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Increased yield of homogeneous HIV-1 reverse transcriptase (p66/p51) using a slow purification approach

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ABSTRACT

A chromatographic procedure to purify recombinant reverse transcriptase (RT) from human immunodeficiency virus-1 is reported. A bacterial system which expressed large amounts of p66 RT polypeptide was used. The purification scheme was optimized for high-yield production of homogeneous p66/p51 RT using a combination of chromatographic matrices in the following order: Q-Sepharose, heparin-Sepharose, phenyl-Sepharose, S-Sepharose, Poly(A)-Sepharose and Q-Sepharose. The p66 polypeptide remained intact after the first chromatographic step on Q-Sepharose, where it was recovered in the non-adsorbed fraction. A high yield of p66/p51 RT was obtained when the time from application to elution of heparin-Sepharose in the second chromatographic step was prolonged. Phenyl-Sepharose was used in the next chromatographic step to separate the heterodimeric forms of RT from p66 RT on the basis of hydrophobicity. The chromatography on S-Sepharose resolved the major heterodimeric form, p66/p51, from other heterodimeric variants. Further purification was done by affinity chromatography on Poly(A)-Sepharose followed by anion-exchange chromatography on Q-Sepharose. Amounts of 25–35 mg of the pure heterodimer p66/p51 RT were recovered from 50 g of bacterial cells.

INTRODUCTION

Reverse transcriptase (RT) from the human immunodeficiency virus (HIV-1) contains RNA-dependent DNA polymerase and DNA-dependent DNA polymerase activities. RT catalyses the synthesis of first single-stranded and then double-stranded DNA from the viral RNA genome on infection of the cells by virus particles. HIV-1 RT has become a subject of intense study aimed at the three-dimensional structure and rational drug design (for a review, see ref. 1).

The RT molecule (p66) is a single polypeptide with a molecular mass of 66 000 (560 amino acids) composed of one N-terminal polymerase domain (p51) and one C-terminal RNAse H domain (p15). The RT molecule has a strong tendency to form a stable heterodimer from the p66/p66 dimer through proteolytic removal of one of the p15 domains.

RT has been expressed in bacterial and yeast cells from the *pol I* gene (encoding for protease, RT and endonuclease) or from the *RT* gene alone, and the purification of RT in the heterodimeric p66/p51 or the p66 form has been reported [2–12]. HIV-1 protease is reported to cleave p66 at the cleavage site Phe 440–Tyr441 [7], whereas bacterial proteases randomly cleave the p66 polypeptide at a susceptible region in the amino acid range 426–446 [1,13,14]. As a result, the carboxyl terminal end of so-called p51 varies and multiple forms of "p51" in association with

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p66 have been observed during the course of purification. The property of p66 to form aggregates with itself and with different degradation products of p66 and also with host cell proteins leads to a lower yield of the final product and increases the complexity of the purification.

Several groups have reported techniques to circumvent the problems caused by bacterial proteolytic processes during the preparation of a homogeneous p66/p51 RT. By gene technology procedures a fusion peptide, *e.g.*, a histidine tail [15,16] or an antigenic peptide [17], was attached to the N- or C-terminal end of the RT chain, which then permitted a rapid purification on a metal affinity or a monoclonal antibody matrix, respectively. Reconstitution of a heterodimer by mixing purified p66 and p51 expressed in separate systems [18–20] or cleaving the purified p66 with HIV-protease [21] are alternative ways which have also been tried to produce homogeneous p66/p51 RT.

The expression of *pol I* gene (encoding for protease, RT and endonuclease) and the mild purification and crystallization of the heterodimer p66/p51 RT have been reported previously [2,3] and reviewed [4]. For crystallization studies, a large amount of pure and homogeneous RT is required. As the yield of RT was fairly low when the *pol I* gene was expressed, we have improved the production of the pure and crystallizable RT material by altering the gene construct (*RT* gene only), bacterial strain and purification scheme.

