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Development of an ELISA for Quantifying Lysozyme in Hen Egg White

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An indirect enzyme-linked immunosorbent assay (ELISA) by inhibition was developed for quantifying lysozyme in hen egg white (HEW), a protein of value in not only the food and pharmaceutical industries but also for poultry research. Various experimental conditions (coating, antibodies dilutions, samples dilutions, preparations, blocking agents, and incubation times) were assayed to optimize this assay to the quantification of HEW in egg white samples. HEW samples were diluted 1:3000 to avoid matrix effects, possibly resulting from lysozyme interaction with other egg white proteins. Assay linearity for lysozyme ranged from 0.38 to 4.8 μg/mL, with intra- and interassay variations of 6.8% and 7.6%, respectively, and the lower detection limit was 0.264 μg/mL. We found that lysozyme concentrations in albumen from eggs laid by a hen cohort ranged from 2.2 to 4.5 mg/mL, thus underlining interhen variability. Overall, these data present an ELISA assay that is simple, quick, sensitive, accurate, and has been specifically designed to determine lysozyme concentrations in egg white samples.

KEYWORDS: Lysozyme; egg white; ELISA; quantification

INTRODUCTION

Lysozyme (EC 3.2.1.17) is a ubiquitous enzyme present in a wide range of biological fluids and tissues within animal and plant kingdoms (*1*−*2*). It has and still receives attention as a model protein for structural, physicochemical, crystallographic, enzymatic, immunological, and evolutionary studies (*3*). It is well-known for its antibacterial properties but also exhibits antiviral, antitumor, and immune modulatory activities (*4*−*6*). Its safety makes it a food preservative of choice as well as an active substance included in pharmaceutical preparations (*7*−*9*). Almost all commercial manufactured lysozyme is extracted from a convenient biological source, the hen egg white (HEW) in which it is relatively abundant (3.4% of total proteins) and easily accessible (*10*). HEW lysozyme is a key factor of the egg natural defense system against bacterial aggression (*11*), and one of our objectives is to explore the possibility of enhancing such defenses via genetic selection and, therefore, to study interhen variability of HEW lysozyme content. This approach requires a quantification method that is adequately sensitive, specific, accurate, reproducible, and convenient for processing hundreds of samples. Several methods have been developed for the quantification of lysozyme content. Some assays relied on the lysozyme lytic action against the cell wall of *Micrococcus lysodeikticus* (*12*, *13*), but evaluations of enzymatic activity need a strict control of temperature, pH, and ionic strength and are time-consuming. Furthermore, the presence of interfering substances or more simply lysozyme denaturation can lead to inaccuracies in quantification (*14*). Finally, enzymatic activity of lysozyme reflects only partly its antibacterial properties as it also shows cationic antimicrobial peptides/domains within its structure, enlarging its antibacterial spectrum to Gram-negative bacteria (*15*−*17*). Therefore, other methods for lysozyme quantification based on the protein itself have been reported in the literature. They included electrophoretic (*18*), chromatographic (*19*−*21*), and immunoenzymatic methods (*22*−*27*). The ELISA technique is particularly promising because of its high sensitivity, high specificity and convenience, especially for analysis of large number of samples, and it is surprising that none of the immunoassays have been applied and adjusted to direct quantification of lysozyme in hen egg white, despite its potential value for egg products industries and poultry researchers. Therefore, we present the optimization and validation of an enzyme-linked immunosorbent assay (ELISA) dedicated to the quantification of lysozyme in hen egg white and using commercial antibodies exclusively.

MATERIALS AND METHODS

Chemicals and Materials. HEW lysozyme was obtained from Ovonor (Annezin-lès-Bethune, France). Bovine serum albumin, ovalbumin, ovotransferrin, ovomucoid, ovoinhibitor, riboflavin binding protein, normal rabbit serum (NRS), PBS (phosphate-buffered saline: 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 7.4), and the OPD (*o*-phenylenediamine) Sigma Fast kit were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France). Purified rabbit polyclonal anti-chicken lysozyme antibody (10 mg/mL) and donkey anti-rabbit IgG horseradish peroxidase-linked whole antibody (1 mL stock solution diluted at 1/5000 as recommended by the manufacturer for detection of IgG down to 300 pg) were obtained from Biodesign (Saco, ME; ref. K59172R) and Amersham Bioscience

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(Orsay, France; ref. NA 934), respectively. Blotting grade blocker nonfat dry milk, EIA grade reagent gelatin, Tween 20, and SDS−PAGE molecular weight standards were from Bio-Rad (Marnes-La-Coquette, France). All other inorganic chemicals were of reagent grade or chemically pure. Flat-bottom 96-well polystyrene microtiter plates (F96 certified Maxisorp) were purchased from Nalge Nunc International (Rochester, NY).

