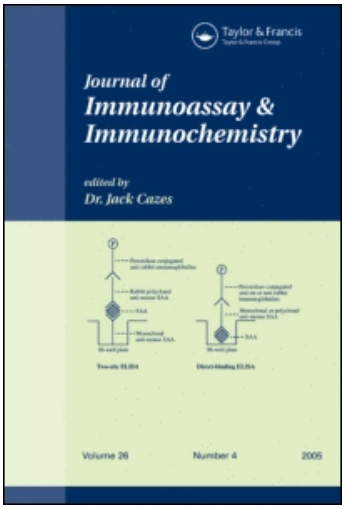

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A HETEROGENEOUS DOUBLE ANTIBODY ENZYME-LINKED IMMUNOASSAY TO MEASURE β-GALACTOSIDASE FUSION PROTEIN

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ABSTRACT

A rapid and sensitive enzyme-linked immunoassay (ELISA) to quantitate recombinant fusion proteins encoded by cloned cDNA in the bacteriophage λgt11 is described. Since the fusion protein is expressed in an equimolar ratio to β-galactosidase, the assay derives the concentration of the recombinant protein in total bacterial lysates or pure preparations from the measurement of β-galactosidase with an enzyme-linked immunoassay. This assay is a useful technique to measure the recombinant proteins for subsequent immunological and biochemical characterization. (KEYWORDS: λgt11, fusion protein, ELISA).

INTRODUCTION

The expression of eukaryotic genes cloned in the bacteriophage λ gt11 has proved to be a useful approach in studying proteins of immunological and biochemical interest. Since cDNA is cloned into the lacZ gene of the λ gt11 DNA, the recombinant protein is only expressed as a fusion hybrid with β-galactosidase. Availability of polyclonal and monoclonal antibodies reactive with β-galactosidase has facilitated the chromatographic purification of the recombinant fusion proteins encoded by the cloned cDNA inserts(1).

Quantitation of the recombinant fusion proteins is, however, hampered by the fact that the β-galactosidase portion of the protein hybrid is enzymatically inactive and therefore, specific and accurate determination of its concentration for subsequent

immunological or biological studies are difficult. Measurement of protein concentration by conventional methods suffers from the disadvantage that fusion proteins of different size may yield inconsistent results. Since the recombinant protein is expressed in an equimolar ratio to β-galactosidase, its measurement quantitates the recombinant portion of the fusion protein in total bacterial lysate or pure preparations. In this paper we present a simple, rapid and specific enzyme-linked immunoassay to measure recombinant fusion proteins. This assay is sensitive, can be performed with small quantities of bacterial lysates or purified fusion protein and is unaffected by interference from bacterial proteins.

MATERIALS AND METHODS

Construction and Screening of Expression cDNA Library in λgt11

A human melanoma cDNA library was constructed in λgt11 and screened with murine anti high molecular weight melanoma-associated antigen antibodies. Details regarding this library and the detected cDNA clones will be described elsewhere. Recombinant λgt11 lysogen was obtained in the *lon* protease deficient *E.coli* strain Y1089 and β-galactosidase fusion protein was induced with isopropyl-b-D-thiogalactopyranoside (IPTG) as described by Huynh et al (2). The bacterial lysate was prepared by repeated freezing and thawing in tris-buffered saline, pH 8.0 containing 0.1% v/v tween 20, phenylmethylsulfonylfluoride 1mmol/L and EDTA 5.0 mmol/L (buffer referred to as lysis buffer) as described (2) and cleared by centrifugation at 10,000 g for 30 min at 4°C.The cleared supernatant was used for subsequent immunoassay

Antibodies

Horseradish peroxidase conjugated goat anti-rabbit Ig antibodies were purchased from Biorad Laboratories (Richmond,CA). Purified mouse anti *E.coli* monoclonal antibody was obtained from Promega Biotech (Madison,WI). Rabbit anti *E.coli* antisera were prepared by monthly immunization of New Zealand White rabbits

with 0.5 mg of β-galactosidase (Boehringer Mannheim) in complete Freund's adjuvant. Following the fourth immunization rabbits were bled, sera extensively absorbed with *E.coli* Y1089 lysate and tested for reactivity and specificity by Western blotting.The antiserum showed strong reactivity with β-galactosidase but not other bacterial proteins.

