Engineering of solvent-exposed loops in *Escherichia coli* β-galactosidase

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Abstract The *Escherichia coli* β-galactosidase is a high molecular mass tetrameric enzyme extensively used as a molecular marker. Despite its proven utility as a partner in fusion proteins, previous attempts to generate insertional mutants rendered inactive or poorly active enzymes, hampering its further engineering for the construction of multifunctional enzymes. We have explored several solvent-exposed loops on the tetramer, namely those spanning residues 246–254, 271–287, 578–584, 770–773, and 793–806, as acceptor sites to accommodate functional protein segments on the surface of active β-galactosidase enzymes. An RGD-containing antigenic peptide positioned in these sites interacts efficiently with specific monoclonal antibodies as well as target integrins on the surface of mammalian cells. The resulting chimeric enzymes are soluble, stable, produced in high yields and enzymatically active. Moreover, the identified insertion sites could be appropriated for the design of promising β-galactosidase-based molecular sensors.

Key words: β-Galactosidase; Peptide display; Protein engineering; Loop structure

1. Introduction

The β-galactosidase enzyme (EC 3.2.1.23) encoded by the *Escherichia coli lacZ* gene, is a high molecular mass multimeric protein composed of four non-covalently linked subunits. This enzyme hydrolyses lactose into glucose and galactose, allowing this sugar to be used as a substrate for heterotrophic bacterial growth. Although this activity has been exploited in food industry to remove lactose from dairy products, the widest application of this enzyme is as a reporter protein or molecular sensor in microbiology and molecular and cell biology. This use has been stimulated by the ability of β-galactosidase to hydrolyse lactose analogues such as *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG), chlorophenol red galactopyranoside (CPRG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), rendering coloured components that can be either spectrophotometrically quantified or visualised in situ. In addition, the development of rapid and simple enzymatic assays [1] has extended the use of *lacZ* (or a 5′ gene segment, encoding the a-complementing β-galactosidase domain) as a universal reporter gene in both prokaryotic and eukaryotic cells.

Although the C-terminus of β-galactosidase is poorly tolerant to protein fusions [2,3], the N-terminus can accept large proteins or protein domains without significant loss of activity [4]. For this reason, this enzyme has also been exploited as a molecular carrier to stabilise heterologous proteins produced in bacteria [5,6], and also to provide either monitoring [7,8] or purification tags [9] for production and downstream processes. More recently, the engineering of β-galactosidase has resulted in the generation of molecular tools for a diversity of purposes. For some of these uses an essential requirement is the insertion of foreign peptides into permissive, solvent-exposed sites, rendering stable chimeric enzymes. As a result of peptide insertions done randomly (or according to structural predictions) along the sequence of β-galactosidase, unstable or extremely poorly active proteins have been generated [10,11]. However, the X-ray structure of the *E. coli* β-galactosidase [12] offers a rationale to explore new candidate peptide acceptor sites. In this work, we have inserted a heterologous protein segment in several solvent-exposed loops of the *E. coli* β-galactosidase to estimate their reactivity and functionality once positioned and the stability and activity of the resulting chimeric enzymes. While the obtained results show a critical role of solvent-exposed loops in the enzyme structure, permissive sites for insertions of functional enzymes have been identified.

2. Material and methods

2.1. Plasmids and bacterial strains

The pJXxxxA series is a collection of vectors derived from pJLACZ [13] encoding modified β-galactosidases. A peptide, reproducing the amino acid sequence (amino acids 134–156) of the FMDV VP1 G-H loop (isolate C-Sta. Pau, Spain/70, clone C-S8c1, serotype C) [14], has been inserted at specific residues whose position is indicated by plasmid reference numbers. *Bam*HI restriction sites were introduced at the desired sites of *lacZ* gene in the plasmid pJLACZ by using appropriate oligonucleotide primers and PCR amplification as described [15]. In these sites we introduced the 70-bp *Bam*HI-*Bam*HI DNA fragment from plasmid pM275VP1, which encodes the VP1 G-H loop segment. For the construction of pJX8CA, pJLACZ was digested with *Eco*RI, the resulting ends filled in with Klenow and the vector ligated with a PCR-amplified segment also encoding the G-H loop sequence. The amino acid sequence at the acceptor sites is depicted in Fig. 1. Plasmids pJLACZ, pM275VP1 and pMV278VP1 have been previously described [13,15]. The expression of the engineered *lacZ* genes is under the control of the CI857^{ts}-repressed p_Rp_L lambda promoters [16]. All the constructs were transformed into the *E. coli* strain MC1061, *hsdR2*, *mcrB*, *araD139*, Δ(*araABC-leu*) *lacX74*, Δ*lacX74*, *galU*, *galK*, *rpsL*, *thi*, *strA* [17], and the proteins produced by temperature shift in the strain BL26, a Lac$^-$ derivative of BL21, *hsdS gal* Lon$^-$ OmpT$^-$ [18].

