Purification, crystallization and preliminary X-ray diffraction studies of the bacteriophage ϕ29 connector particle

Alicia Guasch[a], Antonio Parrága[a], Joan Pous[a], José M. Valpuesta[b], José L. Carrascosa[b], Miquel Coll[a,*]

[a] *Departament de Biologia Molecular i Cel.lular, Centre d'Investigació i Desenvolupament, CSIC, Jordi Girona 18–26, 08034 Barcelona, Spain*
[b] *Centro Nacional de Biotecnología, CSIC, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain*

Received 13 April 1998; revised version received 26 May 1998

Abstract The connector or portal particle from double-stranded DNA bacteriophage ϕ29 has been crystallized. This structure, which connects the head of the virus with the tail and plays a central role in prohead assembly and DNA packaging and translocation, is formed by 12 subunits of the p10 protein and has a molecular weight of 430 kDa. The connector structure was proteolysed with endoproteinase Glu-C from *Staphylococcus aureus* V8, which removes 13 and 18 amino acids from the amino- and carboxy-terminal regions of the p10 protein, respectively. Two crystal forms were grown from drops containing an alcohol solution and paraffin oil. Crystals of form I are monoclinic, space group C2 with cell dimensions $a = 416.86$ Å, $b = 227.62$ Å, $c = 236.68$ Å and $\beta = 96.3°$ and contain four connector particles per asymmetric unit. Crystals of form II are tetragonal, space group $P4_22_12$ with cell dimensions $a = b = 170.2$ Å, $c = 156.9$ Å and contain half a particle per asymmetric unit. X-ray diffraction data from both native crystal forms have been collected to 6.0 and 3.2 Å respectively, using synchrotron radiation. Crystals of form II are likely to have the same packing arrangement as the two-dimensional crystals analyzed previously by electron microscopy.

Key words: Bacteriophage ϕ29; Connector; Portal particle; Crystallization; X-ray diffraction

1. Introduction

ϕ29 is a complex double-stranded DNA bacteriophage that infects *Bacillus subtilis* cells. The viral particles are formed by an elongated eicosahedral capsid, or head, and a tail structure. Between these two structures there is a connecting region called the portal structure or connector. This head-to-tail connector plays an important role in the first steps of head assembly and the packaging of DNA. The connector is also involved in the process of DNA transfer into the host cell [1,2].

Electron microscopy studies based on two-dimensional projections show that the ϕ29 connector is an oligomeric structure built up by multiple copies of the 36 kDa gen 10 product. This structure is composed of two morphological domains: a wider disk-like domain of about 14.5 nm in diameter and 3.2 nm in height with protruding lobules and a narrower domain with a cylindrical shape of 8 nm in diameter and 5 nm in height, which presents a longitudinal channel of 40 nm in diameter along its axis [3]. The symmetry of the connector has been controversial, although there is strong evidence for a 12-fold local symmetry of the particle [1,4].

The connector contains two independent domains for interaction with DNA [5] and with a small RNA of viral origin (prohead RNA or pRNA) which is necessary for DNA packaging into the ϕ29 prohead [6]. Treatment of ϕ29 connectors with endoprotease Glu-C from *Staphylococcus aureus* suppresses the connector DNA binding and abolishes the DNA packaging ability [5].

Although the pRNA seems to play a role in the selection of the DNA to be packaged [7], its role in the DNA translocation process remains unclear. Also, the molecular details of the interaction of both the DNA and the RNA with the connector have not been analyzed in detail due to the lack of a high resolution model of the packaging structure. To understand these complex interactions as well as the molecular basis underlying the DNA translocation during DNA packaging, it is critical to determine the atomic structure of the connector. To this end, we have crystallized proteolysed connector particles; the crystals are suitable for X-ray diffraction and a preliminary crystallographic analysis is described. This is the first crystallographic report of a bacteriophage connector particle.

