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New immunosensor for Lactoferrin determination in human milk and several pharmaceutical dairy milk products recommended for the unweaned diet

Luigi Campanella, Elisabetta Martini, Mauro Tomassetti *

Department of Chemistry, University of Rome "La Sapienza", P.le A. Moro 5, 00185 Rome, Italy

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Abstract

Thorough research was carried out on Lactoferrin immunosensor development. Furthermore, two different competitive procedures were used for Lactoferrin determination, in which either the antigen (Lactoferrin) or the antibody (anti-Lactoferrin) was, respectively, conjugated with horseradish peroxidase enzyme using a biotinylation process. The biotinylation of Lactoferrin and the subsequently used competition procedure for the immunosensor measurement were to get ready. Three different kinds of immunosensors were implemented, in all cases using the peroxidase enzyme as marker and hydrogen peroxide as substrate, but alternatively using as transducers one of the following sensors: (i) an amperometric electrode for H_2O_2, (ii) a Clark electrode and (iii) an iodide electrode. After optimizing the "competitive" measurement procedures and the transducer, the new Lactoferrin immunosensor was used for the determination of Lactoferrin content in human milk and in different types of dried milks or other dairy products, specifically produced and sold in chemist's shops to feed unweaned children in the first few months of life.

Keywords: Immunosensor; Lactoferrin; Human milk; Dried milk; Pharmaceutical dairy products; Unweaned diet

1. Introduction

Milk is known to contain several protective proteins including Lactoferrin that can contribute to the preservation of milk [1–5]. The increasing commercial interest in exploiting the therapeutic value of Lactoferrin has stimulated interest in developing reliable assays for its determination at the endogenous level in milk [6,7].

Milk is an established and healthy food source providing energy, proteins, vitamins, and minerals [1–5]. In addition to its value as a nutrient source, there is growing interest in the ability of milk to kill bacteria and defend human health [8,9]. A number of proteins found in milk under various conditions exhibit antimicrobial activity. For example, immunoglobulin G antibodies, for which we recently fabricated a very original immunobiosensor [10–12], but particularly Lactoferrin, are protective proteins that play an important role in the transfer of passive immunity from the mother to the neonate [13,14]. Lactoferrin, an iron-binding glycoprotein, was first isolated from cow's milk and subsequently from human milk [15,16]. In recent years, several kinds of immunosensors were developed for clinical [17,18] and environmental purposes [19–22] thanks to the possibility of generating a large number of antibodies for the analysis of several chemical species, and owing to the advantage of being able to analyze samples without any need for pretreatment. In this research the development of a new immunosensor for Lactoferrin is described. The study aimed to develop and characterize an immunosensor for the determination of this antibacterial protein (Lactoferrin), with a view to suggesting this method for the routine control of human milk and of dairy milk products for unweaned babies [23–25]. Furthermore, two different competitive procedures were used for Lactoferrin determination, in which the antigen (Lactoferrin), or the antibody (anti-Lactoferrin), respectively, was conjugated with horseradish peroxidase enzyme using a biotinylation process. After optimizing both the transducer and the 'competitive' measurement procedure, the new immunosensor was used to determine Lactoferrin in several types of

* Corresponding author. Tel.: +39 0649913722; fax: +39 06490631.
E-mail address: mauro.tomassetti@uniroma1.it (M. Tomassetti).

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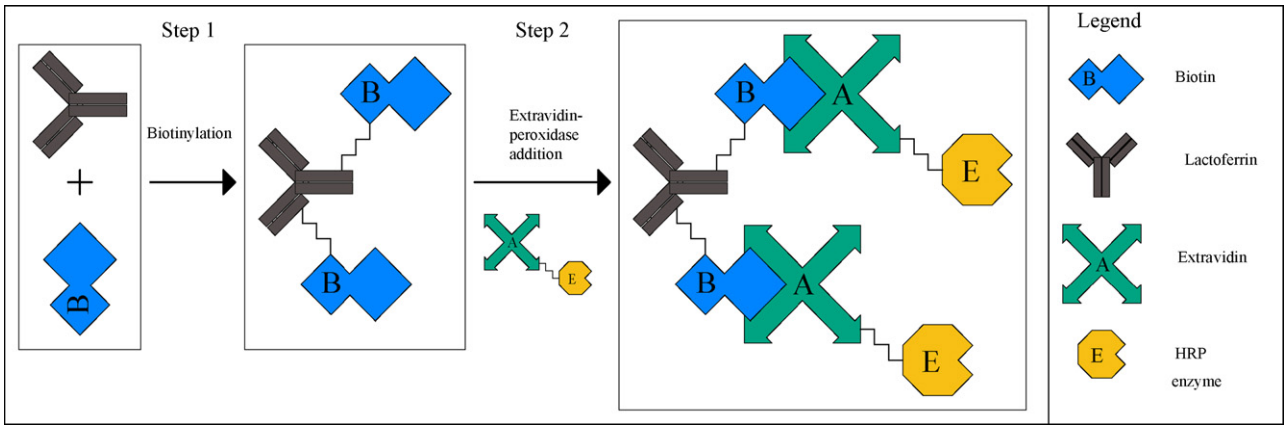

Fig. 1. Biotinylation and conjugation of the Lactoferrin.

powdered milk sold in drugstores specifically for unweaned children's diet and recommended for baby feeding during the first few months. In one of these more recent products, the manufacturer declared that Lactoferrin was actually added to the powdered milk in order to enhance its properties. Lastly two yogurts for neonates and samples of human milk (eighth month after childbirth) were also analyzed and the Lactoferrin concentration of the latter was compared with the concentration found in each of the commercial products for unweaned babies.

2. Experimental

2.1. Apparatus

The amperometric measurements were performed in a 5 ml thermostated glass cell kept under constant stirring. The Clark electrode was supplied by Amel (mod. 332) and the measures were performed using an oximeter (Amel mod. 360), connected to a recorder (AMEL mod. 868). For the amperometric H_2O_2 measures an Amel mod. 551 potentiostat (Milano) was used, coupled with an amperometric hydrogen peroxide electrode by Universal Sensor Inc., New Orleans (USA), Mod. 4006, and connected to an analog recorder Amel mod. 868. The potentiometric measurements were carried out using a potentiometer (Orion model SA 720) connected to a recorder (AMEL mod. 868) and with an iodide electrode by Orion Research Inc., Boston (USA), mod. 94–53.

