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Defined monoclonal antibodies to *Escherichia coli* β-galactosidase as a tool for characterisation of recombinant expression products

Michael Bröker and Hans-Peter Harthus

Research Laboratories of Behringwerke AG, PO Box 1140, D-3550 Marburg, FRG

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Mouse monoclonal antibodies were prepared against β-galactosidase (EC 3.2.1.23) of *Escherichia coli*. The binding sites of these monoclonal antibodies within the β-galactosidase molecule were estimated by immunoblot analyses to various defined peptide regions of β-galactosidase, encoded by expression plasmids. Monoclonal antibodies were characterised, which either bind to the amino-terminal or to the carboxy-terminal region or to an internal section of β-galactosidase. These defined monoclonal antibodies were shown to be a useful tool for characterisation of β-galactosidase fusion proteins expressed in *Escherichia coli*.

Galactosidase, β-; Monoclonal antibody; Recombinant expression product; Epitope mapping; *(E. coli)*

1. INTRODUCTION

Eukaryotic proteins synthesized in *E. coli* were often found to be rapidly degraded in the microbial host unless these proteins were fused to an *E. coli* protein. One of the most common techniques for making fusion proteins is the *E. coli lacZ* system coding for β-galactosidase. Efficient vectors have been constructed to facilitate the expression of bi- or tripartite β-galactosidase hybrid proteins [1,2]. When the fusion protein is used to elicit antibodies against the cloned antigen, it is desirable to reduce the β-galactosidase portion of about 116 kDa of the hybrid protein. This is especially the case, when the cloned peptide is small and the ratio to β-galactosidase therefore is unfavourable. For this purpose, expression plasmids have been constructed that direct the regulated high synthesis of proteins or protein fragments fused to truncated forms of β-galactosidase (βgal$_t$) [3,4]. These vectors facilitate the production of heterologous polypeptides fused to β-galactosidase fragments of only some hundred amino acids instead of more than thousands as encoded by the entire β-galactosidase [5]. The fusion proteins can immunologically be identified by serum, which is specific for the polypeptides fused to the βgal sequences. Whenever such a serum is not available, or when the serum is not reactive with the recombinant counterpart, such a hybrid protein can be detected by serum directed against β-galactosidase. However, polyclonal anti-β-galactosidase sera often react unspecifically, because the material used for immunisation is not totally free of *E. coli* contaminants or the animals have been in contact with *E. coli* and have produced antibodies directed to the bacteria. Therefore, we have prepared monoclonal antibodies (mabs) to β-galactosidase, which are highly specific and sensitive and which show no cross-reactivity with other *E. coli* proteins. The usefulness of these mabs, of which the binding site to β-galactosidase could be identified, is shown in some examples and further applications are discussed.

Correspondence address: M. Bröker, Behringwerke AG, Molecular Biology Department, Postfach 1140, D-3550 Marburg, FRG

2. MATERIALS AND METHODS

2.1. *DNA methods*

DNA manipulations were done according to [6]. DNA fragments were isolated from low melting agarose according to Wieslander [7] and mini prep analyses were carried out as described by Holmes and Quigley [8]. Expression of *lacZ* and *lacZ* coding DNA fragments were carried out in *E. coli* BMH 71-18 (*lac pro*)Δ *thi supE*/F′ *lac*I^{q}Z$^-$ΔM15 pro+) [9].

2.2. *Plasmids*

The following vectors, coding for *lacZ* and truncated *lacZ* sequences, were used in this study: pUR278 [1], pWR590 [3], pBD1, pBD2 and pBD5 [4]. Plasmid pBD2-IC20H is a derivative of pBD2 with a polylinker downstream of the DNA coding for βgal$_t$ [10]. The expression of *lacZ* is under the control of the *lac* promoter and was induced by addition of IPTG to the bacterial cultures.

The plasmid pMB253 codes for a fusion protein consisting of βgal$_t$ and the C domain of the human antihaemophilic factor VIII. The plasmid was made by cloning the 2450 bp *Cla*I-*Xho*I DNA fragment coding for factor VIII [11] into pBD2-IC20H [10] cut with *Sma*I and *Xho*I such that the factor VIII antigen is in frame to the β-galactosidase portion.