2. Materials and methods and results

2.1. Preparation of inactive and proteolysed connectors

The connectors were purified as described [8] with some modifications. Briefly, the protein p10 that builds the connector was overproduced in *Escherichia coli* carrying a recombinant plasmid containing the gene encoding this protein under the control of the PL promoter of phage λ. The overproduced protein was purified in native form, yielding p10 dodecamers that closely resembled the necks extracted from ϕ29 viral particles. The cells were lysed with lysozyme treatment and the connectors isolated from the cell debris by centrifuging the cell extract. The connector solution was loaded onto a DE-32 cellulose (Whatman) column equilibrated with 0.3 M KCl 50 mM Tris-HCl, pH 7.7 and washed with the same buffer to remove the nucleic acids. The pass-through was precipitated with 0.26% ammonium sulfate and redissolved in 5% glycerol, 50 mM Tris-HCl, pH 7.7. The solution was loaded onto a P11 phosphocellulose (Whatman) column equilibrated with 5% glycerol, 50 mM Tris-HCl, pH 7.7 and washed with a 0–1 M NaCl gradient. Pure connectors were obtained at 0.8 M NaCl. The connector solution was then dialyzed against 5% glycerol, 50 mM Tris-HCl, pH 7.7 and subsequently treated with V8 (endoproteinase Glu-C from *S. aureus* V8) (100:1, p10/V8 (w/w)) for 60 min at 25°C. Proteolysis was terminated by addition of 1 mM phenylmethylsulfonyl fluoride. After digestion, the protein solution was loaded onto a p11 phosphocellulose column equilibrated with 5% glycerol, 1 mM DTT, 50 mM Tris-HCl, pH 7.7 and washed with a 0–1 M NaCl gradient. Pure proteolysed connectors, devoid of the proteolytic fragments, were obtained at 0.4 M NaCl.

*Corresponding author. Fax: (34) (3) 2045904.
E-mail: mcccri@cid.csic.es

PII: S0014-5793(98)00672-3

2.2. Sample preparation

The initial protein concentration was 1 mg/ml and the final concentration of 7 mg/ml, used for crystallization experiments, was reached by two different ways, either lowering the volume with Centricon 30 or by precipitation with ammonium sulfate. With the first method, sometimes depending the sample preparation, significant protein loss was observed due to its binding to the Centricon membrane. The second method consisted in an ammonium sulfate precipitation (0.26 g/ml) at 4°C, followed by centrifugation at $4000 \times g$, and dialysis against 0.4 M NaCl, 20 mM Tris pH 7.7, 1 mM DTT. In both cases the same type of crystals were obtained.

The molecular weight of each monomer, determined by mass spectral analysis, was 32 208 Da, which coincides with the theoretical molecular weight of the proteolysed p10 protein. However, in some preparations which resulted in lower quality crystals the molecular mass showed an increase of 30 Da. To prevent it, 1 mM DTT was added to the protein solution.

The initial protein solution for crystallization contained 7 mg/ml of protein in 0.4 M NaCl, 5 mM Tris-HCl, pH 7.7. Higher protein concentrations led to the formation of two-dimensional crystals. High salt concentration was needed to maintain the connectors in solution.

2.3. Crystallization

Initial conditions for crystallization were obtained by the hanging drop vapor diffusion method upon a broad screening following the conditions of Jancarik and Kim [9] and Cudney et al. [10] and included in the Crystal Screening I and II kits (Hampton Research), respectively. The native connector crystallized in 21% *tert*-butyl alcohol, 50 mM $CaCl_2$, 0.1 M Tris-HCl pH 8.0 at 20°C. The proteolysed connector crystallized in 20–30% isopropyl alcohol, 0.2 M $MgCl_2$, 0.1 M HEPES pH 7.5, at 20°C.

A modified batch method [11] using paraffin oil made it possible to increase the crystal size of the proteolysed connector, keeping constant the drop volume. The mixture of protein for crystallization was prepared as follows: 64 μl protein (7 mg/ml) in 0.4 M NaCl, 5 mM Tris-HCl, pH 7.7, 20 μl 0.2 M $MgCl_2$, 0.1 M HEPES, pH 7.5, and 24 μl pRNA 2 mg/ml. The protein solution was filtered through 0.22 μm Ultrafree-MC at 4°C. Crystallization boxes with plastic bridges were used for seating drop experiments. On each bridge depression 10 μl of protein mixture and 50 μl of paraffin oil were applied. The bottom of the box was filled with reservoir solution containing initially 25% isopropyl alcohol. Then, it was progressively increased up to 30% isopropyl alcohol over 2 days. Following this procedure, chunky crystals (form I) appeared after 4 days and reached a maximum size of approx. $0.4 \times 0.3 \times 0.3$ mm (Fig. 1). In some drops, plate-like crystals measuring $0.4 \times 0.4 \times 0.1$ mm (crystal form II) appeared simultaneously.

