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Purification of a viral coat protein by an engineered polyionic sequence

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Abstract

Virus-like particles composed of the polyoma coat protein VP1 were produced as a central building block of an artifical vector system for gene therapy. For this purpose, recombinant VP1 was expressed in *E. coli*. Classical purification schemes resulted only in low yields of protein. Therefore, we developed a new affinity purification procedure. We decided to use a polyionic sequence containing eight glutamic acid residues which allows efficient purification using ion-exchange chromatography. This peptide was inserted in a solvent exposed loop on the surface of VP1. After recombinant expression and cell lysis the first purification and concentration step consisted of a fractionated ammonium sulfate precipitation. The resuspended VP1 was loaded on an anion-exchange column. Elution with ca. 600 m*M* NaCl yielded almost homogeneous protein. Subsequently a size exclusion chromatography was performed to separate the pentameric VP1 from higher oligomeric and aggregated material. In contrast to wildtype VP1 the highly charged mutant form showed no significant tendency to aggregate. To demonstrate the functional state of the VP1 mutant, the in vitro assembly was investigated. At conditions similar to those for wildtype VP1 assembly, the mutant protein could form homogeneous virus-like particles.
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Keywords: Purification; Polyionic sequence; Viral coat proteins; Polyoma VP1

1. Introduction

Polyoma VP1 is the major coat protein of the murine polyoma virus [1]. It is a pentameric protein [2,3] that is able to assemble into virus-like particles (vlp's) in vitro [4,5]. After loading with DNA, such particles were already used as a vector system for in vitro transfection of eucaryotic cells [6]. Recombinant production of VP1 in *E. coli* led to soluble VP1 pentamer. However, purification of recombinant VP1 was performed according to a complex scheme consisting of several chromatographic and precipitation steps [7], resulting in only small amounts of purified VP1. In order to improve the purification procedure we searched for an affinity tag. Besides allowing a simple purification such a tag should enhance the solubility of the recombinant protein, and thus minimize the tendency to aggregate. Therefore, we chose a polyionic peptide sequence. It was shown previously that poly-arginine tails could be used to purify recombinant proteins [8–10] and also its influence on precipitation of proteins was investigated [11]. Furthermore, peptides containing six arginine residues were used before to couple proteins on suitable matrices, thus enabling the construction

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of an enzyme reactor or renaturation of matrix bound proteins [12,13].

Here, we analyzed the interaction of different polyionic peptides consisting of both positively and negatively charged amino acids with commercially available ion-exchange matrices, respectively. Based on these data a fusion of polyoma VP1 with one of these polyionic peptides was constructed and a new simplified purification scheme developed.

2. Experimental

2.1. Construction of the variant VP1-Glu

Mutagenesis of the virus coat protein polyoma VP1 was carried out based on the plasmid pALVP1TAC encoding wt VP1 [7]. Using the Quik-Change site directed mutagenesis kit (Stratagene) a nonapeptide consisting of eight glutamic acid residues and one cysteine (E_8C) was inserted in the VP1 sequence between Asn^{294} and Tyr^{295}. This represents the HI loop of the VP1 structure [2]. The primers used were: forward primer 5′- ATG GGC TGG AGA GTT ACA AGA AAC GAA GAA GAG GAA GAG GAG GAA GAA TGT TAT GAT GTC CAT CAC TGG AGA GGG C-3′ and reverse primer 5′-G CCC TCT CCA GTG ATG GAC ATC ATA ACA TTC TTC CTC CTC TTC CTC TTC TTC GTT TCT TGT AAC TCT CCA GCC CAT-3′.

2.2. Expression of VP1-Glu

The gene of VP1-Glu was expressed in the strain Eco B. The expression strain was cultivated on mineral salt medium at 30°C at a scale of 5 l in a Biostat-Fermenter (Fa. Braun) using the fed-batch technique [14]. The recombinant expression of VP1 was induced by addition of IPTG ad 0.4 m*M* at a cell density of $OD_{600}=20$. Six hours after induction the cells were harvested by centrifugation (8000 *g*, 15 min) and stored at −70°C.