When the earlier reported purification procedure [DEAE-Sepharose, heparin-Sepharose, S-Sepharose and Poly(A)-Sepharose in sequential order] was applied, the resulting RT was a mixture of p66 and different forms of heterodimers. To obtain a high yield of crystallizable heterodimer p66/p51 RT, the purification scheme was modified as follows: (i) instead of a rapid purification, the application and elution times in the second chromatographic step on heparin-Sepharose were prolonged to achieve optimum proteolytic degradation which resulted in an increase in the homogeneous heterodimeric form; (ii) hydrophobic interaction chromatography (phenyl-Sepharose) was introduced to separate undegraded p66 from the heterodimeric forms.

EXPERIMENTAL

Experiment

A fast protein liquid chromatographic (FPLC) system and a PhastSystem were obtained from Pharmacia (Uppsala, Sweden).

Materials

All chromatographic and electrophoretic gel media were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). The electrophoresis gels were PhastGel gel media. The chromatographic matrices were Q-Sepharose Fast Flow, heparin-Sepharose CL-6B and Poly-(A)-Sepharose 4B. HiLoad 26/10 S-Sepharose HP and HiLoad 16/10 phenyl-Sepharose HP prepacked columns were used.

Expression of RT in Escherichia coli

The RT cDNA was isolated by the polymerase chain reaction (PCR) technique from the plasmid pN10E15 [2]. The primers in the PCR were designed so that an NdeI site containing the start codon ATG was introduced at the 5′-terminus. A translation termination stop and BamHI site were introduced at the 3′-terminus of the fragment. The PCR fragment was blunt end ligated into the cloning vector bluescript KS+ at the EcoRV site. The new construct was called pRT.BS. The expression vector was constructed by isolation of the NdeI–BamHI fragment from pRT.BS and ligated to pET11 [22] (Novagene), which previously had been treated with NdeI and BamH1. Expression was performed in the *E. coli* strain BL21(DE3) which is deficient in *lon* and *ompT* protease-deficient. Transformed cells were grown to OD = 1.0 before induction with isopropyl-β-D-galactoside (IPTG) [22]. The cells were harvested 3 h after induction.

Analysis

Determination of protein concentration and analysis by electrophoresis were performed as described by Unge *et al.* [2] and Bhikhabhai *et al.* [3]. Western blotting was performed using antibodies against RT and detection was done using the ECL (Amersham, UK) Western blotting detection system. A Biotinylated SDS-

PAGE standard (Bio-Rad) was used as a molecular mass marker. Activity measurement of RT was done as described by Gronowitz *et al.* [23].

Purification procedure

All chromatographic experiments were performed using the FPLC System at 8°C. SDS-PAGE was used to detect RT in each chromatographic step. Purified heterodimer p66/p51 RT was used as the marker to identify fractions containing RT.

Lysis. Bacterial cells from a 10-l fermenter were harvester by centrifugation, washed twice with 20 m*M* Tris–HCl–100 m*M* NaCl–1 m*M* EDTA (pH 8.0) and then suspended in 150 ml of the lysis buffer (pH 8.0) [20 m*M* Tris–HCl–100 m*M* NaCl–1 m*M* EDTA–1 m*M* dithiothreitol (DTT)–1 m *M* $MgSO_4$] and treated with DNase and RNase. After disruption of the cells using a French pressure cell, the lysate was centrifuged at 23 000 *g* for 30 min. The supernatant was dialysed against buffer A (pH 8.0) (20 m*M* Tris–HCl–100 m*M* NaCl–1 m*M* EDTA–1 m*M* DTT).

Anion-exchange chromatography. After dialysis the solution was diluted with buffer A to 250 ml and applied to a pre-equilibrated Q-Sepharose fast flow column (120 × 50 mm I.D.; flow-rate 4 ml/min). The column was washed with 400 ml of buffer A and RT was recovered in the non-adsorbed fractions (pool I).

Affinity chromatography. The salt concentration of pool I was adjusted to 0.15 *M* NaCl and then applied over a period of about 15 h to the heparin-Sepharose column (70 × 50 mm I.D.; flow-rate 1 ml/min), which was pre-equilibrated with buffer A. The column was eluted with a linear gradient (1.5 bed volumes) of 0.10–0.35 *M* NaCl in buffer A. The RT was recovered in one broad peak (pool II).