2.4. Flash-freezing and heavy atom derivative preparation

Both crystals were extremely unstable under the X-ray at 20 or 10°C and diffracted at a very low resolution (i.e. 10 Å) using a rotating anode source. Under these conditions only a single diffraction image could be taken before complete diffraction decay. However, flash-freezing of the crystals lead to their stabilization and improvement of the diffracting resolution. The flash-frozen procedure was as follows. For crystal form I each crystal was taken directly from the crystallization drop with an appropriated loop attached to a metal tip. Immediately it was introduced in a tube with liquid propane and the tube kept in liquid nitrogen until use. The metal tip fits on a magnetic piece attached to the goniometer head for rapid mounting under the nitrogen stream, were the solid propane evaporates [12]. For crystal form II the crystals were taken from the drop with a loop, soaked on a solution containing 50% glycerol and flash-frozen directly under the nitrogen stream.

Two putative $Ta_6Br_{12}^{2+}$ derivatives of crystal form II were prepared with different soaking times. A few crystals of $Ta_6Br_{12}^{2+}$ were introduced in the crystallization drop containing the connector crystals. After 2 h a pale-green crystal was taken and flash-frozen directly on the nitrogen stream. The same procedure was repeated with a second crystal of intense dark green color after 24 h of soaking.

2.5. X-ray data collection and processing of crystal form I

X-ray intensity data from a single frozen crystal were collected with a MAR Research imaging plate detector, using 0.869 Å synchrotron radiation at the BW7B beamline at the EMBL outstation in the Deutches Elektronensynchrotron (Hamburg). Many crystals had to be tested before higher resolution than 10.0 Å could be observed. A 6.0 Å data set was collected at 120 K with a total of 180 frames of 1° rotation. Data indexing and reduction was performed with DENZO [13]. Crystal data and diffraction statistics are shown in Tables 1 and 2.

2.6. Crystal density determination

For the crystal form I the crystal volume per unit of protein molecular mass V_m [14] varies from 4.84 $Å^3$/Da considering three connectors to 2.44 $Å^3$/Da for six connectors in the asymmetric unit. Because of the size of the particles and their large central channel it was difficult to estimate a reasonable water content in the crystals. For this reason an experimental determination of density was necessary. The density was therefore measured experimentally using a ficoll gradient according to the method of Bode and Schirmer [15]. The protein crystals showed a continuous increase of apparent density during the first 3 min and after this time the crystals descended slowly. The density was determined at the inflection point of the slope as 1.09 g/cm^3 which implies four connector particles in the asymmetric unit of the crystal. With 0.5% glutaraldehyde cross-linked crystals the same value was obtained. The V_m value, assuming four connectors in the asymmetric unit, is 3.61 $Å^3$/Da.

2.7. Data collection and processing of crystal form II and derivatives

X-ray diffraction data for native and $Ta_6Br_{12}^{2+}$ derivatives of soaking crystals of form II were collected with a MAR Research imaging plate detector, using 1.105 Å synchrotron radiation at the BW7B beamline at the EMBL outstation in the Deutches Elektronensynchrotron (Hamburg). A 3.2 Å native data set was collected at 120 K from two crystals with a total of 90° rotation. The detector was set at 385 and 440 mm distance respectively. Data indexing and reduction was performed with DENZO [13]. Crystal data and diffraction statistics are shown in Tables 1 and 2. For crystal form II the calculated crystal volume per unit of protein molecular mass, V_m, is 2.94 $Å^3$/dalton considering half connector in the asymmetric unit of the crystal.

3. Discussion

Three-dimensional crystals of the ϕ29 connector particle can be obtained from alcohol-containing solutions. The strong tendency of the connectors to aggregate in two-dimensional layers has to be prevented by adjusting a precise protein and salt concentration. The removal of micro-aggregates or crystal nuclei by filtering the protein solution is also necessary in order to avoid the formation of large two-dimensional crystal layers in the crystallization drops (Fig. 1).