2.3. Protein purification

All chemicals used during purification and characterization of VP1-Glu were purchased from Merck (Darmstadt, Germany) and were of analytical grade. Chromatographic materials utilized were Poros HQ (PerSeptive Biosystems, Wiesbaden, Germany), heparin Sepharose and Superdex 200 (Pharmacia Biotech, Uppsala, Sweden), and Fraktogel EMD 650 (Merck, Darmstadt, Germany).

For purification of VP1-Glu 15 g cells (wet mass) were suspended in 150 ml buffer A (50 m*M* Tris, pH 7.4, 1 m*M* EDTA, 5% glycerol, 200 m*M* NaCl, 4 m*M* DTT). Cell lysis was performed by high pressure dispersion in presence of 1 unit/ml Benzonase (Merck, Darmstadt, Germany), 20 μg/ml RNase (Roche, Basel, Switzerland) and two tablets of complete protease inhibitor cocktail (Roche, Basel, Switzerland). The soluble fraction of the cell lysate was subjected to a fractionated ammonium sulfate precipitation. The precipitate containing VP1-Glu was formed between 20 and 28% of saturation with ammonium sulfate. It was resuspended in 60 ml of buffer A and dialyzed over night against the same buffer. After dialysis an ion-exchange chromatography (IEX) was performed using an ÄKTA Explorer System (Pharmacia Biotech, Uppsala, Sweden). VP1-Glu was loaded on a Poros 20 HQ column with a bed volume of 1.7 ml. Step elution with 400 m*M* NaCl was carried out for eight column volumes, and then a linear gradient was applied up to 1.2 *M* NaCl using a flow-rate of 4 ml/min. The VP1-Glu containing fractions were pooled. Since VP1 is a DNA binding protein [15], remaining oligonucleotides were digested further by adding 20 μg/ml RNase and 2.5 units/ml Benzonase to the pool of VP1-Glu, supplemented with 10 m*M* $MgSO_4$ and one tablet of EDTA free protease inhibitor. In a last purification step the remaining nucleotides, enzymes, and aggregated material were removed by gel permeation chromatography on a Superdex 200 prep grade column at a flow-rate of 0.7 ml/min. All steps of protein purification were carried out at 4°C.

2.4. Characterization of VP1-Glu

The protein concentration of VP1-Glu was determined photometrically using a calculated extinction coefficient ϵ(280 nm, 1 mg/ml, 1 cm)=1.34 [16,17]. In vitro assembly of VP1-Glu into vlp's was performed by dialyzing the protein against assembly buffer (20 m*M* Tris, pH 7.4, 0.75 *M* ammonium

sulfate, 5% glycerol, 1 mM $CaCl_2$) for two days at 15°C. In order to remove the ammonium sulfate after assembly, the vlp's were dialized against 20 mM Tris, pH 7.4, 200 mM NaCl, 5% glycerol, 1 mM $CaCl_2$ for 1 day at 15°C. In both buffers the vlp's could be stored for several weeks at 4°C.

For electron microscopy a vlp solution with a concentration of 0.2–0.4 mg/ml was adsorbed onto a formvar and carbon-coated copper grid for 2 min. The grid was briefly washed with water and then soaked in 1% uranylacetate for 15 s. The samples were examined using a Tesla BS-500 electron microscope (Tesla Nat. Corp., Brno, Czechia) at a cathode voltage of 1000 eV.

Sedimentation velocity runs were performed in an analytical ultracentrifuge Optima XL-A (Beckman Instruments, Palo Alto, USA). Double sector cells were used at 10 000 and 30 000 rpm in a An60Ti rotor. The data were analyzed using the software provided by Beckman Instruments. For quantitative analyses of the sedimentation equilibria a partial specific volume of 0.734 ml/g was assumed. All measurements were corrected for water viscosity and 20°C.

3. Results and discussion

3.1. Interaction of polyionic peptides with ion-exchange supports

Polyionic interactions between charged peptides and ionic matrices are a powerful tool for purification of recombinant proteins. In order to optimize this affinity purification we first investigated the interaction between polyionic peptides of different length and solid supports used for ion-exchange chromatography. Binding of peptides containing 6, 8 and 10 glutamic acid residues (ACE_{6-10}) to an anion-exchange column (Poros HQ) was analyzed. All peptides bound to the resin in the presence of 50 mM sodium phosphate, pH 7.5. Elution by a linear gradient of NaCl concentration showed the differences in the stability of the interaction of the peptides with the matrix. ACE_6 was eluted at a concentration of 280 mM NaCl (Fig. 1A). With an increasing number of charged amino acids the elution of the polyionic peptides shifted to higher salt concentrations. ACE_8 and ACE_{10} were eluted by 400 and 500 mM NaCl, respectively.