2.2. Bacterial growth and production of recombinant β-galactosidases

Bacterial cultures were grown in 200 ml of LB medium [19], plus 100 μg/ml ampicillin and 50 μg/ml streptomycin (when required), in 1-liter shaker flasks, at 32°C and 250 rpm. When the OD_{550} reached between 0.3 and 0.4 units, the cultures were transferred to a prewarmed bath at 42°C, and further incubated under the same conditions, until steady values of β-galactosidase activity were observed. Then cells were harvested by centrifugation and pellets resuspended in 10 ml of 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl and 10 mM β-mercaptoethanol, pH 7.2, with benzamidine and phenylmethylsulfonyl fluoride at 25 μM and 1 mM, respectively, to prevent proteolysis. Cell disruption was achieved by ultrasonication as described [20], and purification of recombinant proteins was done by affinity chromatography [9]. Briefly, crude cell extracts were applied to a 15-ml *p*-aminophenyl-β-D-galactoside-Sepharose column equilibrated

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with 20 mM Tris-HCl, 10 mM $MgCl_2$, 1.6 M NaCl, pH 7.5. After washing until the A_{280} at the outlet dropped near zero, the retained proteins were eluted with 100 mM $Na_2B_4O_7$, 10 mM β-mercaptoethanol, pH 10, and dialysed against 20 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5. Then the purified enzymes were stored at −80°C until required.

2.3. Determination of protein concentration

Purified proteins were analysed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by either Coomassie blue staining and immunoblotting as described [11]. Protein bands were visualised with polyclonal serum anti-β-galactosidase. Concentration of engineered β-galactosidases was determined spectrophotometrically at 280 nm. Molar absorption coefficients of the mutant proteins were determined as described [21], obtaining values very similar ($\varepsilon = 1.5\times10^6 \pm 1.4\times10^5$ M^{-1} cm^{-1}) to that previously described for *E. coli* β-galactosidase [22]. Other independent methods, namely nitrocellulose scanning followed by image analysis (with BioImage Intelligent Quantifier software), Bradford's colorimetric method [23] and indirect ELISA, were also used to assess the spectrophotometric measurements. Wild-type β-galactosidase (from Boehringer Mannheim) was used as standard.

2.4. Enzymatic assay and kinetics measurements

β-Galactosidase enzymatic activity was analysed according to Miller's method [1]. One enzymatic unit is defined as the amount of protein that produces 1 μmol of *o*-nitrophenol per minute at 28°C. The specific activity was calculated from samples of growing cultures with known enzymatic activity, and the amount of protein was obtained by densitometric analysis of Western blot sheets with appropriate standards. K_m values were measured at different concentrations of substrate (ONPG), by absorbance at 420 nm. Values of ΔA_{420}/min were converted to rate constants, and K_m and V_{max} values determined by using the SigmaPlot Scientific Graphing Software. The first-order rate constant k_{cat} (turnover number) was calculated by dividing V_{max} by the total enzyme concentration in the reaction mixture. Determinations were performed up to five times for each protein.

2.5. Analysis of protein stability

Resistance to heat denaturation was determined by incubating purified proteins at 23 μg/ml in buffer Z at 50°C. Samples were taken at different time intervals, immediately cooled to 4°C and further incubated at 28°C for Miller's assay. Purified proteins at 23 μg/ml were also incubated at 37°C in buffer Z in the presence of 100 μg/ml trypsin. Aliquots were taken at different times and proteolysis was immediately stopped with 10 μM soybean trypsin inhibitor. Enzymatic activity of this mixture was determined as described above in three independent experiments. Half-lives of the active proteins under these conditions were determined and given as t_{50}. In vivo stability was determined by Western blot as described [24]. Briefly, bacterial cultures producing the desired proteins were transferred to 37°C immediately after the addition of 100 μg/ml chloramphenicol. Samples were taken sequentially and ran in PAGE, immunoblotted and submitted to densitometric analysis.

