) were 8.18, 4.09, 2.05, 1.02, 0.51, and 0%. These samples were stored at −20 °C before analysis.

Preparation of Adulterated Skimmed Milk Powders. Skimmed milk was adulterated with each plant protein [soy protein isolate (I and II), pea protein isolate, and SWP100] at levels of 0, 1, 2, and 5% (percent of plant protein in the milk protein content). To 400 L of skimmed milk was added 0.82 kg of the plant protein powder (5% plant protein), and the pH was adjusted to 7.2 by the addition of a 6 M NaOH solution. The solution was stirred overnight at 4 °C and then homogenized three times at 250 bar at 45 °C. No settling out of plant protein was observed after this treatment. Of this milk, 80 L was mixed with 120 L of skimmed milk (2% plant protein) and 40 L was mixed with 160 L of skimmed milk (1% plant protein). The 0% plant protein consisted of skimmed milk only.

The solutions with 0, 1, 2, and 5% plant protein were pasteurized and spray-dried using the pilot plant spray-dryer under low heat conditions, resulting in low-heat spray-dried powder. The same process was repeated for the preparation of high-heat powder, except that an ultrahigh-temperature (UHT) procedure (130 °C, 1 min) was applied before concentration. Care was taken to avoid contamination between the different products. The average protein content of these milk powders was 37.2 ± 0.5%. These samples were stored at −20 °C before analysis.

Biosensor Immunoassay (BIA). Due to the higher protein content (57%) of the calibration standards, 60 mg was weighed in a plastic tube and 9.9 mL of PBS was added. From the adulterated and unknown milk powder samples (protein content ~37%), 100 ± 5 mg was weighed in a plastic tube and 9.9 mL of PBS was added. After 10 s of vigorous mixing, the samples were mixed head over head for 30 min at room temperature. To avoid the injection of particles into the biosensor, 1 mL portions of the dissolved samples were pipetted in vials (Eppendorf) and centrifuged for 5 min at 5000*g*. Of the supernatants, 0.1 mL was pipetted into a microtiter plate, which was sealed with a plate sealer.

In the final format, the four flow channels (Fcs) of the CM5 biosensor chip were coated with total rabbit IgG (Fc1), anti-soy (Fc2), anti-pea (Fc3), and anti-SWP (Fc4). In Fc1 we aimed for a level of 10000 RU, whereas in the other Fcs we aimed for a level of 15000 RU. The BIACORE 3000 operated at a temperature of 25 °C, the running buffer was HBS-EP, with a flow rate of 20 μL min^{-1}, and 20 μL of the samples was injected. The immobilized antibodies were regenerated by the injection of 5 μL of a 10 mM HCl solution. The relative responses measured 10 s before the regeneration started were used for the measurements and calculations. The total run time between samples was 5 min.

RESULTS AND DISCUSSION

Preparation of the Biosensor Chip. During the first experiments, the total IgG fraction of the soy polyclonal antiserum (6 mg mL^{-1} raw serum) was used, of which a small fraction [~10 ng, which corresponded with a response of ~8700 RU (*19*)] was immobilized in one of the flow channels of the biosensor chip. The binding of soy proteins dissolved in buffer, injected at concentrations ranging from 0.01 to 2 mg mL^{-1}, resulted in weak responses only (from 12 to 60 RU). This indicated that the amount of immobilized specific anti-soy antibodies was too low to obtain a sufficient response of binding soy proteins. Therefore, the specific anti-soy antibodies were isolated from the raw serum (0.39 mg mL^{-1} of raw serum) by affinity chromatography on a soy protein column. This confirms the low concentration (6%) of specific antibodies in the total IgG fraction of the anti-soy serum. Using the same procedures, the percentages of specific antibodies in the total IgG fraction of the raw antisera for pea and SWP were determined to be 20 and 7%, respectively. Therefore, due to the presence of irrelevant IgG, the use of the total IgG fraction was unsuitable for the direct BIA concept.

The specific antibodies were immobilized on a second biosensor chip. The anti-soy was immobilized in Fc2 (17900 RU), the anti-pea in Fc3 (17400 RU), and the anti-SWP in Fc4 (17700 RU). Fc1 was activated with EDC/NHS, was directly blocked with ethanolamine, and served as the blank reference Fc. The four Fcs were serially connected, and the response measured in the blank reference Fc (Fc1) was subtracted from the responses measured in the other Fcs.

