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Multiple peak formation from reversed-phase liquid chromatography of recombinant human platelet-derived growth factor

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ABSTRACT

Reversed-phase liquid chromatography of recombinant platelet-derived growth factor (PDGF) results in the appearance of at least four distinguishable peaks. The relative areas of these peaks are, in part, dependent upon the gradient time and the temperature. Isolation and reinjection of each peak gave chromatographic profiles comparable to that obtained from unfractionated PDGF. Increasing the temperature above 60°C resulted in a single peak that, when isolated and reinjected at ambient temperature, produced a chromatogram comparable to PDGF which had not been exposed to elevated temperature. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that all four peaks had the same molecular mass as PDGF and were active as determined by a PDGF mitogenic bioassay. These results indicate that multiple conformations of PDGF are present and we postulate that their appearance may be a result of isomeric structures arising from the presence of Pro–Pro bonds within the primary structure of the protein.

INTRODUCTION

PDGF is one of a growing number of growth factors produced by recombinant protein techniques for possible use in wound healing. It has a molecular mass of *ca.* 28 000 and consists of two polypeptide chains denoted A and B which are about 50% similar in amino acid composition. These chains are linked by interchain disulfide bonds. The dimeric structure is essential for biological activity since upon reduction, PDGF is inactivated. In addition to the heterodimer, homodimers of the A and of the B chains have also been found in nature [1]. The amino acid sequence for PDGF-B monomer is shown in Fig. 1.

Recombinant derived proteins are often subjected to a variety of electrophoretic and chromatographic techniques in order to show that they are homogeneous and devoid of impurities. Commonly used techniques include: sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing, and ion-exchange, hydrophobic-interaction and reversed-phase chromatography. The latter chromatographic technique has been the subject of numerous studies aimed at defining column and eluent parameters in order to better understand the retention process involved in these separations [2–11].

Ser-	Leu-	Gly-	Ser-	Leu-	Thr-	Ile-	Ala-	Glu-	Pro-	10
Ala-	Met-	Ile-	Ala-	Glu-	Cys-	Lys-	Thr-	Arg-	Thr-	20
Glu-	Val-	Phe-	Glu-	Ile-	Ser-	Arg-	Arg-	Leu-	Ile-	30
Asp-	Arg-	Thr-	Asn-	Ala-	Asn-	Phe-	Leu-	Val-	Trp-	40
Pro-	Pro-	Cys-	Val-	Glu-	Val-	Gln-	Arg-	Cys-	Ser-	50
Gly-	Cys-	Cys-	Asn-	Asn-	Arg-	Asn-	Val-	Gln-	Cys-	60
Arg-	Pro-	Thr-	Gln-	Val-	Gln-	Leu-	Arg-	Pro-	Val-	70
Gln-	Val-	Arg-	Lys-	Ile-	Glu-	Ile-	Val-	Arg-	Lys-	80
Lys-	Pro-	Ile-	Phe-	Lys-	Lys-	Ala-	Thr-	Val-	Thr-	90
Leu-	Glu-	Asp-	His-	Leu-	Ala-	Cys-	Lys-	Cys-	Glu-	100
Thr-	Val-	Ala-	Ala-	Ala-	Arg-	Pro-	Val-	Thr-	Arg-	110
Ser-	Pro-	Gly-	Gly-	Ser-	Gln-	Glu-	Gln-	Arg		120

Fig. 1. Amino acid sequence of recombinant human PDGF-BB.

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While developing a reversed-phase liquid chromatography (RPLC) method for PDGF, multiple peak formation was routinely observed regardless of the column source or gradient elution scheme used. This paper describes this unusual behavior for this protein. A proposed explanation for this observation is based upon *cis–trans* isomerization of peptide bonds containing Pro residues taking place within the time course of the chromatographic separation.