Hydrophobic interaction chromatography. The ammonium sulphate concentration of pool II was adjusted to 1.0 *M* and then applied to a phenyl-Sepharose column (100 × 16 mm I.D.; flow-rate 2 ml/min), which had been pre-equilibrated with buffer B (pH 8.0) (1.0 *M* ammonium sulphate–20 m*M* Tris–HCl–1 m*M* DTT–1 m*M* EDTA). The column was eluted with a linear gradient (15 bed volumes) from 1.0 to 0.0 *M* ammonium sulphate buffer. RT eluted at 0.6 *M* ammonium sulphate (Fig. 3, first peak) and the fractions were pooled for further purification (pool III).

Cation-exchange chromatography. Pool III was dialysed against buffer C (pH 6.5) (20 m*M* MES–1 m*M* DTT–1 m*M* EDTA) and applied to an S-Sepharose column (100 × 26 mm I.D.; flow-rate 2 ml/min) pre-equilibrated with buffer C. The column was eluted with a linear gradient (15 bed volumes) from 0.0 to 0.25 *M* NaCl in buffer C. The major peak containing the heterodimer RT eluted at about 0.18 *M* NaCl (Fig. 4A; pool IV).

Affinity chromatography on Poly(A)-Sepharose 4B. Pool IV was dialysed against buffer D (pH 8.0) (20 m*M* Tris–HCl–1 m*M* DTT–1 m*M* EDTA) and applied to a column of Poly(A)-Sepharose 4B (100 × 16 mm I.D.; flow-rate 0.8 ml/min) equilibrated with buffer D. Elution was carried out with a linear gradient (8 bed volumes) from 0.0 to 0.25 *M* NaCl in buffer D. RT was eluted in one broad peak (Fig. 5) and fractions were pooled (pool V).

Final purification on Q-Sepharose. Pool V was dialysed against buffer D containing 75 m*M* NaCl and applied to a Q-Sepharose column (100 × 10 mm I.D.) and RT was collected in the non-adsorbed fractions (pool VI).

Procedure for concentrating the RT. Pool VI was adjusted to 1.0 *M* with respect to ammonium sulphate concentration and applied to a phenyl-Sepharose column equilibrated with buffer B. RT was eluted with low ionic strength buffer D. For crystallization RT was further concentrated to about 15–18 mg/ml by ultrafiltration (Ultrafree-CL filters; Millipore).

RESULTS AND DISCUSSION

A pure and homogeneous p66/p51 in large amounts is a prerequisite for structural studies by X-ray crystallography. RT was expressed in fairly small amounts when the *pol I* gene (encoding for protease, RT and endonuclease) was expressed in bacteria [2, 3]. In this paper we report the expression of only the *p66* gene in a bacterial strain which is deficient in the *lon* protease and *ompT* outer membrane protease. In the lysate RT is present predominantly as p66 (Fig. 1, lane 1). During the early stages of purification, a considerable degradation of p66

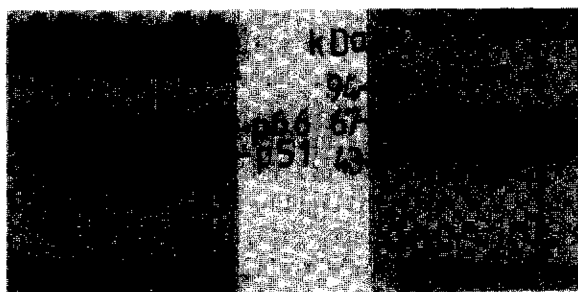


Fig. 1. Western blot analysis of fractions from different chromatographic steps for the purification of recombinant HIV-1 reverse transcriptase. Samples: lane 1 = bacterial lysate; 2 = the non-adsorbed fractions of Q-Sepharose, 3 = pooled fractions containing RT from heparin-Sepharose: 4 = the non-adsorbed fraction for heparin-Sepharose; 5 = the pooled fractions containing the heterodimeric form of RT from the phenyl-Sepharose step; 6 = pure heterodimer RT as marker; 7 = molecular mass markers; 8 = the pooled fractions containing p66 from phenyl-Sepharose; 9 = the major peak containing p66/p51 from S-Sepharose; 10 and 11 = minor peaks from S-Sepharose; 12 = pooled fractions from Poly(A)-Sepharose. kDa = kilodalton.