2.2. Materials

Dialysis membrane (art. D-9777) from Sigma–Aldrich; Ny+ Immobilon affinity membrane (porosity 0.65 μm) from the Millipore Corporation. Anti-Lactoferrin (catalogue number L-3262), Lactoferrin from bovine milk (catalogue number L-9507-50 mg), and the biotinylation kit supplied by Sigma Immunochemicals composed of: biotinylation Reagent (BAC-SulfoNHS, i.e., biotinamido hexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester), 5 M sodium chloride solution, micro-spin Column (2 ml) (practically consisting of a small empty cylindrical vessel pre-packaged with Sephadex G-50), sodium phosphate buffer (pH 7.2; 0.1 M) phosphate buffer saline (PBS) (pH 7.4; 0.01 M) (reconstituted with 1 l of deionized water to give 0.01 M phosphate buffer, 0.138 M NaCl, 2.7 mM KCl, pH 7.4); ExtrAvidin® peroxidase (containing 0.2 ml of ExtrAvidin Peroxidase conjugate at 2.0 mg ml^{-1}, supplied with 0.01% thimerosal).

2.3. Sample analyzed

Two samples of human milk obtained 8 months after birth, one fresh and one frozen, were analyzed. Four different samples

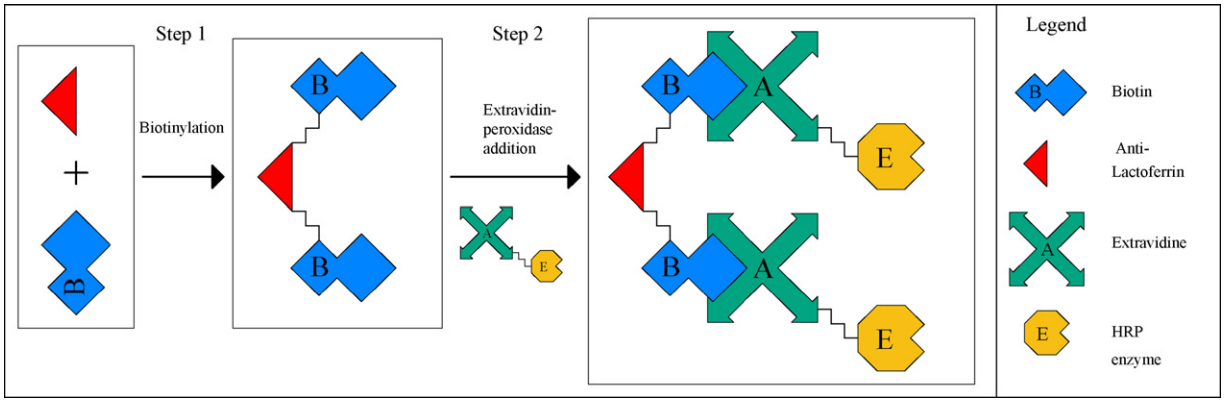

Fig. 2. Biotinylation and conjugation of the anti-Lactoferrin.

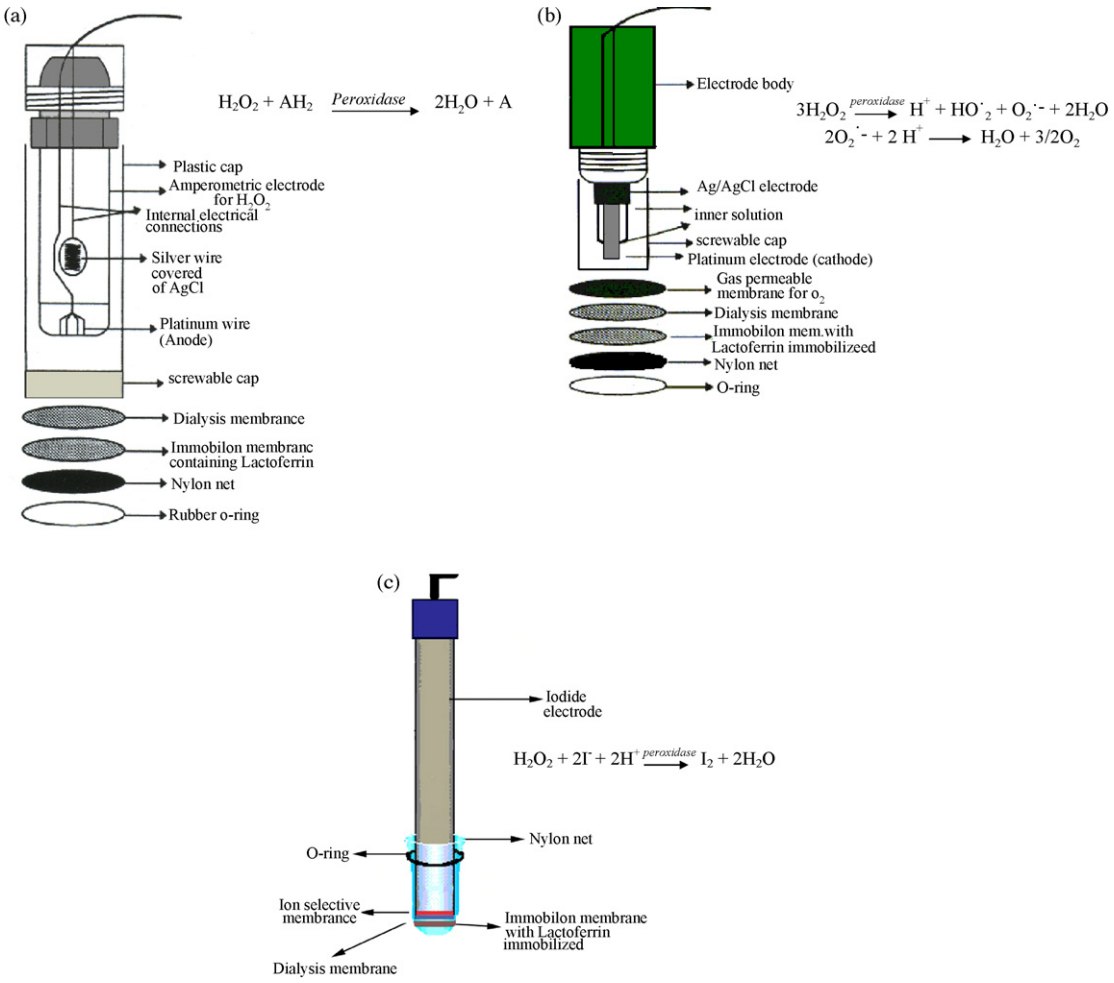

Fig. 3. (a) Immunosensor for Lactoferrin determination using an amperometric hydrogen peroxide electrode as transducer. (b) Immunosensor for Lactoferrin determination using an amperometric gas diffusion electrode (Clark type) as transducer. (c) Immunosensor for Lactoferrin determination using a potentiometric iodide electrode as transducer.

