

Soybean proteins: Alternative blocking agents for immunoassays using nitrocellulose or plastic solid phases

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The values of soybean proteins (SBP) and bovine serum albumin (BSA) as blocking agents were compared in immunoradiometric (IRMA), western immunoblotting (WIBA) and immunodot (IDA) assays. Protein purification, from soybean flour, only involved two aqueous extractions at alkaline pH and a precipitation at acid pH. About 100 g protein per kg of flour were obtained. For IRMA and IDA, the wells of PVC microtitration plates or nitrocellulose dots were sensitized with sonicated Trypanosoma cruzi epimastigotes and, as first antibody (Ab) source, sera from Chagasic or normal humans were used, followed by an anti-human [¹²⁵I]IgG, as a second Ab. Using SBP, the background in IRMA was at least as good as that obtained with BSA. Proportional decreases in the reactivity of the Chagasic sera were observed. The same antigenic preparation and a total extract from sonicated Mycobacterium tuberculosis were analyzed in WIBA, comparing BSA, "BLOTTO" and SBP as blocking agents. Clearly, SBP was more efficient at decreasing the non-specific staining and allowed the detection of bands otherwise masked by the background. It is possible that, unlike SBP, both BSA and "BLOTTO" contain variable amounts of contaminating bovine Abs which may cross react with the conjugate used as second Ab. Moreover, contamination of the first or second Abs with traces of immunoglobulins directed against BSA and milk proteins (i.e. casein) is also conceivable.

INTRODUCTION

Western immunoblotting (WIBA) (Towbin *et al.*, 1979), immunoradiometric (IRMA) (Catt and Tregear, 1967) and immunodot (IDA) (Hawkes *et al.*, 1982) assays require binding of the antigen (Ag) or antibody (Ab) to solid phases such as nitrocellulose and polyvinylchloride (PVC). Prior to probing the Ag, the remaining active protein binding sites on the solid phase must be blocked.

The efficiency of the blocking procedure is essential to minimize background signals, specially if high sensitivity is required (Burnette, 1981; Muillerman *et al.*, 1982). Two groups of blocking agents are generally

used: proteins (such as bovine serum albumin, BSA) (Towbin *et al.*, 1979); skimmed milk or purified casein and gelatin (Johnson *et al.*, 1984) and non-ionic detergents (such as Tween-20 and Nonidet P-40) (Spinola and Cannon, 1985; Batteiger *et al.*, 1982).

To our knowledge, the use of vegetable proteins as blocking agents is not conventional in immunoassays. We show here that soybean proteins (SBP) are a better and cheaper alternative to animal products.

MATERIALS AND METHODS

Materials: Goat immunoglobulin G (IgG) anti-rabbit IgG, goat IgG anti-human IgG

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(both reagents affinity purified) were obtained from Sigma Chemical Co. (St. Louis, MO). Radioactive iodine (^{125}I) was purchased from the Chilean Nuclear Energy Commission. Radiolabeling of proteins was performed by the Iodogen method (Fraker and Speck, 1978). Nitrocellulose membranes were from Bio-Rad (Richmond, CA), and PVC microtiter plates were obtained from Falcon, Becton Dickinson Co. (Oxnard, CA). Human sera, positive and negative for the presence of antibodies (IgG) against *Trypanosoma cruzi*, were obtained from our University Blood Bank (Hospital Clínico de la Universidad de Chile "José Joaquín Aguirre"). A sonicated extract of *T. cruzi* was obtained from cultured forms (epimastigotes) of the Tulahuén strain, kindly donated by Dr. Antonio Morello, Department of Biochemistry, Faculty of Medicine, University of Chile. *Mycobacterium tuberculosis* was obtained from a culture of 60 days, and the bacteria were concentrated by centrifugation followed by sonication. All other common reagents were of analytical grade.

Preparation of rabbit antiserum: Rabbits were injected three times, one week apart, with 250 μg of a whole sonicated extract of *Mycobacterium tuberculosis*, emulsified 1:1 with complete Freund adjuvant. The first injection was administered in the rear foot pads, and the following two, subcutaneously on the back. Preimmune bleeding was obtained a week before the first injection.

