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Renaturation of heterodimeric platelet-derived growth factor from inclusion bodies of recombinant *Escherichia coli* using size-exclusion chromatography

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Abstract

A procedure for renaturation of heterodimeric platelet-derived growth factor (PDGF-AB) from inclusion bodies of recombinant *Escherichia coli* using size-exclusion chromatography is described. Either prepurified or crude PDGF-AB inclusion bodies solubilized with guanidinium hydrochloride were subjected to buffer exchange from denaturing to renaturing conditions during chromatography. Renaturation of PDGF-AB involves folding of the solubilized and unfolded molecules into dimerization competent monomers during size-exclusion chromatography and subsequent dimerization of folded monomers into the biologically active heterodimeric growth factor. Optimized conditions result in an overall yield of 75% active PDGF-AB with respect to size-exclusion chromatography and subsequent dimerization. The described approach allows renaturation at high protein concentrations and circumvents aggregation which is observed when refolding is carried out by dilution. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *Escherichia coli*; Growth factors; Proteins

1. Introduction

Platelet-derived growth factor (PDGF) is a very potent mitogen for cells of mesenchymal origin – e.g. smooth muscle cells, connective tissue cells, and blood cells [1–4]. It is a non-glycosylated all-β sheet protein with a molecular mass of M_r 30 000 and composed of two subunits linked together by two intermolecular disulfide bonds. In addition to intermolecular disulfide bonds, each monomer contains a knot-like arrangement of three intramolecular disulfide bridges [5–7].

Two different monomers, denoted as A- and B-chains, are known to exist in the three natural occurring isoforms PDGF-AA, -AB, and -BB which have apparently different biological functions [3,8–10]. A comparative study on the mitogenic activities of the three different dimeric isoforms revealed the lowest activity for PDGF-AA [11]. The BB and AB isoforms showed similar stimulatory effects on cell proliferation with a slightly higher activity of the heterodimeric form of the growth factor [11]. In addition, the majority of PDGF purified from human platelets is the heterodimeric growth factor [10,12]

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indicating that it may have a higher importance in wound healing processes [4].

The wound healing activity of PDGF renders it to a potential therapeutic agent of pharmaceutical importance [13–16]. Therefore, many efforts have been undertaken to develop a therapeutic agent for non-healing chronical wounds based on the recombinant production of this growth factor using mammalian [17], yeast [18] or bacterial expression systems [11,19–21]. Due to obvious greater difficulties involved in the recombinant production of a heterodimeric protein, the majority of clinical studies on recombinant PDGF has been carried out using the BB homodimer [4]. PDGF-BB has now been approved by the US Food and Drug Administration for treatment of diabetic foot ulcers [22].

While production of PDGF with yeast and mammalian expression systems leads to very low levels of a biologically active protein [17,18], the utilization of bacterial expression systems results in substantially higher yields of the growth factor which is exclusively found as biologically non-active protein in inclusion bodies [11,19–21]. Previously described renaturation procedures of homo- or heterodimeric PDGF from inclusion bodies of recombinant *E. coli* have been carried out with *S*-sulfonated monomers [11,20,21]. It was shown that PGDF-AB heterodimers are almost exclusively formed when renaturation was carried out using an equimolar mixture of the *S*-sulfonated A- and B-chains [11,21].

The objective of this study was the development of a renaturation process for the heterodimeric form of the growth factor which circumvents *S*-sulfonation and allows renaturation at high yields and high protein concentrations. Following a procedure for the preparation of biologically active PDGF-AB produced as inclusion bodies in recombinant *E. coli* using size-exclusion chromatography is described. The production of the recombinant growth factor is directed from a bicistronic expression vector which allows equimolar synthesis of the A- and B-chains in the recombinant bacteria. The renaturation process involves the folding of the solubilized and unfolded molecules at high concentrations into dimerization competent monomers during size-exclusion chromatography and the subsequent dimerization of the folded monomers into the biologically active heterodimeric growth factor.

2. Experimental

2.1. Production and recovery of PDGF-A/B inclusion bodies

The high-cell density cultivation for temperature-inducible production of PDGF-A/B with recombinant *E. coli* TG1:pBS/PDGF-BA2 was performed as described before [23]. The expression vector carrying a bicistronic operon encoding the mature A- and B-chains of human PDGF under the control of the temperature-inducible λ-P_R promoter, pBS/PDGF-BA2, has also been described previously [21]. Product formation in high-cell density cultures was induced at 50 g l^{-1} cell dry mass by a temperature-shift from 30 to 42°C. The culture broth was transferred directly 8 h post-induction from the bioreactor to a high pressure homogenizer for cell disruption (800 bar, SHL 05, Bran-Lübbe). The resulting homogenate was diluted four-fold with 0.05 mol l^{-1} sodium phosphate buffer (pH 7.0) and subsequently passed at a flow-rate of 75 l h^{-1} through a continuously working centrifuge (Westfalia Separator CSA8). The insoluble cell fraction (inclusion bodies and cell debris) was washed three times with 0.02 mol l^{-1} Tris–HCl (pH 8.5), 0.5 mmol l^{-1} EDTA, and 2% (v/v) Triton X-100. After each washing step, the suspension was centrifuged at 8500 *g* and 4°C for 40 min (Cryofuge 20-3, rotor 7790, Heraeus Sepatech). The last centrifugation step was carried out with 25 ml aliquots of the suspension at 10 000 *g* and 4°C for 40 min (Sorvall SS34, DuPont). The remaining pellet fraction containing PDGF-A/B inclusion bodies was stored at −70°C.