occurs through the proteolytic activity of bacterial proteases. We therefore optimized the purification process in order to obtain a maximum yield of the heterodimeric p66/p51 RT and to separate it from the unprocessed p66 RT. About 25–35 mg of pure and homogeneous p66/p51 RT, suitable for crystallization experiments, can now be purified from about 50 g of bacterial cells. Instead of rapid purification we extended the time of interaction between bacterial proteases and RT by slowing the purification process in the second chromatographic step. SDS-PAGE followed by silver staining was used to detect the microheterogeneities and other impurities during the purification. Western blotting experiments were done to detect the RT at different stages of purification (Fig. 1).

Purification of the heterodimer p66/p51 RT

The details of the purification are described under Experiment. The purification procedure is summarized in Table I. The purification scheme consists of chromatographic steps in the following order: Q-Sepharose, heparin-Sepharose, phenyl-Sepharose, S-Sepharose, Poly(A)-Sepharose and Q-Sepharose. The presence of protease inhibitors, phenylmethylsulphonyl fluoride (PMSF) and benzamidine, had negligible effect on the proteolytic activity of the bacterial proteases and were therefore excluded during the lysis of the cells. The lysed supernatant was dialysed and then applied to Q-Sepharose fast flow column. RT was obtained in the non-adsorbed fractions and 75% of the bacterial proteins were bound to Q-Sepharose. RT was detected mainly as p66 (Fig. 1, lane 2). In order to

TABLE I

PURIFICATION OF THE HETERODIMER p66/p51 OF HIV REVERSE TRANSCRIPTASE

The chromatographic matrix used in each step in the purification is given in italics. Volume, yield, concentration and percentage of protein recovered in each step are shown. Protein concentration was measured by UV absorbance (1 mg/ml solution of RT has an absorbance of 2.2 at 280 nm) except where indicated by the Bradford method.

Pool	Purification step	Volume (ml)	Total protein (mg)	Protein concentration (mg/ml)	% of total protein
	E. coli lysate	250	3400[a]	13.0	100
I	*Q-Sepharose* non-adsorbed fraction	590	940[a]	1.6	28
II	*Heparin-Sepharose* pool	280	370[a]	1.3	11
III	*Phenyl-Sepharose* peak 1	230	140	0.6	4
IV	*S-Sepharose* major peak	57	51	0.9	1.5
V	*Poly(A)-Sepharose* pool	66	46	0.7	1.4
VI	*Q-Sepharose* non-adsorbed fractions	86	43	0.5	1.3
	Phenyl-Sepharose	10	34	3.4	1.0
	Ultrafiltration	1.6	29	18.0	0.9

[a] Bradford method used.

recover RT in non-adsorbed fractions, the equilibration buffer for Q-Sepharose was optimized to pH 8.0 and to the conductivity equal to that of 0.1 *M* NaCl.

The non-adsorbed fractions were packed with heparin-Sepharose. The column was eluted with a linear gradient of NaCl and the majority of RT was eluted in one broad peak (Fig. 1, lane 3). SDS-PAGE of a number of fractions along the peak showed that the fractions contained both p66 and different forms of the heterodimer (Fig. 2, lanes 2–5). In the non-adsorbed fractions from heparin-Sepharose (Fig. 1, lane 4), Western blot analysis revealed the presence of p66 RT. This was not due to overloading of the column. The lack of affinity for the heparin by some of the p66 RT could instead be due to conformational differences in the RT.

Separation of the heterodimer from p66 fractions was achieved in the next chromatographic step on a phenyl-Sepharose high performance column (Fig. 3). The heterodimeric form eluted earlier than p66 (Fig. 2, lanes 6 and 7), indicating that the heterodimer is less hydrophobic than p66. The amount of the heterodimer material was compared with the p66 material and correlated with the time from the application to the elution from the heparin-Sepharose column.

