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ARTICLE

Advantages of Soybean Peroxidase over Horseradish Peroxidase as the Enzyme Label in Chemiluminescent Enzyme-Linked Immunosorbent Assay of Sulfamethoxypyridazine

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An indirect competitive chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) of sulfamethoxypyridazine (SMP) was developed. The conjugates of streptavidin with cationic horseradish peroxidase (HRP) and anionic soybean peroxidase (SbP) were used in CL-ELISA for the detection of biotinylated anti-SMP antibodies. For streptavidin-HRP conjugate-catalyzed chemiluminescence measured 20 s after the initiation of the enhanced chemiluminescence reaction (ECR), the limit of detection (IC_{10}), the IC_{50} value, and the working range in CL-ELISA of SMP are 0.3, 12.4, and 1.2−85.0 ng/mL, respectively. An increase in the time interval between the ECR initiation and the luminescence measurement results in the loss in the quality of analytical measurements because of the time-dependent quenching of chemiluminescence typical of the HRP-catalyzed ECR. In the case of SbP-based CL-ELISA of SMP, the limit of detection, the IC_{50} value, and the working range (0.025, 0.17, and 0.045−0.63 ng/mL, respectively) are better than those for HRP-based CL-ELISA. Furthermore, the analytical parameters of SbP-based CL-ELISA remain unchanged during a long period of time (for at least 30 min). The recovery values from four spiked milk samples with different concentrations of SMP in SbP-based CL-ELISA vary from 70 to 130%.

KEYWORDS: Peroxidase; soya; horseradish; sulfamethoxypyridazine; chemiluminescent ELISA; milk

INTRODUCTION

Sulfonamides belong to a group of antimicrobial agents that are actively applied in livestock and poultry for the prophylaxis and therapy of bacterial infections causing the accumulation of sulfonamides in meat, eggs, and milk (*1−3*). The presence of sulfonamides in foodstuffs may cause allergic or toxic reactions in humans and the appearance of resistant strains of pathogenic microorganisms (*3*). The maximum residue limits for sulfonamides were set to 100 μg/kg in edible animal tissues and 100 μg/L in milk (valid for the European Union and the United States) (*4*, *5*). Moreover, because sulfonamides are among the most frequently prescribed pharmaceuticals, they were found in wastewater (*6−8*). The remediation of the environment requires knowledge of the concentration of these chemicals.

Consequently, it is important to develop sensitive, rapid, and robust methods for the effective control of sulfonamide residues in different samples. The current techniques of the sulfonamide detection are commonly based on bacterial growth inhibition or liquid chromatography (*3*, *9*, *10*). These methods are either laborious or slow for massive screening. Furthermore, the complexity of the instrumentation and the requirement for thorough sample cleanup render the chromatography unsuitable for wide use.

During the past years, various enzyme immunoassays for different sulfonamides have been developed and successfully applied as an alternative to chromatographic methods (*11−14*). The cationic isoenzyme *c* of the peroxidase from horseradish roots (HRP, EC 1.1.11.7) is widely used as a label in enzyme-linked immunosorbent assay (ELISA) with colorimetric, fluorimetric, and luminescent detection. Although the colorimetric detection is commonly used in ELISA, the most sensitive ELISA kits are based on chemiluminescence measurement (*15−17*).

Luminol is the peroxidase substrate commonly used in chemiluminescence assays. However, because the HRP-catalyzed oxidation proceeds with low efficiency, enhancers are added to a solution of luminol and H_2O_2 (*18−20*). *p*-Iodophenol is the most popular enhancer. Kinetic studies of the HRP-catalyzed enhanced chemiluminescence reaction (ECR) showed that the chemiluminescence intensity increases sharply during the first few minutes, reaches a maximum, and then decreases (*21*, *22*). The emission decay was related to the HRP inactivation as a result of the interaction of the enzyme with the radical products of substrate oxidation (*22*).