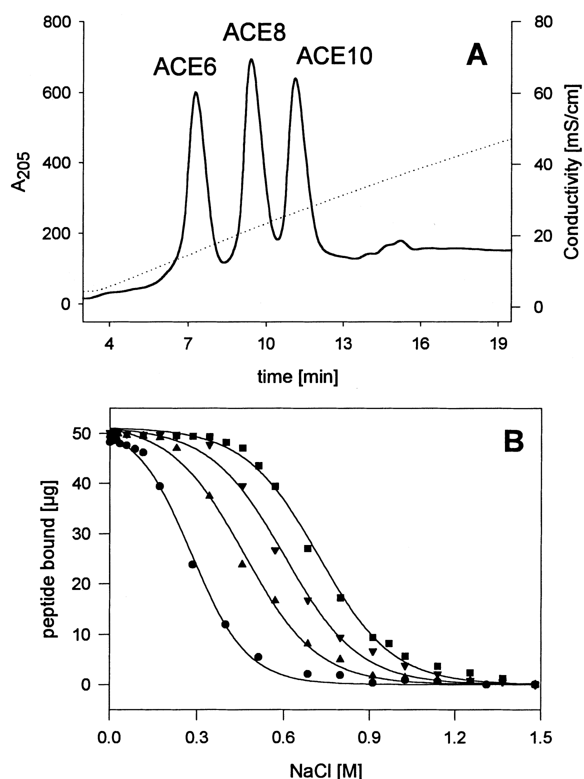

Fig. 1. Interaction of polyionic peptides with ion-exchange matrices. (A) Oligo-glutamic acid of different length (E6, E8, E10 75 µg each) were loaded on a Poros HQ column (bed volume 1.7 ml). Elution was performed using a linear gradient of NaCl concentration between 0 and 1 M. Identification of each peptide in the chromatogram was achieved by comparison of the elution characteristics of the respective isolated peptides. The peptides eluted at 280 (E6), 400 (E8) and 500 (E10) mM NaCl, respectively. (B) Oligo-lysine (K4 (●), K6 (▲), K8 (▼), K10 (■) 50 µg each) were separately incubated with Fraktogel EMD 650 for 30 min at different NaCl concentrations in a batch. Afterwards the samples were centrifuged and washed with water. The bound peptide was eluted with 1.5 M NaCl and the amount of peptide was quantified spectroscopically.

Similar experiments were performed to investigate the binding of positively charged peptides to cation-exchange supports like heparin Sepharose (data not shown) and Fraktogel EMD 650. To this end the matrix was suspended in 260 µl buffer containing the peptide at a concentration of 50 µg/ml at various NaCl concentrations. After an incubation of 30 min at 20°C the matrix was twice washed with water.

Peptide bound to the matrix was eluted with 1.5 *M* NaCl and quantified spectroscopically at 205 nm. In Fig. 1B the fraction of different oligo-lysine peptides bound to Fraktogel EMD 650 is shown as a function of the ionic strength of the buffer. As in the case of negatively charged peptides the binding was strongly dependent on the number of charges presented by the oligo-lysine peptides.

These data showed that a polyionic tag used for purification of recombinant proteins should consist of at least four charged residues. However, since most buffers used in protein chemistry have a basal level of salt of 50 – 150 m*M* a tag of six to eight charged residues should be preferred.

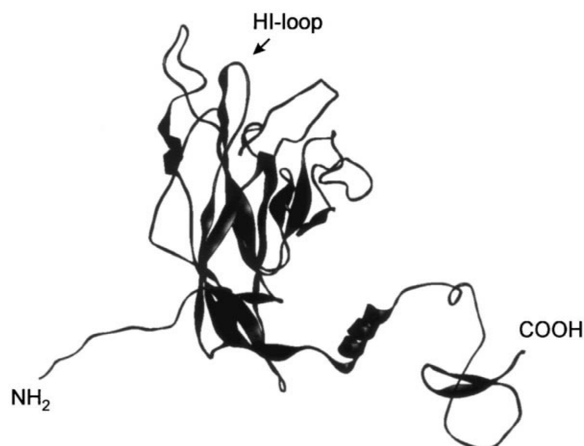

Fig. 2. Structure of polyoma VP1. A monomer of the pentameric VP1 [3] is displayed. The HI loop used for the insertion of the polyionic peptide is marked by an arrow.

