Application Note

Comparison of Blocking Agents for ELISA

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Introduction

The high sensitivity of ELISA implies a stringent limitation to the acceptable background signal due to non-specifically bound reactants. Low background is usually achieved by thorough "blocking" of the test wells with an inert or irrelevant protein.

This work demonstrates a variation in efficiency of blocking agents dependent on their molecular weight.

Methods and Results

Flat bottomed 96 well polystyrene plates (Thermo Scientific Nunc Immuno Plate MaxiSorp F96) were used in all experiments.

We compared the three blocking agents: bovine serum albumin (BSA), newborn calf serum (NBCS), and casein (i.e. sodium caseinate: 25 g casein powder (Sigma) dissolved in 800 mL 0.3 M NaOH by overnight stirring at 37°C, then titrated to pH 7.0 with HCI and made up to 1000 mL). After incubation of the wells with a dilution series in PBS of the respective blocking agents overnight at 4°C, and subsequent incubation with 1:4 PBS diluted rat serum for 90 min. at 4°C, each followed by washing with PBS + 0.05% Tween 20, the leakiness of the blocking layer for rat Ig was detected using the signal producing reagents: anti-rat rabbit F(ab')2 conjugated with horse radish peroxidase (HRP), and H_2O_2/ tetramethyl-benzidine (TMB) enzyme substrate.

The results, which are shown in Fig. 1, confirm the superiority of casein for blocking agent.

In order to investigate the nature of the blocking leakiness, the two enzymes HRP and alkaline phosphatase (AP) were used. The wells were coated with one enzyme, blocked with NBCS or casein, and incubated with a dilution series of the other enzyme followed by addition to separate wells of the respective substrates: H_2O_2/TMB and para-nitrophenyl phosphate (PNPP).

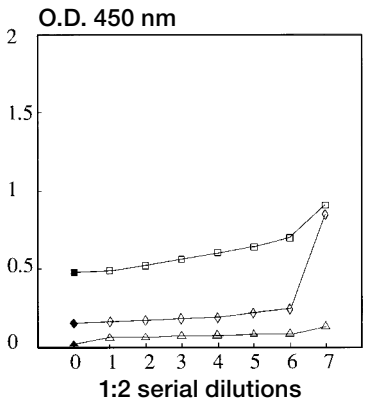

Fig. 1

Efficiency of three blocking agents.

Neither 100 mg/mL BSA (■) nor neat NBCS (◆) block the immobilization of rat Ig from 1:4 diluted serum as effectively as 25 mg/mL casein (▲). On dilution (open symbols) the failure of blocking is also more striking with the former agents than with casein.

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Substrate for 1st stage enzyme was added to detect any displacement of that enzyme, whereas substrate for 2nd stage enzyme was added to detect penetration of the blocking layers by that other enzyme.

The results, which are shown in Fig. 2, indicate that blocking leakiness is rather due to penetration than to displacement of the blocking layers.

Again, attention was drawn to casein, specifically, why was it a better blocking agent?

HPLC gel filtration of casein and NBCS in PBS on a TSK HW 55S column (LKB, Sweden) showed that NBCS consists of protein species > 60 kD while casein contains a heterogeneous collection of molecular species from > 60 kD to < 10 kD (Fig. 3).

Fractionation of casein by ultrafiltration into three molecular weight (MW) grades, MW < 10 kD, MW > 30 kD, and 10 kD < MW < 30 kD, enabled us to show that the high blocking efficiency of casein was entirely due to its content of small MW proteins, and that the blocking potency was "inversely proportional" to the MW of the component proteins (Fig. 4).

Summary

Blocking leakiness seems to be due to penetration and not to displacement of blocking layers by 2nd stage reagents.

Casein appears to be an effective blocking agent due to its content of small protein species.

BSA and NBCS contain relatively large MW components so that random close packing of these molecules leaves bare patches of unblocked plastic surface to which 2nd stage reagents can bind, leading to higher background characteristics of these blocking agents.

