FEBS 17146

FEBS Letters 388 (1996) 99–102

Recombinant barnase as a label in ELISA

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Received 5 April 1996; revised version received 23 April 1996

Abstract Recombinant barnase was proposed as a label in the enzyme-linked immunosorbent assay (ELISA). Barnase-conjugated pig transferrin was prepared by the periodate oxidation procedure. Solid-phase bound barnase activity was determined from the change in RNA-ethidium bromide complex fluorescence upon RNA hydrolysis. The sensitivity of transferrin-barnase conjugate determination in ELISA was no less than 5 ng per well. The conjugate was applied in competition ELISA for free transferrin determination.

Key words: ELISA; Barnase; Transferrin; Monoclonal antibodies; Fluorescence; Ethidium bromide

1. Introduction

General requirements for enzyme labels in ELISA include high specific activity, allowing detection in low concentrations; enzyme availability; storage stability; retention of activity upon chemical modification during preparation of conjugates with antigens or antibodies; simplicity and sensitivity of enzyme assay. At present a great number of marker enzymes are used in enzyme immunoassay, and the list is constantly supplemented with new entries [1].

Barnase, a ribonuclease from *Bacillus amyloliquefaciens*, was conjugated with a model antigen (transferrin) for its further application in ELISA. Barnase is a small single domain protein (110 amino acids, 12 382 Da) with a well known three-dimensional structure [2]. The barnase gene has been cloned, and the enzyme is available in large amounts due to highly effective expression in *Escherichia coli* [3]. Barnase is an enzyme that is uniquely resistant to different denaturing factors and highly stable during storage. The absence of SH groups and disulfide bridges in the barnase molecule allows for its application in different schemes for synthesis of conjugates with antibodies and antigens [4].

Because periodate oxidation is widely used in labelling antibodies and antigens, this technique was applied to make the transferrin-barnase conjugate. Terminal carbon hydrates in the transferrin molecule were oxidized to dialdehydes, which subsequently interacted with barnase ε-amino groups to form covalent bonds. The conjugate was characterized immunochemically both by its binding with immobilized monoclonal antibodies against transferrin and inhibition of this binding by free transferrin. The solid-phase bound ribonuclease activity was determined by a novel method based on a change in the fluorescence of the RNA-ethidium bromide complex on RNA hydrolysis by barnase. Highly sensitive instruments for direct measurement of fluorescence in microplate wells are available for investigators, and the sensitivity of enzyme determination with fluorogenic substrates is essentially higher than that of spectrophotometric methods [1]. ELISA using barnase as marker enzyme as well as other immunoassay techniques are promising highly sensitive procedures for determination of antigens and antibodies.

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Abbreviations: BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; Mab, monoclonal antibody; OPD, *o*-phenylenediamine; TE, 10 mM Tris-HCl buffer with 1 mM EDTA pH 7.4

2. Materials and methods

2.1. Reagents

Sodium periodate was obtained from Sigma (USA); sodium borohydride, bovine serum albumin (BSA), Tween-20, and glycerol from Serva (Germany); ethidium bromide from Boehringer Mannheim (Germany); sheep antibodies against rabbit IgG labelled with horseradish peroxidase from Amersham (UK); total yeast RNA from Reachim (Russia).

2.2. Materials

Highly active recombinant barnase from *E. coli* was obtained from Prof. I.B. Leshinskaya (Kazan' State University, Russia); pig transferrin and monoclonal antibodies against pig transferrin of PTF-02 hybridoma were obtained from Prof. F. Franek (Institute of Molecular Genetic, Prague, Czech Republic). Polyclonal rabbit antibodies against barnase were obtained from Prof. R. Hartley (National Institutes of Health, Bethesda, MD, USA). The Nunc Immune Maxisorb plates of Nunc (Denmark) were used in this work.