2.2. Solubilization of PDGF-A/B inclusion bodies

After thawing, the PDGF-A/B inclusion bodies (from 25 ml aliquots) were resuspended in 5 ml solubilization buffer composed of 6 mol l^{-1} guanidinium hydrochloride (Gnd–HCl), 0.1 mol l^{-1} Tris–HCl (pH 8.5), 0.1 mol l^{-1} dithiothreitol (DTT), and 1 mmol l^{-1} EDTA. The resulting suspension was sonicated for 5 min (50 W, Labsonic U 40T, Braun). After overnight incubation at room temperature, the solution was centrifuged at 26 000 *g* and

4°C for 45 min (Biofuge 28 RS HFA 16.15, Heraeus Sepatech) and the supernatant containing 1.2 mg ml^{-1} PDGF-A/B (=27% of the total protein in the soluble fraction) was immediately subjected to size-exclusion chromatography.

2.3. Size-exclusion chromatography

Chromatography was carried out at room temperature using a size-exclusion column (High Load Superdex 75 pg XK 26/60, Pharmacia) with a bed volume of 348 ml (FPLC system BioLogic, Bio-Rad). All eluents were treated by microfiltration (nitrocellulose filtersheet, 0.1 μm cutoff, Sartorius) and degassed prior to chromatography.

Size-exclusion chromatography for prepurification of solubilized PDGF-A/B inclusion bodies was carried out under denaturing conditions [0.05 mol l^{-1} glycine/H_3PO_4 (pH 3.0), 2 mol l^{-1} Gnd–HCl]. The Gnd–HCl concentration was determined according to Nozaki [24]. The column was loaded with 10 ml of the above inclusion body solution (2.8% of the bed volume) via an injection loop and eluted at 4 ml min^{-1}. The PDGF-A/B containing fractions were collected and concentrated by ultrafiltration (2.5 mg ml^{-1} PDGF-A/B; Centriprep-3, Amicon) prior to renaturation.

Size-exclusion chromatography for renaturation of solubilized and denatured PDGF-A/B was carried out with the following standard buffer system based on reduced (GSH) and oxidized glutathion (GSSG) as redox system to allow disulfide bond formation during renaturation: Eluent A [0.1 mol l^{-1} Tris–HCl (pH 7.8); 4 mol l^{-1} Gnd–HCl] and Eluent B [0.1 mol l^{-1} Tris–HCl (pH 7.8); 10 mmol l^{-1} GSH; 0.25 mmol l^{-1} GSSG].

The equilibrated column (0.5 mol l^{-1} Gnd–HCl; 12% eluent A, 88% eluent B) was loaded with 1 ml solubilized and denatured PDGF-A/B (0.28% of the bed volume) via an injection loop. Samples were allowed to penetrate the column using 10 ml of 2 mol l^{-1} Gnd–HCl, elution conditions were changed afterwards to 0.5 mol l^{-1} Gnd–HCl at a flow-rate of 2 ml min^{-1} to allow renaturation of the growth factor. Solubilized PDGF-A/B inclusion bodies were subjected either directly or prepurified to size-exclusion chromatography under renaturing conditions.

2.4. Heparin chromatography

Heparin chromatography was applied for small-scale final polishing of renatured PDGF-AB (e.g. removal of monomeric PDGF). The column (HiTrap, Pharmacia) with a total volume of 5 ml was equilibrated at room temperature with 0.05 mol l^{-1} Tris–HCl (pH 7.0) using four column volumes. Injection of 1 ml of the renaturation mixture was carried out via an injection loop and the column was washed with four additional column volumes of 0.05 mol l^{-1} Tris–HCl (pH 7.0). A linear gradient of 0–1 mol l^{-1} NaCl in 0.05 mol l^{-1} Tris–HCl (pH 7.0) was used for elution of heterodimeric PDGF-AB (flow-rate of 1 ml min^{-1}, six column volumes).

2.5. Blocking of free thiol groups

Disulfide-bonded heterodimeric PDGF-AB was distinguished from non-disulfide-bonded monomers through disulfide trapping by irreversible blocking of free thiol groups and subsequent gel electrophoresis under non-reducing conditions. Blocking of free thiol groups was carried out by the addition of 0.2 mol l^{-1} iodoacetate (pH 8.7) to an equal volume of the PDGF-A/B containing sample [25].