MATERIALS AND METHODS

The liquid chromatographic equipment consisted of a Model 8700 pump (Spectra-Physics), a Model SPG 6A variable-wavelength UV detector (Shimadzu) and a Chromjet integrator (Spectra-Physics). The columns used included a C_4 Vydac (250 × 4.6 mm I.D.; The Separations Group) and a TSK gel Octadecyl-NPR (35 × 4.6 mm I.D.; Toso Haas). The flow-rate was 1.0 ml/min in all experiments. Temperature control during the heating and cooling experiments was carried out using a column temperature controller (Anspec). Cooling experiments used an independent Nesslab Model RTE110 to regulate the temperature. PDGF used in this study is a homodimer of the B chain (human sequence, Fig. 1) and is derived from a bacterial expression system. SDS-PAGE was performed in 15% gels according to Laemmli [12]. A mitogenic assay was used to detect bioactivity of PDGF [13].

RESULTS

A number of procedures were used to established the homogeneity of PDGF and to aid in its characterization. These included SDS-PAGE, hydrophobic interaction chromatography, and size exclusion chromatography. Under these conditions a single component was obtained. When PDGF was chromatographed under RPLC conditions, however, a heterogeneous elution profile with typically four peaks, was present regardless of the source of the column, pore size, length, or whether the ligand was C_4 or C_{18} and was reproducible over a prolonged period. Fig. 2A and B show the chromatograms obtained when Toso Haas NPR C_{18} and Vydac C_4 columns were used. Peak ratios were dependent on the length of time for the gradient with longer gradient times favoring an increase in the slowest eluting peak.

The Toso Haas C_{18} column allowed for greater flexibility in manipulating conditions of gradient time and temperature, and all subsequent studies

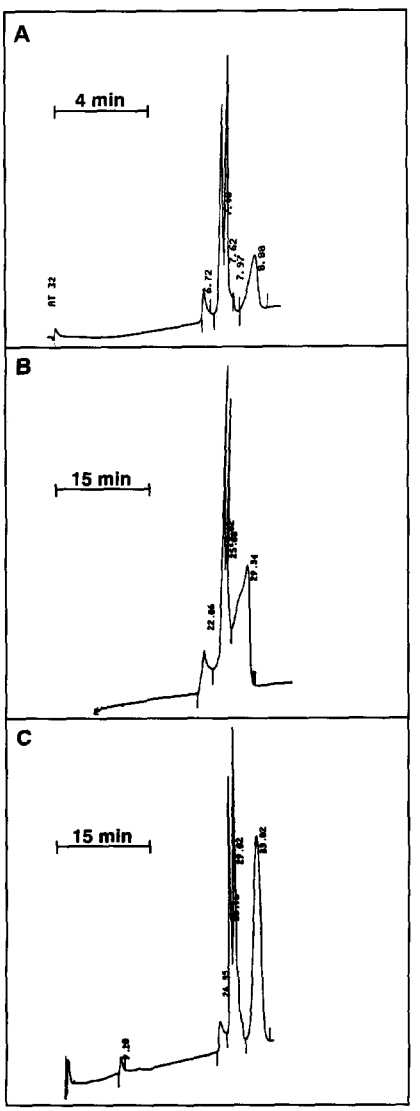

Fig. 2. RPLC profiles of PDGF-BB as a function of column type and length of gradient. (A) Toso Haas NPR C_{18}, gradient time 10 min; (B) Vydac C_4, gradient time 40 min; (C) Toso Haas NPR C18, gradient time 40 min. eluent A, 0.1% trifluoroacetic acid in water; B, 0.1% trifluoroacetic acid in acetonitrile, gradient from 12% to 15% B.

were carried out using this column. The Toso Haas C_{18} column is a polymer based micropellicular support with a relatively low binding capacity for proteins. A series of increasing amounts of PDGF from 5 to 23 μg did not affect the elution profile to any significant extent establishing that column overloading was not a contributing factor to multiple peak formation.