BIA with the Plant Proteins Dissolved in Buffer. Solutions with different concentrations (0.02−2000 μg mL^{-1}) of the soy, pea, and SWP proteins were injected (20 μL at flow rates of 5 and 20 μL min^{-1}). For the regeneration of the immobilized antibodies, 5 μL of a 10 mM HCl solution was injected. Typical sensorgrams obtained during this experiment for the binding of soy, pea, and SWP proteins to the anti-soy (Fc2), anti-pea (Fc3), and anti-SWP (Fc4) are shown in Figure 1. The total run time between two samples, for 20 μL injections at 20 μL min^{-1}, was ~5 min. At concentrations of 200 μg mL^{-1}, maximum responses of 450 RU due to the binding of soy proteins and 550 RU due to the binding of pea and SWP proteins were obtained. The responses obtained 10 s before the regeneration started (R in Figure 1) were plotted against the different concentrations of the proteins, and the dose−response curves are shown in Figure 2. Increased responses with increasing concentrations were observed, and higher responses were obtained at lower flow rates (5 μL min^{-1}) due to the longer contact time between antibody and antigen. The limits of detection (LOD) for the three plant protein solutions in buffer (defined as 3 times average response of the zero standard) were between 0.2 and 0.5 μg mL^{-1} (4−10 ng of protein per injection) at a flow rate of 20 μL min^{-1} and ~10 times lower (0.4−1 ng of protein) at a flow rate of 5 μL min^{-1}. As shown in the inset of Figure 2, the dose−response curves were most sensitive at lower concentrations (<200 μg mL^{-1}), whereas at higher concentrations the immobilized antibodies became saturated with proteins, as visualized by flattening of the curves.

BIA in Milk Powders. The calibration standards prepared in skimmed milk powder had concentration ranges from 0 to 8% of the plant proteins in the milk

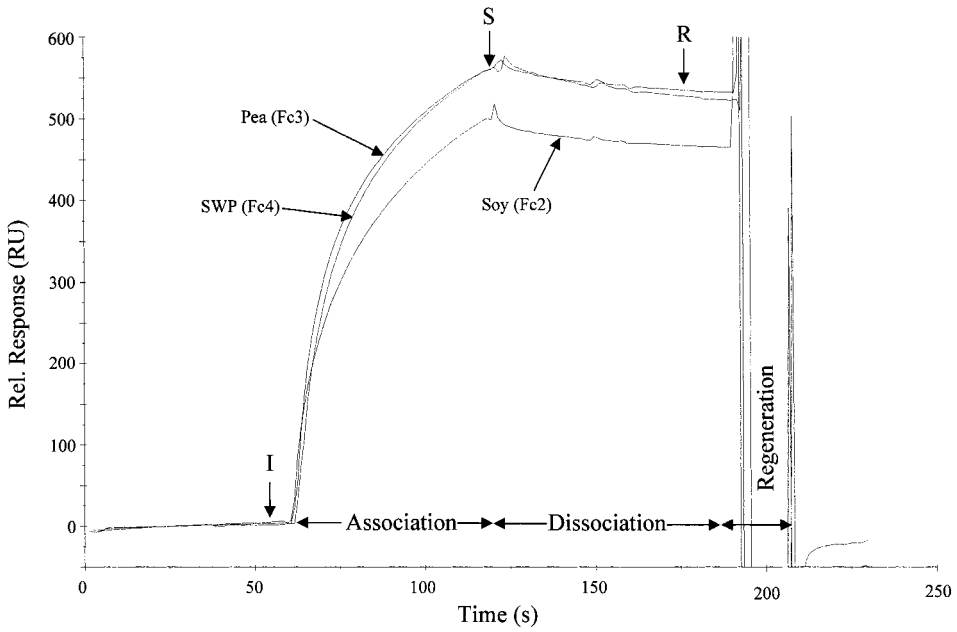

Figure 1. Sensorgrams obtained after the injection (20 μL at a flow rate of 20 μL min^{-1}) of the soy, pea, and SWP proteins dissolved in buffer (200 μg mL^{-1}) in Fc2 (anti-soy), Fc3 (anti-pea), and Fc4 (anti-SWP). I, start injection; S, stop injection; R, used response.