2.6. Gel electrophoresis

Protein analysis was done by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli [26]. Samples were mixed with sample buffer consisting of 7% (w/v) SDS, 40% (v/v) glycerol, 0.25 mol l^{-1} Tris–HCl (pH 6.8), 0.0005% (w/v) bromophenol blue and 0.15 mol l^{-1} DTT (in some cases when non-reducing conditions were necessary, DTT was not added to the sample buffer). After 10 min boiling, electrophoresis was carried out using precast gels (EXCEL-Gel 8-18, Pharmacia). Gels were stained with Coomassie Brilliant Blue R250 and quantification was carried out by densitometry (Hirschmann elscript 400).

2.7. Fluorescence spectroscopy

Fluorescence emission spectra were obtained at 25°C with a Perkin-Elmer LS50B fluorescence spec-

trophotometer (Perkin-Elmer, Überlingen, Germany) using 0.6 ml quartz cuvettes ($d=0.5$ cm). The excitation wavelength was 280 nm, emission was determined from 300–400 nm.

2.8. N-terminal sequencing

Automated N-terminal sequence analysis was performed on a protein sequencer (470A, Applied Biosystems) with on-line HPLC (12A, Applied Biosystems) via automated Edman degradation.

2.9. Bioassay

Mitogenic activity of PDGF-AB was assayed by the stimulation of [^{3}H]thymidine incorporation into the DNA of serum-depleted BALB 3T3 cells as described previously [27]. The assay was done in triplicate using PDGF-AB in concentrations ranging from 0.5 to 40 ng ml^{-1}. Commercial available PDGF-AB (SIGMA P6684) was used as standard.

2.10. Endotoxin analysis

Endotoxin concentrations in PDGF-AB samples were determined using a commercially available endotoxin test kit (COATEST Endotoxin LAL-test, Endosafe) according to the manufacturers instructions. Since negatively charged endotoxins bound to basic proteins (PDGF, p*I* 9.8–10.2 [2]) are not accessible by this test, samples were treated prior to analysis with proteinase K for the determination of the total endotoxin content [28].

3. Results and discussion

3.1. Purification of unfolded and reduced PDGF-A and -B monomers using size-exclusion chromatography

Inclusion bodies were harvested, washed with detergents, and solubilized by Gnd–HCl as described in Section 2. Subsequently, solubilized and reduced inclusion body proteins were subjected to size-exclusion chromatography under denaturing conditions for further purification of PDGF-A and -B monomers. A typical chromatogram indicates effective separation of monomeric PDGF-A/B from protein contaminants present in the solubilized inclusion bodies, as well as from the additives EDTA and DTT (Fig. 1). In addition, SDS–PAGE analysis of the PDGF-A/B containing fractions from the different steps of the purification procedure confirmed that no other protein contaminants were present in the PDGF-A/B containing eluate fraction collected after size-exclusion chromatography under denaturing conditions (Fig. 2).

The prepurified inclusion bodies containing pure unfolded and reduced PDGF-A/B monomers in 2 mol l^{-1} Gnd–HCl were subjected to native conditions either by dilution or by buffer exchange using size-exclusion chromatography to study the renaturation of the growth factor in the absence of contaminating proteins.

3.2. Studies on renaturation of heterodimeric PDGF-AB using the dilution method

Experiments on the renaturation of PDGF-AB were first carried out using the dilution method. Prepurified PDGF-A/B monomers (in 2 mol l^{-1} Gnd–HCl) were rapidly diluted into standard renaturing buffer (0.5 mol l^{-1} Gnd–HCl, 25°C) at varying final protein concentrations. These experiments revealed that aggregation was a major side reaction at all protein concentrations studied. Even at protein concentrations as low as 10 μg ml^{-1} PDGF-A/B, only 45% of the expected total protein fluorescence (corresponding to approximately 45% soluble growth factor) was present in the renaturation mixture after 20 h of incubation and subsequent removal of aggregates by ultrafiltration (M_r 100 000 cutoff filter, Microcon-100, Amicon). These discouraging results stimulated us to search for another procedure allowing renaturation at higher protein concentrations.