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On the other hand, anionic peroxidases isolated from *Arthromyces ramonus*, soybean hull, palm leaves, and sweet potato efficiently catalyze the oxidation of luminol in the absence of enhancers (*23−25*). In the chemiluminescence method, the limits of detection for soya, palm, and sweet potato peroxidases are 0.3, 1.0, and 0.01 pM, respectively (*23−25*). Moreover, these peroxidases are not inactivated by reaction products, thus forming a long-term chemiluminescent signal. Note that the stability of anionic plant peroxidases, which is one of the main characteristics determining the usefulness of enzymes in immunoassay, is also substantially higher than that of HRP (*26*, *27*). On the basis of the aforesaid, anionic peroxidases can be considered to be promising enzymes in immunoassays with chemiluminescence detection. In the present study, we used soybean peroxidase (SbP) because it is presently the only commercially available anionic peroxidase.

Herein, we describe the development of chemiluminescent ELISA (CL-ELISA) for the detection of sulfamethoxypyridazine (SMP) using SbP and HRP as enzyme labels. The SbP-based CL-ELISA was used to measure SMP in milk.

MATERIALS AND METHODS

Materials. Peroxidases from soya bean *Glycine max* (RZ 1.5) and horseradish root *Armoracia rusticana* (RZ 3.0) were purchased from Sigma (United States) and Biolar (Latvia) and used without additional purification. Luminol, *p*-iodophenol, SMP, Triton X-100, and Tris base were obtained from Sigma (United States). Poly(ethyleneglycol) (M_w = 6000), H_2O_2 (30% w/v), and $NaBH_4$ were from Merck (Germany). Streptavidin was from Imtek (Russia). Biotin *N*-hydroxysuccinimide ester and $NaIO_4$ were from ICN Biomedicals (United Kingdom). All other reagents were of analytical grade. The concentration of H_2O_2 was estimated by measuring the absorbance at ε_{240} = 43.6 M^{-1} cm^{-1}. All solutions were prepared using Milli-Q water.

Immunoreagents. The antiserum K2 was produced by immunization of rabbits with the conjugate of *N*-sulfanyl-4-aminobutyric acid (SAB) and soybean trypsin inhibitor as described previously (*28*). The IgG fraction was isolated from the antiserum using the PEG precipitation. The concentration of IgG solutions was determined spectrophotometrically at ε_{280} = 0.77 mg^{-1} mL cm^{-1}. The SAB−ovalbumin conjugate was synthesized by the succinimide/carbodiimide activation method as described previously (*12*).

Streptavidin was conjugated with HRP and SbP as follows: Each peroxidase (5 mg/mL) was oxidized with sodium periodate (6.8 or 27 mM for SbP and 6.8 mM for HRP) for 20 min in the dark at 25 °C (*29*, *30*). The enzyme samples were dialyzed against a 1 mM acetic buffer, pH 4.8, at 4 °C overnight. The solutions of oxidized SbP and HRP were mixed with streptavidin (0.5 mg) dissolved in 50 mM carbonate buffer (75 μL), pH 9.6, and stirred for 2 h at ambient temperature. To reduce the azomethine bonds, a 10% $NaBH_4$ solution (10 μL) was added to the reaction mixture, and the stirring was continued for 1 h in the dark. Finally, the conjugates were intensively dialyzed against a 50 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). The conjugates were stored in 50% glycerol at −20 °C.

Biotinylation of Antibody. Covalent binding of biotin to rabbit anti-SMP antibody was carried out as described previously (*31*). Twenty-five microliters of biotin *N*-hydroxysuccinic ester dissolved in dimethyl sulfoxide (3.1 mg/mL) was mixed with 0.5 mL of the purified antibody (6.8 mg/mL) and incubated at room temperature for 2 h followed by the dialysis against PBS. The biotinylated antibody contained nine biotin moieties per antibody molecule, which was estimated by the titration of free amino groups using 2,4,6-trinitrobenzenesulfonic acid (*32*). The biotinylated antibody was stored in 50% glycerol at −20 °C.