The vector pBO3 codes for the synthesis the herpes simplex virus type 1 envelope glycoprotein gC. The gC antigen is made as a tripartite fusion protein and is sandwiched between the amino-terminus of cI repressor protein and the entire β-galactosidase (cI::gC::βgal). The expression of the pBO3 encoded hybrid protein is controlled by the *tac* promoter [2].

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2.3. *Imuunological procedures*

Extracts of *E. coli* cells expressing β-galactosidase or β-galactosidase fusion proteins were separated in 8–12% SDS-PAGE and were transferred to nitrocellulose sheets (Schleicher and Schuell). Western blot analyses were done according to Burnette [12]. The concentration of the individual mabs was 10 μg/ml in the buffer solution. Alkaline phosphatase coupled anti-mouse antibodies were used as conjugate with Fast blue B salt (Serva) and naphthol AS-MX Phosphate (Sigma) as substrates. Extracts of the β-galactosidase deficient *E. coli* strain MC4100 (*araD*139, Δ(*argF-lac*) U169, rpsL150, relA1, flb5301, deoC1, ptsF25rbsR) [13] served as negative control antigen.

2.4. *Preparation of monoclonal anti-enzyme antibodies*

4–6-week-old Balb/c mice were immunized subcutaneously and/or intraperitoneally with 20 μg of β-galactosidase in Freund's complete adjuvant. Four weeks later, mice were boostered with 10 μg of β-galactosidase in incomplete Freund's adjuvant. Prior to fusion, mice were boostered intraveneously 4 times at daily intervals. Spleen cells were isolated aseptically and were fused with the myeloma cell line SP 2/o using polyethylene glycol (PEG) according to standard methods. The final cell pellet was resuspended in DMEM containing 20% calf serum and HAT (0.1 mM hypoxanthin, 0.004 mM aminopterin, 0.016 mM thymidine) and added to 24-well culture plates (Nunc). About 14 days later, individual cell clones were picked out and were transferred to a new well. Three days later, supernatants were tested for antibody content as well as for the presence of β-galactosidase-specific antibodies. Positive cell cultures were grown up and were frozen in liquid nitrogen. In parallel, positive cell lines were cloned using a single cell manipulator.

2.5. *Screening of monoclonal antibodies*

Supernatants were screened using β-galactosidase-coated microtitration plates. Mabs bound to the solid phase were demonstrated by a second incubation with horseradish peroxidase (HRP) labelled rabbit anti-mouse antibodies. Relative affinity of anti-β-galactosidase mabs was determined by incubating defined concentrations of different antibody containing supernatants on β-galactosidase-coated microtitration plates.

2.6. *Production and purification of mabs*

Cell lines producing anti β-galactosidase mabs were propagated as ascites fluids using IFA-primed Balb/c mice. Immunoglobulins were purified by affinity chromatography using Protein A-Sepharose (Pharmacia/LKB) according to the manufacturer. Purity was monitored by HPLC and SDS-PAGE (Phast System, Pharmacia/LKB). Protein concentration was determined by optical density measurement at 280 nm. Class and subclass isotypes were determined by double diffusion techniques (Miles).

3. RESULTS

3.1. *Identification of anti-β-galactosidase mabs*

Fifteen anti-β-galactosidase mabs were isolated, which recognized β-galactosidase bound in microtiter plates and which also reacted with β-galactosidase in Western blots. Seven mabs were further analyzed with respect to the binding site within the β-galactosidase molecule. The mabs 87-55/60, 87-134/202, 87-134/238, 87-179/03, 87-179/20 and KG12 belong to the subclass IgG_1, while 87-176/103 was of IgG_{2a} subtype.