Radioimmunoassay

E.coli β-galactosidase was labelled with ^{125}I to a specific activity of 17.5 mCi/mg using the iodogen techniques(3). Serially diluted rabbit anti-β-galactosidase antiserum was titrated with this label as described previously(6). Dilution of the antiserum (1:1000)which bound 50% of the added label was used in the RIA using *E.coli* β-galactosidase as the standard. The procedure for the RIA was identical to that described (4).

Double Antibody Enzyme Immunoassay (DAIA)

Wells of a flexible microtiter plate ('U' plates, 96 well vinyl, Dynatech Laboratories, Alexandria,VA) were coated for 16hr at 4°C with 100 ml of the mouse anti-β-galactosidase monoclonal antibody(100 mg/L of $NaHCO_3$ buffer 100 mmol/L, pH 9.5, referred to as the coating buffer). The liquid was discarded by flicking of the assay plate and wells were washed 4 times with tris-buffered saline containing 0.1% tween 20(buffer referred to as TBST).The plate was filled with TBST containing 10% v/v calf serum and incubated at 37°C for 2hr or stored at -20°C until used. To quantitate the concentration of β-galactosidase fusion protein in the bacterial lysates, either 100 ml of the IPTG induced bacterial lysate, or extract of *E.coli* Y1089, or known concentrations of β-galactosidase diluted in TBST were placed in each well and incubated for 2hr at room temperature with shaking. Liquid was discarded and the plate was washed three times with TBST. Each well was filled with 100 ml of the rabbit anti-β-galactosidase antiserum diluted with TBST and incubated at room temperature for 1hr with shaking. Liquid was discarded, the plate was washed 4 times with TBST and each well was filled with the peroxidase conjugated goat anti rabbit IgG antibodies diluted 1:5000 in TBST. The plate was

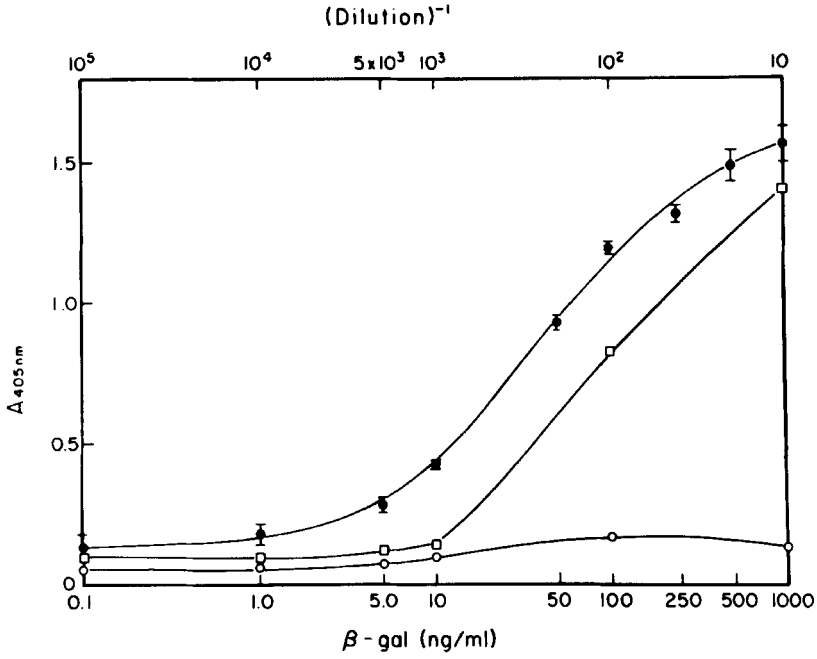

FIGURE 1. Sensitivity and specificity of the DAIA to quantitate recombinant fusion proteins in bacterial lysate.

A standard curve was constructed by measuring known concentrations of pure β-galactosidase(●) in the DAIA. Serial dilutions of the λ gt11 clone D-1 induced with IPTG(□) or of extract of *E.coli* Y1089 treated with IPTG(○) were tested in parallel. Each point in the chart is the average of four replicate determinations ± standard deviation (bar).

incubated at room temperature for 1hr with shaking and washed 4 times with TBST. Each well was filled with 100 ml of the peroxidase substrate solution (o-phenylenediamine dihydrochloride, 5 mg; 5 ml of 30% hydrogen peroxide dissolved in 10 ml of Na_2HPO_4 125 mmol/L and citric acid 36 mmol/L, pH 6.0) and the absorbance at 405 nm was measured after 15 min using a Titertek Multiskan MC (Flow Laboratories, McLean,VA)