Chemiluminescent ELISA. Ninety-six-well black nontransparent polystyrene microtiter plates (Nunc, Denmark) were coated by adding 100 μL of SAB−ovalbumin (3 μg/mL) dissolved in 50 mM phosphate buffer, pH 7.4 into each well and incubated at 4 °C overnight. The plates were washed four times with 0.1% Triton X-100 in PBS (PBST). Then, 50 μL of biotinylated anti-SMP antibody (1:800 dilution) and 50 μL of a SMP solution were added into the wells and incubated for 1 h at 37 °C. The SMP concentration was varied in the range from 0 to 500 ng/mL. The plates were washed again as described above. Then, the conjugates of streptavidin with SbP or HRP (1:2000 dilution) were added into the wells (100 μL). The plates were incubated for 1 h at 37 °C and then washed with PBST four times. Finally, 100 μL of a 100 mM Tris-HCl buffer, pH 8.5, containing 2 mM hydrogen peroxide, 1 mM luminol, and 2 mM *p*-iodophenol (unless otherwise stated) was added into each well and stirred. Finally, the chemiluminescence intensity was monitored vs time using a Zenyth 1100 plate reader (Anthos Labtec Instruments, Austria).

Preparation of Spiked Milk Samples. Four cow milk samples purchased from a local dairy farm were mixed with a SMP solution (1 mg/mL in methanol). Using each milk sample, four spiked solutions with an SMP concentration equal to 210, 70, 23, and 8 ng/mL were prepared. To eliminate the matrix effect, the spiked samples were centrifuged at 8000*g* for 15 min at 4 °C. The upper layer (fat) was discarded, and the bottom layer was mixed with a trichloroacetic acid solution (1 g/mL) to precipitate the milk proteins. The concentration of trichloroacetic acid in each sample was 1.7% (w/v). The resulting solutions were centrifuged for 15 min at 10000*g* at 4 °C. Prior to the assay, the supernatants were diluted 50 times using PBST.

Data Analysis. Standards and samples were run in triplicates, and the mean values were processed. Standard curves were obtained by plotting the light intensity against the logarithm of the analyte concentration and fitted to a four-parameter logistic equation using the Origin 7.5 software (OriginLab Corp., United States):

$$Y = \{(A-D)/[1+(x/C)^B]\}+D$$

where A is the asymptotic maximum (intensity in the absence of an analyte, I_{max}), B is the curve slope at the inflection point, C is the x value at the inflection point, and D is the asymptotic minimum (background signal).

RESULTS AND DISCUSSION

Recently, we have developed an indirect competitive ELISA with the colorimetric detection for the determination of SMP in milk using SbP as a label (*33*). The limit of detection, the IC_{50} value, and the working range were 0.4, 4.8, and 1.3−63.0 ng/mL, respectively.

The chemiluminescence detection of the enzymatic activity of conjugates of HRP (the enzyme commonly employed in ELISA) is often used to increase the sensitivity of ELISA (*15−17*). On the basis of the indirect competitive CL-ELISA (**Figure 1**), where the steptavidin−HRP conjugate is employed as the revealing conjugate and luminol and *p*-iodophenol (enhancer) serve as the components of the substrate solution, we developed the sensitive immunoassay for the determination of SMP. The substrate mixture contains 1 mM luminol and 2 mM *p*-iodophenol; that is, this mixture is identical to the optimal substrate mixture for the SbP conjugate (see below). In the separate experiment, we showed that the use of a 1.0 mM luminol/2.0 mM *p*-iodophenol solution instead of a 0.5 mM luminol/1.0 mM *p*-iodophenol solution (the substrate mixture usually employed in CL-ELISA with HRP conjugates) has no adverse effect on the analytical parameters of the SMP determinations.