2.6. Fluorescence spectroscopy

Intrinsic fluorescence was determined at 28°C in a luminescence spectrophotometer LS50 (Perkin Elmer) with a thermostated cell holder, by using 15 μg/ml-protein solutions in Z buffer. Quartz suprasil cuvettes of 10 mm light path were employed. The excitation wavelength was set to 295 nm with a 2.5-nm slit. Emission spectra were recorded from independent samples in triplicate, between 300 and 420 nm, with a 10-nm slit in 0.5-nm wavelength increments.

2.7. Competitive ELISA

Competitive ELISA was performed in triplicate by slight modification of a standard protocol [11]. Briefly, 20 pmol of the KLH-coupled synthetic peptide GH23 reproducing the inserted segment sequence (TTYTASARGDLAHLTTTHARHLP), were adsorbed to ELISA microtiter plates. After blocking, competition for mAb SD6 was done as described [11] with increasing amounts of pure proteins.

2.8. Cell binding assay

Binding of chimeric proteins to baby hamster kidney (BHK) cells was performed as previously described [25] in ELISA microtiter plates. In each well, we adsorbed 2.5 pmol of mutant β-galactosidases as cell ligands and 5×10^4 cells were added for the analysis. After 2 h incubation at 37°C, cell staining was done with crystal violet. The absorbance was determined at 620 nm and the values given as referred to that obtained by using human vitronectin as immobilised cell ligand.

3. Results

3.1. Design and production of chimeric β-galactosidases

A peptide of 27 amino acids, representing the G-H loop amino acid sequence of foot-and-mouth disease virus (FMDV) VP1 protein, has been inserted into different sites of protein LACZ, a pseudo-wild-type *E. coli* β-galactosidase lacking the 8 N-terminal amino acids. In FMDV serotype C, this peptide contains several overlapping B-cell epitopes forming the antigenic site A [26], and comprises an evolutionarily conserved, integrin-binding RGD motif responsible for cell attachment and infection [27]. This segment was inserted by recombinant DNA procedures into four intra-domain, surface-exposed regions without regular structure, which are not involved in contact interfaces and are far from the neighbouring domains and monomers in the folded tetramer. These sites are distributed on different areas of the monomer surfaces, and they were chosen to display the inserted peptides separated on the assembled tetramer. The minimal distance between insertion sites in proteins JX249A, JX581A, JX772A and JX795A is 57 Å, in general being higher than 90 Å. These values were measured as the atomic distances between the Cα of the residues indicated in the protein notation. Within the loops, the precise insertion sites were selected to generate minimal distortions, avoiding the vicinity of amino acids that restrict the loop conformation, such as proline, tyrosine and tryptophan, and looking for the proximity of glycine that could promote more flexibility of the inserted segment. When possible, medial sites in the target loop were favoured. Two additional insertion proteins (M275VP1 and M278VP1), containing the FMDV G-H loop segment posi-

Table 1
Enzymatic properties of modified β-galactosidases

Protein	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	Specific activity (U/μg)
LACZ	0.2222 ± 0.0114	18 300 ± 436	82 345 ± 4658	2765 ± 347
JX8CA	0.2455 ± 0.0074	19 097 ± 484	77 800 ± 3065	2840 ± 218
JX249A	0.2444 ± 0.0027	15 065 ± 396	61 650 ± 1758	2030 ± 256
JX581A	0.2670 ± 0.0038	10 810 ± 269	40 490 ± 1161	960 ± 78
JX772A	0.1043 ± 0.0091	2870 ± 198	27 530 ± 3062	515 ± 31
JX795A	0.0951 ± 0.0076	2646 ± 115	27 800 ± 2529	675 ± 102
M275VP1	0.4299 ± 0.0043	2132 ± 58	4960 ± 144	375 ± 72
M278VP1	0.3100 ± 0.0970	1496 ± 173	4825 ± 1610	170 ± 43
β-Galactosidase	0.2332 ± 0.0033	19 501 ± 122	83 623 ± 1294	2950 ± 189