(i.e., A, B, C, D) of powdered milk for newborns, produced by different pharmaceutical firms, were analyzed, in one of which Lactoferrin had also been added by the producer; finally two different samples of yogurt for babies (yogurt and pear, yogurt and apricot), all purchased at a drugstore, were tested.

3. Methods

3.1. Lactoferrin and anti-Lactoferrin biotinylation

The avidin-biotin peroxidase technique is based on the use of a biotinylated antigen or antibody and an avidin horseradish peroxidase conjugate as part of the labeling system. The technique exploited the high affinity binding of biotin to avidin. The BiotioTag kit is specially designed for the small scale labeling of antibodies using biotinamido hexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester (BAC-SulfoNHS) as the labeling reagent. This reagent is particularly useful when mild reaction conditions are required for the biotinylation of sensitive biomolecules such as antibodies, enzyme and surface proteins. Following the labeling reaction, the biotinylated protein is separated from unreacted or hydrolyzed reagent by a fast gel-filtration step using G-50 microspin columns. BAC-SulfoNHS reacts with free amino groups of proteins to form stable amide bonds. The full procedure is illustrated in Fig. 1 for the antigen biotinylation. Briefly: 0.1 ml of 1.0 mg ml^{-1} Lactoferrin solution in sodium phosphate buffer (pH 7.2; 0.1 M) was prepared. Separately a BAC-SulfoNHS solution 5 mg ml^{-1} was also prepared, dissolving 5 mg of biotinamido hexanoic acid 3-sulfo-*N*-hydroxysuccinimide ester in 30 μl DMSO and adding sodium phosphate buffer (pH 7.2; 0.1 M) to a final volume of 1 ml. Immediately 10 μl of BAC-SulfoNHS solution were added to the Lactoferrin solution with gentle stirring and incubated under stirring for 30 min at room temperature.

Then the resin was re-suspended in the column by vortexing, the column was equilibrated with 0.2 ml of PBS (pH 7.40; 0.01 M), (this buffer is needed as an equilibration buffer of the microspin G-50 column and for the elution of the labeled protein from the column). The biotinylation reaction mixture was applied to the top-center of the resin and the column was centrifuged for 5 min at 700 × *g*. The purified sample was collected at the bottom in an Eppendorf test tube. This step was repeated twice more and a total of three fractions were collected. ExtrAvidin peroxidase solution (20 μl, 2.0 mg ml^{-1}) was diluted 1:100

in PBS containing 1% BSA, incubated for 1 h at room temperature and rinsed gently with PBS (pH 7.4; 0.01 M).

The biotinylation of the antibody was carried out with the same procedure. The anti-Lactoferrin protein biotinylation is illustrated in Fig. 2.

3.2. Anti-Lactoferrin immobilization on Immobilon membrane

The Immobilon Ny+ membrane (a positively charged nylon membrane with polyester reinforcement optimized for reliable and reproducible transfer, immobilization, hybridization, and subsequent reprobing) was cut into approximately 1 cm^2 surface area disks and 100.0 μl of a 1.0 mg ml^{-1} anti-Lactoferrin conjugated was directly deposited on the membrane surface. The Immobilon membranes were then dried at room temperature for about 24 h and stored at 4 °C.

3.3. Lactoferrin immobilization on Immobilon membrane

Lactoferrin, 50.0 μl of a 2 mg ml^{-1} solution, was directly deposited on the surface of the same membrane (Immobilon Ny+) cut into approximately 1 cm^2 area disks. The Immobilon membranes were then dried at room temperature for about 24 h and stored at 4 °C.

3.4. Immunosensor assembly

For this purpose, three different types of electrochemical transducers were used: an amperometric electrode for H_2O_2 determination, a gaseous diffusion amperometric electrode for O_2 determination (Clark type electrode) and an ion selective electrode for iodide (Fig. 3a–c, respectively).

The first transducer consisted of an amperometric electrode for H_2O_2 determination, with a Pt anode and an Ag/AgCl/Cl^- cathode, provided with a plastic cap filled with 0.1 M KCl solution and screwed onto the body of the electrode, at the lower end of which a dialysis membrane was positioned. The Immobilon membrane with the immobilized anti-Lactoferrin or Lactoferrin overlapped the dialysis membrane. Finally, a nylon net overlapped the latter membrane. The three membranes and the net were secured by a rubber O-ring to the plastic cap, as shown in Fig. 3a.

The second transducer used consisted of a gaseous diffusion amperometric electrode for O_2 determination (Clark type electrode).

In practice, three membranes were mounted on the head of the Clark electrode, in the following order: gas-permeable membrane, dialysis membrane and Immobilon membrane with Lactoferrin or anti-Lactoferrin immobilized on it. The membranes were kept in place by a nylon net and a rubber O-ring (Fig. 3b).

The third transducer consisted of a potentiometric ion selective electrode (iodide electrode) (Fig. 3c). The iodide electrode was constructed with a sensing pellet containing a suitable mixture of silver halide and silver sulfide, which is bound to the tip of the epoxy electrode body, as the pellet is in contact with the iodide solution. The potential variation is measured against a constant reference potential with a digital mV-meter.

Three membranes were mounted on the head of the electrode, in the following order: ion selective membrane, dialysis membrane and Immobilon membrane with Lactoferrin or anti-Lactoferrin immobilized on it. Finally a nylon net and an O-ring were superimposed on the head of the electrode.