The calibration curve for the SMP determination in HRP-based CL-ELISA is presented in **Figure 2** (top curve). In this case, the light intensity was measured 20 s after the initiation of the oxidation of luminol (the dead time of the reader used in the study). The chemiluminescence detection of HRP activity instead of the colorimetric detection allowed the extension of the working range (1.2−85.0 ng/mL) as well as the improvement of the lower detection limit (0.3 ng/mL) (**Table 1**, line a). The coefficient of variation (CV) for SMP concentrations within the working range of the assay varies from 0.7 to 10%. The CV values for the intra- and interassay (n = 3) are smaller than 12%.

However, the kinetic study showed that HRP-induced chemiluminescence drops quickly (**Figure 2**). This fact is in accordance with the previously reported data (*22*, *30*). These results provide

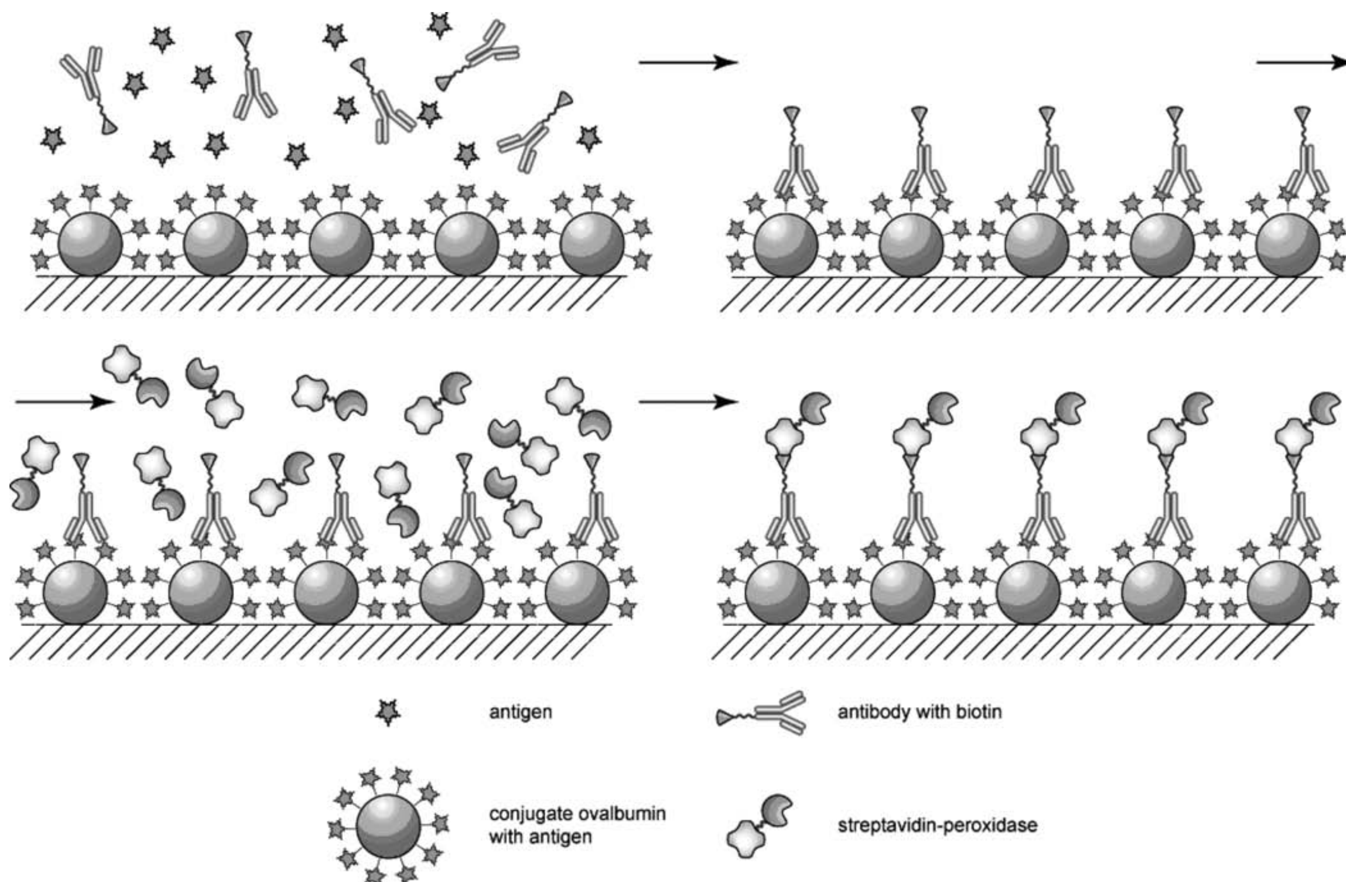

Figure 1. Scheme of indirect competitive CL-ELISA of SMP.