3.3. Renaturation of heterodimeric PDGF-AB using size-exclusion chromatography

Renaturation of the growth factor was initiated by subjecting prepurified PDGF-A/B monomers (2.5 mg ml^{-1} PDGF-A/B in 2 mol l^{-1} Gnd–HCl) to size-exclusion chromatography under renaturing conditions. At first, the protein solution was allowed to

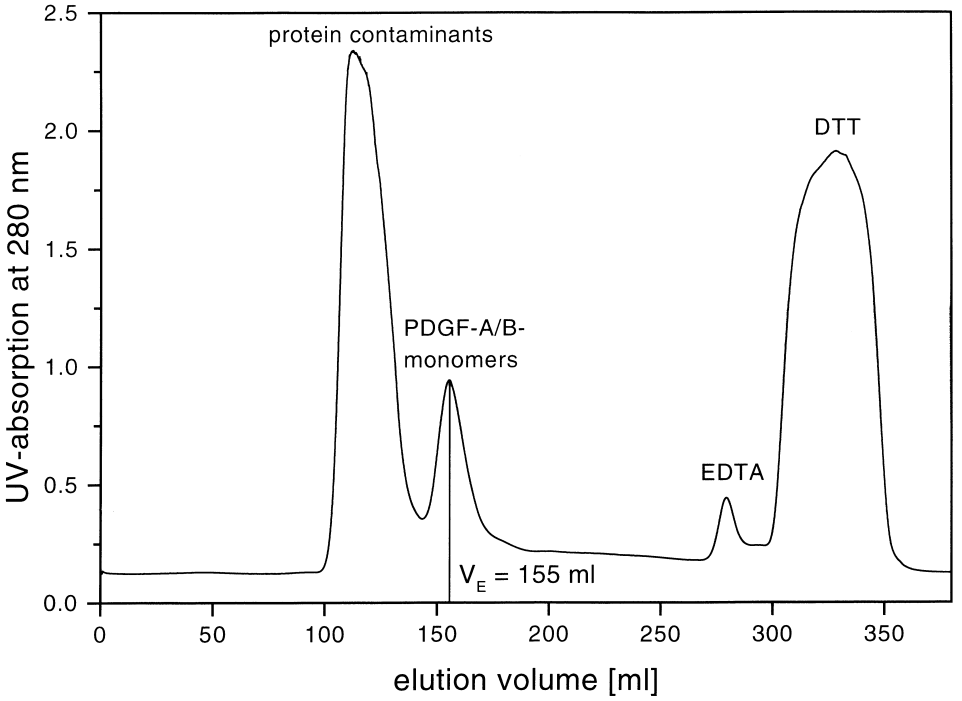

Fig. 1. Prepurification of Triton X-100 washed PDGF-A/B inclusion bodies under denaturing conditions (2 mol l^{-1} Gnd–HCl) using size-exclusion chromatography. The amount injected was 10 ml of solubilized, non-prepurified inclusion bodies containing 1.2 mg l^{-1} PDGF-A/B (=27% of the total protein in the soluble fraction). Conditions were as described in Section 2.

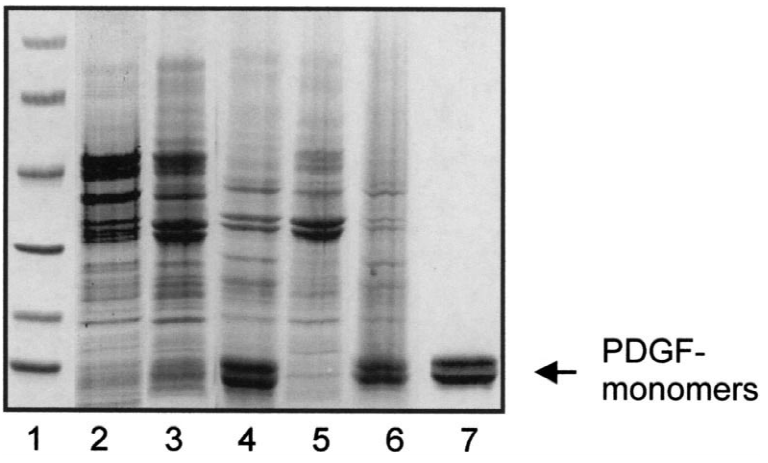

Fig. 2. Prepurification of PDGF-A/B inclusion bodies. SDS–PAGE analysis of PDGF-A/B containing fractions from different stages of the inclusion body purification procedure. Lanes: 1=Low-molecular-mass marker proteins (M_r): 94 000 (top), 67 000, 43 000, 30 000, 20 100, 14 400; 2=Total cell protein; 3=Crude inclusion bodies (insoluble cell protein); 4 and 5=Insoluble and soluble fractions, respectively, after washing crude inclusion bodies two times with Triton X-100; 6=Soluble fraction after solubilizing Triton X-100 washed inclusion bodies with 6 mol l^{-1} Gnd–HCl; 7=PDGF-A/B containing eluate fraction after subjecting Triton X-100 washed and Gnd–HCl solubilized inclusion bodies to size-exclusion chromatography under denaturing conditions (see Fig. 1).