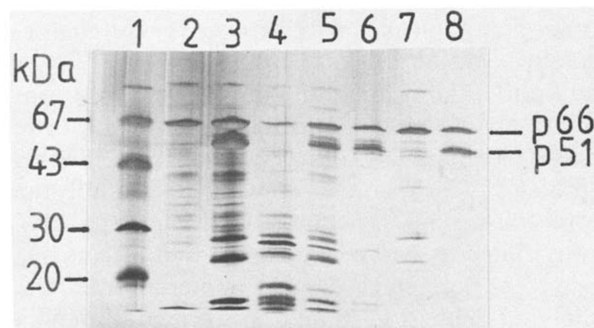


Fig. 2. Analysis of fractions from heparin-Sepharose and phenyl-Sepharose by SDS-PAGE (12.5%). Gel, PhastGel homogeneous 12.5. Samples: lane 1 = molecular mass marker; 2–5 = fractions from eluted peak from heparin-Sepharose (lane 2 = fraction from the start of the peak; 3 = top fraction of the peak; 4 = fraction from the end of the peak; 5 = pooled fractions); 6 = heterodimeric fraction from phenyl-Sepharose (Fig. 3, arrow 6); 7 = p66 fraction from phenyl-Sepharose (Fig. 3, arrow 7); 8 = pure heterodimer RT as marker.

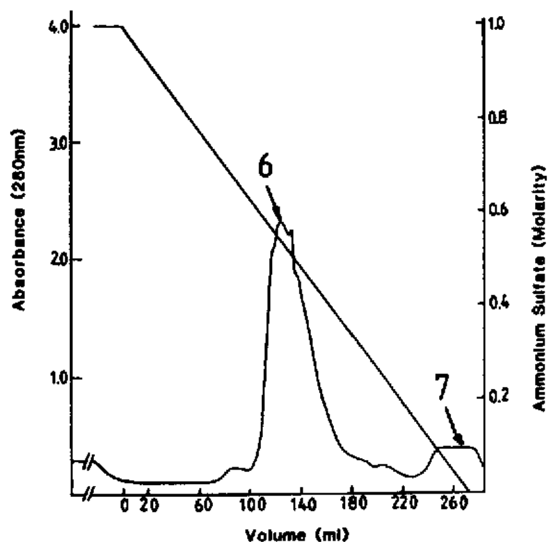
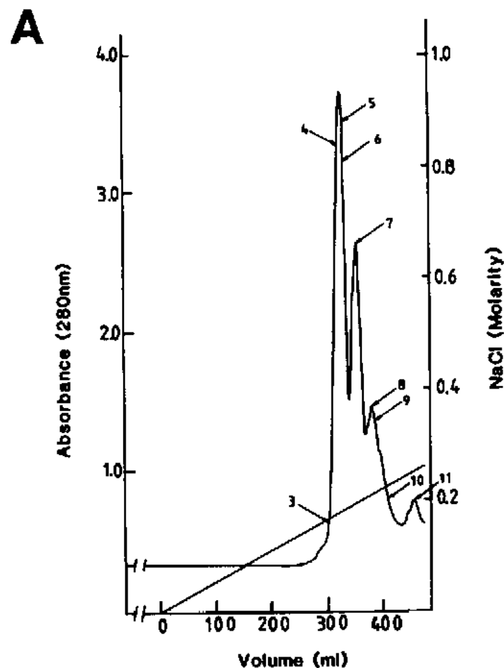


Fig. 3. Hydrophobic interaction chromatography using HiLoad 16/10 phenyl-Sepharose HP. Column, 100 × 16 mm I.D.; flow-rate, 2 ml/min; sample, pooled peak from the heparin-Sepharose column. Elution was performed with a linear gradient (300 ml) from 1.0 to 0.0 *M* ammonium sulphate in the buffer [20 m*M* Tris–HCl–1 m*M* EDTA–1 m*M* DTT (pH 8.0)].

When the heparin-Sepharose step was carried out rapidly within 3 h, the ratio of heterodimer peak to the p66 peak in the subsequent hydrophobic chromatography was estimated to be about 60:40, whereas prolonged application and elution (about 15 h or overnight) of the sample on the heparin-Sepharose column resulted in a ratio of about 80:20.