3.2. *Localization of the antigenic determinants recognized by the mabs*

To localize the binding site of the mabs within the β-galactosidase molecule, we made use of expression vectors, which code for the entire β-galactosidase or for defined β-gal_t fragments of different length (fig.1). Cells carrying the plasmid pUR278 synthesize the total β-galactosidase protein, while the vectors pBD1, pWR590 and pBD2 code for truncated forms beginning from the amino-terminus until amino acid Leu^{651}, Asn^{583} or Asp^{375}, respectively. The vector pBD5 codes for a fusion protein in which the carboxy-terminal portion of β-galactosidase from Glu^{650} to Phe^{1007} is fused to a short β-galactosidase leader [4]. These two β-galactosidase segments are separated by a stretch of the herpes simplex virus glycoprotein D antigen (indicated by a line in fig.1). These 5 polypeptides, carrying different but in part overlapping β-galactosidase antigenic regions, were expressed in *E. coli*. Total cell extracts from these transformants and extracts from the plasmid-free and β-galactosidase-deficient *E. coli* strain MC4100 and a protein molecular weight mixture including β-galactosidase as antigen were separated by

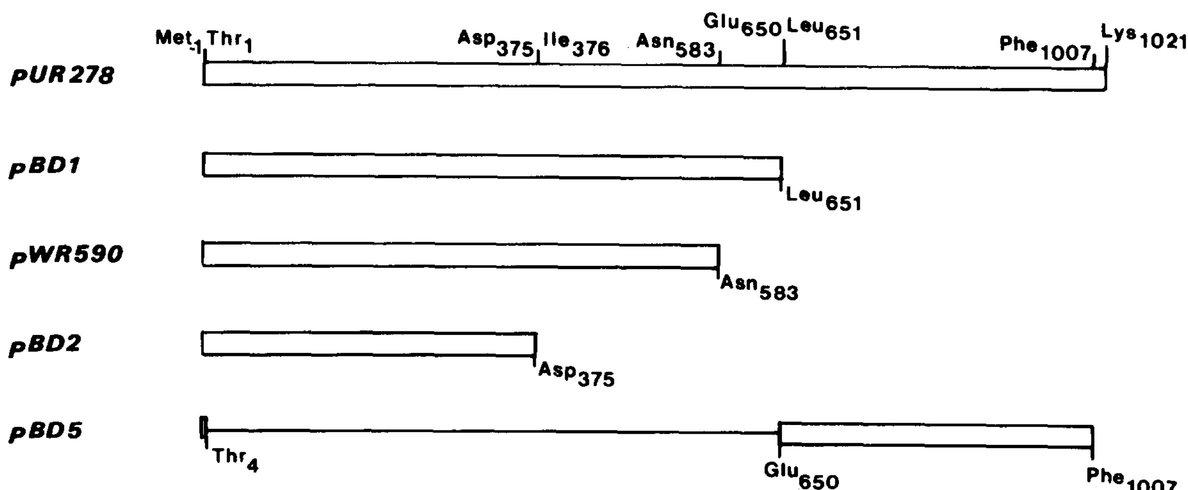

Fig.1. Scheme of the β-galactosidase polypeptide portions which are encoded by the various expression plasmids. The thin line in the pBD5-encoded protein represents a polypeptide stretch of the herpes simplex virus glycoprotein gD which is in frame with the amino-terminal and the carboxy-terminal portion of β-galactosidase [4].

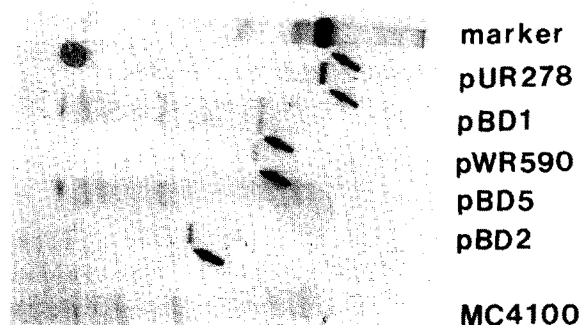

Fig.2. Reaction pattern of mab 87-55/60 in Western blots with defined β-galactosidase portions. Cell extracts from *E. coli* BMH-71-18, expressing different β-galactosidase segments, extracts from *E. coli* MC4100 and molecular weight marker proteins (Sigma) were blotted and incubated with mab 87-55/60. The lane with the marker proteins contains myosin, β-galactosidase, phosphorylase, bovine albumin, egg albumin and carboanhydrase. The β-galactosidase polypeptides, which were recognized by the mab, are indicated by an arrow.