Preparation of Egg White Samples. Eggs of 100 38-week old Isabrown hens belonging to different families of the same pedigree line were collected during a 1-week period and stored at 4 °C. The following week, five eggs per hen were broken and the albumen collected. This albumen was pooled and homogenized for 1 min at 8000 rpm using an Ultra-Turrax T25 (IKA, Staufen, Germany). This mixture was divided in aliquots and stored at −20 °C until further analysis.

SDS−PAGE, Western Blotting, and Protein Concentration. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) was performed according to ref *28* using a 12.5% resolving gel and a 4% stacking gel. Purified lysozyme and one egg white sample were diluted in sample buffer (62.5 mM Tris HCl pH 6.8, 10% glycerol, 2% SDS, 0.005% bromophenol blue and 5% β-mercaptoethanol), heated 4 min at 95 °C, and loaded onto the gel. After migration, proteins were stained with Coomassie Brilliant Blue R250 or electroblotted onto a 0.45 μm nitrocellulose membrane in 25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3. For western blotting analysis, membranes were first soaked for 30 min in TBS (20 mM Tris HCl, 0.5 M NaCl, pH 7.5) and blocked for 1 h in TTBS (TBS plus 0.05% Tween 20) containing 5% nonfat dry milk. They were then washed (2 × 5 min) in TTBS, before incubation (2 h) with rabbit polyclonal anti-chicken lysozyme antibody diluted 1:10 000 in 1% BSA−TTBS (TTBS plus 1% bovine serum albumin). After washing in TTBS (1 × 15 min and 3 × 5 min), membranes were then incubated for 1 h with donkey anti-rabbit IgG horseradish peroxidase-linked antibody diluted 1:75 000 in 1% BSA−TTBS. They were washed in TTBS as previously described with two additional 5 min washes in TBS. Immunoreactive bands were then revealed by the enhanced chemiluminescence method ECL+ (Amersham Biosciences, Orsay, France).

Concentrations of egg white total proteins were measured according to ref *29* using the Uptima Coo Protein Assay Kit (Interchim, Montluçon, France). The standard was one egg white sample, the protein concentration of which was previously determined using the Kjeldahl method (*30*).

Initial Indirect ELISA Procedure. Incubations were performed at room temperature (18−22 °C) on a rotating microplate shaker Titramax 100 (Heidolph Instruments, Cinnaminson, NJ). Microplates were washed four times with PBS between every step of the assay (100 μL/well except after blocking: 200 μL/well). Reactant volumes were 50 μL/well, except for the blocking solution (200 μL/well). Microplates were initially coated for 2 h with HEW lysozyme in PBS and then nonspecific sites were blocked for 1 h using 3% BSA in PBS. Polyclonal rabbit anti-chicken lysozyme antibody (AB1) and donkey anti-rabbit IgG horseradish peroxidase-linked antibody (AB_2) in 1% BSA−TPBS (1% BSA−PBS plus 0.05% Tween 20) were sequentially added and incubated for 2 h. The substrate (OPD at 0.4 mg/mL in 0.05 M phosphate−citrate buffer containing 0.4 mg/mL urea hydrogen peroxide) for peroxidase was placed in the wells for 20 min. The reaction was stopped by the addition of 1 M H_2SO_4. Absorbance was read at 490 nm using a microplate reader Argus 300 (PerkinElmer, Courtaboeuf, France).

ELISA Optimization. Checkerboard titrations (CBT) were performed to determine the optimal lysozyme concentration for coating (5−0.000 08 μg/mL in PBS) under various incubation conditions (from 15 min to 3 h at room temperature or overnight at 4 °C, with or without agitation). Optimal dilutions for AB_1 (1:50 to 1:204 800) and AB_2 (1:625 to 1:40 000) were tested at various incubation times (from 30 min to 2 h). For each pair of AB_1−AB_2 dilutions, the ratio between absorbance for AB_1 and absorbance for normal rabbit serum (NRS) was calculated (binding ratio: BR). Four different blocking buffers (1% BSA−PBS, 1% nonfat dry milk (NFDM)−PBS, 1% gelatin−PBS, 0.2% Tween 20−PBS) were screened. The antibody dilution buffers were identical with the notable addition of Tween 20 (0.05% final), and OPD incubation times from 5 to 35 min were also tested. Finally, indirect ELISA by competition and indirect ELISA by inhibition were assessed to define the optimal combination of the previous conditions for quantifying lysozyme in egg white samples, and the interest of preincubating lysozyme solution (standard and samples) in glass tubes with one equal volume of AB1 to promote AB1-free lysozyme binding was evaluated.

