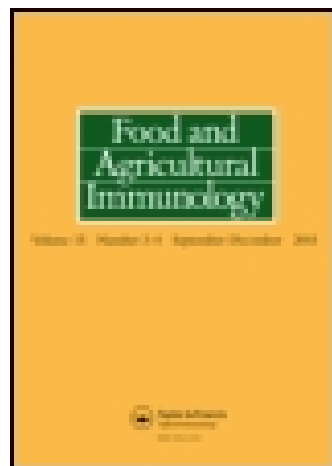




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P. Rauch^a, I. Hochel^a, I. Vodrážka^a, Z. Rumlová^a, J. Káš^a & J.-L. Guesdon^b

^a Department of Biochemistry and Microbiology, Institute of Chemical Technology, 166 28, Prague, Czechoslovakia

^b Institut Pasteur, 75724, Paris Cédex 15, France

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Comparison of Hen Egg Lysozyme Immunoassays Based on Different labels

P. RAUCH, I. HOCHÉL, I. VODRÁŽKA, Z. RUMLOVÁ, J. KÁŠ AND J.-L. GUESDON¹

Department of Biochemistry and Microbiology, Institute of Chemical Technology, 166 28 Prague, Czechoslovakia; ¹Institut Pasteur, 75724 Paris Cédex 15, France

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To check the concentrations of hen egg lysozyme in foodstuffs, added as a bacteriostatic agent, immunoassays based on different labels have been developed. The following detection limits (defined as non-specific binding increased by three standard deviations) were achieved using antibody labelled with either peroxidase, ¹²⁵I or a biotin-streptavidin system: 0.8; 0.75 and 0.13 ng/ml, respectively. Only the most sensitive lysozyme immunoassay was likely to be suitable for application to analysis of cheese because matrix interference effects mean that sample extracts need to be diluted prior to assay.

INTRODUCTION

Hen egg lysozyme may be used as a food preservative during so-called 'cold sterilization' owing to its bacteriostatic effect (Chander *et al.*, 1984). At present, egg lysozyme can be used for the preservation of spices, sausages, unpasteurized milk, fresh fruits and vegetables (Roberts & Kruser, 1984). It can be added to milk during cheese making (Brickjaer *et al.*, 1982). Thus sufficiently specific and sensitive methods are needed to be able to check the lysozyme content of foods in which it is used as a preservative. Immunoassays fulfil in general the requirement of specificity and sensitivity (Clifford, 1985; Morgan *et al.*, 1985) and thus are rapidly becoming the methods of choice.

The development of sensitive methods requires, however, much searching and testing of various procedures. Unfortunately, data in the literature do not always allow easy comparison of the sensitivity of various immunoassay formats obtained using the same antibody (Tijssen, 1987; Vaag, 1988). The high sensitivity possible with immunoassay also allows the frequently encountered interfering substances present in food samples to be overcome by suitable dilution. In such cases, high assay sensitivity of the immunoassay is a big advantage eliminating the need for sample clean-up stages. Accordingly we have looked at sandwich immunoassays each using the same antibody preparation, but using different labels (peroxidase, ¹²⁵I, biotin-streptavidin system) in order to find the most sensitive procedure.

MATERIALS AND METHODS

Materials

Egg white lysozyme was from Fluka, Switzerland, peroxidase (RZ 3) and biotin-N-hydroxy-succinimide ester were supplied by Boehringer, FRG, streptavidin-peroxidase conjugate was purchased by Amersham, UK, and Na ^{125}I , carrier free, was supplied by the Institute of Nuclear Research, Swierck, Poland. The other chemicals of analytical grade were from Lachema, Czechoslovakia. Polystyrene microplates for enzyme immunoassay were purchased from Koh-i-noor, Dalečín, Czechoslovakia. Different types of cheese were obtained from the local market.

Methods

The procedure for the preparation of the rabbit antiserum and the isolation of IgG fraction from the immune serum was described elsewhere (Beránková *et al.*, 1987). The conjugate IgG-peroxidase (IgG-Px) was synthesized according to Wilson & Nakane (1978) and used without further purification. Its protein content was 1.3 mg/ml and Px:IgG ratio 1.9.

IgG was labelled with the radionuclide ^{125}I by the chloramine T procedure as described elsewhere (Rauch *et al.*, 1984).

The conjugate of IgG-biotin was synthesized according to Guesdon *et al.* (1979).