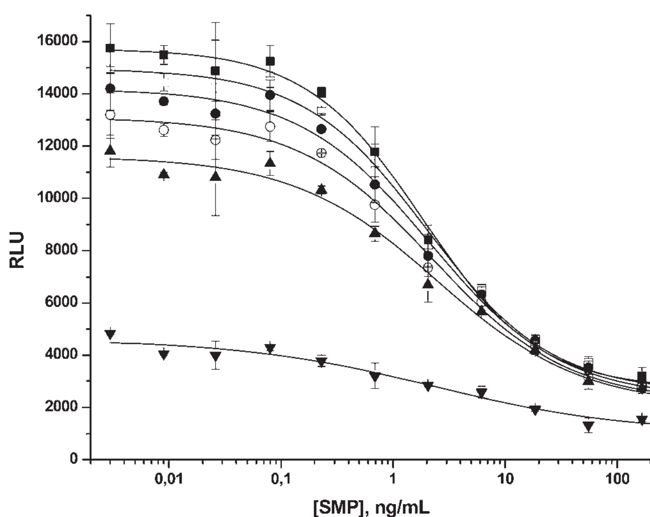

Figure 2. Indirect HRP-based CL-ELISA of SMP. The chemiluminescence intensity was recorded 20 (■), 160 (□), 300 (●), 440 (○), 580 (▲), and 1160 (▼) s after the initiation of the oxidation of luminol, respectively.

evidence that the observed change in the intensity adversely affects the analytical parameters of the SMP assay. The key issue is the time interval between the initiation of the HRP-catalyzed oxidation of luminol and the chemiluminescence measurement. An increase of the time interval resulted in an increase in the lower detection limit and the IC_{50} values (**Table 1**, lines a−d). Moreover, the working range of the SMP assay became narrower. Besides, the oxidation of luminol during a longer period of time led to an increase in the CV values (**Table 2**).

Table 1. Analytical Parameters of CL-ELISA for the SMP Determination as a Function of the Time of the Peroxidase-Catalyzed Oxidation of Luminol

			ng/mL		
	enzyme	duration of peroxidase-catalyzed oxidation of luminol (s)	IC_{50}	working range (IC_{20}−IC_{80})	limit of detection (IC_{10})
a	HRP	20	12.4	1.2−85	0.3
b	HRP	160	16.0	2.6−80	0.8
c	HRP	300	22.0	4.0−85	1.6
d	HRP	440	35.0	8.0−125	2.6
e	SbP	20	0.17	0.045−0.63	0.02
f	SbP	160	0.22	0.045−1.10	0.02
g	SbP	300	0.28	0.07−1.00	0.03
h	SbP	440	0.35	0.08−1.50	0.03
k	SbP	580	0.34	0.08−1.20	0.03
l	SbP	1160	0.45	0.08−2.50	0.03
m	SbP	1740	0.45	0.08−2.30	0.03

Table 2. Coefficients of Variation for SMP Concentrations Measured by CL-ELISA as a Function of the Time of HRP-Catalyzed Oxidation of Luminol[a]

[SMP] (ng/mL)	CV values calculated from the chemiluminescence data measured at different times after the initiation of HRP conjugate-catalyzed oxidation of luminol (%)			
	20 s	160 s	300 s	440 s
55.7	8.2	9.7	10.2	11.3
18.6	8.5	9.4	15.3	19.1
6.2	3.4	6.9	9.5	11.5
2.1	4.8	7.7	12.0	ND

[a] ND, not determined.