In the next purification step, on an S-Sepharose high performance column (Fig. 4A), SDS-PAGE (Fig. 4B) indicated that the major peak contained the heterodimer p66/p51 and the remaining peaks consisted of heterodimers where the size of "p51" differed. About 45% of RT was recovered as the exact heterodimeric p66/p51 RT. The other "p51s" were identified as being a fragment of RT by Western blot analysis (Fig. 1, lanes 10 and 11).

When either the lysate or the non-bound-fraction from the Q-Sepharose column was allowed to stand for about 15 h and then subjected to subsequent purification steps, an increase in the heterodimeric products was observed in the hyrophobic interaction chromatographic step. However, further purification of the heterodi-



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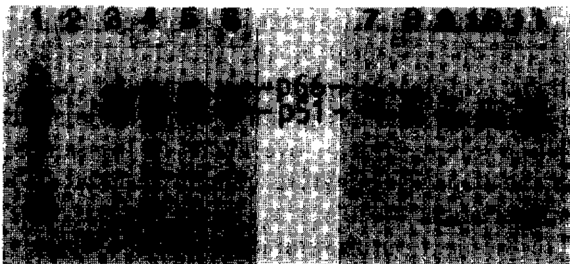

Fig. 4. (A) Cation-exchange chromatography using HiLoad 26/10 S-Sepharose HP. Sample, pooled fractions from phenyl-Sepharose; column, 100 × 26 mm I.D.; fraction volume, 10 ml. Elution was performed with a linear gradient (750 ml) of from 0.0 to 0.25 *M* NaCl in buffer [20 m*M* MES–1 m*M* EDTA–1 m*M* DTT (pH 6.5)]. The fractions indicated by arrows were analysed by SDS-PAGE. (B) Analysis of the fractions from S-Sepharose by SDS-PAGE (12.5%). Gel, PhastGel homogeneous 12.5. Samples: lane 1 = pooled fractions from phenyl-Sepharose; 2 = non-bound fractions of S-Sepharose; 3–11 = different fractions from the S-Sepharose step. The fractions are indicated by arrows 3–11 in the elution profile of S-Sepharose in (A).

meric peak fraction resulted in multiple peaks spread all over the chromatogram from the S-Sepharose column. Each peak fraction con-

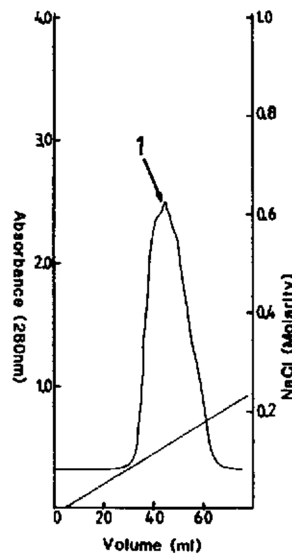

Fig. 5. Affinity chromatography of Poly(A)-Sepharose. Column, 100 × 16 mm I.D.; flow-rate, 0.8 ml/min; sample, pooled peak from S-Sepharose column. Elution was carried out with a linear gradient from 0.0 to 0.25 *M* NaCl in buffer [20 m*M* Tris–HCl–1 m*M* DTT–1 m*M* EDTA (pH 8.0)]. Total volume = 8 × bed volume. RT was eluted in one broad peak (Fig. 6, lane 1).

tained a heterodimer where the size of "p51" varied. The degree of the proteolytic degradation was judged by the chromatographic pattern on S-Sepharose. With the slow purification approach the heterodimer was recovered in only one major peak on S-Sepharose chromatography.

Further purification on Poly(A)-Sepharose was done mainly to separate the p66/p51 RT from small amounts of other DNA/RNA binding proteins which co-eluted with RT in all the preceding steps. The heterodimer p66/p51 RT was eluted as one peak (Figs. 5 and 6, lane 1).