2.3. Determination of barnase activity

To 100 μl of 0.1 M sodium citrate buffer, 0.2 M NaCl, pH 6.5, containing 50–300 μg/ml of total yeast RNA, 10 μl of barnase solution in TE was added and the mixture was incubated at 50°C for 1 h. Following the incubation, 200 (100) μl of ethidium bromide solution in TE (1 μg/ml) was added to each probe and the fluorescence was measured in a Hitachi F-4000 fluorimeter at $\lambda_{ex} = 365$ nm and $\lambda_{em} = 592$ nm.

2.4. Preparation of transferrin-barnase conjugate

To 700 μl of transferrin solution (1.0 mg/ml) in 0.01 M Na-phosphate buffer, pH 7.4 (buffer A) 20 μl of sodium periodate solution (40 mg/ml in water) was added and the mixture was incubated for 5–15 min in the dark at room temperature. After this, 15 μl of glycerol and 300 μl of barnase solution (2 mg/ml) in buffer A were added and incubation continued for 1 h at room temperature. Finally, 100 μl of sodium borohydride solution (4 mg/ml in water) was added, the mixture was kept for 30 min and then dialyzed overnight against buffer A at 4°C.

2.5. Indirect ELISA

Mab immobilization was carried out in 0.05 M Na-carbonate-bicarbonate buffer, pH 9.6, containing 100 μg/ml Mabs; the plates were incubated overnight at 4°C. The plates were washed three times and free binding sites were blocked with 1% BSA solution in 0.01 M Na-phosphate buffer, 0.15 M NaCl, pH 7.4 for 2 h at room temperature. The plates were washed and different amounts of transferrin-barnase conjugate in 0.01 M Na-phosphate buffer, 0.15 M NaCl, 0.2% BSA and 0.05% Tween-20, pH 7.4 (buffer B) were added to the wells. The plates were kept for 1 h at room temperature, washed three times and incubated for 1 h under similar conditions with a solution of rabbit polyclonal antibodies against barnase in buffer B. After washing, rabbit antibodies were detected with horseradish peroxidase-labeled sheep

PII S0014-5793(96)00535-2

polyclonal antibodies against rabbit IgG (1:500 in buffer B, incubation for 1 h at room temperature). After washing, peroxidase activity was determined using OPD Enzymatic kit (Diaplus, Russia). Optical density at 492 nm was measured in a Multiscan Plus multichannel spectrophotometer (Labsystems, Finland).

2.6. Inhibition analysis using transferrin-barnase conjugate

90 μl of a sample containing different concentrations of transferrin (25–200 μg/ml) in buffer B and 10 μl of transferrin-barnase conjugate diluted 1:200 with buffer B were added to the wells containing anti-transferrin Mabs immobilized as described above. After incubation for 1 h at room temperature, the plates were washed and the solid-phase bound ribonuclease activity was determined as described above.

3. Results and discussion

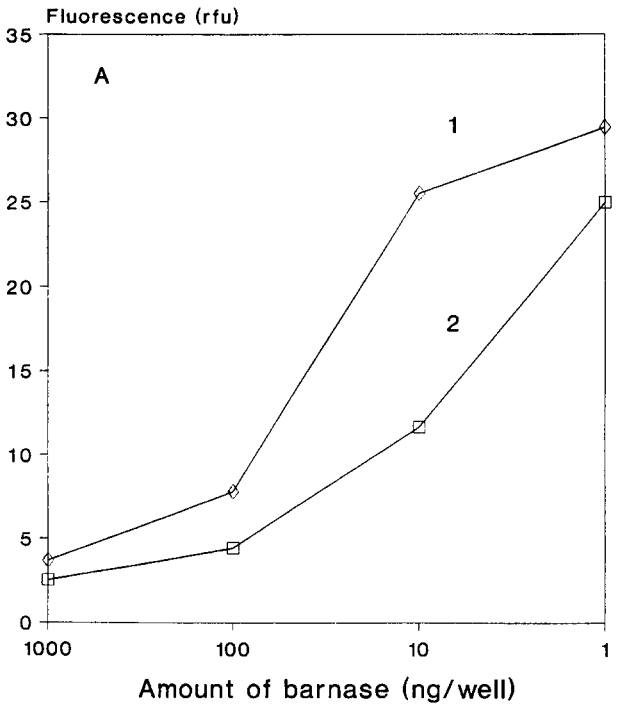
The major reason limiting ribonuclease application in immunoassays was the absence of a sensitive detection method. The methods based on absorbance measurements do not provide the required sensitivity [5]. However, two fluorogenic substrates have been synthesized recently, allowing ribonuclease determination in concentrations below the picomolar by changes in fluorescence [6,7]. Our method is based on the known ability of ethidium bromide to intercalate between nucleic bases with formation of an intensively fluorescent complex (λ_{ex}=365 nm and λ_{em}=592 nm). Upon RNA degradation by ribonuclease the number of sites available for ethidium bromide intercalation decreases in proportion to the amount of added enzyme. Thus, the fluorescence intensity recorded after incubation and addition of ethidium bromide to the reaction mixture will be in inverse proportion to the amount of added ribonuclease.