For comparison, the SbP–streptavidin conjugate was also used in CL-ELISA of SMP. Previously, the preferable composition of the substrate mixture for the SbP-catalyzed oxidation of luminol has been reported (*23*). The optimization has been carried out for the homogeneous reaction. In the present study, we found that the nonenzymatic chemiluminescence increases by a factor from two to three in the presence of an adsorbed complex of SAB–ovalbumin and the biotinylated antibody in the wells. This forced us to re-evaluate the composition of the substrate mixture under conditions similar to those used in CL-ELISA (heterogeneous reaction).

The substrate composition was optimized using black plates, where the SbP–steptavidin conjugate involved in the triple complex with SAB–ovalbumin and the biotinylated antibody K2 catalyzed the oxidation of luminol. As can be seen from **Figure 3A**, in the absence of *p*-iodophenol in the substrate solution, the variation of the luminol concentration has virtually no effect on the luminescence intensity. In contrast, in the presence of *p*-iodophenol, the intensity increases with increasing luminol concentration. Moreover, the addition of *p*-iodophenol to the substrate mixture also leads to an increase in the intensity. However, the observed enhancement effect is negligible as compared to that observed upon the addition of *p*-iodophenol to the HRP substrate mixture.

The effect of the composition of the substrate mixture on the background noise of chemiluminescence (wells with the complex of SAB–ovalbumin and the biotinylated antibody K2 but without SbP–streptavidin) was also evaluated. It was shown that an increase in the concentration of luminol leads to an increase in the intensity of the noise signal (**Figure 3B**). Interestingly, we observed that the noise signal is *p*-iodophenol-dependent. Thus, an increase in the enhancer concentration in the luminol solution resulted in the sharp quench of background chemiluminescence. Previously, it has been reported that benzothiazole enhancers also depress the background luminescence upon the nonenzymatic oxidation of luminol (*19*).

To determine the optimal composition of the substrate mixture in the case of SbP-based CL-ELISA, we evaluated the effect of the *p*-iodophenol and luminol concentrations on the ratio of SbP-induced chemiluminescence to the background. As can be seen from **Figure 3C**, the highest values of the ratio were obtained with the use of substrate mixtures containing 1 mM luminol and 2 mM *p*-iodophenol (mixture a) or 1 mM luminol and 3 mM *p*-iodophenol (mixture b). Taking into account that the most stable chemiluminescent signal was observed for the mixture a (data are not presented), in the subsequent work with the SbP–streptavidin conjugate, we used the following substrate mixture: 100 mM Tris-HCl buffer, pH 8.5, 2 mM H_2O_2, 1 mM luminol, and 2 mM *p*-iodophenol.

The calibration curves for the determination of SMP using SbP-based CL-ELISA are presented in **Figure 4**. By measuring the chemiluminescence intensity 20 s after the initiation of the oxidation of luminol, we evaluated the limit of detection, the IC_{50} value, and the working range, which are 0.025, 0.175, and 0.045–0.63 ng/mL, respectively (**Table 1**, line e). These results clearly show that the replacement of HRP with SbP in CL-ELISA leads to a substantial improvement of detectability of SMP and to an extension of the working range of the assay. In the case of the SbP conjugate, the CV values for SMP concentrations measured within the working range vary from 0.7 to 11%. The CV values for the intra- and interassay ($n = 3$) are lower than 12%. Therefore, the precision of the SMP immunoassay is independent of the nature of peroxidase (**Tables 2** and **3**).