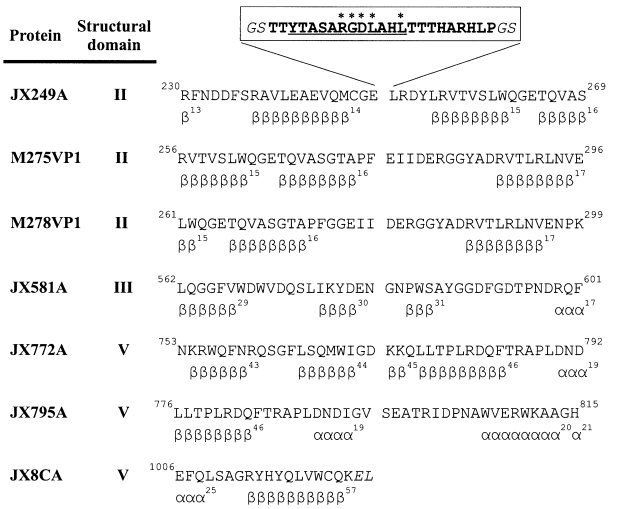

Fig. 1. Amino acid sequences at the insertion sites of the mutant β-galactosidases (listed on the left). The inserted peptide (boxed) contains 23 residues from the wild-type FMDV VP1 protein, spanning positions 134–156 [39] (in bold), and 4 extra flanking amino acids encoded by the *Bam*HI restriction sites (in italics). The underlined stretch indicates the extent of the continuous SD6 B-cell epitope [40], and the residues labelled with asterisks are those critical for the interaction of the whole FMDV particles with the cell surface receptor [41]. Residues in β-galactosidase are numbered as indicated [42], and the structure of the native enzyme is indicated by α (alpha helices) and β (beta strands), according to published data [12]. The β-galactosidase domain receiving the viral peptide is also indicated.

tioned in the β-galactosidase activating interface [11], were also included in this study, as well as a C-terminal fusion which was constructed as a control. Details of all these proteins are given in Fig. 1.

These chimeras were produced by temperature shift in the *E. coli* strain BL26. β-Galactosidase enzymatic activity was detected in all the cultures immediately after the expression of the *lacZ* mutant genes. Inhibition of cell growth was not observed, and the obtained yield was similar to that of parental β-galactosidase, indicating absence of toxicity over the cells (not shown). Western blot analysis of cell extracts revealed the presence of bands immunoreactive with both SD6 anti-VP1 monoclonal antibody (mAb) and anti-β-galactosidase sera, with a mobility indistinguishable from that of the native β-galactosidase (not shown). The purification of these proteins was efficiently done by *p*-aminophenyl-β-D-thiogalactoside (TPEG)-Sepharose columns as described [9].

3.2. Solvent exposure of the displayed peptides

The solvent exposure of the displayed peptides on purified proteins was assessed by competitive ELISA. All the soluble proteins competed with a bound synthetic peptide (with the same amino acid sequence as inserted into the enzymes) for mAb SD6 (Fig. 2A). Since the inserted peptide also carries an RGD motif that serves the virus to infect target cells, probably by binding to integrin $a_{V\beta3}$ [25,28,29], cell attachment was also explored as an indication of the steric availability of the FMDV cell ligand motif. Fig. 2B shows significant binding of FMDV-target cells to all the constructs.

3.3. Enzymatic activities of modified β-galactosidases

The influence of peptide insertions on the catalytic properties of the carrier protein is shown in Table 1. Neither the specific activity nor the specificity constant (k_{cat}/K_m) are altered by the fusion at the C terminus, and parameters are only moderately affected by the insertion at position 249. In other modified enzymes the viral peptide inactivates the enzyme by at least 50%. At positions 275 and 278 the insertion affects monomer-monomer contacts in the activating interface and it is very close to the active site. However, the enzymatic activity of the resulting proteins is still measurable by conventional protocols. The catalytic constant (k_{cat}) is affected in all the modified enzymes, but an important reduction is observed in JX772A, JX795A and especially M275VP1 and M278VP1. However, K_m increases only in M275VP1 and M278VP1, and the specificity for the substrate (k_{cat}/K_m) is also dramatically impaired in these two proteins. These results prove that peptide accommodation at the tested sites, although affecting at different extent the activity of the receiving enzyme, does not prevent either tetramer formation or substrate hydrolysis, the latter being reduced by only 3-fold for insertions in domain V, and about 15-fold when the activating interface of domain II is involved.