Three-dimensional crystals of both native and proteolysed connectors are obtained this way, but the later grow to larger size and can be stabilized for X-ray data collection. The proteolytic cleavage removes 13 residues of the N-terminus and 18 residues of the C-terminus. The N-terminal region of p10 contains a stretch of basic charged residues that could hinder

Table 1
Crystal data

Crystal form	Space group	Cell dimensions (Å)	V_m ($Å^3$/Da)	Molecules/connectors in the asymmetric unit
I	C2	$a = 416.8$ $b = 227.6$ $c = 236.7$ $\beta = 96.3$	3.61	48/4
II	$P4_22_12$	$a = b = 170.2$ $c = 156.9$	2.94	6/0.5

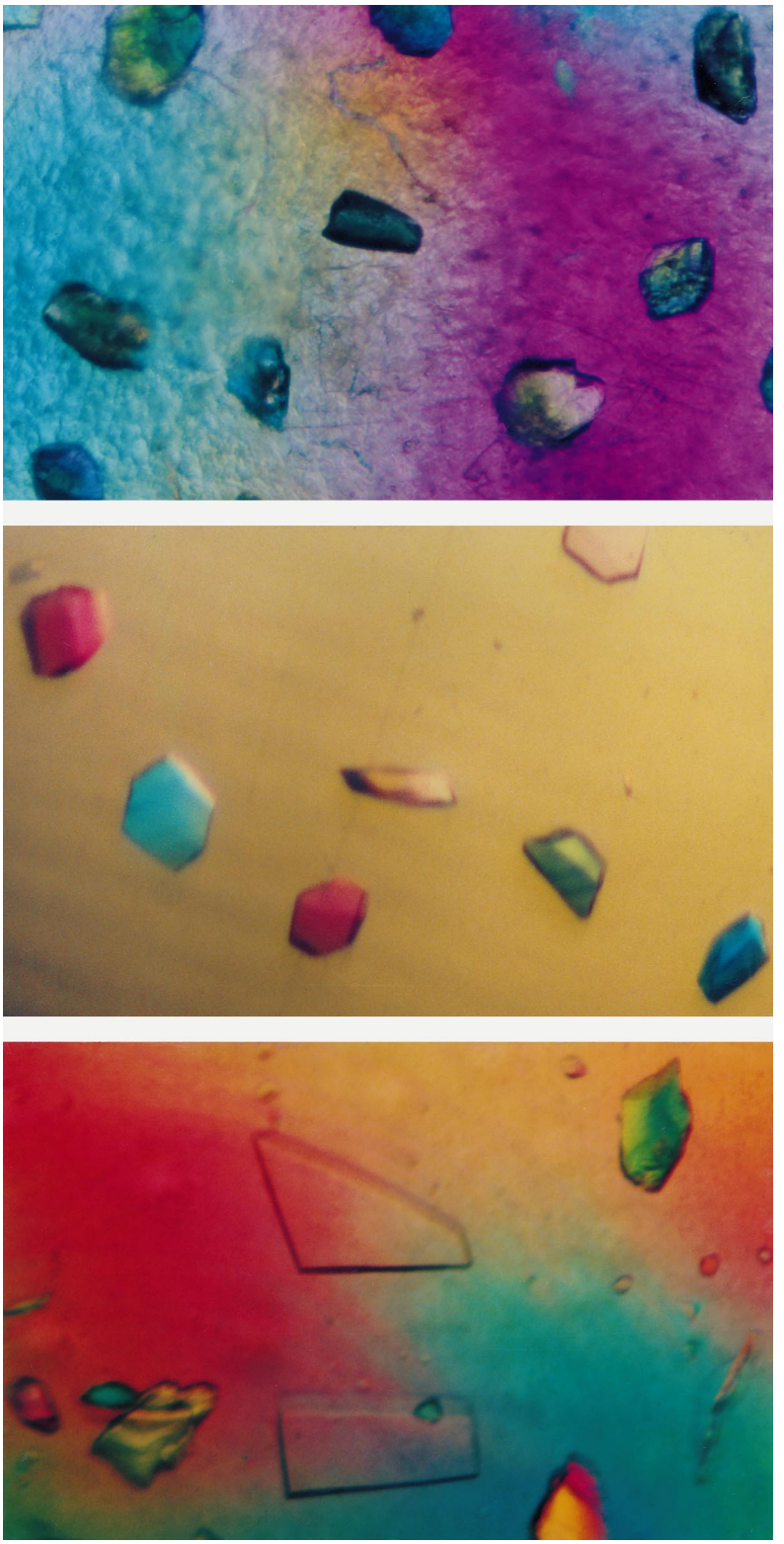

Fig. 1. Crystals of the bacteriophage ϕ29 connector obtained with the proteolyzed p10 protein. Top: Crystals of form I obtained without filtering the protein solution. A background of two-dimensional layers as the ones used for EM studies covers the bottom of the drop. Middle: After filtering the initial p10 protein solution crystals of form I grow from clear drops and show faces and sharp edges. Bottom: Plate-like tetragonal crystals of form II coexist in some drops with crystals of form I.