In SbP-based CL-ELISA of SMP, a comparison of the calibration curves obtained at different times after of the onset of the oxidation of luminol showed that the intensity of SbP-induced chemiluminescence changes negligibly, as opposed to that observed for HRP. As can be seen from **Figure 4**, the intensity increases during the first short period of time, and then, the intensity remains virtually unchanged during the period of time

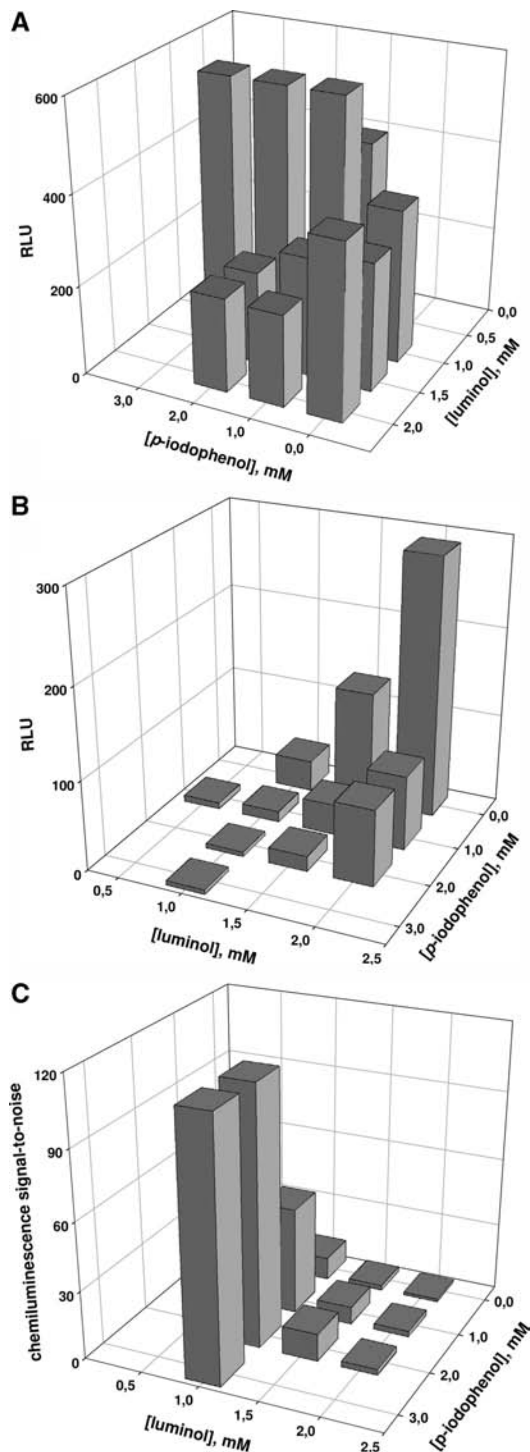

Figure 3. Influence of the luminol and *p*-iodophenol concentrations on the chemiluminescence intensity generated in the course of oxidation of luminol catalyzed by the SbP-streptavidin conjugate in the triple complex with SAB-ovalbumin and the biotinylated antibody K2 in black plate wells: (**A**) SbP-induced chemiluminescence, (**B**) the background, and (**C**) the ratio of the SbP-induced chemiluminescence to the background. The chemiluminescence intensity was recorded 440 s after the initiation of the oxidation of luminol. The CV values for the CL intensity and the ratio of the SbP-induced chemiluminescence to the background ($n = 3$) are lower than 7%.

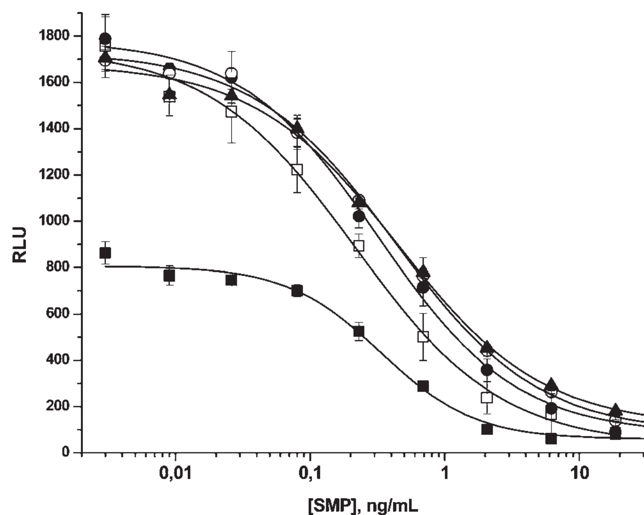

Figure 4. Calibration curves for the determination of SMP in SbP-based CL-ELISA. The chemiluminescence intensity was recorded 20 (■), 160 (□), 580 (●), 1160 (○), and 1740 (▲) s after the initiation of the oxidation of luminol, respectively.