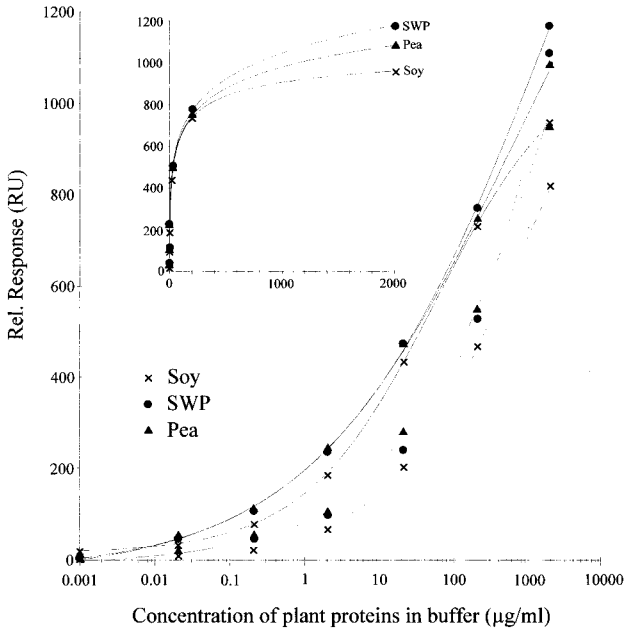

Figure 2. Dose−response curves obtained in the three Fcs after the injections (20 μL) of different concentrations of plant proteins (soy, pea, and SWP) dissolved in buffer and at two flow rates [(—) 5 μL min^{-1}; (- - -) 20 μL min^{-1}]. Inset: Responses for the three plant proteins (5 μL min^{-1}) plotted against the linear concentration.

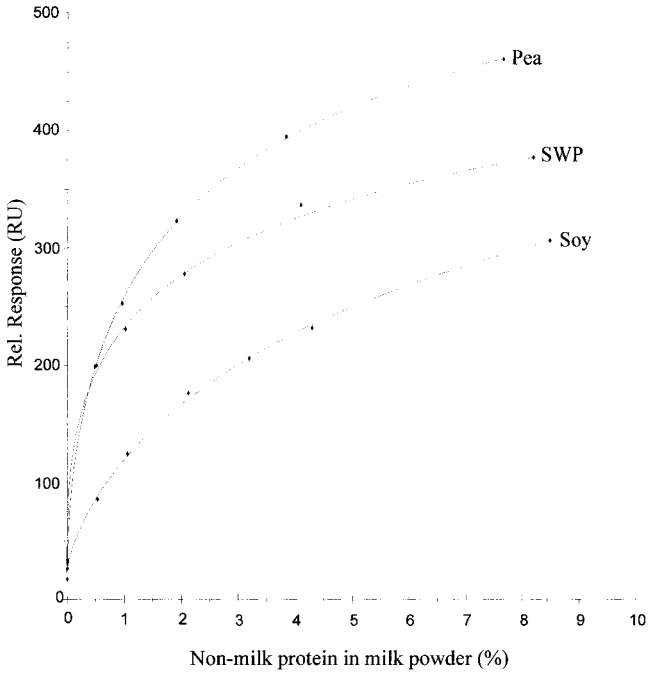

Figure 3. Dose−response curves obtained in the three Fcs with the calibration standards in milk powder (0−8% of plant protein in the milk protein). Twenty microliter injections were made at a flow rate of 20 μL min^{-1}.

protein content (average protein content of the calibration standards was 57%) and were dissolved in HBS-EP buffer (60 mg in 9.9 mL). After the injection (20 μL) of the zero calibration standards, nonspecific binding (90 RU at a flow rate of 20 μL min^{-1}) was observed in the Fcs with immobilized antibodies (probably protein−protein interactions) and almost no nonspecific binding was observed in the blank reference Fc (Fc1). To correct for this nonspecific binding, a new biosensor chip was prepared, in which immobilized total rabbit IgG was used as reference in Fc1 (final response of 9000 RU). The anti-plant protein antibodies were once again immobilized in the other three Fcs (final response in each Fc of ~14000 RU). With this biosensor chip, the response due to nonspecific binding from the zero calibration standard in Fc1 was ~40 RU, which was a little less than the responses in the other Fcs (~60 RU). The responses obtained after the injections of the calibration standards in milk powder and corrected for the reference Fc were plotted against the percentages of nonmilk protein (see Figure 3). Similarly as obtained with the standard curves in buffer (Figure 2), the highest sensitivity was obtained at the lower concentrations (<1% of plant protein in the total protein content of milk powder).