ELISA Validation. Linearity limits (i.e. the assay working range) and detection limits (DL) were estimated from 10 lysozyme curves obtained from different assays and from different samples. DL was defined as the lowest lysozyme concentration outside the range of two standard deviations over background. Different egg white dilutions corresponding to the linear part of the egg white curve were prepared (1:3000, 1:4000, and 1:5000) and analyzed by ELISA to determine the accuracy of the assay. Lysozyme concentrations in diluted samples were plotted against egg white dilutions, and a linear regression was performed to assess parallelism. Diluted egg white samples were fortified with different lysozyme amounts (from 0.05 to 1 μg/mL) for recovery studies. Recoveries were estimated by considering both added and total lysozyme concentrations (R and R_t) and were calculated as follows

$$R\,(\%) = (L_t - L_{EW})/L_a \times 100 \quad R_t\,(\%) = L_t/(L_{EW} + L_a) \times 100$$

where L_{EW} is the lysozyme concentration measured in diluted egg white, L_a is the concentration of lysozyme added in diluted egg white, and L_t is the total concentration of lysozyme measured in fortified diluted egg white.

Specificity was estimated using calibration curves prepared (1) in egg white ultrafiltrate (5000 Da cutoff) devoid of larger proteins and (2) in the same ultrafiltrate supplemented with several protein components of egg white at the commonly encountered concentrations in this fluid (*10*): ovalbumin (54 mg/mL), ovotransferrin (12 mg/mL), ovomucoid (11 mg/mL), ovoinhibitor (1.5 mg/mL), and riboflavin binding protein (0.8 mg/mL). Each lysozyme solution was diluted in 1% gelatin−TPBS, along with the added egg white proteins when present.

Reproducibility was estimated using 10 replicates performed for several dilutions of three different egg white samples and for several lysozyme concentrations. Intra-assay variation was determined with standard lysozyme using six replicates of the standard curve on the same microplate. Intra-assay variation was also determined for egg white samples using an optimized dilution of one egg white with five replicates/dilution. The assay was repeated five times. Interassay variation was estimated on 10 and 20 different assays in triplicate performed on different days by the same operator for the standard curve and the same egg white sample, respectively.

Statistics. Fitting of the curves and statistical analyses were performed with the GraphPad Prism software (version 3.02 for Windows, GraphPad software, San Diego, CA). Linearity in the median part of standard (lysozyme) and experimental (albumen) curves was estimated by a linear regression and then the identity between slopes was checked with covariance analysis (ANCOVA). A two-way variance analysis (ANOVA) completed by a Bonferroni test for comparisons of means was performed for the comparisons between usual and experimental standard curves used for the estimation of specificity. The *t*-test was used to analyze the difference in recovery relative to 100%.

RESULTS AND DISCUSSION

Control of Lysozyme Purity and Antibody Specificity. Purity of the commercial lysozyme and anti-HEWL specificity were assessed by SDS−PAGE and western blot. A protein band of 14.3 kDa corresponding to chicken lysozyme was clearly identified in both egg white and also in protein standard solution as expected, since lysozyme is one of the proteins composing the protein marker (**Figure 1**).

A minor protein band of 18 kDa was also detected after SDS−PAGE when a large amount of standard lysozyme was loaded (50 μg). This band was immunodetected in both deposits

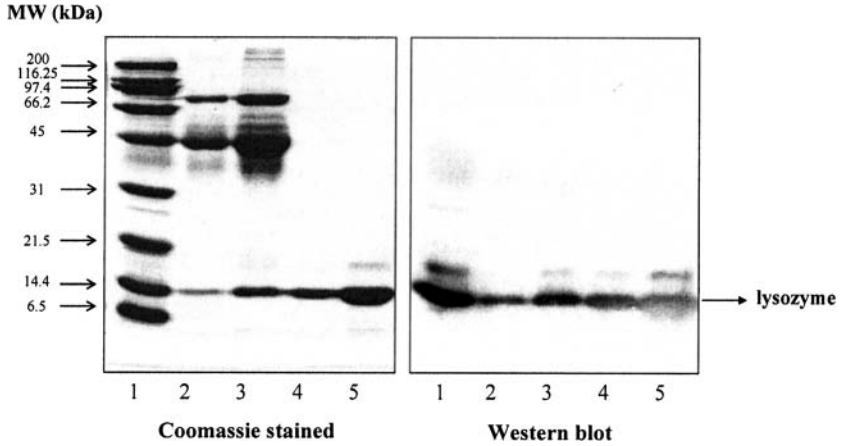

Figure 1. SDS–PAGE and western blotting for assessing standard lysozyme purity and AB_1 specificity. Lane 1, molecular weight markers; lanes 2 and 3, 7.5 and 35 μg of hen egg white (HEW); lanes 4 and 5, 10 and 50 μg of HEW lysozyme.