SANDWICH IMMUNOASSAY OF LYSOZYME

(a) With Peroxidase as a Label

Antibody (IgG fraction) solution (0.1 ml; 2 $\mu\text{g}/\text{ml}$) in 0.05 M-carbonate buffer, pH 9.6, was pipetted into appropriate wells of the polystyrene microplate. The immobilization of antibody to the surface of the polystyrene wells occurred overnight at 2°C. The unbound IgG fraction was then removed by aspiration and the wells washed three times with the carbonate buffer. Aliquots (0.1 ml) of sample or standard solutions of lysozyme were pipetted into the washed wells with human serum albumin added to a final concentration 1% prior to incubation for 2 h at 37°C. The well contents were again removed by aspiration prior to washing three times with 0.01 M-phosphate buffer (pH 7.4) containing 0.8% NaCl and 0.05% Tween 20 (solution PBS). IgG-peroxidase conjugate (IgG-Px), diluted 1:1000 with PBS containing 1% human serum albumin was then added to washed wells and left to interact for 2 h at 37°C. The unbound conjugate was removed by aspiration. Wells were washed three times with PBS (containing 1% human serum albumin) prior to adding 0.1 ml of peroxidase substrate (50 mg o-phenylenediamine in 100 ml of 0.1 M-sodium citrate containing 0.03% hydrogen peroxide). The indicator reaction was stopped after 30 minutes of incubation at 37°C with 50 μl of 2 M- H_2SO_4 . The colour density of the reaction mixture was measured directly in the wells by a microplate reader (MR700 Dynatech) at 492 nm.

(b) With ^{125}I Label

The procedure described above was followed, except the reaction took place in polystyrene tubes (instead of the wells of a microplate) and IgG- ^{125}I conjugate (specific radioactivity 200 kBq/ μg) was used instead of IgG-Px. The radioactivity of ^{125}I in the test tubes was measured as described earlier (Rauch *et al.*, 1986).

(c) With Biotin-streptavidin System

The first two steps for the method for peroxidase label were followed as described. In the following step IgG-biotin (1 $\mu\text{g}/\text{ml}$) and subsequently streptavidin-peroxidase (diluted 1:1000) were incubated for 2 hrs at 37°C. All individual steps were followed by triple washings with PBS.

PREPARATION OF CHEESE EXTRACT

Cheese was gently grated, and a sample (1 g) was suspended in 10 ml PBS. After being mixed for 30 minutes at 20°C, the suspension was filtered over chromatographic paper (Whatman No. 1). The pH of the filtrate was adjusted to 7.0 with 0.066 M-Britton-Robinson buffer (pH 10.0). This extract, further diluted in PBS, was taken for immunoanalysis.

RESULTS AND DISCUSSION

Immunoprecipitation methods are time-consuming and are often not sufficiently sensitive. The methods based on double immunodiffusion, radial immunodiffusion and rocket immunoelectrophoresis, are more sensitive making it possible to detect analyte to 10 µg/ml. Sandwich immunoassays utilizing a single antibody offer much higher sensitivity and they are also relatively simple and fast. However, the possible advantages depend also on the type of label employed. Figure 1 shows the calibration curves obtained for the sandwich immunoassays described, based on antibody labelled with peroxidase, ^{125}I and biotin. The detection limits of these three procedures were

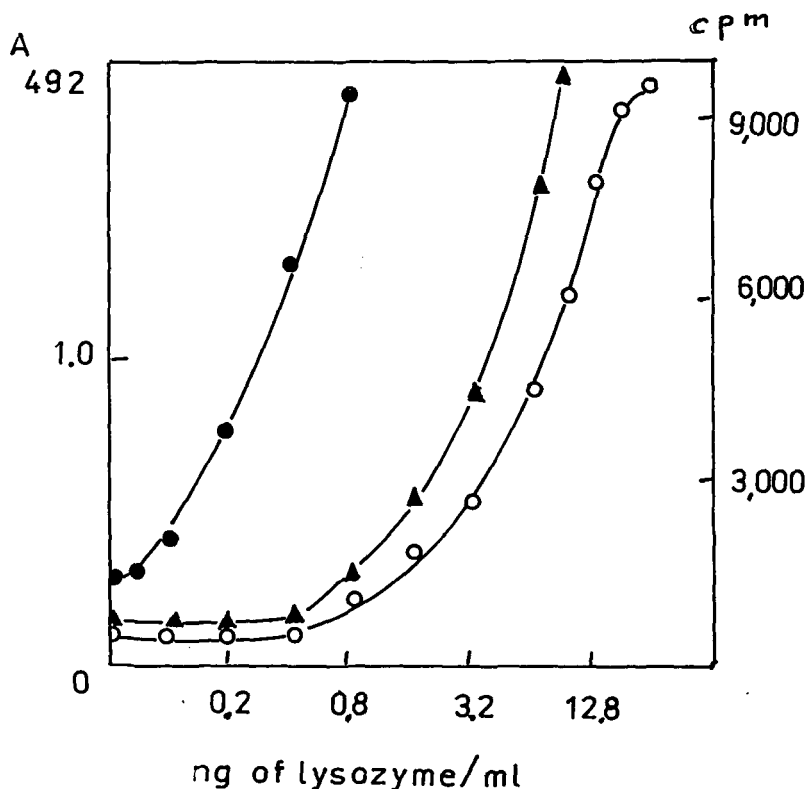


FIG. 1. Calibration curves for sandwich immunoassay of hen egg lysozyme using different antibody labels.