Table 1. Average Percentages of Nonmilk Proteins (Soy, Pea, and SWP) in the Adulterated Milk Powders (Pasteurized and UHT) As Determined with the BIAs in Duplicate, on Five Separate Days, Using the Calibration Standards in Milk Powder

adulterated milk powder with	nonmilk protein in adulterated milk powder (%)					
	pasteurized			UHT		
	average	SD	CV (%)	average	SD	CV (%)
soy I 1%	1.06	0.16	15.1	0.98	0.15	15.3
soy II 1%	0.95	0.12	12.6	1.03	0.14	13.6
soy I 2%	1.82	0.29	15.9	1.80	0.44	24.4
soy II 2%	2.02	0.23	11.4	2.41	0.27	11.2
soy I 5%	4.56	0.47	10.3	5.12	0.64	12.5
soy II 5%	5.19	0.59	11.4	6.10	0.67	11.0
pea 1%	0.95	0.17	17.9	0.71	0.15	21.1
pea 2%	1.66	0.23	13.9	1.14	0.09	7.9
pea 5%	4.03	0.77	19.1	2.56	0.30	11.7
SWP1%	1.32	0.39	29.5	0.52	0.18	34.6
SWP 2%	1.92	0.25	13.0	1.01	0.30	29.7
SWP 5%	5.07	0.36	7.1	1.53	0.24	15.7

Quantification of Nonmilk Proteins in Adulterated Milk Powders. The calibration standards in milk powder were injected on five successive days, and the curves were used to calculate the percentages of nonmilk proteins in the adulterated milk powders dissolved in PBS (100 mg in 9.9 mL), which were injected on five different days as well. The results are summarized in Table 1. The calculated percentages for the soy-containing samples matched with the expected percentages and the CVs were fair (between 10 and 24%). Comparable results were obtained with the pasteurized samples containing pea proteins and SWP. The percentages of pea and SWP in the UHT samples were highly underestimated. This might be caused by denaturation of the plant proteins during the heat treatment process, which might make them less soluble or might change the antigenic properties.

Analysis of Nonadulterated Milk Powders. Different milk powders ($n = 33$), obtained from the Dutch General Inspection Service in 2000, were analyzed according to the same procedure and were all found to be negative with regard to the proteins involved in this study. The average background responses obtained in the Fcs with anti-soy, anti-pea, and anti-SWP were 27 ± 3, 29 ± 3, and 27 ± 2 RU, respectively. Using the calibration standards in milk powder, the calculated backgrounds were 0.02 ± 0.01, 0.001 ± 0.001, and 0.001 ± 0.001%, respectively. This means that the LOD (= average background + 3 SD) were 0.05% for the soy proteins and 0.004% for the pea proteins and SWP (percent of plant protein in the total milk protein content).

Differences between Adulterated and Nonadulterated Milk Powders. For the screening of milk powders for adulteration with nonmilk proteins, the differences between responses obtained with nonadulterated and adulterated samples are important. The responses (RU) measured several times, spread over 8 days, with the blank calibration standards (0%), the 1% calibration standards (C) as well as the 1% adulterated samples, which were pasteurized (P) or high heated (UHT), are shown in Figure 4. The differences between the blank and 1% samples were highest for pea and SWP and could be increased by using the same injection volumes (20 μL) at a lower flow rate (5 μL instead of 20 μL min^{-1}). Although the differences for soy samples were less, the 1% adulterated samples could easily be distinguished from the blanks.

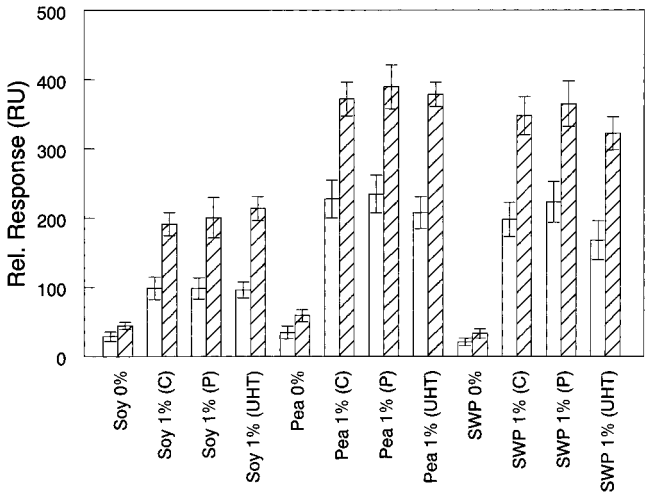

Figure 4. Response differences between blank milk powders (0%) and milk powders containing 1% of the nonmilk proteins [20 μL injections at flow rates of 5 (▨) and 20 μL min^{-1} (□)]. C, calibration standard; P, pasteurized milk powder; UHT, heat-treated milk powder.