Polyacrylamide gel electrophoresis and Western blotting: Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% discontinuous buffer system (Laemmli, 1970). Then, these were electrophoretically transferred at 10°C to nitrocellulose in an electroblot apparatus (Bio Rad), according to standard methodology (Towbin *et al.*, 1979). After transfer, the nitrocellulose was cut into 5 mm strips. These were blocked with one of three different blocking agents. The blocking solutions used were 5% w/v "BLOTTO" (Bovine lacto transfer technique optimizer, skimmed milk) (Johnson *et al.*, 1984), 3% w/v BSA and 0.5% w/v SBP, all of them in phosphate-buffered saline (PBS). The strips were incubated for 2 h in 2 ml of a solution containing 1/100 diluted antiserum. Controls were incubated

with sera from normal humans or preimmune rabbits. Strips were washed 5 times with PBS-0.05% v/v Nonidet P-40 (PBS-NP40), 10 min for each change, incubated with a goat ^{125}I IgG anti-human or anti-rabbit IgG, followed by radioautography.

Immunoradiometric assay: Twenty five μl of sonicated epimastigotes (20 μg total protein per ml), in 0.1 M carbonate buffer (pH 9.6), were placed into the wells of PVC plates. The plates were then incubated overnight at 4°C; washed three times with PBS-NP40; saturated for 1 h with 1% w/v BSA or 0.5% w/v SBP; and incubated at room temperature in triplicates for 2 h with Chagasic or normal human sera. Then, they were washed three times with PBS-NP40; incubated for 2 h at room temperature with 10⁵ cpm of a goat ^{125}I IgG anti-human IgG, washed three times with PBS-NP40 and the radioactivity associated with the individual well was measured.

Extraction of soybean proteins: A simple and inexpensive procedure was used. 200 g of soybean flour were soaked in a liter of water at 70°C, adjusting the pH to 8.6. Temperature was lowered to 50°C and the suspension stirred for 30 min, centrifuged at 1,800 x g for 10 min and pellet and supernatant separated. The pellet was extracted with 600 ml of water at pH 8.6. Both supernatants were pooled and the pH adjusted to 4.5, with continued stirring while the proteins precipitated. The precipitate was washed with water pH 4.5 and redissolved with 200 ml of PBS. The final concentration of protein was about 10% w/v (Bradford, 1976; Spector, 1978). When analyzed under reducing conditions in SDS-PAGE, the protein extract showed four major polypeptides with apparent molecular weights ranging from 30 to 200 kDa (results not shown).

RESULTS AND DISCUSSION

Figures 1 and 2 show the results of WIBAs where the blocking efficiency of "BLOTTO" (tracks 1,2), 3% w/v BSA (tracks 3,4) and 0.5% w/v SBP (tracks 5,6) are compared. A *T. cruzi* epimastigote (Figure 1) and *M. tuberculosis* (Figure 2) sonicates were electroblotted in two continuous front gels and the nitrocellulose strips were reacted with se-