3.4. Structural stability of modified β-galactosidases

The peptide display at the selected sites of β-galactosidase results in an increase of temperature and proteolytic sensitivity in vitro, in general both parameters being affected simultaneously (Table 2). Whereas both JX8CA and JX249A are barely less resistant to trypsin than the parental LACZ protein, the rest of the mutants are more sensitive, suggesting a moderate destabilisation of the tetramer. However, this proteolytic sensitivity was not observed in vivo (not shown). The sensitivity to trypsin treatment of JX581A is remarkable. This does not correspond to the high thermal stability and enzymatic activity exhibited by this construct. This rapid degradation appears to be caused by reasons other than loss of protein structure, since no displacement in the μ_{max} is observed in the fluorescence analysis of this mutant protein. Position 278

Table 2
Stability and tryptophan fluorescence peak of modified β-galactosidases

Protein	Proteolytic stability ($t_{50\%}$, min)	Thermal stability ($t_{50\%}$, min)	λ_{max} (nm)
LACZ	109.4 ± 10.2	141.6 ± 12.7	340.75 ± 1.00
JX8CA	90.4 ± 5.4	67.5 ± 15.4	341.00 ± 0.50
JX249A	73.0 ± 3.2	11.1 ± 1.1	340.75 ± 0.75
JX581A	1.2 ± 0.1	21.8 ± 5.7	340.25 ± 0.25
JX772A	27.1 ± 3.3	18.2 ± 7.2	339.75 ± 0.50
JX795A	22.1 ± 1.6	28.7 ± 9.7	340.25 ± 0.25
M275VP1	7.0 ± 0.7	9.4 ± 0.2	341.25 ± 1.00
M278VP1	5.6 ± 0.1	8.2 ± 0.2	342.00 ± 0.25
β-Galactosidase	> 270	> 240	340.50 ± 0.50

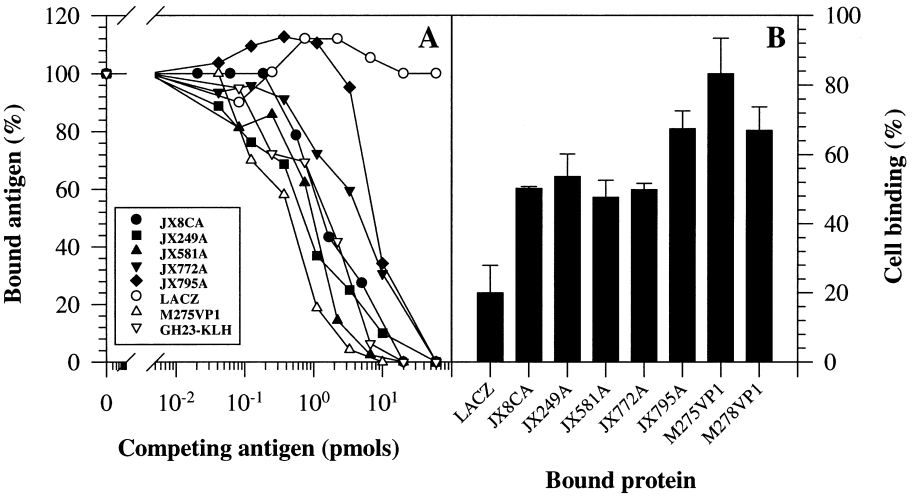

Fig. 2. A: Competition of recombinant β-galactosidases for mAb SD6, with the KLH-coupled synthetic peptide GH23 in indirect ELISA. The same peptide has been included as a control of competing, soluble antigen. B: Binding of BHK cells to bound recombinant proteins. Absorbances of stained cells are referred to that obtained with human vitronectin.

is the only site among those tested in which peptide insertion causes a clear although minor destabilisation of the protein structure as monitored by shift of the fluorescence spectrum peak.

Whereas the enzymatic activity of the parental, pseudo-wild-type LACZ protein is indistinguishable from that of the native enzyme (Table 1), the lack of the eight N-terminal residues in LACZ makes this protein more sensitive to temperature denaturation and proteolytic attack (Table 2).