Table 1. Summary of Characteristics of the Optimized Indirect ELISA by Inhibition

volume/well	reaction volume	50 μL
	washing volume	100 μL
washing	buffer	PBS, pH 7.4
coating	buffer	PBS, pH 7.4
	lysozyme concn	0.02 μg/mL
	incubation	2 h, room temp (18–22 °C), agitation
inhibition	buffer	1% gelatin, 0.05% Tween 20 in PBS, pH 7.4
	AB_1[a] final dilution	1:1300
	egg white dilution	1:3000
	lysozyme concn for standard curve	0.08 to 12 μg/mL
	preincubation (in tubes)	overnight, 37 °C, agitation
	incubation (on microplate)	1.5 h, room temp (18–22 °C), agitation
AB_2	buffer	1% gelatin, 0.05% Tween 20 in PBS, pH 7.4
	AB_2 dilution	1:5000
	incubation	2 h, room temp (18–22 °C), agitation
reaction and color development	buffer	0.05 M phosphate–citrate with 0.4 mg/mL urea hydrogen peroxide
	OPD concn	0.4 mg/mL
	incubation	30 min, room temp (18–22 °C), agitation
	H_2SO_4 concn	1 M
reaction measurement	wavelength	490 nm

[a] Antigen–AB_1 mixture made by addition of one volume of egg white sample or standard lysozyme to the same volume of AB_1 at 1:650 (final AB_1 dilution: 1:1300).

of standard solution, but also in egg white (35 μg) and among the molecular weight markers. This minor protein could correspond to avidin, which is composed of four 15.6-kDa subunits (*31*). Avidin and lysozyme are difficult to separate by conventional chromatographic methods, mainly because of their close basic isoelectric point (10 and 10.7, respectively) (*31*, *32*). Therefore, the commercial lysozyme preparation could have been contaminated with avidin. Likewise, the antibody could have been raised against avidin-contaminated lysozyme, leading to the detection of this minor protein after western blotting. Another candidate for contamination could be a minor glycosylated form of egg white lysozyme with an apparent molecular mass of 18 kDa, as reported by Trudel et al. (*33*).

ELISA Optimization. Table 1 summarizes the characteristics and the different steps of the optimized ELISA for quantification of lysozyme in egg white. We have first optimized lysozyme concentration for microplate coating and AB_1 and AB_2 dilutions taking into account the specific conditions needed for an ELISA by competition or inhibition (*34*, *35*). An AB_2 dilution of 1:5000 was initially selected, since a 1:2000 dilution induced a strong background signal. This dilution was confirmed after optimizing AB_1 dilution and lysozyme coating, because the ratio between absorbance for AB_1 and that of normal rabbit serum was high (not maximal) but avoided that the related AB_1 titration curves had absorbance higher than 2.

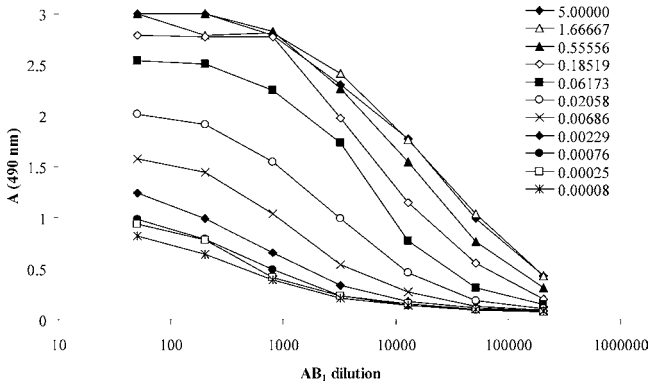

Figure 2. AB_1 titration curves for coating lysozyme concentrations ranging from 5 to 0.000 08 μg/mL. AB_2 dilution was 1:5000. Incubation times for coating and antibody reactions were 2 h.