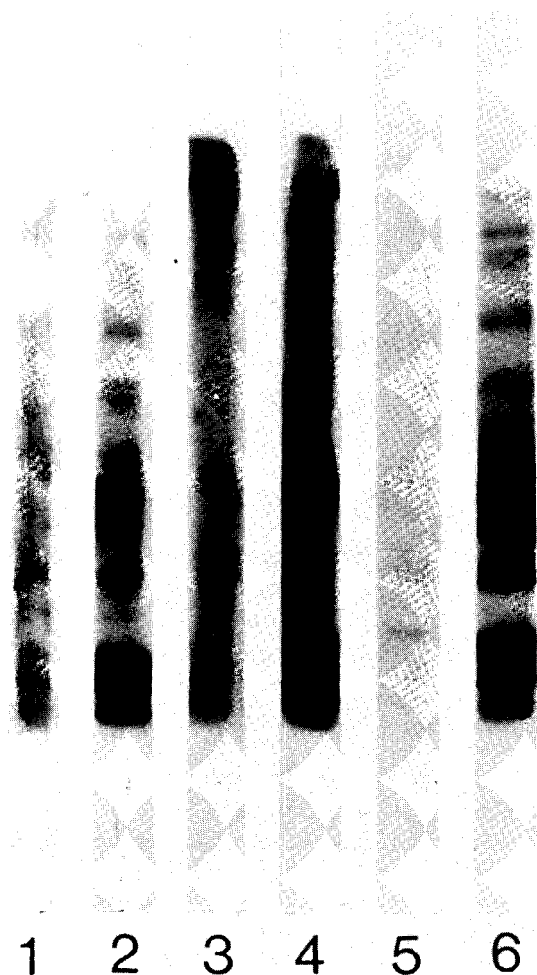


Fig. 1: Effect of BSA, "BLOTTO" and SBP as blocking agents in WIBA. Sonicated *T. cruzi* epimastigotes analyzed by SDS-PAGE, transferred to a nitrocellulose sheet and blocked. Nitrocellulose strips reacted with a sera from a Chagasic human (tracks 2,4,6) or from a normal human (tracks 1,3,5). Goat [125 I]IgG anti-human IgG used as second antibody. Blocking buffers: 5% w/v "BLOTTO" (tracks 1,2), 3% w/v BSA (tracks 3,4) and 0.5% w/v SBP (tracks 5,6).

rum from a Chagasic individual (Figure 1; tracks 2,4,6) or rabbit hyperimmune serum (Figure 2; tracks 2,4,6). As negative controls a normal human serum (Figure 1; tracks 1,3,5) and a rabbit preimmune serum (Figure 2; tracks 1,3,5) were used. All strips were reacted with a goat [125 I]IgG anti-human IgG or anti-rabbit IgG. The strongest background in both cases was obtained with BSA, a slight reduction is observed with "BLOTTO" and a clear cut decrease is obtained with SBP. The benefits of a low background can be appreciated, if the definition of three lower molecular weight bands from *T. cruzi* (Figure 1; tracks



Fig. 2: Effect of BSA, "BLOTTO" and SBP as blocking agents in WIBA. Sonicated *M. tuberculosis* analyzed by SDS-PAGE, transferred to a nitrocellulose sheet and blocked. Nitrocellulose strips incubated with a rabbit hyperimmune serum (tracks 2,4,6) or a rabbit preimmune serum (tracks 1,3,5). Goat [125 I]IgG anti-rabbit IgG used as second antibody. Blocking buffers: 5% w/v "BLOTTO" (tracks 1,2), 3% w/v BSA (tracks 3,4) and 0.5% w/v SBP (tracks 5,6).

2,4,6) and the main band from *M. tuberculosis* extract (Figure 2; tracks 2,4,6) are compared. The low background obtained with SBP also allows detection of two polypeptides by the normal serum (Figure 1; track 5). These are difficult, if not impossible to detect in the homologous tracks blocked with "BLOTTO" or BSA (Figure 1; tracks 1,3).

The three blocking agents were also compared in IDA, using the same reagents as those used in WIBA. This time, the Ag (*T. cruzi* epimastigote sonicate) was titrated. The overall background was clearly smaller with SBP and the detectable end-point dilution of the Ag was easier to discern (results not shown).

It was also interesting to test the blocking quality of SBP with a different solid phase. In an IRMA, the wells of PVC microtitration plates were coated with serial dilutions of sonicated *T. cruzi* epimastigotes and developed as in the WIBA described above. Figure 3 shows that, by using SBP instead of BSA as blocking agents, a decrease in the background radioactivity obtained with the normal serum was achieved. Thus, when the