Fig. 1A shows ethidium bromide fluorescence as a function of barnase amount at two RNA concentrations in the substrate buffer. It is seen that the higher the enzyme amount the lower the fluorescence intensity. The position of the point where the curves reach a plateau depends on the RNA concentration in the substrate buffer, since at higher concentrations of RNA more enzyme is necessary for its effective hydrolysis. The method allows one to detect down to 10 pg barnase per well at 50°C.

Most popular enzyme labels permit visual evaluation of the results without sophisticated equipment. In our case the barnase-induced changes in fluorescence could be seen in a standard TF-20 M transluminator (Vilber Lourmat, France) (Fig. 1B). Maximum sensitivity, observed at an RNA concentration of 75 μg/ml, was about 10 pg of enzyme per well. At lower RNA concentrations in the substrate buffer, the fluorescence intensity is insufficient for highly sensitive visualization.

The recombinant barnase was cross-linked to the model antigen glycoprotein transferrin by the periodate method as described in Section 2. The transferrin-barnase conjugate was tested in indirect ELISA wherein the conjugate was bound to anti-transferrin mouse Mabs. After washing, rabbit polyclonal anti-barnase antibodies were added. The rabbit antibodies were detected using antibodies against rabbit IgG labelled with peroxidase (Fig. 2A). The results in Fig. 2A show that the conjugation technique used makes possible effective transferrin cross-linking with barnase. The conjugate exhibited insignificant non-specific binding with immobilized BSA, in all cases not exceeding 0.13 ± 0.01 optical units.

In the next group of experiments, the titer of transferrin-barnase conjugate in ELISA was determined from ribonuclease activity (Fig. 2B). Decreasing amounts of conjugate



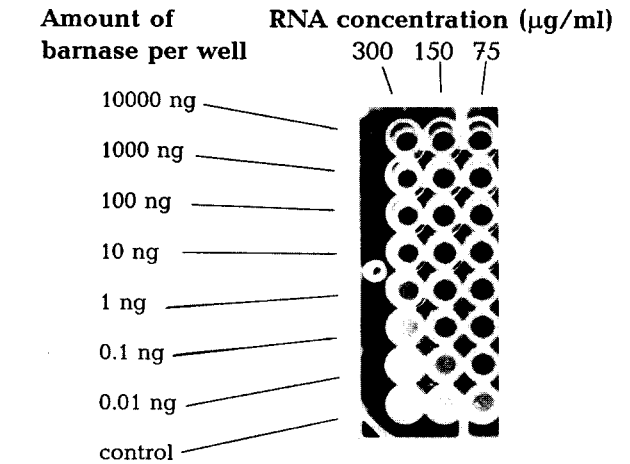


Fig. 1. A: Dependence of fluorescence on barnase content in the sample. The sample (10 μl) was incubated for 1 h at 50°C with substrate buffer containing 300 μg/ml (curve 1) and 150 μg/ml (curve 2) of RNA. Fluorescence was measured after addition of ethidium bromide solution as described in Section 2. B: Visual detection of barnase activity using the RNA-ethidium bromide complex fluorescence. The RNA concentration in substrate buffer for wells of each vertical row is shown at the bottom. Barnase amounts in wells of horizontal rows are shown to the left. The photograph was done after incubation of the plates with samples for 1 h at 50°C and addition of ethidium bromide solution as described in Section 2.