3.5. Determination of Lactoferrin by immunosensor

First competitive procedure: Competition between Lactoferrin biotin-avidin-peroxidase conjugated and non-conjugated Lactoferrin, both free in solution, for anti-Lactoferrin immobilized in membrane.

To this end, the Immobilon membrane, on which anti-Lactoferrin was immobilized, was fixed to the head of the amperometric electrode for hydrogen peroxide as described in Section 3.4. Before measurement, the immunosensor was dipped into a Tris–HCl buffer solution (pH 8.0; 0.1 M), containing 0.05% Tween-20 by weight and 2.5% BSA by weight (bovine albumin was used to minimize non-specific absorption on the membrane). The Lactoferrin to be determined was added in 5 ml of Tris–HCl buffer solution (pH 8.0; 0.1 M) contained in the measurement cell, together with a fixed supply of Lactoferrin biotin-avidin-peroxidase conjugated, i.e., 20 μl (2.0 mg ml^{-1}) of conjugated Lactoferrin. The peroxidase-conjugated Lactoferrin was allowed to compete with the non-conjugated Lactoferrin, both free in solution, in binding with the anti-Lactoferrin immobilized on the Immobilon membrane. After washing with the same buffer solution to remove all the unbound Lactoferrin, the specific substrate of the enzyme, i.e., 20 μl of H_2O_2 solution 1% (v/v) was added to the renewed buffer solution in which the immunosensor was dipped, under stirring. The measured signal (as nA) of the transducer correlated directly with the Lactoferrin concentration to be measured. In this case, the higher the concentration of non-conjugated Lactoferrin free in solution, the stronger the signal produced by the hydrogen peroxide. Indeed, the lower the conjugated Lactoferrin bound to the antibody immobilized on Immobilon membrane, the lower the H_2O_2 consumed in the enzymatic reaction, and therefore, the higher the signal of the H_2O_2 oxidized at the amperometric electrode. The sequence for measuring the Lactoferrin by this procedure is schematized in Fig. 4. Using this procedure a calibration curve was constructed and employed to determine the unknown concentration of Lactoferrin contained in the sample.

The same sequence used in constructing the calibration curve described above was used to construct the calibration curve for Lactoferrin when the amperometric electrode for O_2, or the potentiometric iodide electrode, were, respectively, employed as transducers. But when the Clark electrode was the transducer, the measured signal (as ppm O_2) correlated inversely with the quantity of Lactoferrin to be measured.

3.6. Determination of Lactoferrin by immunosensor

Second competitive procedure: Competition between Lactoferrin immobilized in membrane and Lactoferrin free in solution,

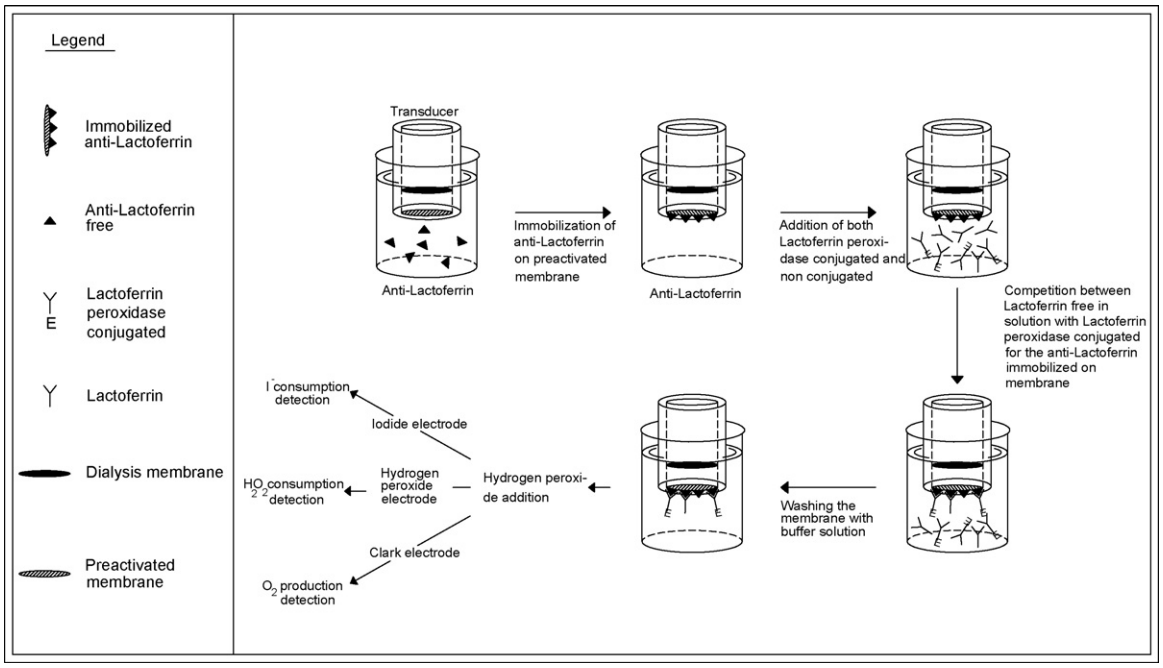

Fig. 4. Determination of Lactoferrin by immunosensor. First procedure: competition between Lactoferrin biotin-avidin-peroxidase conjugated and Lactoferrin free in solution for anti-Lactoferrin immobilized in membrane Immobilon.

for anti-Lactoferrin biotin-avidin-peroxidase conjugated free in solution.