penetrate the column under denaturing conditions as described in Section 2. Thereafter, elution conditions were changed to renaturing conditions. A typical chromatogram under standard renaturing conditions (0.5 mol l^{-1} Gnd–HCl, 10 mmol l^{-1} GSH and 0.25 mmol l^{-1} GSSG) is depicted in Fig. 3. The main peak (V_e=161±1 ml) corresponds to monomeric PDGF-A/B (see also Fig. 4). Elution of PDGF-A/B as monomeric protein after subjecting the unfolded and reduced monomers to size-exclusion chromatography under renaturing conditions was additionally confirmed when a mixture of the monomeric and heterodimeric form of the growth factor was injected and eluted using the described conditions (data not shown). A further PDGF containing fraction was eluted at a volume of V_e=108 ml corresponding to the void volume of the column (V_0=122 ml, determined with Blue dextran). This fraction was attributed to PDGF-A/B aggregates formed during chromatography on the column which are too large to penetrate the pores and, moreover, are too large to occupy the entire interparticle space. However, aggregation of PDGF-A/B during size-exclusion chromatography under renaturing conditions was only observed to a low extent (≈10% aggregation at 2.5 mg ml^{-1} PDGF-A/B), in contrast to renaturation of the growth factor using the dilution method (≈55% aggregation at 10 μg ml^{-1} PDGF-A/B). In addition, aggregates were removed efficiently from the folded monomeric growth factor during chromatography. The recovery of PDGF-A/B (soluble monomers and aggregates) collected after size-exclusion chromatography under renaturing conditions was always higher than 90% of the protein that was loaded onto the column.

Aggregation was influenced by the amount of protein loaded onto the column, e.g. doubling the protein concentration from 2.5 to 5 mg ml^{-1} increased aggregation from 10 to 20%, respectively. Also, replacing Gnd–HCl by NaCl (both at 0.5 mol l^{-1}) increased aggregate formation from 10 to 60%, respectively. However, variations in the con-

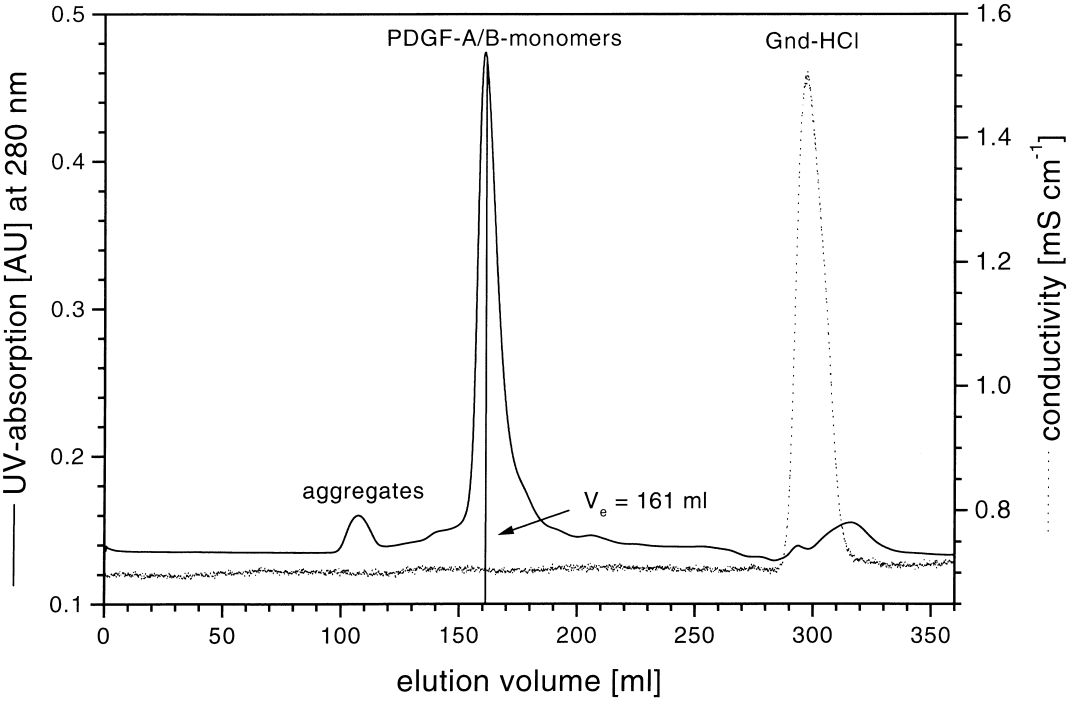

Fig. 3. Renaturation of prepurified PDGF-A/B inclusion bodies under standard renaturing conditions (0.5 mol l^{-1} Gnd–HCl) using size-exclusion chromatography. The amount injected was 1 ml of prepurified inclusion bodies containing 2.5 mg l^{-1} PDGF-A/B. Conditions were as described in Section 2.