SDS-PAGE and were blotted onto nitrocellulose membranes.

Fig.2 documents the reactivity of mab 87-55/60 to the various β-galactosidase polypeptides encoded by the expression vectors. Mab 87-55/60 recognized β-galactosidase antigens specified by the plasmids pUR278, pBD1, pWR590 and pBD2, but did not react with the β-galactosidase segment encoded by pBD5 neither did it bind to proteins from the β-galactosidase-deficient *E. coli* strain MC4100. From this reaction pattern it can be concluded, that mab 87-55/60 binds to an epitope within the amino-terminal region from Thr^{1} to Asp^{375} of the β-galactosidase molecule (table 1). The binding sites of the other 6 mabs were determined in the same way and the results are overviewed in table 1. Three binding classes of β-galactosidase-specific mabs were defined by this procedure: class 1 mabs bind to the β-galactosidase amino-terminus from Thr^{1} to Asp^{375}, class 2 mabs recognize epitopes within the central part of β-galactosidase from Ile^{376} to Leu^{651} and class 3 mabs react with the carboxy-terminal portion specified by the amino acid sequence from Glu^{650} to Phe^{1007}.

3.3. *Characterization of the tripartite cI::gC:: βgal fusion protein*

We have previously made use of the expression vector pMF2 [2] to synthesize tripartite fusion proteins of the order cI::gene X::βgal in *E. coli*. When gene X within these hybrid proteins was represented by a long stretch of the herpes simplex virus type 1 (HSV-1) glycoprotein C (gC), the expression products encoded by the vector pBO3 (see section 2) were rapidly degraded. This cI::gC::βgal fusion protein could not be identified in *E. coli* extracts by SDS-PAGE separation and staining with Coomassie brilliant blue. The entire fusion protein could not be detected in Western blots but only by radio-immunoprecipitation, when a polyclonal serum directed to HSV-1 was used. The high sensitivity of 3 mabs, representing either class 1, class 2 or class 3 (table 1) is documented in fig.3. The mab 87-55/60, mab 87-179/03 and mab 87-176/103 reacted with the pUR278 encoded β-galactosidase (lane 1) and with the small amount of the entire cI::gC::βgal fusion protein of molecular mass 182 kDa encoded by pBO3 (lane 2). In addition, a few degradation products can be recognized by mab 87-55/60 (fig.3A/2), some more can be seen by mab 87-179/03 (fig.3B/2) and the bulk is visualized by mab 87-176/103 (fig.3C/2). We assume that β-galactosidase sequences in the neighbourhood of the gC antigen are preferentially degraded by proteases and therefore epitopes in the amino-terminal region of β-galactosidase are lost faster than those located in the middle or carboxy-terminal region. This would explain the increasing number of bands which appear in the blots by using the mabs in the order: mab 87-55/60, mab 87-179/03 and mab 87-176/103 (fig.3A,B,C, lanes 2).

The mabs recognized not only β-galactosidase, βgal_t or fusion proteins of type gene X::β-galactosidase, but also *E. coli* expression products of type βgal::gene X and βgal_t::gene X. All fusions tested so far were identified in Western blots with a variety of viral or human polypeptide stretches represented by gene X (unpublished results).

Table 1

Reaction pattern of the defined β-galactosidase segments with 7 individual mabs and the deduced binding sites of the mabs

mab	Immunoreaction					binding site	class
	pUR278	pBD1	pWR590	pBD5	pBD2		
87-55/60	+	+	+	–	+	Thr^{1}-Asp^{375}	1
87-134/202	+	+	+	–	+	Thr^{1}-Asp^{375}	
87-134/238	+	+	+	–	+	Thr^{1}-Asp^{375}	
87-179/03	+	+	+	–	–	Ile^{376}-Leu^{651}	2
87-179/20	+	+	+	–	–	Ile^{375}-Leu^{651}	
87-176/103	+	–	–	+	–	Glu^{650}-Phe^{1007}	3
KG12	+	–	–	+	–	Glu^{650}-Phe^{1007}	