The coating of lysozyme concentrations ranging from 5 to 0.000 08 μg/mL combined with AB1 dilutions ranging from 1:50 to 1:204 800 revealed that the AB_1 titration curve with a plateau region reaching a maximum absorbance of 2 and a low background corresponded to a coating lysozyme concentration of 0.02 μg/mL (**Figure 2**). The AB_1 titration curve was fitted with the sigmoid dose−response (variable slope) model (**Figure 3**), and the optimal AB_1 dilution, which corresponded to the

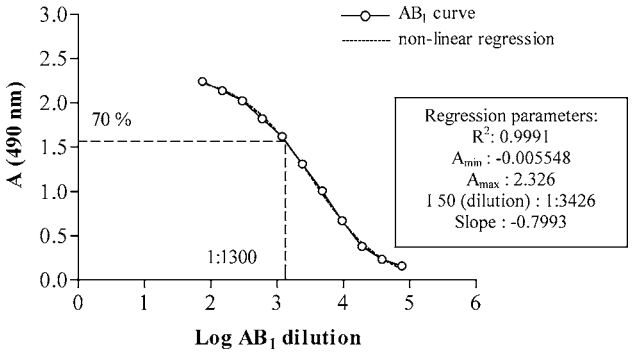

Figure 3. Determination of optimal AB_1 dilution for indirect ELISA by competition or inhibition. The AB_1 titration curve obtained with a coating lysozyme concentration of 0.02 μg/mL and an AB_2 dilution of 1:5000 was fitted with the sigmoid dose–response (variable slope) model. The optimal AB_1 dilution (1:1300) is related to an absorbance (*A*) corresponding to 70% of the maximal one. Incubation times for coating and antibody reactions were 2 h. A_{min} (minimal absorbance), A_{max} (maximal absorbnce), I_{50} (50% of inhibition).

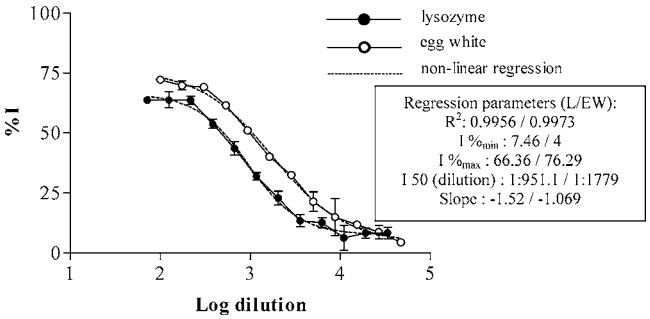

Figure 4. T Standard lysozyme (L) and egg white (EW) curves obtained with indirect ELISA by inhibition. Curves ($n = 3$ for L, $n = 2$ for EW) were fitted with the sigmoid dose–response (variable slope) model. To achieve comparison between curves, lysozyme concentrations were converted into dilutions (1:71 to 1:33674) on the basis of an initial arbitrary concentration of 1000 μg/mL. Egg white dilutions ranged from 1:100 to 1:47 143. Coating lysozyme concentration was 0.02 μg/mL; final AB_1 dilution in the mixture was 1:1300 and AB_2 dilution was 1:5000. Incubation times were 2 h for coating, overnight at 37 °C under agitation (preincubation) followed by 1.5 h on the microplate at room temperature for the antigen–AB_1 mixture, and 2 h for AB_2. $I\,\%_{min}$ (minimal percentage of inhibition), $I\,\%_{max}$ (maximal percentage of inhibition), I_{50} (50% of inhibition).

dilution leading to 70% of the maximal signal as described previously (*34*), was determined: 1:1300.

We then tested different blocking agents, both for an eventual blocking step and for the antibody dilution solution. We have considered that an additional blocking step to the protocol was not necessary, since under our conditions, the presence of the blocking agent in the antibody dilution buffer was sufficient to obtain a good signal/background ratio for most of the tested conditions. Among the four different agents tested (1% BSA−PBS, 1% nonfat dry milk (NFDM)−PBS, 1% gelatin−PBS, 0.2% Tween 20−PBS), BSA and gelatin were the best blocking agents for the antibody dilution buffer. Both have been used in several immunoassays for quantifying HEW lysozyme in different food matrices (*25*, *27*). As BSA and lysozyme could interact due to their opposite charges at the working buffer pH of 7.4 (p*I* of 10.7 and 5.5 for lysozyme and BSA, respectively), as described by Steiner et al. (*36*), we selected gelatin to avoid interference with the assay. Thus, subsequent optimization steps were performed in the absence of a blocking step, using 1% gelatin−TPBS as dilution buffer for antibodies, lysozyme standard solution, and egg white samples.

Different incubation times were tested for coating, antibody reactions, and OPD reaction, and the optimized times of incubation are shown in **Table 1**. If considered more practical by the operators, coating could be performed overnight at 4 °C under static conditions.

When testing indirect competitive ELISA for lysozyme quantification, no consistent sigmoid curve was obtained and the maximal percentage of competition (%*C*) did not exceed 20% whatever the conditions (data not shown). This could result from a difference in lysozyme conformation and/or accessibility of epitopes to AB_1 due to denaturation when interacting with plastic surfaces (*37*). To favor AB_1 binding to the free antigen, we performed an inhibition assay in which sample or standard solution and AB_1 were premixed and preincubated before being deposited onto the coated microplate, as recommended by Crowther et al. (*34*) and Harlow et al. (*35*). After testing various temperatures and times of incubation, an increase in the assay sensitivity was observed when preincubation of the antigen−AB1 mixture took place overnight (18 h) at 37 °C under agitation.