3.2. Design of a fusion of VP1 and a polyionic peptide

For the construction of a variant of VP1 containing a polyionic fusion peptide we first considered whether a positively or negatively charged tag should be used. Since VP1 in its native state is a pentameric protein the fusion with a polyionic peptide tag would introduce a high charge density in the protein. Using a tag based on positively charged lysine or arginine residues might result in an increase in binding and co-purification of DNA and RNA. Therefore, we decided not to use a positively charged peptide, but a sequence containing eight glutamic acid residues. Besides glutamic acid residues, a cysteine was introduced thus enabling coupling of fluorescence dyes that should allow a sensitive detection of VP1 particles in further cell culture experiments.

Since part of the N-terminus and the C-terminus of VP1 are of functional importance regarding the assembly process and the stability of these particles [18,19] the glutamic acid containing peptide was inserted within the sequence of VP1 instead of fused to one of the termini. VP1 possesses three large protuding loops, each connecting two strands of a β-sheet (DE-, HI- and BC2-loop, Fig. 2). From crystal structure analyses it has been shown, that the DE-loop is involved in monomer monomer interactions within the pentamer. The two other loops seemed to allow an insertion of a peptide without strongly disturbing the VP1 structure. In this first approach we chose the HI-loop at the position Asn^{294} for the insertion of a nonapeptide containing eight glutamic acid residues and one cysteine.

3.3. Expression and purification of VP1-Glu

Based on the expression plasmid for wt VP1, pALVP1TAC [7], the mutant VP1-Glu was constructed using site directed mutagenesis. Thus, like wt VP1 the expression of the mutant was under control of the same tac promotor, allowing a direct comparison of the expression of wt VP1 and VP1-Glu. As shown in Fig. 3 expression of VP1-Glu resulted in a large amount of recombinant product. The yield of VP1-Glu was only slightly lower than that of wt VP1 under identical conditions. The partitioning between soluble and insoluble product did not change significantly compared to wt VP1, thus allowing production of soluble VP1-Glu in large quantities.

Starting with the soluble fraction of the cell lysate the first purification step consisted of a fractionated ammonium sulfate precipitation. This step proved to be very efficient for two reasons. In the pentameric form of VP1-Glu N- and C-terminus were prone to proteolysis. Addition of ammonium sulfate stabilized VP1, thus preventing proteolytic cleavage of VP1-Glu by proteases present in the cell lysate. Cleavage products already formed during expression precipitated at an ammonium sulfate saturation of about

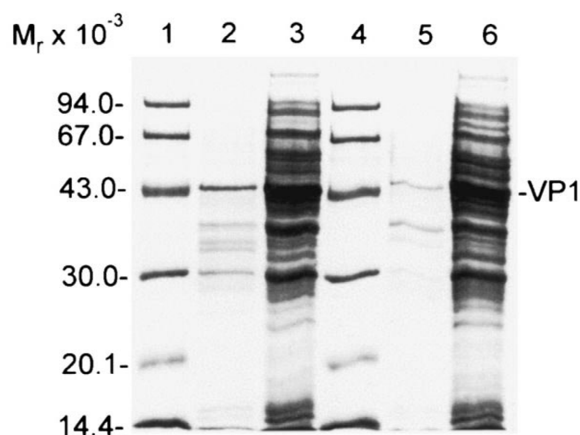

Fig. 3. Expression of VP1-Glu. Cells producing either VP1-Glu or wt VP1 were harvested 6 h after induction of expression. Soluble and insoluble fractions of the cell lysate were analyzed. Lane 1,4 – Molecular mass standard; lane 2 – insoluble fraction of wt VP1 expression; lane 3 soluble fraction of wt VP1 expression; lane 4 – insoluble fraction of VP1-Glu expression; lane 5 – soluble fraction of VP1-Glu expression