4. Discussion

The identification of permissive sites for presentation of foreign peptides has been achieved successfully in different *E. coli* proteins, like LamB [30], MalE [31], lactose permease [32], ClpG [33] and PhoE [34] among others. When using enzymes, the insertion of functional protein segments offers an interesting approach for the design of new generation molecular sensors [35,36]. Because of the simplicity of its quantitative analysis, *E. coli* β-galactosidase is one of the most appropriate candidates as carrier framework for the construction of multifunctional enzymes. However, empirical insertional approaches have been barely successful in the identification of tolerant acceptor sites [10,11]. By using crystallographic coordinates [12] we have explored the tolerance to peptide insertion of five selected unstructured segments of the *E. coli* β-galactosidase, which we presumed to be sufficiently exposed, far from the active site and from monomer-monomer interfaces, to act as competent acceptors. The produced chimeric monomers are stable in vivo, they fold correctly and associate into active tetramers that can be purified by standard affinity chromatography. Moreover, on the surface of the assembled enzyme, the inserted protein segment is recognised by an mAb directed against an internal epitope, and it also interacts efficiently with RGD-dependent cell surface integrins.

The introduction of peptides does not cause important alterations on the tertiary structure of the proteins (Table 2), confirming the appropriate choice of the target loops. Protein M278VP1, in which the enzymatic activity is significantly affected, only shows a small shift of tryptophan fluorescence peak (Table 2), that could represent a local destabilisation of the enzyme in the activating interface. This observation is in agreement with the lower enzymatic activity and stability of this mutant (Tables 1 and 2). This region of *E. coli* β-galactosidase (amino acids 271–287) extends from one monomer to another forming the activating interface [12], and it was selected on the basis of secondary structure predictions [15]. The analysis of the insertional effects confirms that this loop is involved in critical monomer-monomer interactions.

The analysis of the mutant enzymes reveals that the positioning of the viral peptide has impairing effects on substrate processing (Table 1). Interestingly, JX772A and JX795A show a higher affinity for the ONPG than the wild-type protein (note the lower K_m values in Table 1, suggesting a higher accessibility of the active site), eclipsed by a dramatic reduction in the hydrolysis rate. In mutants JX249A and JX581A, β-galactosidase activity and stability are only moderately affected. The unexpected trypsin sensitivity of JX581A is probably due to an efficient presentation of a trypsin target site present in the viral peptide [37,38], since the Western blot analysis of trypsin-inactivated protein samples reveals two stable protein fragments of 70 and 45 kDa, respectively (not shown), compatible with a limited digestion at the insertion site. It is worthy to stress that the peptide fusion at the C-terminus (in JX8CA) and the lack of a short homologous N-terminal peptide in the pseudo-wild type (LACZ) have dramatic effects on the thermal stability of the enzyme (Table 2) but little influence on the enzymatic activity. These results reveal the participation of both protein termini in stabilising the tetramer. The identification of 249 and 581 as permissive insertion sites is a promising result for the generation of insertional active mutants of *E. coli* β-galactosidase as the basis for new molecular sensors.