When testing egg white dilutions, sigmoid curves were obtained for dilutions greater than 1:100. For dilutions lower than 1:100 and lysozyme concentrations greater than 20 μg/mL, we noticed a high-dose "hook effect" which is characterized by a decrease in assay response at high antigen concentration. This phenomenon is a well-known artifact attributable to cooperative interactions occurring generally in sandwich immunoassays (*38*, *39*). In our case, it could be explained by the presence of an excess of free antigens preventing AB_1 from binding through steric hindrance. An identity between slopes of linear parts of egg white and standard curves was observed and is presented in **Figure 3**.

ELISA Validation. *Linearity and Sensitivity.* From calibration curve shown in **Figure 3**, we determined that the assay working range was from 0.38 ± 0.07 to 4.8 ± 1.4 μg/mL. The detection limit, which is defined as the lowest lysozyme concentration outside the range of two standard deviations over background, was 0.264 ± 0.086 μg/mL. Linearity limits were also calculated for six different egg white samples. All the curves shared a common linear interval between dilutions of 1:2800 and 1:5000 (data not shown), corresponding to lysozyme concentrations of 1.18 and 0.68 μg/mL in the case of an initial theoretical lysozyme concentration of 3.4 mg/mL (*10*).

Accuracy. The parallelism of the assay was assessed for egg white sample dilutions falling within the working range. It was acceptable ($r^2 = 0.9667$), though variability was high for sample diluted 1:5000 (data not shown).

Specificity. The specificity of the ELISA was evaluated by two complementary approaches, either by altering the lysozyme concentration in the egg white and looking at lysozyme recovery or by looking at the effect of adding various major egg white proteins on the lysozyme standard curve.

Recovery Studies. This approach was settled upon to analyze the impact of any interaction between lysozyme and other egg white proteins (matrix effect) (**Figure 4**). Egg white sample diluted 1:3000 to 1:5000 was fortified with different lysozyme amounts in such a way that total lysozyme concentration fell within the linear part of the standard curve. Recoveries were estimated by considering both added and total lysozyme concentrations (R and R_t) to limit the effect of high variability in lysozyme levels measured in basal and supplemented egg white samples. The best recoveries were obtained for egg white

Table 2. Recovery Test with Different Dilutions of Hen Egg White (HEW) Fortified with Several Concentrations of Lysozyme (lys.) ($n = 3$)[a]

HEW dilution ([lys.])	theoretical added [lys.] (μg/mL)	recovered [lys.] (μg/mL)	R[b] (% ± SD)	theoretical total [lys.] (μg/mL)	total recovered [lys.] (μg/mL)	R_t[b] (% ± SD)
	1	0.868	87 ± 9	1.751	1.619	92 ± 5
	0.75	0.672	90 ± 9	1.501	1.423	95 ± 4
3000	0.5	0.458	92 ± 27	1.251	1.209	97 ± 11
(0.751 μg/mL)	0.25	0.304	121 ± 12	1.001	1.055	105 ± 3
	0.1	0.038	38 ± 6[c]	0.851	0.789	93 ± 1[c]
	0.05	0.000	0 ± 0[c]	0.801	0.696	87 ± 6
	1	0.670	67 ± 13	1.603	1.273	79 ± 8
	0.75	0.758	101 ± 14	1.353	1.361	101 ± 8
4000	0.5	0.344	69 ± 2[c]	1.103	0.947	86 ± 1[c]
(0.603 μg/mL)	0.25	0.220	88 ± 38	0.853	0.823	96 ± 11
	0.1	0.091	91 ± 47	0.703	0.694	99 ± 7
	0.05	0.000	0 ± 0[c]	0.653	0.476	73 ± 5
	1	0.789	79 ± 1[c]	1.494	1.283	86 ± 1[c]
	0.75	0.525	70 ± 3[c]	1.244	1.019	82 ± 2[c]
5000	0.5	0.253	51 ± 3[c]	0.994	0.747	75 ± 2[c]
(0.494 μg/mL)	0.25	0.166	66 ± 6	0.744	0.660	89 ± 2
	0.1	0.076	76 ± 34	0.594	0.570	96 ± 6
	0.05	0.000	0 ± 0[c]	0.544	0.371	68 ± 8