20%, whereas full length VP1-Glu precipitated at higher ammonium sulfate concentrations. However, this separation of full length and partially degraded products was not complete since VP1-Glu is a pentameric protein and precipitation might depend on how many monomers of VP1-Glu within a pentamer were partially degraded. A small amount of full length VP1-Glu was also observed in the precipitate at 20% ammonium sulfate saturation and, on the other hand, a thin band at ca. 36 KDa corresponding to the partially degraded VP1-Glu was still present in the precipitate at 28% ammonium sulfate saturation (Fig. 4C). The partial degradation and the existence of aggregated VP1-Glu in the lysate might be responsible for the loss of protein during this separation step (Table 1).

In the second step of purification the solubilized ammonium sulfate precipitate was loaded on a Poros HQ column. Most of the contaminating proteins were found in the flow through. A step of 400 m*M* NaCl resulted in the elution of additional weakly bound proteins. Elution of VP1-Glu was achieved by a linear gradient from 0.4 to 1.2 *M* NaCl (Fig. 4A). Almost homogeneous protein was recovered at NaCl concentrations of 600 m*M*. Fractions containing VP1-Glu eluting above 700 m*M* NaCl were discarded because of high contaminations with nucleic acids, which eluted in a sharp peak at 800 m*M* NaCl.

The NaCl concentrations neccessary for elution of VP1-Glu were significant higher then those used for the elution of the peptide ACE_8 on the same matrix. This indicated that in the case of pentameric VP1-Glu more than one polyionic sequence contributed to the interaction with the matrix (cf. Fig. 1A).

Although VP1-Glu seemed to be purified to homogeneity after anion-exchange chromatography (the additional thin band with an apparent molecular mass of 36 KDa represents a degradation product still integrated in the pentameric structure of VP1) there might be some oligomeric or aggregated species present, since the association of pentamers to vlp's is an intrinsic property of VP1. Thus in a last purification step a preparative gel permeation chromatography on a Superdex 200 prep grade column was performed (Fig. 4B). It showed that about 20% of VP1-Glu was aggregated, whereas the remaining VP1-Glu was in its native pentameric state. Analyses of the pentamers after different times of storage by gel permeation yielded only the pentamer, indicating that the native pentameric form is a stable, non-aggregating protein.

3.4. Characterization of VP1-Glu

Since the fusion peptide was inserted into the sequence of VP1 it remained in the protein after purification, thus presenting a high density of additional negative charges on the surface of VP1-Glu. In order to analyze whether this insertion influences the functional properties of VP1 we characterized VP1-Glu in more detail. Its structural integrity as a pentamer was already demonstrated by gel permeation chromatography during protein purification (Fig. 4B). This result was confirmed by analytical ultracentrifugation. The s-value of s(w,20)=7.2 S determined for VP1-Glu (Fig. 5) is identical to that of pentameric wt VP1 within experimental error (data not shown, [4,20]). However, the stability of VP1-Glu is slightly decreased compared to that of wt VP1 as measured by temperature denaturation (Fig. 6). Whereas the midpoint of the thermal denaturation transition was ca. 48°C for wt VP1, it shifted to Tm=37°C for VP1-Glu. This destabilization might