were added to the wells with immobilized anti-transferrin Mabs and the plates were incubated for 1 h at room temperature. After washing, the solid-phase bound barnase activity was determined by the above-described procedure. As seen in Fig. 2B, the conjugate titer is no less than 1:8000 and the sensitivity is no less than ~5 ng of conjugate per well. To

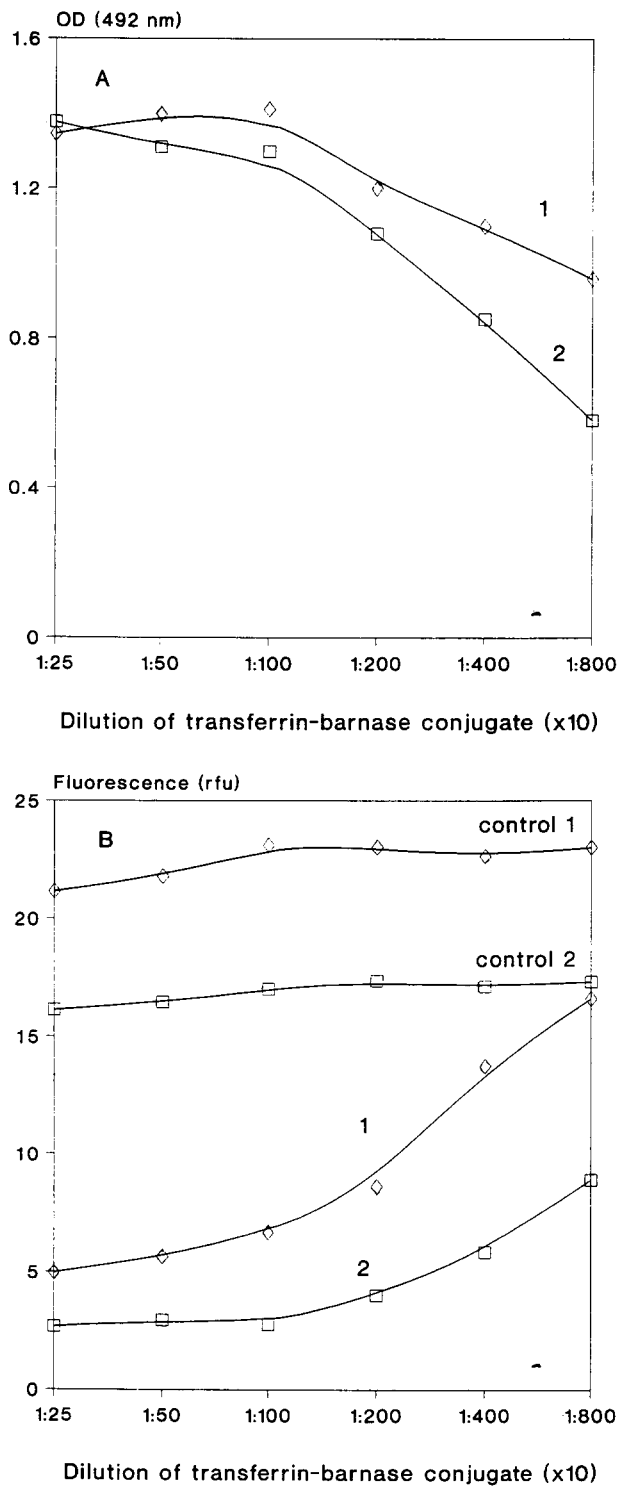


Fig. 2. A: Determination of the transferrin-barnase conjugate titer by indirect ELISA. The conjugate bound with the immobilized anti-transferrin Mabs was detected by the anti-barnase rabbit polyclonal antiserum diluted 1:250 (curve 1) and 1:500 (curve 2). After washing, rabbit antibodies were detected using anti-rabbit IgG sheep antibodies conjugated with peroxidase, as described in Section 2. B: Determination of transferrin-barnase conjugate titer from ribonuclease activity using direct ELISA. The solid-phase bound ribonuclease activity was detected as described in Section 2. The substrate buffer contained 160 μg/ml (curve 1) and 80 μg/ml (curve 2) RNA. The curves 'control 1' and 'control 2' (160 and 80 μg/ml RNA in substrate buffer) show non-specific binding of the transferrin-barnase conjugate to immobilized BSA.