[a] Measured lysozyme concentrations for each HEW dilution are noted in brackets. Coating lysozyme concentration was 0.02 μg/mL; final AB_1 dilution in the mixture was 1:1300 and AB_2 dilution was 1:5000. Incubation times were 2 h for coating, overnight at 37°C under agitation (preincubation) followed by 1.5 h on the microplate at room temperature for the antigen–AB_1 mixture and 2 h for AB_2. [b] R and R_t: recovery/added lysozyme concentration and recovery/total lysozyme concentration, respectively. [c] value significantly different from 100% ($P < 0.05$), as estimated by the t-test.

diluted 1:3000 and 1:4000 (**Table 2**). Low or no recovery of added lysozyme was obtained for concentrations lower or equal to 0.1 μg/mL. On the other hand, total recovery was acceptable, whatever the added lysozyme. Low recoveries were observed when egg white, diluted 1:1000 or 1:2000, was reinforced with lysozyme in the range of 0.05−1 μg/mL (results not shown). This emphasizes the problem of matrix effects in egg white when trying to quantify one of its constituent proteins, especially lysozyme, which possesses a highly basic pI. The initial pH of egg white (7.6), as well as that of our ELISA buffers (7.4), is intermediate to the basic pI of lysozyme and the pI of most of egg white proteins, e.g. ovalbumin, 4.5; ovotransferrin, 6.1; ovomucoid, 4.1; ovoinhibitor, 5.1; ovomucin, 4.5−5 (*10*), thus promoting electrostatic interactions (*40*−*42*). As a result, some epitopes may be masked and inaccessible to AB_1. Consequently; AB_1 binds preferentially to immobilized lysozyme, despite the presence of NaCl (0.137 M) in the buffer, which is known for its ability to decrease such interactions. The only way to avoid such interference is to dilute the sample while remaining within the assay linear working range. This objective was attained in our assay at egg white dilutions around 1:3000. High sensitivity was a prerequisite to deal with such dilution, avoiding matrix effects. In our case, with a detection limit of 264 ng/mL, the sensitivity of the ELISA was acceptable and suitable for the measurement of lysozyme in egg white. A higher sensitivity would have required dilution of egg white to a level at which it would have been difficult to obtain good reproducibility (e.g., egg white dilution of about 1:1,000,000 for a 1−10 ng/mL working range). Such high sensitivity was also obtained in several ELISA, which have been developed to quantify HEW lysozyme in food matrix (*24*, *25*, *27*) or human lysozyme in biological fluids (*22*, *26*), milieu where the lysozyme can be present at very low concentration. However, to date, no ELISA assay has been optimized for investigating lysozyme in egg white samples where matrix effects can be particularly strong assuming the high viscosity of this milieu.

Effect of major egg white proteins: to estimate possible interference with the most abundant egg white proteins, standard curves were obtained from a lysozyme solution prepared in egg white filtrate initially enriched or not with the main albumen proteins, at concentrations that were representative of those naturally present in egg white (ovalbumin, ovotransferrin, ovomucoid, ovoinhibitor, riboflavin binding protein). No significant effect was observed on the sigmoid shape of the curves, nor between slopes and ordinate intercepts of the linear parts of the curves (data not shown). Thus, no matrix effect due to the egg white filtrate or to the added egg white proteins was detected. As a matrix effect was previously observed for egg white dilutions lesser than 1:3000, i.e. falling into the dilution range performed to obtain a calibration curve from the lysozyme solution supplemented with albumen proteins (from 1:34 to 1:170 000), it is likely that the added proteins—despite their acidic pI—did not interact with lysozyme to an extent sufficient to impair its binding with AB_1. Therefore, it is possible that matrix effects arose mainly from interaction between lysozyme and another egg white protein, ovomucin. This protein (pI 4.5−5) represents 1.5% of egg white total protein and is largely responsible for the gel structure of the albumen. Its association with lysozyme was previously reported and was partially attributed to electrostatic interactions (*42*, *43*). However, as it is difficult to obtain pure and soluble ovomucin, we could not verify the effect of this protein in our assay.

Reproducibility. Precision profiles were obtained for different lysozyme concentrations and egg white dilutions. Variability increased at lower lysozyme concentrations, while remaining acceptable (<10%) within the working linear range (data not shown). The same trend was observed with different egg white samples, with coefficient of variation (CV) exceeding 10% between dilutions 1:3000 and 1:5000. Considering the previous results (egg white linear working range, recovery studies) an egg white dilution of 1:3000 was considered as the most suitable for the assay. Concerning the standard curve-fitting parameters (minimal absorbance, A_{min}; maximal absorbance, A_{max}; slope, and I_{50}; **Figure 2**), all CV values were below 10% for intra-assay variation, whereas day-to-day variability was larger, especially for A_{max} and I_{50}, but did not exceed 15%. Intra- and interassay variations for the lysozyme concentration in the same egg white sample were 2.334 ± 0.159 mg/mL (CV of 6.8%) and 2.791 ± 0.211 mg/mL (CV of 7.6%), respectively. Some of these variations could be attributed to the variability inherent in the standard curve.