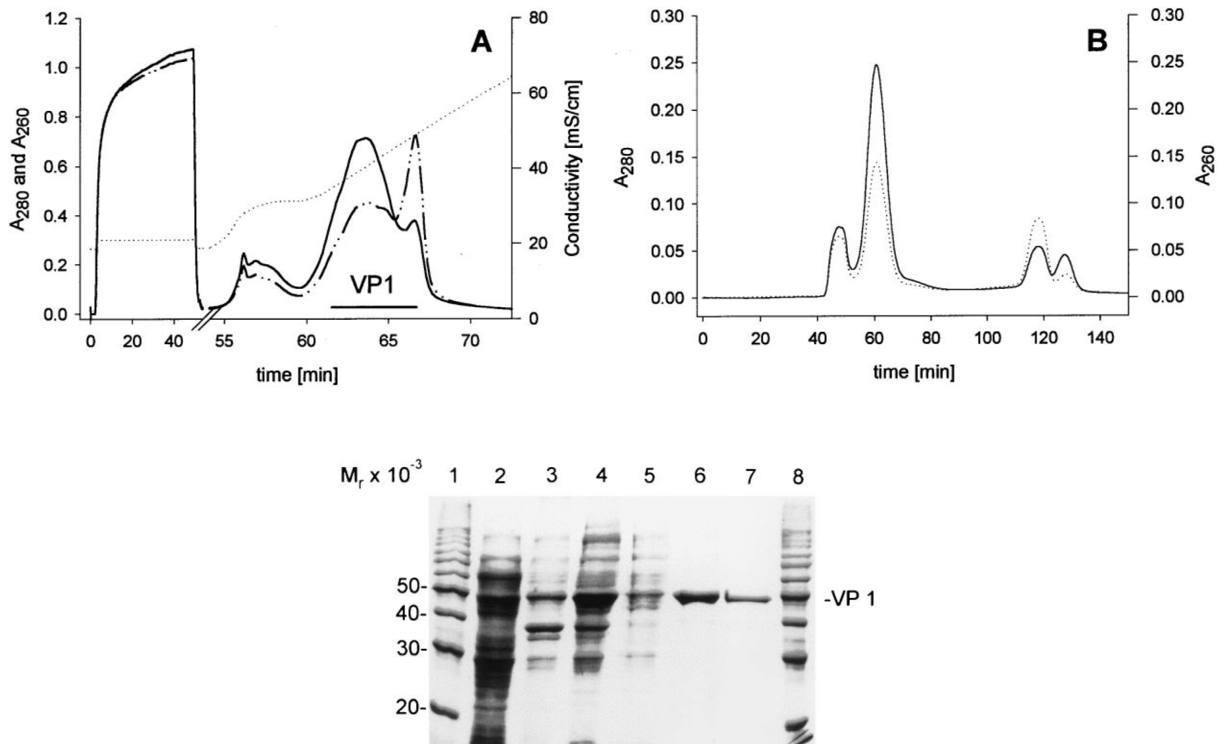

Fig. 4. Purification of VP1-Glu. VP1-Glu obtained after a fractionated ammonium sulfate precipitation was applied on a Poros HQ column (1.7 ml bed volume) equilibrated in buffer A. The column was then washed with 15 ml of buffer A supplemented with 400 m*M* NaCl. Elution of VP1-Glu was achieved by applying a linear gradient of NaCl between 0.4 and 1.2 *M*. VP1-Glu eluted at 600 m*M* NaCl. The pool of VP1-Glu was further purified by gel filtration (Superdex 200 prep grade), equlibrated in buffer A. (A) Elution profile of the IEX using a Poros HQ column. Absorption at 280 nm (–), at 260 nm (---) and the conductivity (···) were monitored. (B) Elution profile of the gel permeation on a Superdex 200 prep grade column, monitoring the absorption at 280 nm (–) and 260 nm (---). The time of elution of VP1-Glu of about 60 min corresponds to the molecular weight of a pentamer. (C) SDS-PAGE of VP1-Glu purification. lane 1, 8 – Molecular mass standard; lane 2 – cell extract; lane 3 – precipitate after 20%-$(NH_4)_2SO_4$-saturation; lane 4 – precipitate after 28%–$(NH_4)_2SO_4$–saturation; lane 5 – flowthrough from Poros HQ; lane 6 – eluate of Poros HQ at 600 m*M* NaCl; lane 7 – VP1 pentamer after gel permeation on a Superdex 200 prep grade.

be due to electrostatic repulsion on the surface of VP1-Glu. The insertion itself seemed not to be responsible for this destabilization, since insertion of another sequence at the same position in VP1 did not lead to changes in VP1 stability (unpublished data).