The non-conjugated Lactoferrin free in solution at different concentrations, to be determined, was each time allowed to compete with the same antigen, but immobilized on the Immobilon membrane attached to the head of the amperometric transducer for hydrogen peroxide in order to produce the antigen–antibody reaction with a fixed supply of antibody, free in solution and labeled with biotin-avidin-peroxidase. Before measurement, the electrode was immersed in 5 ml of 0.1 M Tris–HCl buffer solution containing 0.05% Tween-20 and 2.5% by weight BSA (in order to minimize non-specific absorption on the membranes); then, in the measurement cell containing 5 ml of the Tris–HCl buffer solution (pH 8.0; 0.1 M) of Lactoferrin to be determined, together with a fixed supply of the enzyme-labeled anti-Lactoferrin, i.e., 25 μl (1.0 mg ml^{-1}), were allowed to incu-

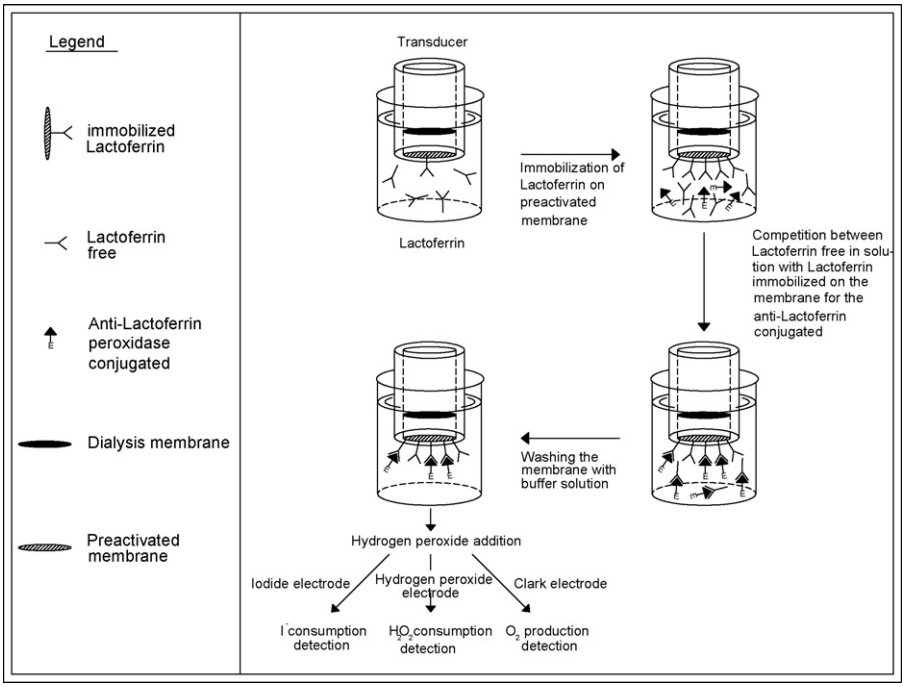

Fig. 5. Determination of Lactoferrin by immunosensor. Second procedure: competition between Lactoferrin immobilized in membrane Immobilon and Lactoferrin free in solution, for anti-Lactoferrin biotin-avidin-peroxidase conjugated.

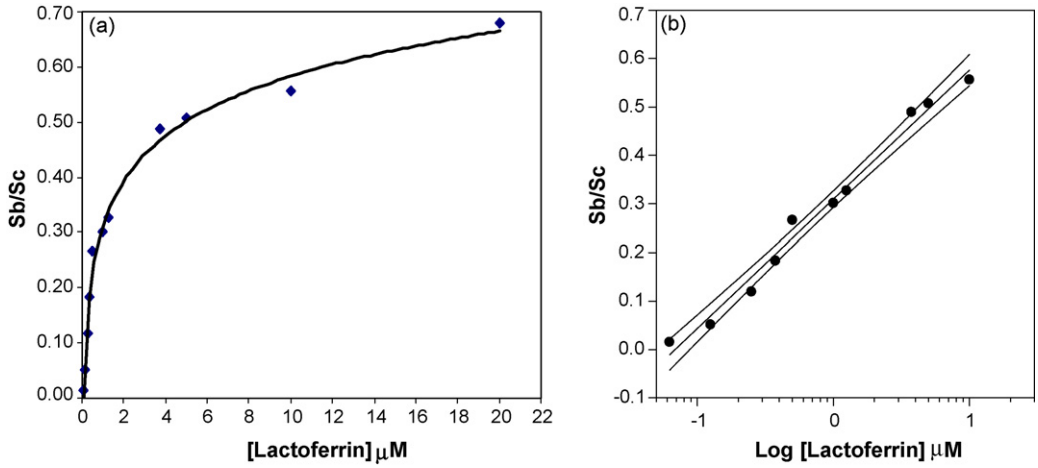

Fig. 6. First procedure: (a) behavior of the immunosensor response as a function of increasing Lactoferrin concentration, using Immobilon membrane and an amperometric electrode for H_2O_2 as transducer and (b) corresponding calibration curve and confidence interval for Lactoferrin determination, obtained using a semilogarithmic scale (Sc = sample signal/nA; Sb = blank signal/nA).

bate at 25 °C for 1 h. The free in solution Lactoferrin competed with the Lactoferrin immobilized on the membrane in bonding the labeled anti-Lactoferrin. On adding the enzyme substrate (20 μl of H_2O_2 solution 1%, v/v) to the renewed buffer solution, after washing with the same buffer to remove all the unlabelled anti-Lactoferrin not bound to the Lactoferrin, the recorded signal was correlated with the quantity of labeled immunocomplex formed on the surface of the membrane. In practice the sequence of events occurring during the Lactoferrin assay is outlined in Fig. 5. The calibration curve obtained by plotting the current signal versus the final Lactoferrin concentration was then used to determine the concentration of the unknown Lactoferrin.

The same schematic sequence was used for constructing calibration curves to determine the Lactoferrin when the amperometric electrode for O_2, or iodide electrode, were, respectively, utilized as transducers.

4. Results and discussion

4.1. Optimization of procedure

First of all it was necessary to optimize the method for the construction of the immunosensor for Lactoferrin determination using two different "competitive" procedures, as shown in Figs. 4 and 5, and three different transducers, illustrated in Section 3.4.

The behavior of the response in the Lactoferrin determination, obtained using the first competitive procedure and the amperometric immunosensor equipped with hydrogen peroxide transducer, is shown in Fig. 6(a) and analogous responses for the immunosensor, equipped with a Clark electrode, or with an iodide electrode, are shown, respectively, in Figs. 7(a) and 8(a), while the calibration straight lines, obtained from the same data but using the semilogarithmic scale, are shown in Figs.