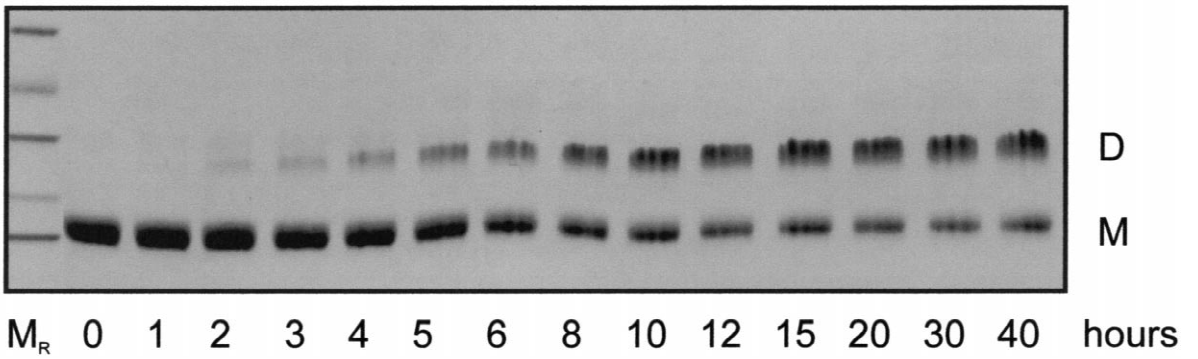

Fig. 4. Dimerization of PDGF-A/B monomers after renaturing size-exclusion chromatography. SDS–PAGE analysis of the PDGF-A/B containing eluate fraction after size-exclusion chromatography of prepurified PDGF-A/B inclusion bodies under standard renaturing conditions. Aliquots from the eluate were taken at the indicated times and free thiol groups were immediately blocked by the addition of iodoacetate. Samples were electrophoresed under non-reducing conditions to monitor the time-dependent formation of disulfide-bonded heterodimeric PDGF-AB. M and D denote the positions of the monomeric and dimeric form of the growth factor and M_r the lane of the low-molecular-mass marker (M_r): 67 000 (top), 43 000, 30 000, 20 100, 14 400.

centration or ratio of the redox system components or the pH (pH 7.8–8.7) did not show any effect on aggregation during chromatography. Following chromatography, aggregation of PDGF-A/B in the eluate fraction corresponding to the monomeric growth factor did not occur at any of the conditions studied.

When size-exclusion chromatography under renaturing conditions was carried out with Gnd–HCl concentrations below 0.5 mol l^{-1} (without substituting Gnd–HCl by NaCl), multiple peak formation was observed with a main peak (e.g. V_e=169 ml, 0.25 mol l^{-1} Gnd–HCl) and multiple minor tailing peaks (data not shown). All peak fractions analyzed correspond to the monomeric form of the growth factor which is able to dimerize to heterodimeric PDGF-A/B.

Treatment of aliquots taken at different time points from the PDGF-A/B containing eluate fraction with iodoacetate for irreversible blockage of free thiol groups and subsequent SDS–PAGE analysis under non-reducing conditions revealed that the eluted monomeric form of the growth factor was able to dimerize via disulfide bridge formation to yield heterodimeric PDGF-AB (Fig. 4). However, dimerization of monomeric PDGF-A/B to the heterodimeric form of the growth factor is a slow process occurring in the time-scale of hours.

A number of variables such as protein concentration, temperature, concentration of Gnd–HCl, and ratio and concentration of reduced to oxidized glutathion were analyzed with respect to their impact on the formation of heterodimeric PDGF-AB in the eluate fraction (Table 1). The highest yield of heterodimeric PDGF-AB (≈90%) was obtained in the absence of Gnd–HCl or with a 10:1 ratio of reduced to oxidized glutathion. Low temperature and high Gnd–HCl concentrations resulted in poor dimerization yields, whereas the initial concentration of PDGF-A/B monomers in the eluate fraction did not affect the final yield of the heterodimeric growth factor.

Considering that around 90% of the unfolded and reduced PDGF-A/B applied to size-exclusion chromatography under renaturing conditions elutes as folded monomeric protein, a yield of about 75% heterodimeric PDGF-AB can be reached by combining chromatography with subsequent dimerization.

A further simplification of the renaturation procedure was achieved by subjecting Gnd–HCl solubilized and reduced, but non-prepurified PDGF-A/B inclusion body proteins to size-exclusion chromatography under standard renaturing conditions (Fig. 5). The chromatogram and the SDS–PAGE analysis of the different eluate fractions show that the majority of host cell proteins elute prior to the refolded PDGF-A/B monomers, but the PDGF-A/B containing eluate fraction is not completely devoid of contaminating proteins (Fig. 5). However, enhanced

Table 1
Comparison of different parameters on the extent of dimerization of PDGF-A/B monomers after size-exclusion chromatography under renaturing conditions

Parameter[a]	Value	Dimerization yield (%)[b]
PDGF-A/B (μmol l^{-1})	1.9	59
	8.9	64
	47.0	63
Temperature (°C)	4	17
	25	64
Gnd–HCl (mol l^{-1})	0	93
	0.25	73
	0.50	64
	0.80	35
	1.20	17
	1.50	13
Redox system	10, 0.25; 40:1	64
GSH (mmol l^{-1}), GSSG (mmol l^{-1});	5, 0.25; 20:1	62
ratio GSH:GSSG	5, 0.5; 10:1	89
	5, 1; 5:1	57