The most important functional parameter of VP1

Table 1

Summary of purification of VP1-Glu. VP1-Glu was purified starting with 15 g (wet mass) of biomass. The amount of total protein was measured by Bradford analyses [21]. Since VP1-Glu does not possess any enzymatic activity the amount of VP1-Glu at different stages of purification was estimated by quantification of Coomassie stained gels using the program Phoretics (Pharmacia Biotech, Sweden)

Stage of purification	Volume [ml]	Total protein [mg]	VP1-Glu [mg]	Yield [%]
Cell extract	150	1005	50.25	100
Fractionated ammonium sulfate precipitation	50	72.25	32.16	64
Poros 20 HQ	18	28.87	23.43	47
Superdex 200	36	18.56	18.56	37

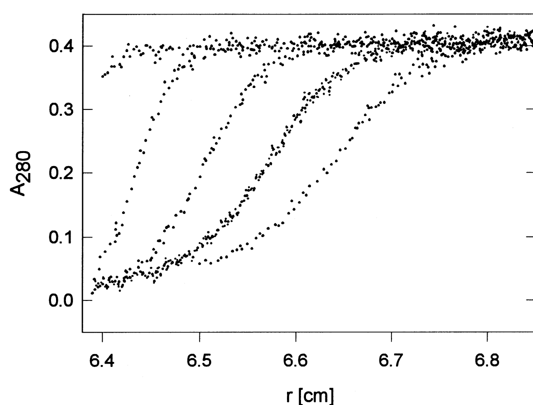

Fig. 5. Analytical ultracentrifugation of pentameric VP1-Glu. The sedimentation velocity run was performed at 30 000 rpm, 20°C in buffer A. Scans were taken every 10 min (displayed every 30 min). The s-value was calculated to be s(w,20)=7.2 S.

is its ability to assemble into vlp's [4]. Therefore, we studied the assembly of VP1-Glu. The protein was dialyzed against assembly buffer containing 0.75 *M* ammonium sulfate. Ammonium sulfate was previously shown to induce assembly of VP1 [5]. Under these conditions VP1-Glu also assembled into virus-like particles as judged by gel permeation chromatography and electron microscopy (Fig. 7). The electron micrograph of assembled VP1-Glu showed

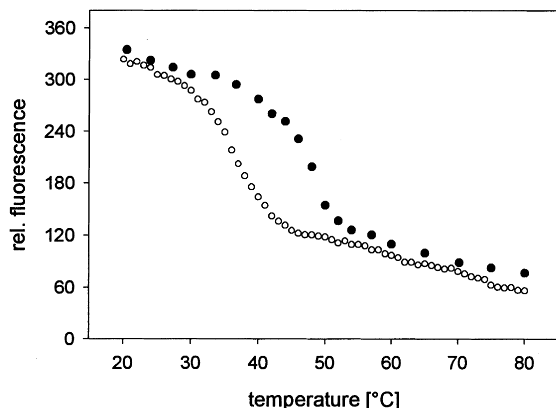

Fig. 6. Thermal denaturation of VP1-Glu. 50 μg/ml VP1-Glu and wt VP1 in buffer A were used, respectively. The heating rate was 0.3°C/min. The denaturation was measured using fluorecence in a stirred and temperature controlled cuvette. A wavelength of 295 nm was set for excitation and 338 nm for emission. The temperature transition of wt VP1 (●) had an apparent midpoint of Tm=48°C whereas VP1-Glu (○) showed a transition with Tm=37°C

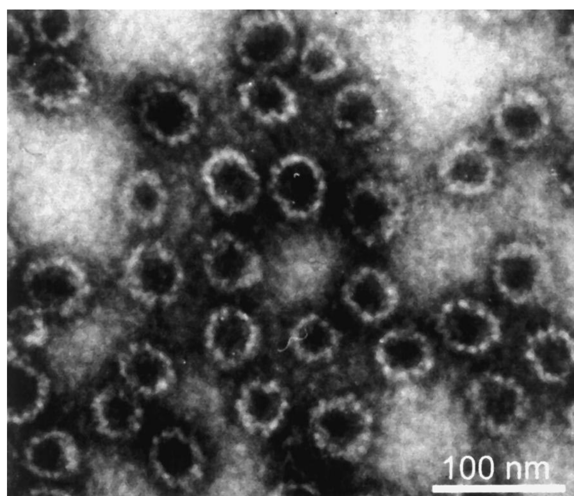

Fig. 7. Electron micrograph of virus-like particles of VP1-Glu. VP1-Glu at 0.36 mg/ml were dialyzed against assembly buffer for two days at 15°C. Afterwards the protein was adsorbed onto a formvar and carbon coated grid and negatively stained using uranyl acetate. Nominal magnification was 100 000-fold.

that virus-like particles of different sizes were formed, a phenomenon also known for vlp's composed of wt VP1 under these conditions [5]. Thus, the basic functional characteristics of VP1-Glu were shown to be identical to those of wt VP1.