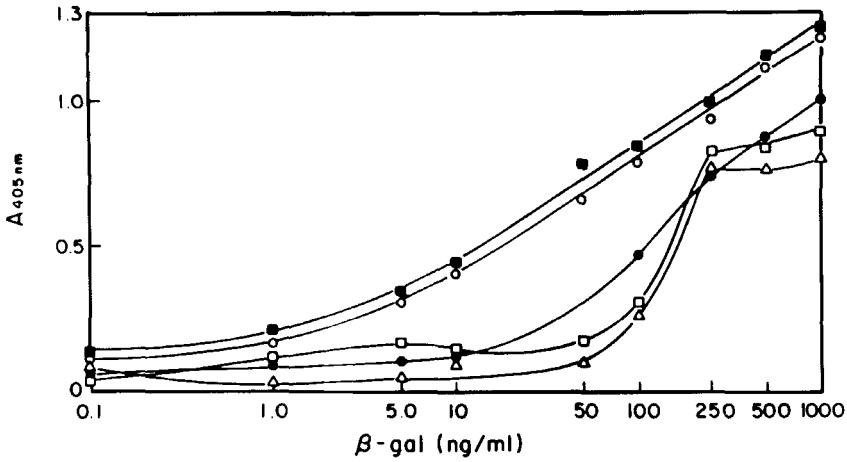

FIGURE 2. Effect of the coating IgG concentration on the sensitivity of DAIA to quantitate recombinant fusion proteins.

Plates were coated with 100 ml of an anti β-galactosidase monoclonal antibody solution at the concentration of 5 mg/L(△), 10 mg/L(□), 25 mg/L(●), 50 mg/L(○) or 100 mg/L(■). Each plate was then used to measure known concentrations of β-galactosidase in the DAIA. Each point is the average of three replicate determinations.

RESULTS AND DISCUSSION

A dose response curve was constructed by incubating increasing concentrations of pure β-galactosidase (0.1-1000 μg/L) in an anti-β-galactosidase monoclonal antibody coated microtiter plate. In repetitions,the standard curve so obtained was always linear in the range 5-250 μg/L (Fig.1).The lower and the upper detection limits of the assay was 5 μg/L and 350 μg/L, respectively.

In order to examine the cross reactivity of the bacterial lysate with the anti-β-galactosidase antibodies, extracts of a bacterial culture infected with the recombinant λgt11 clone (D-1) and induced with IPTG were tested. As shown in figure 1, the serial dilutions of the extract from clone D-1 produced a curve parallel to the standard curve. An extract from wild type *E.coli* strain Y1089 treated with IPTG however, did not show a significant cross reactivity with the anti-β-galactosidase antibodies (Fig.1).

TABLE 1

Comparison of measurement with the DAIA and with a RIA of the concentration of β-galactosidase in the lysate of *E.coli* Y1089 infected with recombinant λgt11 clones.

cDNA clone	β-galactosidase ng./ml (mean + 1 SD)	
	DAIA	RIA
B-2	472 ± 25	461 ± 32
B-3	157 ± 13	175 ± 15
B-4	407 ± 36	448 ± 35
B-5	172 ± 14	189 ± 17
D-1	250 ± 17	262 ± 20
A-13-1	100 ± 15	125 ± 10
Y1089 [a]	5.2 ± 1.5	5.7 ± 1

a.used as negative control.

Next, we examined the effect of the concentration of coating monoclonal antibody on the range and the sensitivity of the assay. To this end, concentrations of monoclonal antibody ranging from 5 mg/L to 100 mg/L were applied to the plate. As shown in Figure 2, the most linear and consistent standard curves were obtained at 50 mg/ml and 100 mg/L of monoclonal antibody. The working concentration of the monoclonal antibody was therefore chosen at 100 mg/L in the subsequent assays. The aberrant shape of the standard curves obtained from the assay plates coated with 5 mg/L and 10 mg/L of anti-β-galactosidase monoclonal antibody (Fig.2) is likely to be due to the inefficient and non-homogeneous coating of the plates. Clearly, these concentrations of the coating antibody are unsuitable for this assay.

The validity of the assay was tested by comparing the results obtained by DAIA to those derived by RIA. As shown in Table 1, there is good agreement between the two measurements.

The DAIA presented in this paper is a simple, rapid and sensitive technique to quantitate recombinant fusion proteins encoded by cDNA inserts cloned in λgt11. These proteins can be subsequently used in various immunological and biochemical studies, where an accurate estimation of the recombinant protein concentration is of importance.

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