In the last step, the RT material from the Poly(A)-Sepharose column was passed through a Q-Sepharose column (Fig. 7). About 95% of the RT did not bind to the Q-Sepharose (Fig. 6, lanes 3–6), whereas the remaining part of RT was bound to the matrix probably as another heterodimeric form (Fig. 6, lane 7). The non-adsorbed fraction from the Q-Sepharose column was first concentrated on the phenyl-Sepharose column and then further concentrated to 15–18 mg/ml by ultrafiltration. This heterodimeric p66/

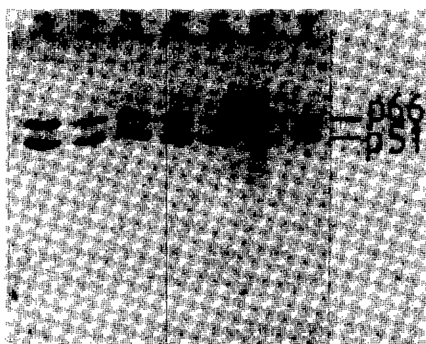

Fig. 6. Analysis of fractions from Poly(A)-Sepharose and Q-Sepharose step by SDS-PAGE (12.5%). Gel, PhastGel homogeneous 12.5. Samples: lane 1 = pooled fractions containing RT from Poly(A)-Sepharose; the fractions analysed in lanes 2–7 are indicated by arrows 2–7 in Fig. 7 (2–6 = non-bound fractions in the second Q-Sepharose step; 7 = bound fractions from Q-Sepharose).

p51 RT that has been processed from p66 by the bacterial proteases had an activity equal to that of RT material from the *pol I* polypeptide, processed by HIV-1 protease [2,3].

This pure and active RT was crystallized in a complex with $tRNA^{Lys3}$, the natural primer of HIV-1 RT. A three-dimensional crystallographic

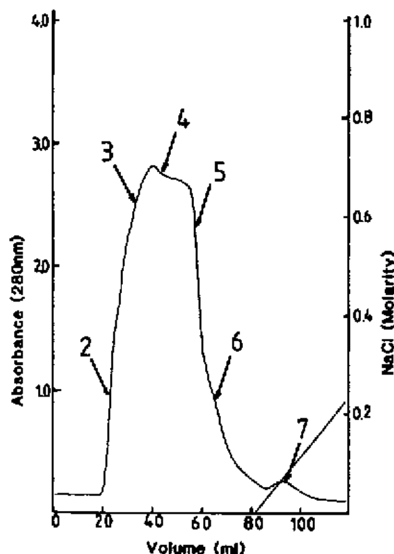

Fig. 7. Anion-exchange chromatography using Q-Sepharose. Sample, pooled fractions from Poly(A)-Sepharose; column, 100 × 10 mm I.D.; fraction volume, 10 ml. RT was recovered in the non-adsorbed fractions. The fractions indicated by arrows were analysed by SDS-PAGE (Fig. 6).

study is in progress using a synchrotron radiation X-ray source at the EMBL outstation in Hamburg. The presence crystals diffract to about 3.8 Å resolution using the X 31 beam in Hamburg. Crystal structure investigations are also in progress in other laboratories and crystals of the heterodimer complexed with a Fab fragment and double-stranded DNA diffracting to about 3.5 Å resolution have been reported [24]. Recently, the crystal structure to 3.5 Å resolution of the heterodimer in a complex with an inhibitor has been presented [25]. However, this structure is not detailed enough to allow for a proper positioning of the amino acid chains. Therefore, all efforts (such as that presented in this paper) which can result in a more homogeneous and thus a better heterodimer material are well justified.

CONCLUSIONS

We have shown that by using a combination of chromatographic techniques the homogeneous p66/p51 RT could be separated (i) from p66 by using hydrophobic interaction chromatography, (ii) from various heterodimeric forms of RT where the size of "p51" differed by cation-exchange chromatography on S-Sepharose and (iii) from various RT forms with different conformations by anion-exchange chromatography on Q-Sepharose. By slowing the purification process in the heparin-Sepharose step, an increase in the yield of the crystallizable heterodimer p66/p51 was achieved. With the present "slow" purification scheme the recovery yield is about 1% of the total protein or about 12% of the expressed p66. The activity of the heterodimer p66/p51, which is processed from p66 only by bacterial proteases, is equal to that of the heterodimer which is obtained by processing the *pol I* polypeptide by HIV-protease in the bacteria. This purification procedure has also been shown to be suitable for various mutants of RT produced in our laboratory.

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