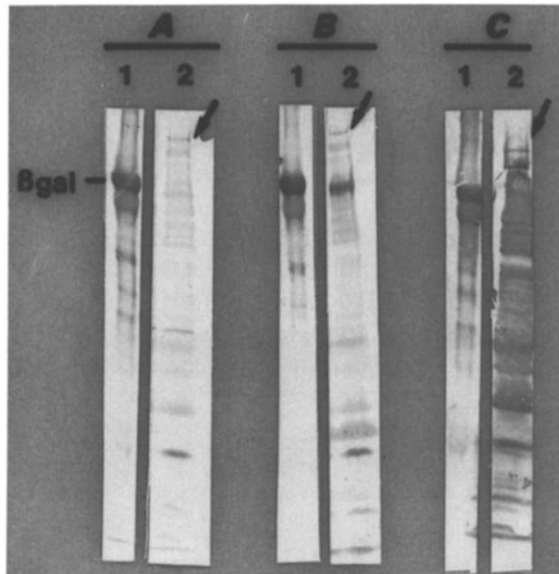

Fig.3. Sensitivity of three individual mabs. Western blot analyses with extracts from *E. coli* [pUR278], expressing entire β-galactosidase (lane 1) and *E. coli* [pBO3], expressing the hybrid protein cI::gC::βgal (lane 2) with mab 87-55/60 (A), mab 87-179/03 (B) and mab 87-176/103 (C). The reaction with the entire fusion protein is indicated by an arrow.

3.4. *Differentiation of proteolytically degraded β-galactosidase and factor VIII specific antigenic determinants derived from a βgal$_t$::factor VIII fusion protein*

In order to rise polyclonal antisera to the C domain of the human blood coagulation protein factor VIII, we cloned the cDNA coding for factor VIII from Glu^{1649} to Tyr^{2332} in frame to the βgal$_t$ encoded by pBD2-IC20H [11] to yield the vector pMB253 (for detailed cloning procedure see section 2). The βgal$_t$::factor VIII fusion protein (≈117 kDa) is relatively unstable in *E. coli* and degradation products of the fusion protein can be detected when total cell extracts of *E. coli* [pMB253] are separated by SDS-PAGE and are stained with Coomassie brilliant blue (data not shown). Western blot analyses with polyclonal anti factor VIII serum and anti β-galactosidase mab 87-55/60 were helpful to analyze the expression of recombinant factor VIII antigens in *E. coli* (fig.4). Without induction of the *lac* promoter, no pMB253-encoded fusion protein could be detected neither in Coomassie brilliant blue-stained gels (data not shown) nor in Western blots by using the polyclonal anti-factor VIII rabbit serum. Nevertheless, the mab 87-55/60 detected the fusion protein (fig.4, filled circle) and the βgal$_t$ (fig.4, filled square) encoded by pBD2 in such cell extracts, indicating a basal expression, because the *lac* promoter was not totally repressed in this system, although the expression host *E. coli* strain BMH 71-18 is an overproducer of the *lac* repressor protein.

However, the mab 87-55/60 did apparently not respond in proportion to the induced level of the fusion protein (fig.4B, lane 3 versus lanes 4,5). The reason for this failure is not known yet.

In any case, degradation products predominantly carrying epitopes specific for β-galactosidase (fig.4, open star) or factor VIII (fig.4, triangle) could be