Table 2. Relative Responses [Response Protein/Response Reference Protein (Soy, Pea, or SWP) × 100%] Obtained in Fc2 (Anti-soy), Fc3 (Anti-pea), and Fc4 (Anti-SWP) after Injection (20 μL at a Flow of 20 μL/min) of Different Protein Solutions (100 μg/mL)

protein	relative responses (%)		
	Fc2 (anti-soy)	Fc3 (anti-pea)	Fc4 (anti-SWP)
soy isolate	100	10	1
pea isolate	24	100	1
SWP 100	3	1	100
soybean	150	9	1
marrowfat (pea)	20	105	5
green pea (fresh)	31	87	7
green pea (split)	26	81	6
rye	3	2	36
walnut	22	12	<1
Brazil nut	20	3	<1
cashew	14	5	<1
hazelnut	13	4	<1
almond	6	5	7
peanut	15	7	<1
sesame seed	8	3	1
pine nut	7	2	<1
sunflower seed	4	5	2
pumpkin seed	24	9	2
maize	10	3	1
barley	3	2	15
brown bean (kidney bean)	18	5	<1
pistachio	14	7	7
pecan	5	2	1
lupine (total)	9	12	<1

Binding of Plant Proteins from Other Sources. Proteins from some other food products (see Table 2), obtained from the local market, were extracted according to the same procedure as described for the soy, pea, and SWP products (see Materials and Methods). Protein solutions (100 μg mL^{-1}) were injected, and the responses obtained due to binding to the antibodies in the three Fcs were compared with the responses obtained with the soy, pea, and SWP protein solutions injected at the same concentration (see Table 2). The anti-soy antibodies reacted strongly with protein extracts from soybeans and less with proteins from green pea, pea isolate, nuts (walnut, Brazil nut, cashew, hazelnut, and pistachio),

pumpkin seed, marrowfat, brown bean, peanut, and maize. The anti-pea antibodies reacted strongly with protein extracts from marrowfat and green pea and slightly with soy isolate, walnut, and total lupine. The anti-SWP antibodies showed binding with rye and barley proteins only. These findings show that the BIAs, using these particular antibodies, were not restricted to detecting adulterations with pea, soy, and SWP proteins but could be used for tracing a much broader range of nonmilk proteins.

Reuse of the Biosensor Chip. In direct BIAs, the performance of the immobilized antibodies during the successive injections is important. Calibration curves (plant proteins in milk powder) obtained directly after the preparation of the chip and after 250 and 650 injections were compared (data not shown). Although losses of activity were found with all three immobilized antibodies, acceptable calibration curves could still be obtained after 650 injections.

ABBREVIATIONS USED

BIA, biosensor immunoassay; SPR, surface plasmon resonance; PAGE, polyacrylamide gel electrophoresis; CZE, capillary zone electrophoresis; ELISA, enzyme-linked immunosorbent assay; FPLC, fast protein liquid chromatography; SWP, soluble wheat proteins; BSA, bovine serum albumin; IgG, immunoglobulin G; PBS, phosphate-buffered saline; NHS, *N*-hydroxysuccinimide; EDC, *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide; Fc, flow channel; RU, response units; LOD, limit of detection; LDM, limit of determination; SD, standard deviation; CV, coefficient of variation; P, pasteurized; UHT, ultrahigh temperature; C, calibration standard; Tris, tris(hydroxymethyl)aminomethane.

ACKNOWLEDGMENT

We thank the following partners in one of the projects for their helpful discussions: Rosina López-Alonso Fandiño (Instituto de Fermentaciones Industriales, Madrid, Spain); Lourdes Sánchez Paniagua (Facultad de Veterinaria, Zaragoza, Spain); Gary Brett (Institute of Food Research, Norwich, U.K.); and Tiziana Cattaneo (Istituto Sperimentale Lattiero-Caseario, Lodi, Italy). We also thank the following co-workers at RIKILT: Jolanda du Pré, Nathalie Smits, and Anniek Kemmers-Voncken for their work on protein extraction and antisera preparation and Louise Lindsey for correcting the grammar of the manuscript.

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Received for review April 3, 2001. Revised manuscript received August 1, 2001. Accepted August 15, 2001. This research was performed within two EU Projects (SMT4-CT97-2205 and GRD1-2000-25801). We thank the European Committee for funding the projects and the Dutch Ministry of Agriculture, Nature Management and Fisheries, for cofunding.

JF010440P