4. Conclusion

Here we show that polyionic fusion peptides can be used for the efficient purification of recombinant proteins. According to its affinity to standard commercial matrices, both positively and negatively charged peptides are suitable. Efficient binding is promoted by octapeptides of basic or acidic amino acids. In this first approach, we used an octaglutamic acid peptide as purification tag, inserted in a surface loop of pentameric VP1 of polyoma virus. By this way, we could establish a fast and efficient purification procedure for functional VP1-Glu. For more general applications such polyionic peptides could be fused to the N- or C-terminus of the respective proteins. Introducing a specific cleavage site for proteases would allow a processing after purification to get the unmodified mature protein. In this context it should be noted that the cleavage site of enterokin-

ase consists of four aspartic acid residues. Elongation of this sequence might directly lead to a peptide sequence combining both, a polyionic affinity tag and a specific proteolytic cleavage site.

5. Abbreviations

DTT	dithiothreitol
IEX	ion-exchange chromatography
IPTG	isopropyl-β-thiogalacto-pyranosid
PAGE	polyacrylamide gel electrophoresis
vlp	virus-like particle
wt VP1	wildtype of the polyoma virus coat protein 1
VP1-Glu	mutant VP1 with an insertion consisting of a CE_8 peptide in the HI-loop of VP1

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References

[1] W. Eckhardt, in: B.N. Fields, D.M. Knipe (Eds.), Fundamental Virology, Raven Press, New York, 1991, p. 727.
[2] T. Stehle, Y. Yan, T.L. Benjamin, S.C. Harrison, Nature 369 (1994) 160.
[3] T. Stehle, S.C. Harrison, Structure 4 (1996) 183.
[4] D.M. Salunke, D.L. Caspar, R.L. Garcea, Cell 46 (1986) 895.
[5] D.M. Salunke, D.L. Caspar, R.L. Garcea, Biophys. J. 56 (1989) 887.
[6] J. Forstova, N. Krauzewicz, V. Sandig, J. Elliott, Z. Palkova, M. Strauss, B.E. Griffin, Hum. Gene Ther. 6 (1995) 297.
[7] A.D. Leavitt, T.M. Roberts, R.L. Garcea, J. Biol. Chem. 260 (1985) 12803.
[8] J.C. Smith, R.B. Derbyshire, E. Cook, L. Dunthorne, J. Viney, S.J. Brewer, H.M. Sassenfeld, L.D. Bell, Gene 32 (1984) 321.
[9] S.J. Brewer, H.M. Sassenfeld, United States Patent, Patent Number 4,532,207 (1983)
[10] H.M. Sassenfeld, Trends. Biotechnol. 8 (1990) 88.
[11] M.Q. Niederauer, I. Suominen, M.A. Rougvie, C.F. Ford, C.E. Glatz, Biotechnol. Prog. 10 (1994) 237.
[12] G. Stempfer, B. Höll-Neugebauer, R. Rudolph, Nat. Biotechnol. 14 (1996) 329.
[13] G. Stempfer, B. Höll-Neugebauer, E. Kopetzki, R. Rudolph, Nat. Biotechnol. 14 (1996) 481.
[14] A. Teich, H.Y. Lin, L. Andersson, S. Meyer, P. Neubauer, J. Biotechnol. 64 (1998) 197.
[15] R.B. Moreland, L. Montross, R.L. Garcea, J. Virol. 65 (1991) 1168.
[16] D.B. Wettlaufer, Adv. Protein Chem. 17 (1962) 375.
[17] C.N. Pace, F.X. Schmid, in: T.E. Creighton (Ed.), Protein Structure. A Practical Approach, Oxford University Press, Oxford, 1997, p. 253.
[18] R.L. Garcea, D.M. Salunke, D.L. Caspar, Nature 329 (1987) 86.
[19] T. Stehle, S.C. Harrison, EMBO J. 16 (1997) 5139.
[20] A. Kosukegawa, F. Arisaka, M. Takayama, H. Yajima, A. Kaidow, H. Handa, Biochim. Biophys. Acta 1290 (1996) 37.
[21] M.M. Bradford, Anal. Biochem. 72 (1976) 248.