the crystallization process. It is also possible that because both N- and C-terminal ends are presumably located at the narrow edge of the connector cylinder [1], the elimination of the two tails in this region must favor the packing of the particles in the *c* axis direction, that is, the piling of the connectors along their 12-fold axis. Thus, three-dimensional crystals are favored

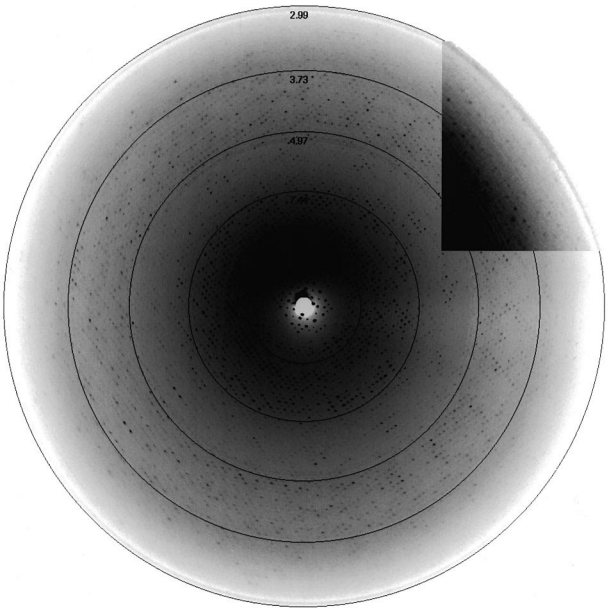

Fig. 2. Diffraction image obtained from a frozen crystal of form II, using synchrotron radiation. The crystal is oriented with the *c* axis approximately parallel to the X-ray beam. Resolution rings are labeled in Å.

over two-dimensional layers, which appear easily in the crystallization drops containing the native protein.

The use of paraffin oil covering the crystallization drops is also critical in order to enlarge the crystals for X-ray diffraction analysis. Without oil, the volume of the drop increases during the crystallization process because a volatile precipitant is used and therefore the protein is diluted during the vapor diffusion experiment. Being the protein concentration already low in the initial solution, the small crystals obtained in drops without oil are unable to grow further. The use of paraffin oil is an efficient alternative crystallization method to dialysis buttons, which were also tried unsuccessfully in order to prevent the increase in the drop volume. The paraffin oil also retards nucleation by slowing the alcohol diffusion into the crystallization drop.

The p10 monomer contains two cysteine residues that form a disulfide bridge (J.J. Calvete, J.M. Valpuesta and J.L. Carrascosa, unpublished results), and seven methionines. Preventing oxidation – most probably at a methionine residue – is also important in order to reproduce crystallization. The protein sample was treated with *N*-methylmercaptoacetamide, an effective reducing agent that converts methionine sulfoxide to methionine under relatively mild conditions [16].

Two crystal forms have been obtained, appearing sometimes in the same crystallization drops. Although both crystals diffract to high resolution, they display very different diffraction patterns. Crystals of form I diffract anisotropically and weakly beyond 6 Å. Even though reflections can be observed up to 3.4 Å resolution, no useful data can be collected in the 6–3.4 Å resolution range. An attempt to do so resulted in low completeness, high R-merge values and unacceptable signal to noise ratio. On the other hand, for crystal form II diffraction intensities can be observed up to 3.2 Å, with good completeness in the last resolution shell and reasonable $< I/\sigma I >$ values (Table 2). The diffraction pattern (Fig. 2) does not show the strong anisotropy and dramatic intensity drop after 6 Å seen in crystal form I. In crystal form II a packing arrangement as that described for the two-dimensional sheets of ϕ29 connectors is expected to occur. Electron diffraction patterns obtained from frozen-hydrated thin crystals show unit cell parameters of $a = b = 165$ Å and 422 symmetry [3], similar to those found in three-dimensional crystals of form II. An analysis of the internal symmetry of the particle will be published elsewhere [17]. Crystal of form II will be used for further crystallographic analysis, including the search for new heavy atom derivatives suitable for phasing.