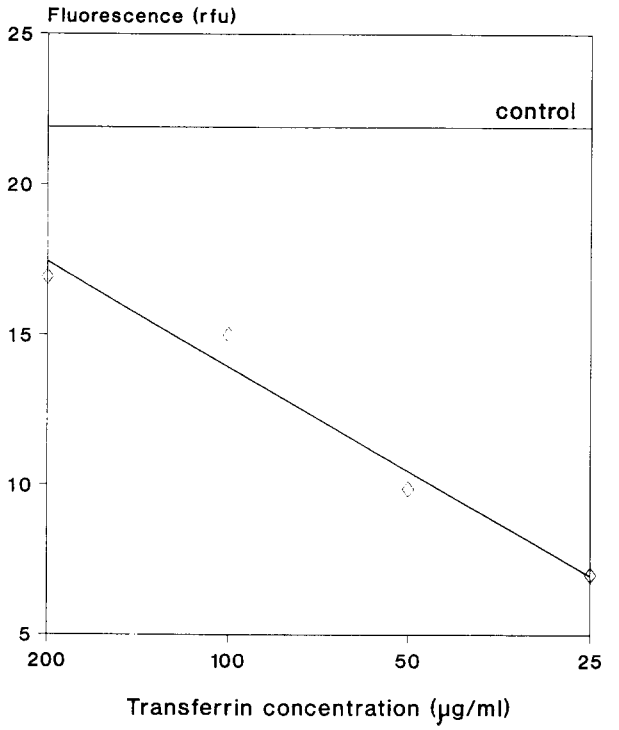


Fig. 3. Inhibition by free transferrin of transferrin-barnase conjugate binding in the competition ELISA. The curve marked 'control' corresponds to the fluorescence of the substrate buffer containing 160 μg/ml of RNA.

assay the solid-phase bound barnase we used two different RNA concentrations (160 and 80 μg/ml). The sensitivity increased slightly at low RNA concentration (Fig. 2B, curve 2), in accordance with the above results. It is possible to estimate the effect of RNA concentration by comparison of the mean values of fluorescence intensity obtained for the end point (1:8000) with those of the control. For curve 1, the control values exceed the experimental ones by a factor of 1.47, and for curve 2 by a factor of 1.9. Thus, using a substrate buffer with low RNA concentration allows one to increase the sensitivity by a factor of ~1.3. In these experiments no remarkable non-specific binding of transferrin-barnase conjugate was observed within a dilution range from 1:250 to 1:8000 with immobilized BSA (Fig. 2B).

The conjugate was used for transferrin determination by competition ELISA. Free pig transferrin and transferrin-barnase conjugate competed with each other for solid-phase immobilized anti-transferrin Mabs. After washing, ribonuclease activity was assayed as described in Section 2. We used an immunosorbent with large amounts of immobilized antibodies (100 μg/ml in coating buffer). Thus, for effective competition the conjugate and antigen to be determined should be present in equimolar concentrations. The results of the analysis are shown in Fig. 3. To summarize, recombinant barnase has a number of merits as a marker enzyme in ELISA. These are high activity, availability in large amounts, low cost, extreme stability, the possibility to use common labelling techniques for preparation of immunoreagents, the possibility of enzyme activity modulation and highly sensitive assay.

Acknowledgements: The authors are grateful to Prof. Robert W. Hartley from the National Institute of Health (Bethesda, MD,

USA) for useful discussions during the preparation of the manuscript. This research was supported by grants from the Russian Foundation for Basic Research and Human Genome, New Methods in Bioengineering and Frontiers in Genetics project.

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