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References

[1] Miller, J.H. (1972) in: Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[2] Corchero, J.L., Viaplana, E., Benito, A. and Villaverde, A. (1996) J. Biotechnol. 48, 191–200.
[3] Viaplana, E., Feliu, J.X., Corchero, J.L. and Villaverde, A. (1997) Biochim. Biophys. Acta 1343, 221–226.
[4] Ullmann, A. (1992) BioEssays 14, 201–205.
[5] Marston, F.A.O. (1986) Biochem. J. 240, 1–12.
[6] Hellebust, H., Murby, M., Abrahmsen, L., Uhlén, M. and Enfors, S.-O. (1989) Bio/Technology 7, 165–168.
[7] Benito, A., Valero, F., Lafuente, J., Vidal, M., Cairó, J., Solà, C. and Villaverde, A. (1993) Enzyme Microb. Technol. 15, 66–71.
[8] Kracke-Helm, H.A., Rinas, U. and Bailey, J. (1991) Enzyme Microb. Technol. 13, 554–564.
[9] Ullmann, A. (1984) Gene 29, 27–31.
[10] Baum, E.Z., Bebernitz, G.A. and Gluzman, Y. (1990) Proc. Natl. Acad. Sci. USA 87, 10023–10027.
[11] Benito, A., Mateu, M.G. and Villaverde, A. (1995) Bio/Technology 13, 801–804.
[12] Jacobson, R.H., Zhang, X.-J., DuBose, R.F. and Matthews, B.W. (1994) Nature 369, 761–766.
[13] Benito, A., Vidal, M. and Villaverde, A. (1993) J. Biotechnol. 29, 299–306.
[14] Sobrino, F., Dávila, M., Ortín, J. and Domingo, E. (1983) Virology 128, 310–318.
[15] Benito, A. and Villaverde, A. (1994) FEMS Microbiol. Lett. 123, 107–112.
[16] Schauder, B., Blöcker, H., Frank, R. and McCarthy, J.E.G. (1987) Gene 52, 279–283.
[17] Casadaban, M.J., Martínez-Arias, A., Shapira, S.K. and Chou, J. (1983) Methods Enzymol. 100, 293–300.
[18] Studier, F.W. and Moffat, B.A. (1986) J. Mol. Biol. 189, 113–130.
[19] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[20] Feliu, J.X. and Villaverde, A. (1994) Biotech. Tech. 8, 509–514.
[21] Gill, S.C. and von Hippel, P.H. (1989) Anal. Biochem. 182, 319–326.
[22] Fasman, G.D. (1990) in: Practical Handbook of Biochemistry and Molecular Biology, CRC Press, Boca Raton, FL.
[23] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[24] Corchero, J.L., Cubarsí, R., Enfors, S.O. and Villaverde, A. (1997) Biochem. Biophys. Res. Commun. 237, 325–330.
[25] Villaverde, A., Feliu, J.X., Harbottle, R.P., Benito, A. and Coutelle, C. (1996) Gene 180, 101–106.
[26] Mateu, M.G., Martínez, M.A., Capucci, L., Andreu, D., Giral, E., Brocchi, E. and Domingo, E. (1990) J. Gen. Virol. 71, 629–637.
[27] McKenna, T.S.C., Lubroth, J., Rieder, E., Baxt, B. and Mason, P.W. (1995) J. Virol. 69, 5787–5790.
[28] Berinstein, A., Roivainen, N., Hovi, T., Mason, P.W. and Baxt, B. (1995) J. Virol. 69, 2664–2666.
[29] Jackson, T., Sharma, A., Ghazaleh, R.A., Blakemore, W.E., Ellard, F.M., Simmons, D.L., Newman, J.W.I., Stuart, D.I. and King, A.M.Q. (1997) J. Virol. 71, 8357–8361.
[30] Charbit, A., Ronco, J., Michel, V., Werts, C. and Hofnung, M. (1991) J. Bacteriol. 173, 262–275.
[31] Betton, J.M., Martineau, P., Saurin, W. and Hofnung, M. (1993) FEBS Lett. 325, 34–38.
[32] Manoil, C. and Bailey, J. (1997) J. Mol. Biol. 267, 250–263.
[33] Der Vartanian, M., Méchin, M.C., Jaffeux, B., Bertin, Y., Félix, I. and Gaillard-Martinie, B. (1994) Gene 148, 23–32.
[34] Agterberg, M., Adriaanse, H., van Bruggen, A., Karperien, M. and Tommassen, J. (1990) Gene 88, 37–45.
[35] Benito, A., Feliu, J.X. and Villaverde, A. (1996) J. Biol. Chem. 271, 21251–21256.
[36] Betton, J.M., Jacob, J.P., Hofnung, M. and Broome-Smith, J.K. (1997) Nat. Biotechnol. 12, 1276–1279.
[37] Bolwell, C., Brown, A.L., Barnett, P.V., Campbell, R.O., Clarke, B.E., Parry, N.R., Ouldridge, E.J., Brown, F. and Rowlands, D.J. (1989) J. Gen. Virol. 70, 45–57.
[38] Hernández, J., Valero, M.L., Andreu, D., Domingo, E. and Mateu, M.G. (1996) J. Gen. Virol. 77, 257–264.
[39] Villanueva, M., Dávila, M., Ortín, J. and Domingo, E. (1983) Gene 23, 185–194.
[40] Verdaguer, N., Mateu, M.G., Andreu, D., Giralt, E., Domingo, E. and Fita, I. (1995) EMBO J. 14, 1690–1696.
[41] Mateu, M.G., Veleron, M.L., Andreu, D. and Domingo, E. (1996) J. Biol. Chem. 271, 12814–12819.
[42] Fowler, A. and Zabin, I. (1978) J. Biol. Chem. 253, 5521–5525.