Acknowledgements: This work was supported by the Ministerio de Educación y Ciencia (Grants PB95-0224 to M.C. and PB96-0818 to J.L.C.), by the CSIC (Acciones especiales 1993–95) and the Generalitat de Catalunya (Centre de Referència en Biotecnologia and Grant 1997SGR-275 to M.C.). Data collection at EMBL-DESY was supported by the EU Large Installations Project CHGECT93-0040. We thank R. Huber for providing the $Ta_6Br_{12}^{2+}$ compound and for useful discussion.

References

[1] Valpuesta, J.M. and Carrascosa, J.L. (1994) Q. Rev. Biophys. 27, 107–155.
[2] Casjens, S. and Hendrix, R. (1988) in: The Bacteriophages (Calendar, R.A., Ed.), Vol. 1, pp. 15–91, Plenum, New York.
[3] Valpuesta, J.M., Carrascosa, J.L. and Henderson, R. (1994) J. Mol. Biol. 240, 281–287.
[4] Müller, D.J., Engel, A., Carrascosa, J.L. and Vélez, M. (1997) EMBO J. 16, 2547–2553.
[5] Donate, L.E., Valpuesta, J.M., Rocher, A., Méndez, E., Rojo, F., Salas, J.L. and Carrascosa, J.L. (1992) J. Biol. Chem. 267, 10919–10924.
[6] Donate, L.E., Valpuesta, J.M., Mier, C., Rojo, F. and Carrascosa, J.L. (1993) J. Biol. Chem. 268, 20198–20204.

Table 2
Data collection statistics

Crystal form	I	II	TaBr derivative
Resolution range (Å)	40–6.0	40–3.2	40–7.0
Number of unique reflections	41 193	35 898	3 437
Completeness (%)			
All data	75.7	89.0	88.1
Highest resolution shell (range in Å)	64.9 (6.21–6.0)	80.8 (3.31–3.2)	87.6 (7.25–7.0)
Average redundancy	3.3	3.5	2.7
$< I/\sigma I >$			
All data	3.9	6.1	8.0
Highest resolution shell (range in Å)	1.2 (6.21–6.0)	2.3 (3.31–3.2)	4.1 (7.25–7.0)
R_{merge} [a]	11.3	15.4	11.5
Highest resolution shell (range in Å)	15.7 (6.21–6.0)	20.3 (3.31–3.2)	19.8 (7.25–7.0)

[a] $R_{merge} = \Sigma_1 I_h - < I_h > _1 / \Sigma < I_h >$ where $< I_h >$ is the average I of reflection h and their symmetry related reflections.

[7] Valpuesta, J.M., Donate, L.E., Mier, C., Herranz, L. and Carrascosa, J.L. (1993) EMBO J. 12, 4453–4459.
[8] Ibáñez, C., García, J.A., Carrascosa, J.L. and Salas, M. (1984) Nucleic Acids Res. 12, 2351–2356.
[9] Jancarik, J. and Kim, S.H. (1991) J. Appl. Crystallogr. 24, 409–411.
[10] Cudney, R., Patel, S., Weisgraber, K., Newhouse, Y. and McPherson, A. (1994) Acta Crystallogr. D 50, 414–423.
[11] Blow, D.M., Chayen, N.E., Lloyd, L.F. and Saridakis, E. (1994) Protein Sci. 3, 1638–1644.
[12] Rodgers, D.W. (1994) Structure 2, 1135–1140.
[13] Otwinowski, Z. (1993) DENZO: an Oscillation Data Processing Program for Macromolecular Crystallography, Yale University, New Haven, CT.
[14] Matthews, B.W. (1968) J. Mol. Biol. 33, 491–497.
[15] Bode, W. and Schirmer, T. (1985) Biol. Chem. Hoppe-Seyler 366, 287–295.
[16] Houghten, R.A. and Li, C.H. (1979) Anal. Biochem. 98, 36–46.
[17] Guasch, A., Pous, J., Párraga, A., Valpuesta, J.M., Carrascosa, J.L. and Coll, M. (1998) J. Mol. Biol. (in press).