● IgG-biotin

▲ IgG- ^{125}I

○ IgG-peroxidase

A 492: absorbance at 492 nm for peroxidase label (●, ○)

cpm counts per minute for ^{125}I -label (▲).

TABLE 1. Comparison of detection limits of hen egg lysozyme immunoassays based on different labels

Label	Non-specific binding $\pm$ S.D. A 492 nm or cpm	Detection limit of the assay ng/ml
Peroxidase	0.104 $\pm$ 0.020	0.80
IgG-B-S-Px	0.305 $\pm$ 0.042	0.13
¹²⁵ I	630 $\pm$ 124	0.75

Note: IgG-B-S-Px is IgG-biotin-streptavidin-peroxidase.

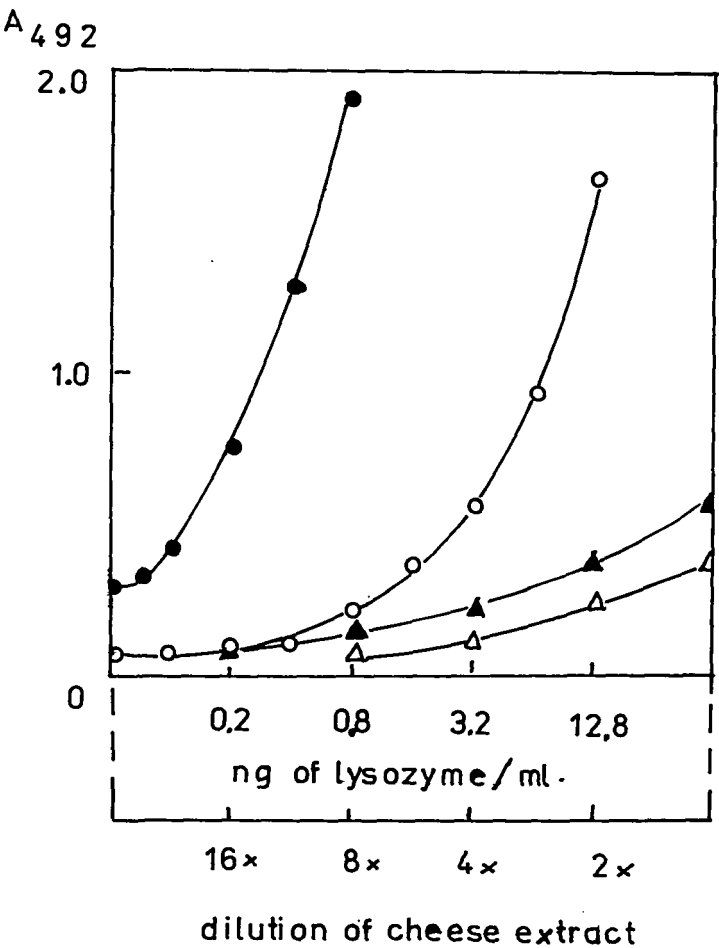


FIG. 2. Interference of cheese extracts of various dilutions in lysozyme immunoassay (IA).
● IA with IgG-biotin (control)
○ IA with peroxidase (control)
▲ interference of the extract of Edam
△ interference of the extract of Emmenthal.

determined as the appropriate blank values increased by three standard deviations. The detection limits are summarized in Table 1.

The method based on IgG-Px conjugate would evidently be the method of choice based on its simplicity. It also has a low detection limit, if only the analysis of standards is taken into account, of suitable magnitude for food analysis. Similar detection limits were achieved with the procedure based on using ^{125}I -conjugate but it was much less convenient than the enzyme assay. In addition, of course, the problems of working with radioisotopes have been well documented.

The application of the streptavidin-biotin system demonstrated a remarkable increase in assay limit of detection (by six times) when IgG-biotin and subsequently the conjugate streptavidin-peroxidase (Table 1) was used. The higher non-specific binding apparent with this label was probably caused by the non-homogeneity of the IgG fraction (which had not been purified by affinity chromatography prior to use).

Frequently in food analysis, interfering substances in the sample (Darley *et al.*, 1988; Rauch *et al.*, 1988) may cause considerable inaccuracy. Figure 2 shows that undiluted extracts of both tested cheeses (Emmenthal, Edam) cause dramatic interference in the sandwich immunoassays described. Dilution of the cheese extracts (8 times for Emmenthal and 16 times for Edam) appears to go some way to overcome the problem. The suppression of matrix interference effects by sample dilution is the simplest remedy to the problem and an effective one because it does not involve significant extra time or cost. However, a limitation to universal application is the sensitivity of the available assay. In the present case, the use of the streptavidin-biotin system would seem to fulfil this requirement and would therefore be suitable for assaying added lysozyme in cheese. Further work will establish the feasibility of the approach. Alternative sample purification procedures and/or the raising of new antibodies not affected by matrix interferences are not always viable propositions.

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