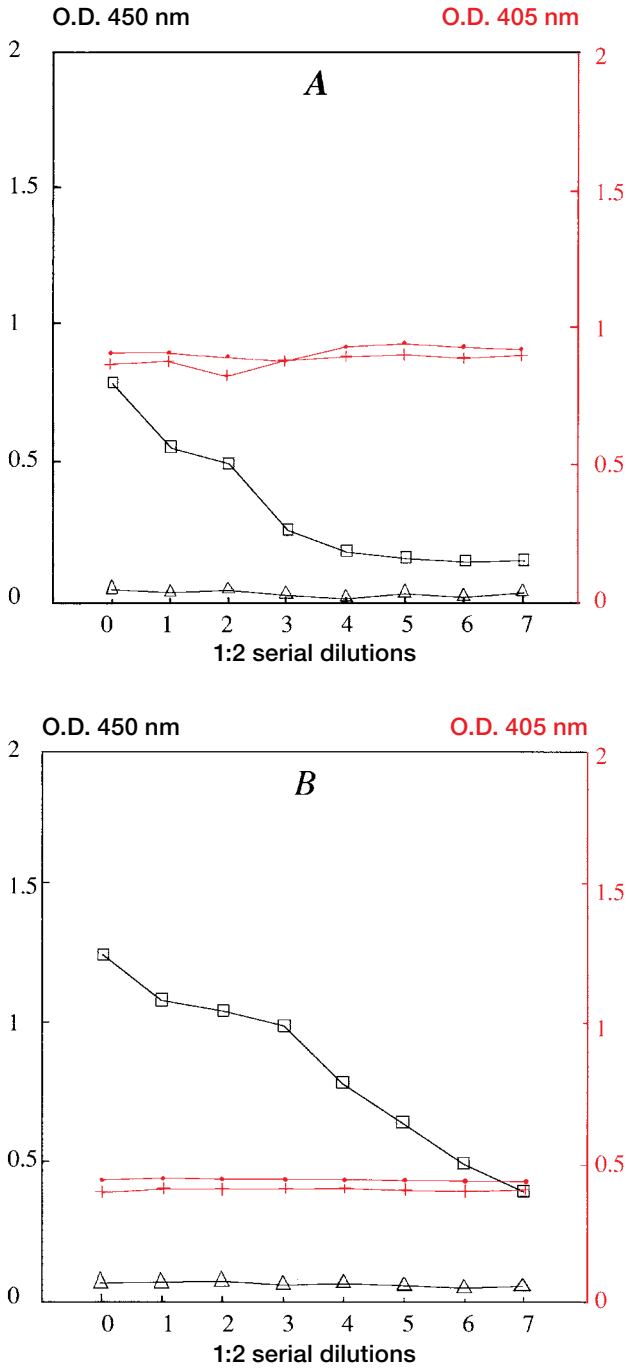


Fig. 2

Penetration of "blocking" AP by HRP (A), and vice versa (B).

A saturating layer of 1st stage enzyme (■), supplemented with a secondary blocking with 10% v/v NBCS, was penetrated by 2nd stage enzyme in a dose-dependent manner (□). When the secondary blocking was with 25 mg/mL casein, no penetration occurred at any dose of 2nd stage enzyme (△). There was no significant displacement of 1st stage enzyme by the secondary reagents (+). Converted HRP and AP substrates were measured at 450 and 405 nm, respectively.

Fig. 3

Gel filtration analysis of NBCS and sodium caseinate showing that whereas there are very few molecules smaller than 45 kD in NBCS, the size of the casein components is very heterogeneous with many molecules smaller than 6 kD. The MW standards refer to BSA (67 kD), ovalbumin (45 kD), a-lactalbumin (12.8 kD), and insulin (6 kD).