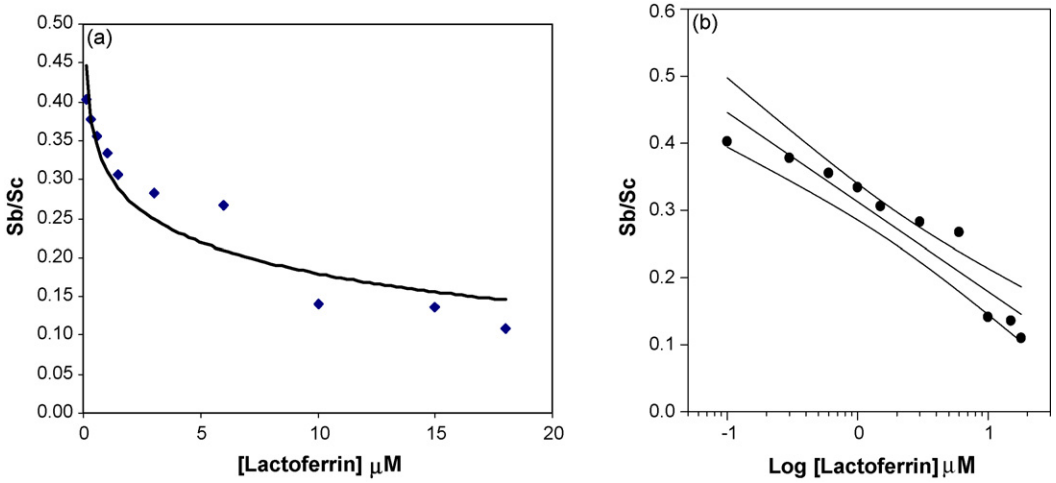

Fig. 7. First procedure: (a) behavior of the immunosensor response as a function of increasing Lactoferrin concentration using Immobilon membrane and a Clark electrode as transducer and (b) corresponding calibration curve and confidence interval for Lactoferrin determination, obtained using a semilogarithmic scale (Sc = sample signal/ppm O_2; Sb = blank signal/ppm O_2).

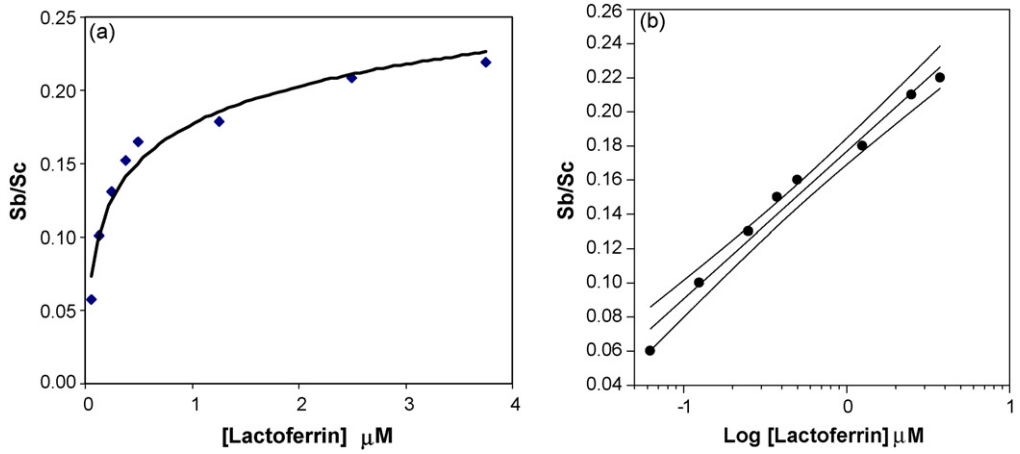

Fig. 8. First procedure: (a) behavior of the immunosensor response as a function of increasing Lactoferrin concentration, using Immobilon membrane and an iodide electrode as transducer and (b) corresponding calibration curve and confidence interval for Lactoferrin determination, obtained using a semilogarithmic scale (Sc = sample signal/mV; Sb = blank signal/mV).

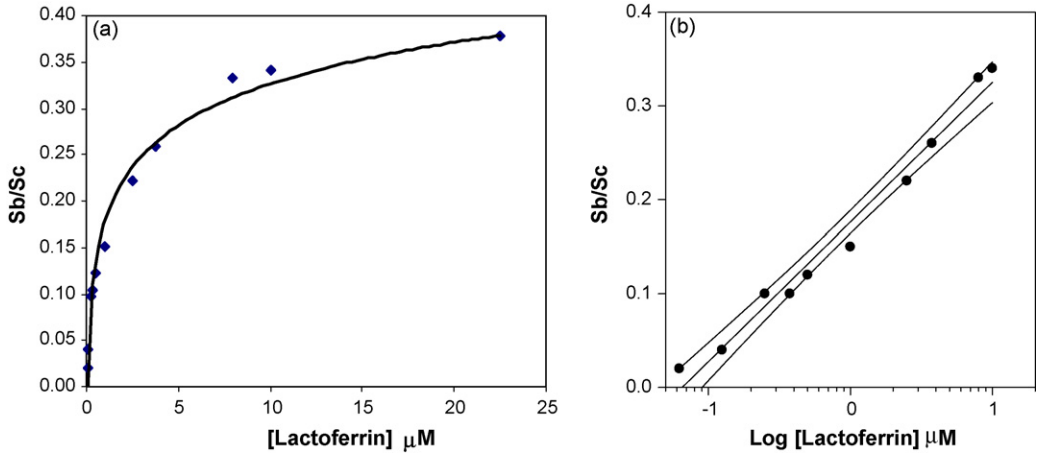

Fig. 9. Second procedure: (a) behavior of the immunosensor response as a function of increasing Lactoferrin concentration, using Immobilon membrane and an amperometric electrode for H_2O_2 as transducer and (b) corresponding calibration curve and confidence interval for Lactoferrin determination, obtained using a semilogarithmic scale (Sc = sample signal/nA; Sb = blank signal/nA).