[a] Standard conditions: 8.9 μmol l^{-1} PDGF-A/B, 0.5 mol l^{-1} Gnd–HCl, 10 mmol l^{-1} GSH, 0.25 mmol l^{-1} GSSG, 25°C, pH 7.8 (temperature and PDGF-A/B concentrations refer to the conditions during dimerization in the eluate fraction after size-exclusion chromatography). The column was loaded with 2.5 mg ml^{-1} prepurified PDGF-A/B monomers (in 2 mol l^{-1} Gnd–HCl).
[b] The standard deviation was less then 5%.

aggregation of PDGF-A/B was not observed when non-prepurified but solubilized and reduced inclusion body proteins were subjected directly to size-exclusion chromatography under renaturing conditions. In addition, PDGF-A/B monomers in the eluate fraction were able to dimerize in the same manner and to the same extent as shown for the prepurified monomers indicating that the contaminants do not influence the correct folding to the biologically active protein. Reduced renaturation yields which have been reported for the refolding of hen egg white lysozyme in the presence of inclusion body contaminants during renaturation by dilution [29] were not observed during renaturation of PDGF-A/B using size-exclusion chromatography.

3.4. Characterization of renatured heterodimeric PDGF-AB

Characterization of renatured PDGF-AB was carried out after small-scale polishing using Heparin chromatography as described in Section 2.

N-terminal sequence analysis demonstrated a homogenous N-terminus for the PDGF-A and -B chains which were identical with the sequence of the authentic human PDGF-A and -B chains and devoid of the leading bacterial methionine (PDGF-A chain: SIEEAVPAVCKT; PDGF-B chain: SLGSLTIAEPAM).

Fluorescence emission spectra obtained from denatured PDGF-A and -B chains in 6 mol l^{-1} Gnd–HCl and renatured heterodimeric PGDF-AB revealed the same red-shift from 347 to 351 nm, respectively, in the maximum of fluorescence emission (data not shown) as has been reported previously for the BB homodimer [18].

The renatured recombinant PDGF-AB was also tested for its mitogenic activity on 3T3 cells. For comparison, commercial available PDGF-AB (Sigma P6684, Germany) was used as standard. Half maximal stimulation of [^{3}H]thymidine incorporation into the DNA of serum-depleted cells was observed at 3.5 and 5.5 ng ml^{-1} PDGF-AB for the renatured growth factor of this study and the commercial standard, respectively (Fig. 6). These activities are in accordance with those reported previously for PDGF-AB (3–5 ng ml^{-1} [11,30]).