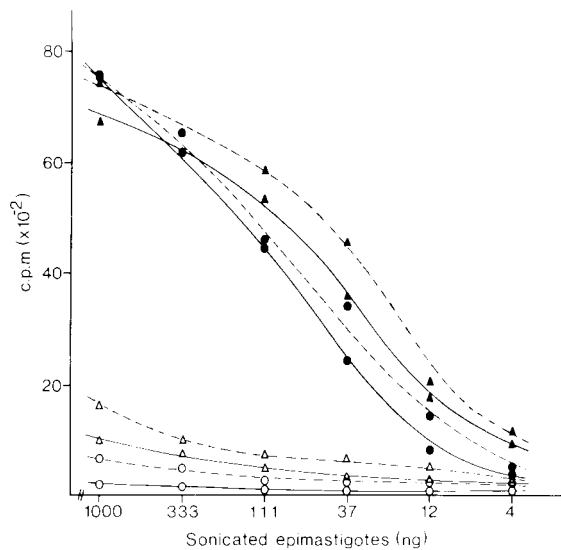


Fig. 3: Comparison of BSA and SBP as blocking agents for IRMA. PVC microtitration plates incubated with dilutions of sonicated *T. cruzi* epimastigotes. Plates saturated with 1% w/v BSA (\blacktriangle) and 0.5% w/v SBP (\bullet) in PBS. Dilutions of sera from Chagasic (\bullet, \blacktriangle) and normal (\circ, \triangle) humans were added. Plates washed and treated with goat [125 I]IgG anti-human IgG. Each value, mean of triplicate measurements. Experiment 1: dashed lines. Experiment 2: solid lines.

amount of Ag per well was 333 ng, the background was about 17 and 8%, when BSA and SBP were used as blocking agents, respectively. With minor variations, these figures are valid along the Ag titration curves. As expected, the decrease in background is also reflected in modest but proportional decreases of the signals obtained with the Chagasic sera. Similar results were obtained when dilutions of the Chagasic and normal sera were tested in an IRMA against a constant Ag concentration (Figure 4).

The intensity of background may be a crucial factor for the sensitivity and specificity of immunoassays so widely used as WIBA, IDA and IRMA. In WIBA, besides sensitivity, resolution power is an important issue. In this respect, our results show that SBP, even at lower protein concentration than BSA or "BLOTTO", are more efficient. It is possible that, unlike SBP (a vegetable protein mixture), both BSA and powdered milk, may contain variable amounts of contaminating bovine immunoglobulins which may be recognized, in a crossreactive way, by the conjugate used as second antibody (Thean and Toh, 1989). It is even conceivable that the Abs used as probes may be contaminated with trace amounts of immunoglobulins against the

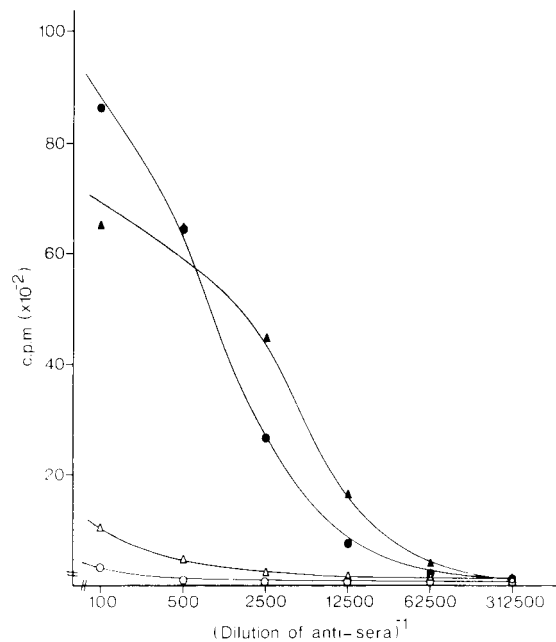


Fig. 4: Comparison of BSA (\blacktriangle) and SBP (\bullet) as blocking agents for IRMA. Experiment performed as described in legend of Fig. 3, except that sera dilutions were tested against a constant (1 μ g) Ag concentration.

blocking animal protein. As a consequence, a disturbing high background may be obtained.

Non-ionic detergents, when used as blocking agents, are known to remove proteins from the solid phase (Lin and Kasamatsu, 1983; Hoffman and Jump, 1986) and to create rather stringent conditions for the Ag-Ab interaction. Thus, a low background may be obtained but, frequently, with unacceptable losses in specific signals.

Our results indicate that SBP stringency is greater than BSA and "BLOTTO". This has been advantageous in the systems described here, when human or rabbit antibodies are used as probes. However, when a new detection system is to be standardized, it is advisable to test different blocking agents.

Finally, SBP represents an economically advantageous alternative to BSA. The soybean flour is extremely cheap and the production of amounts of blocking material takes a few steps using only inexpensive reagents. This aspect may be important when large numbers of assays are performed.

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