Table 1
Analytical characterization of immunosensor method for Lactoferrin determination, using the first competitive procedure and alternatively one of three different transducers

Methods	Determination of Lactoferrin by means of immunosensor. Test geometry: competition between Lactoferrin biotin-avidin-peroxidase conjugated and Lactoferrin free in solution for anti-Lactoferrin immobilized in membrane		
Employed transducer	H_2O_2 electrode	Clark electrode	Iodide electrode
Regression equation (Y = a.u., X = μM)	$Y = 0.27\ (\pm 0.04)\ \log X + 0.31\ (\pm 0.02)$	$Y = -0.13\ (\pm 0.09)\ \log X + 0.31 (\pm 0.05)$	$Y = 0.09\ (\pm 0.03)\ \log X + 0.18\ (\pm 0.05)$
Confidence level $(1-\alpha) = 0.95$;	$(n-\nu) = 9$; $(t = 2.26)$	$(n-\nu) = 8$; $(t = 2.31)$	$(n-\nu) = 6$; $(t = 2.44)$
Linear range (μM)	0.07–10	0.08–14	0.07–3.7
Correlation coefficient	0.9891	0.8979	0.9782
Repeatability of the measurement (as pooled S.D.%)	5.5	7.7	6.8
Low detection limit (LOD) (μM)	0.035	0.040	0.035
Instrumental response time (min)	5	5	7

(a.u. = nA when using H_2O_2 as transducer; a.u. = ppm O_2 when using Clark electrode as transducer; a.u. = mV when using iodide electrode as transducer).
Operating conditions: buffer solution: Tris (pH 8.0, 0.1 M); incubation temperature 25 °C; incubation time: 60 min. Employed membrane: Immobilon membrane.

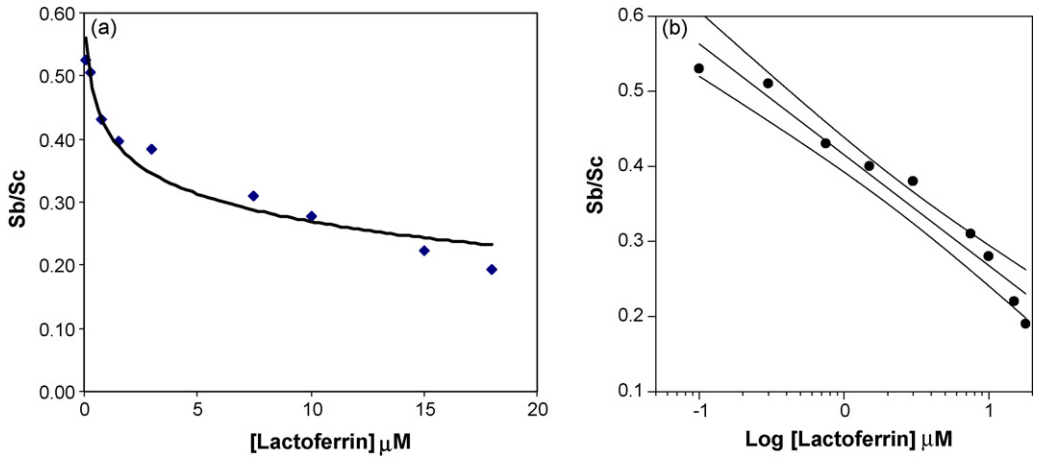

Fig. 10. Second procedure: (a) behavior of the immunosensor response as a function of increasing Lactoferrin concentration, using Immobilon membrane and a Clark electrode as transducer and (b) corresponding calibration curve and confidence interval for Lactoferrin determination, obtained using a semilogarithmic scale (Sc = sample signal/ppm O_2; Sb = blank signal/ppm O_2).

6(b), 7(b) and 8(b). The comparison of results (summarized in Table 1) referring to the analytical characterization and the respective equations of calibration straight lines reported in Figs. 6(b), 7(b) and 8(b) shows that the low detection limit (LOD) for Lactoferrin is of the order of 35 nM, in all cases; the sensitivity, expressed as the slope of the calibration straight line, is found to be higher (for the measures carried out using the first competitive procedure) and the R.S.D.% lower if the hydrogen peroxide electrode is employed as transducer than for the measurements carried out using the Clark electrode or the iodide electrode. Lastly the linear range is about three decades in all the cases.

Response behavior in Lactoferrin determination, obtained using the second competitive procedure and the amperometric immunosensor equipped with hydrogen peroxide transducer, is shown in Fig. 9(a), while the corresponding response curves when the Clark electrode or iodide electrode were, respectively, used as transducers, are shown in Figs. 10(a) and 11(a). Lastly, the calibration straight lines obtained using the same data as for the previous response curves, but employing the semilogarithmic scale, are shown in Figs. 9(b), 10(b) and 11(b), respectively. Table 2 summarizes the main analytical data for the determination of Lactoferrin, by the second competitive procedure, respectively, obtained using the above three different transducers, as proposed in the present research. The linear range is, in all cases, about three decades for the three different immunosensors, but, using the hydrogen peroxide electrode as transducer, the linear range and the sensitivity are a little larger, the correlation coefficient greater and R.S.D.% lower than for the corresponding data obtained using the two other transducers considered. The low detection limit (LOD) for all three immunosensors is of the order of 25–30 nM. It can also be observed that with the second competitive method used to determine Lactoferrin, LOD values are usually comparable to that of the first "competitive" procedure and precision values (R.S.D.% = 5.3–7.1) are slightly lower than with the method using the first competitive procedure (in this case R.S.D.% was = 5.5–7.7). Lastly the correlation coefficient values of the calibration curves are comparable to those of the first competitive procedure although, if the first competitive procedure and

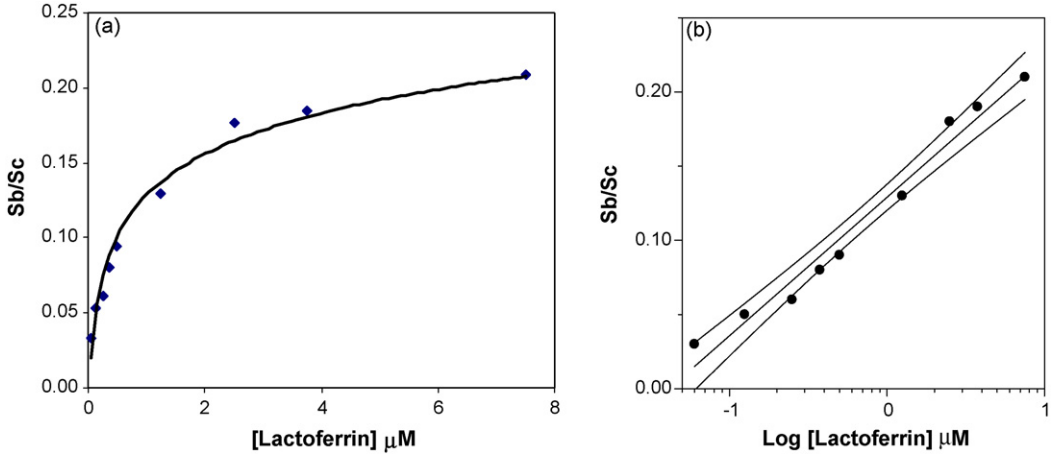

Fig. 11. Second procedure: (a) behavior of the immunosensor response as a function of increasing Lactoferrin concentration, using Immobilon membrane and an iodide electrode as transducer and (b) corresponding calibration curve and confidence interval for Lactoferrin determination, obtained using a semilogarithmic scale (Sc = sample signal/mV; Sb = blank signal/mV).