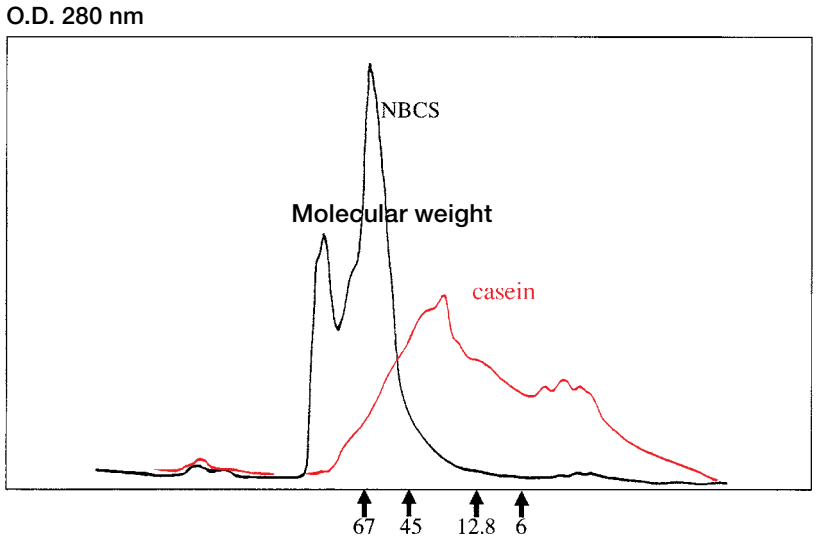
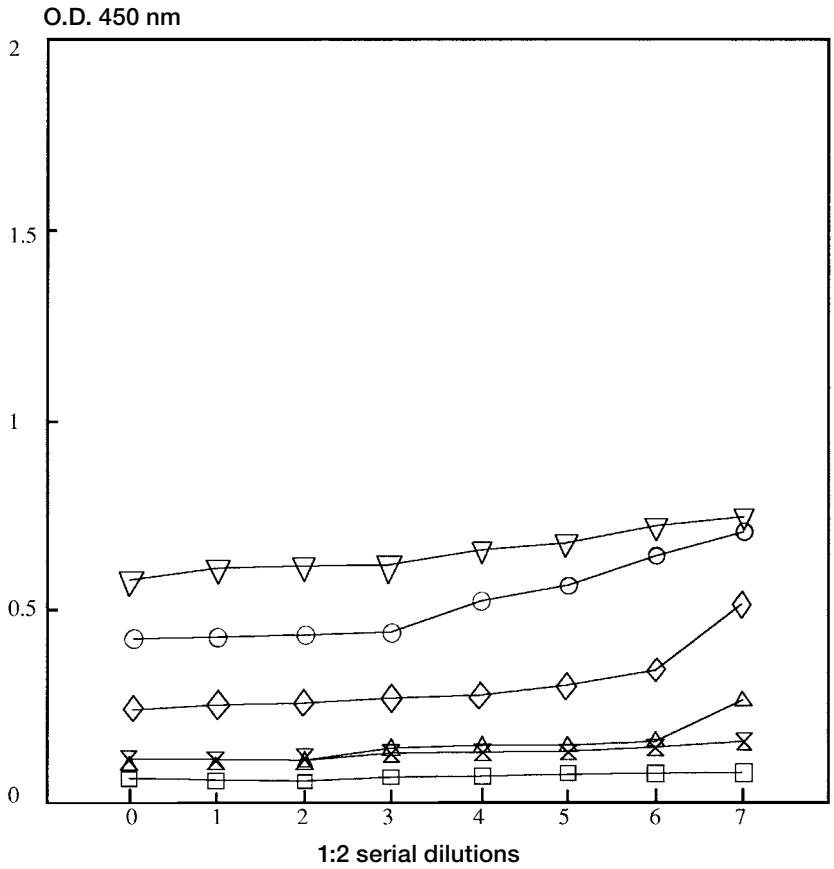


Fig. 4

Molecular size and blocking efficiency. Blocking of rat Ig immobilization with casein components from size fractionation confirms that smaller molecules block better. Rat Ig penetrated blocking layers of BSA (▽) and NBCS (○) giving high plateau background levels and early failure of blocking with dilution. Casein molecules › 30 kD (◇) were much less effective than molecules ‹ 10 kD (□). Casein molecules between 10 and 30 kD were intermediate in effectiveness (△), comparable with unfractionated casein (X). The initial concentration of each blocking agent corresponds to E280 = 4.0.



Further Reading

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Submitted.

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