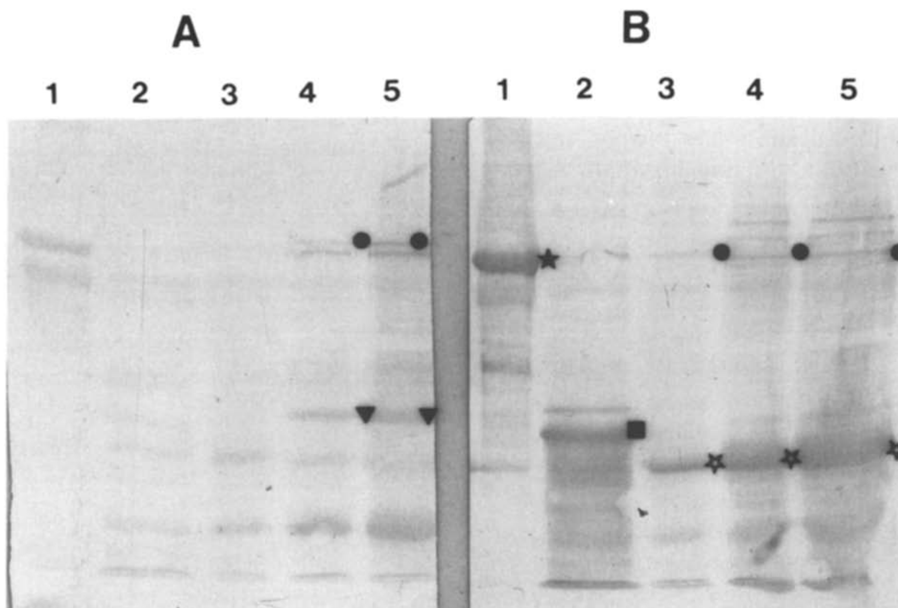

Fig.4. Differentiation between β-galactosidase-specific and factor VIII-specific polypeptide stretches. Western blot analyses with polyclonal anti-factor VIII serum (A) and mab 87-55/60 (B). Molecular weight marker proteins (lane 1); *E. coli* [pBD2]-induced culture expressing gal$_t$ (lane 2); *E. coli* [pMB253], uninduced culture (lane 3); *E. coli* [pMB253], induced culture, expressing the βgal$_t$::factorVIII fusion protein, harvested 1 h after induction (lane 4), or after 4 h (lane 5). The filled square indicates the position of the βgal$_t$ encoded by pBD2, the filled star the entire β-galactosidase, the filled circle marks the position of the entire βgal$_t$::factor VIII fusion protein and a partial degradation product of about 116 kDa, the open star indicates the β-galactosidase-specific degradation products, and the filled triangle factor VIII-specific degradation products.

distinguished by using the different antibodies. This specific analysis enabled us to unequivocally identify factor VIII-specific degradation products and to isolate such immunostained bands from the nitrocellulose sheet in order to raise polyclonal monospecific anti-factor VIII antibodies by the procedure described by Sandkamp et al. [14].

4. DISCUSSION

In this paper, we present mabs which have been raised against the *E. coli* β-galactosidase. By use of Western blot analyses with distinct recombinant β-galactosidase polypeptide fragments, we could identify the binding regions within the β-galactosidase molecule. Due to the cloned β-galactosidase segments, 3 classes of binding regions were determined: the amino-terminal region, the central part and the carboxy-terminal region. Construction of further plasmids, that direct the synthesis of even smaller truncated β-galactosidase segments and synthetic peptides could allow a still more precise epitope mapping.

The mabs were shown to be highly sensitive and specific and can improve the identification of recombinant expression products by Western blot analyses. Furthermore, the mabs can be used for immunoprecipitation studies, in immunofluorescence techniques (unpublished observations) and possibly for purification of β-galactosidase fusion proteins by immunoaffinity chromatography. Whenever the synthesis of the *E. coli lacZ* gene in bacteria, yeast, plant or animal tissue culture cells has to be monitored as a means of marker for transcriptional/translational control, the immunological detection of β-galactosidase with the aid of these mabs may be an alternative for the measurement of enzymatic activity of β-galactosidase.

In addition, monoclonal antibodies recognizing intact β-galactosidase without affecting enzymatic activity can be used to develop highly sensitive detection systems based on β-galactosidase/anti-β-galactosidase complexes bridged with an unlabelled anti-mouse antibody.

These complexes could be an alternative for the preparation of covalent antibody/enzyme conjugates or can be used very effectively in combination with bispecific monoclonal antibodies having one binding site for the analyte and the other one for β-galactosidase. In addition, the sensitivity of enzyme immunoassays can be increased by amopification steps using repeated incubations of enzyme/anti-enzyme complexes.

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