Table 3. Coefficients of Variation for SMP Concentrations Measured by CL-ELISA as a Function of the Time of SBP-Catalyzed Oxidation of Luminol[a]

[SMP] (ng/mL)	CV values calculated from the chemiluminescence data measured at different times after the initiation of SbP conjugate-catalyzed oxidation of luminol (%)						
	20 s	160 s	300 s	440 s	580 s	1160 s	1740 s
2.06	ND	ND	ND	ND	12.0	5.8	0.4
0.69	ND	2.0	11.0	9.2	11.0	1.0	7.8
0.23	9.0	5.7	4.5	8.8	4.8	0.7	2.3
0.08	3.0	8.0	4.5	5.0	5.3	4.4	2.8

[a] ND, not determined.

Table 4. Recovery of SMP from Spiked Milk Samples Using SbP-Based CL-ELISA (n = 6)

	milk 1		milk 2		milk 3		milk 4	
[SMP] (ng/mL)	recovery (%)	CV (%)	recovery (%)	CV (%)	recovery (%)	CV (%)	recovery (%)	CV (%)
8	115	6.4	113	7.6	105	4.7	130	6.4
23	94	5.1	71	7.0	69	5.1	86	3.1
70	99	4.5	77	9.9	78	9.7	79	4.5
210	98	2.9	84	5.9	80	3.1	82	0.7

from 160 to 1740 s, and some quenching of chemiluminescence is observed only after 1740 s. The above results show that the analytical parameters of SbP-based CL-ELISA of SMP remain virtually independent of the instant at which the chemiluminescence is measured (**Table 1**, lines f−m). In this case, the CV values also remain virtually unchanged and low, which allows the determination of the SMP concentration with high accuracy (**Table 3**).

The conjugates of streptavidin with HRP and SbP used in the above experiments were synthesized at different concentrations of sodium periodate (see the Materials and Methods). To estimate the possible effect of the oxidant concentration on the immunochemical properties of the conjugates, two preparations of the SbP−streptavidin conjugate were obtained. One preparation was synthesized under the conditions optimal for the HRP conjugation (i.e., 6.8 mM $NaIO_4$), and another preparation was synthesized under the conditions chosen for SbP (27.0 mM $NaIO_4$). It was shown that a decrease in the oxidant concentration leads to a decrease in the yield of the conjugate. Nevertheless, the same analytical parameters for the SMP assay were obtained for both conjugates. Therefore, the modification of the conditions of the SbP−streptavidin synthesis has no adverse effect on the immunochemical properties of the SbP conjugate and the performance of the assay; hence, the described differences are associated with the enzymatic properties rather than with the protocols of conjugation.

To sum up, in CL-ELISA anionic SbP has advantages over the commonly used cationic isozyme *c* of HRP. The SbP-based CL-ELISA developed in the present study was used to determine SMP in milk. For this purpose, the spiked samples containing SMP at different concentrations (210, 70, 23, and 8 ng/mL) were prepared using four milk preparations purchased in a local dairy farm. It is well-known that milk is a complex colloidal system, and the matrix effect is often observed in the analysis of milk samples by ELISA (*33*−*35*). To prevent the matrix effect, the spiked milk samples were pretreated with trichloroacetic acid followed by the 100-fold dilution with PBST. The results of the spiked sample analysis by SbP-based CL-ELISA showed that the recovery values vary from 70.5 to 130% (**Table 4**). This is evidence that the CL-ELISA developed in the present study is suitable for the determination of SMP in real milk samples.

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