Finally, the renatured PDGF-AB was analyzed for

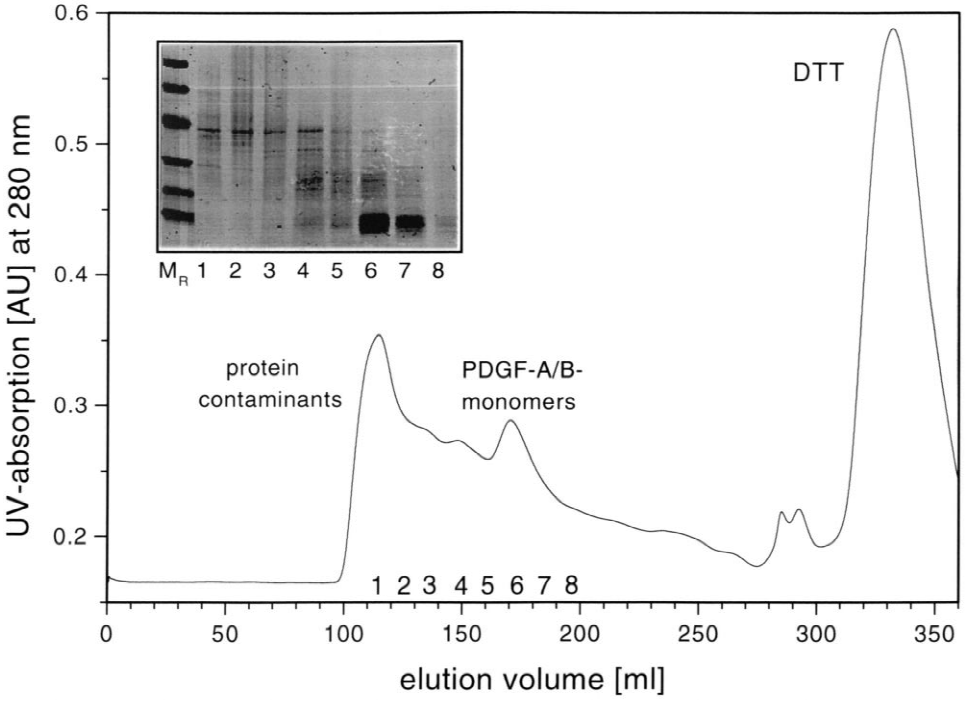

Fig. 5. Renaturation of Triton X-100 washed, non-prepurified PDGF-A/B inclusion bodies by size-exclusion chromatography under standard renaturing conditions (0.5 mol l^{-1} Gnd–HCl). The amount injected was 1 ml of solubilized, non-prepurified inclusion bodies containing 1.2 mg l^{-1} PDGF-A/B (=27% of the total protein in the soluble fraction). Conditions were as described in Section 2. Insert: SDS–PAGE analysis of eluate fractions. M_r denotes the lane of the low-molecular-mass marker (M_r): 94 000 (top), 67 000, 43 000, 30 000, 20 100, 14 400. The other lanes correspond to eluate fractions marked in the chromatogram with the same numbers.

the presence of endotoxins. Free endotoxin concentrations were found to be 3 ng mg^{-1} PDGF-AB (0.2 mg ml^{-1} PDGF-AB). Proteolytic digestion of PDGF-AB with proteinase K and subsequent determination of the total endotoxin concentration revealed 17 ng mg^{-1} PDGF-AB indicating that the majority of endotoxins was tightly bound to the growth factor. Since the upper limit for endotoxins are 0.5 ng kg^{-1} bodyweight [31] and clinical administrations of PDGF are in the μg range [14], the remaining contamination of the renatured growth factor with endotoxins appears to be acceptable.

4. Conclusions

Size-exclusion chromatography under renaturing conditions represents a two-step procedure for the renaturation of heterodimeric PDGF-AB (or probably more general for multimeric proteins) produced as inclusion bodies in recombinant *E. coli*. The first step represents the folding of solubilized and unfolded protein chains into dimerization competent monomers during chromatography. The second step involves the dimerization of competent monomers into the heterodimeric growth factor after chromatography. This two-step procedure offers independent possibilities for optimizing both steps (minimal aggregation at high protein concentrations during chromatography and maximal dimerization after chromatography).

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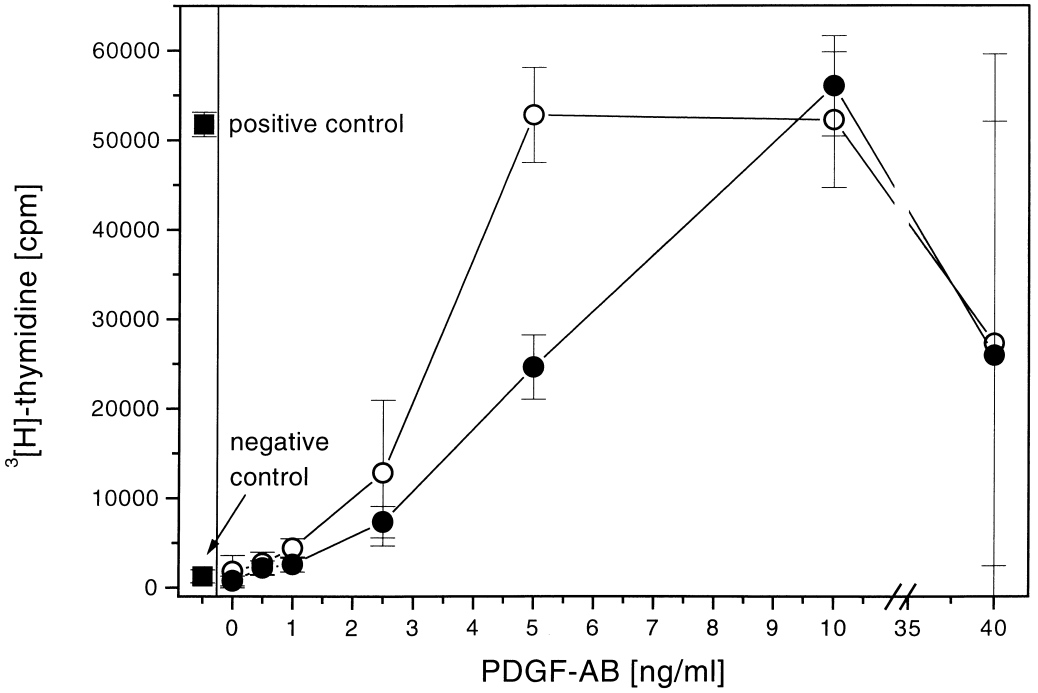

Fig. 6. Determination of the biological activity of PDGF-AB. Stimulation of [^{3}H]thymidine incorporation into the DNA of serum-depleted BALB 3T3 cells by PDGF-AB. Comparative studies on the mitogenic activity of PDGF-AB after fine-polishing using Heparin chromatography (○) and of commercial available PDGF-AB (Sigma P6684) (●). Positive and negative controls for the mitogenic activity measurements were the stimulation of [^{3}H]thymidine incorporation into the DNA of serum-depleted BALB 3T3 cells by 20% fetal calf serum and phosphate buffered saline, respectively.

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