Table 2
Analytical characterization of immunosensor method for Lactoferrin determination, using the second competitive procedure and alternatively one of three different transducers

Methods	Determination of Lactoferrin by means of immunosensor. Test geometry: competition between Lactoferrin immobilized in membrane and Lactoferrin free in solution, for anti-Lactoferrin biotin-avidin-peroxidase conjugated free in solution		
Employed transducer	H_2O_2 electrode	Clark electrode	Iodide electrode
Regression equation (Y = a.u., X = μM)	$Y = 0.15\ (\pm 0.04)\ \log X + 0.18\ (\pm 0.03)$	$Y = -0.14\ (\pm 0.07)\ \log X + 0.42\ (\pm 0.06)$	$Y = 0.09\ (\pm 0.03)\ \log X - 0.13\ (\pm 0.09)$
Confidence level $(1-\alpha) = 0.95$;	$(n-\nu) = 9$; $(t = 2.45)$	$(n-\nu) = 9$; $(t = 2.45)$	$(n-\nu) = 7$; $(t = 2.36)$
Linear range (M)	0.05–25	0.06–15	0.06–7.5
Correlation coefficient	0.9861	0.9520	0.9735
Repeatability of the measurement (as pooled S.D.%)	5.3	7.1	6.4
Low detection limit (LOD) (M)	0.025	0.030	0.030
Instrumental response time (min)	5	5	7

(a.u. = nA when using H_2O_2 as transducer; a.u. = ppm O_2 when using Clark electrode as transducer; a.u. = mV when using iodide electrode as transducer).
Operating conditions: buffer solution: Tris (pH 8.0, 0.1 M); incubation temperature 25 °C; incubation time: 60 min. Employed membrane: Immobilon membrane.

Table 3
Determination by immunobiosensor of lactoferrin in human milk and in several pharmaceutical dairy milk products recommended for the unweaned children's diet

Biological matrix	Found Lactoferrin concentration (ppm) $n = 5$; R.S.D.% ≤ 5	Found Lactoferrin concentration (μM) $n = 5$; R.S.D.% ≤ 5
Human milk (freeze conservation)	84.1	1.05
Human milk	98.5	1.23
Dried milk (A)	7.68	0.096
Dried milk (B)	10.24	0.128
Dried milk (C)	8.32	0.104
Dried milk (D)[a]	80.8	1.01
Yogurt and pear	0.77	0.009
Yogurt and apricot	0.86	0.01

Values expressed both as ppm (mg l^{-1}) and as μM.
(A, B, C, D) = are several commercial powdered milk for unweaned diet produced by different pharmaceutical firm.
[a] Product with Lactoferrin added by producer.

the hydrogen peroxide electrode were used instead of the second procedure with the same transducer, the sensitivity is about twice as high.

4.2. Sample analysis

To this end, the hydrogen peroxide transducer and the first procedure were used to determine Lactoferrin in all the analyzed human milk samples, in infant formulas and yogurts because, by using this immunosensor assembly and the first procedure, we found a wide linear range and a usually low detection limit but, above all, a higher sensitivity.

The Lactoferrin concentrations determined using the optimized immunosensor to analyze two samples of human milk—one fresh and one deep-frozen, four different samples of powdered baby's milk, one to which Lactoferrin had been added by the manufacturer, and two fruit yogurts specifically manufactured for children are set out in Table 3. As can be seen, compared with the human milk samples, three of the powdered milk samples for unweaned babies have a Lactoferrin concentration that

Table 4
Recovery tests of added Lactoferrin in human milk and in several pharmaceutical dairy milk products recommended for unweaned children

Biological matrix	Found Lactoferrin concentration (μM) ($n = 5$); R.S.D.% ≤ 5	Added Lactoferrin concentration (μM)	Experimental Lactoferrin concentration (μM) ($n = 5$); R.S.D.% ≤ 5	Recovery % Lactoferrin concentration in biological matrix
Human milk (Diluted 1:10)	0.121	0.10	0.238	107.7
Human milk (Diluted 1:5)	0.235	0.20	0.492	113.1
Dried milk (D)[a] (Diluted 1:3)	0.351	0.10	0.476	105.5
Dried milk (D)[a] (Diluted 1:2)	0.454	0.20	0.633	96.8

[a] Product with Lactoferrin added by producer.

is about one order of magnitude lower; only the powdered milk sample with the manufacturer's Lactoferrin addition has a Lactoferrin concentration of about the same order of magnitude as that of human milk and, in the two samples of human milk analyzed, the frozen one has a concentration of lactoferrin that is 15% lower than in fresh milk. Lastly, the yogurt samples were found to have very low lactoferrin levels, about 1000 times lower that in human milk. Table 4 also shows the "recoveries" achieved by applying the standard addition method; the overall results were rather good (recoveries are always between 97 and 113%).

4.3. Linearity, precision, accuracy and robustness

In conclusion, the method showed good "robustness", as it was practically the working temperature rather the room temperature, and the working pH buffered by 0.1 M buffer solution, that maintain the pH value strictly constant, whatever the sample analyzed. In addition, the amount of antibody or marked antigen used for the competition measures, though constant, is nevertheless not critical, as it is always in reasonable excess in all the measures included in the linear range.

Finally Tables 3 and 4 show that the analytical method developed had a good reproducibility; the accuracy evaluation, carried out by the standard addition method in the analyzed samples, demonstrated that the recoveries for all these samples were at least acceptable.

5. Conclusions

The immunosensor developed proved suitable for measuring Lactoferrin concentration both in human milk and in finding commercial pharmaceutical products. The highest Lactoferrin concentration in all the samples tested was found in fresh human milk.

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