Variability of Lysozyme Level in Albumen from Eggs Laid by a Hen Cohort. To assess the ELISA for routine analysis, we estimated the variability of lysozyme level in albumen of eggs laid by 97 hens of the same pedigree line. The concentration of egg white proteins and lysozyme (**Figure 5**) followed a normal distribution, as estimated with the Kolmogorov−Smirnov test. Total protein concentrations ranged from 99.5 to 131.4 mg/mL of egg white, corresponding to an average level of 10.9 wt % of whole albumen. Lysozyme concentrations ranged approximatively from 2.2 to 4.4 mg/mL (experimental mean, 3.080 mg/mL) and from 2 to 4% (experimental mean, 2.723%) of total egg white proteins. All these values are similar to those usually reported for hen egg white, i.e. total proteins, 11 wt % of whole albumen (*44*); lysozyme, 3.4% of total proteins (*10*). The interhen variability of lysozyme levels has been previously described (*45*). Values obtained were higher than the present study and ranged from 3.5 to 5.8 mg/mL. These discrepancies may be inherent to the assay methodology like turbidimetric method, which can be affected by

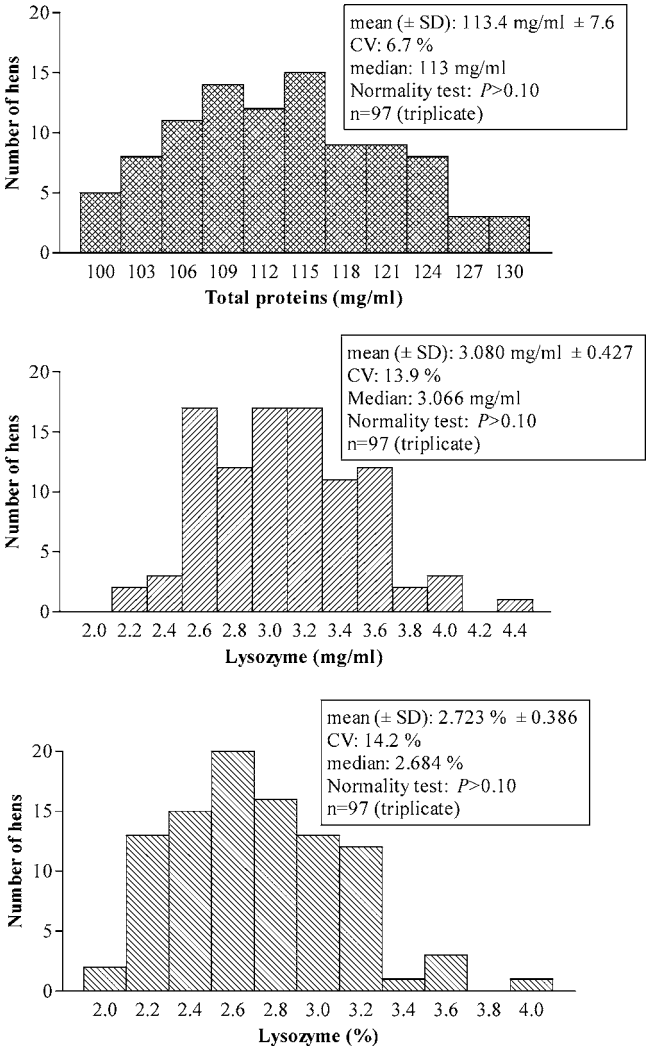

Figure 5. Distribution of (**a**) total protein concentrations (in mg/mL of egg white) and (**b, c**) lysozyme levels (in mg/mL and % of total proteins) in egg white from eggs (5 eggs/hen) laid by 97 38-week old Isabrown hens. Normality of the distribution was assessed by performing the Kolmogorov–Smirnov test: distribution was considered normal for $P > 0.10$. Assay parameters were as previously optimized.

many factors (*14*) and/or genetic characteristics of the studied hens (White Leghorns hens versus Isabrown hens in our study). The first study has been carried out 50 years ago and since then selection of laying hens has more than double the yearly egg production, resulting in a specialized line with more homogeneous genetic background. However, the comparison of egg quality of modern lines to old types reveals a limited change in egg composition (*46*).

From these results, we conclude that this optimized ELISA, described in **Table 1**, is sufficiently linear, sensitive, specific, accurate, reproducible, and convenient for quantifying lysozyme as low as 0.26 μg/mL in hen egg white, especially when many samples have to be analyzed.

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