To examine the effect of column contact time, PDGF was injected and after a period of 30 s the flow-rate was maintained isocratically for 60 min at which time the gradient elution was started. The results showed that the relative amount of peak 4 increased by a factor of two while both peak 1 and peak 2 almost decreased in half. The influence of the column eluent on PDGF was also evaluated. This was performed in several ways. First, PDGF was incubated in 0.1% TFA for periods up to 24 h. Comparable chromatograms were obtained at all times examined indicating that acidic conditions did not effect the chromatographic behavior of PDGF. Second, PDGF was incubated in trifluoroacetic acid–water–acetonitrile (0.1:62.9:37), corresponding to the eluent composition where peak 4 eluted. After a period of 60 min, the sample was treated in one of two ways. In the first, dilution with 0.1% trifluoroacetic acid was made to achieve approximate initial eluent composition. In the second, the sample was injected directly. Evaluation of the elution profiles showed that no change had occurred.

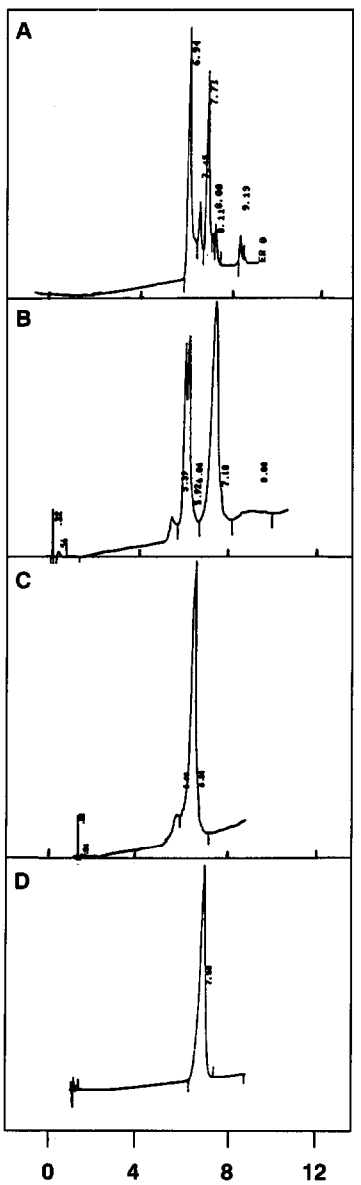

Fig. 3. RPLC profiles of PDGF-BB as a function of temperature. (A) −5°C, (B) 40°C, (C) 60°C, (D) 99°C.

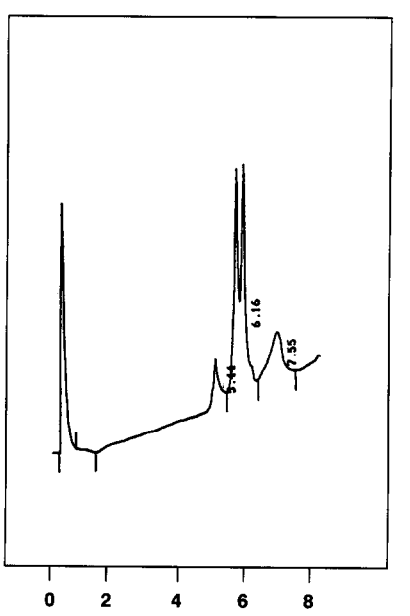

Fig. 4. Elution profile of PDGF-BB peak collected from 60°C chromatography (see Fig. 2C).

More dramatic changes were observed when temperature was varied. At −5°C, the profile shifted in favor of early eluting peaks. Because of the shifts in retention times, it was difficult to ascertain which of the early eluting peaks present at room temperature was increasing in intensity. As temperatures increased to 25, 40 and 60°C, there was a slight decrease in the retention times of the peaks with the peak profile shifting in favor of the later eluting peaks until at 60°C, a single peak was present and at 99°C this peak sharpened considerably (Fig. 3). Throughout these temperature evaluations, the total peak area remained essentially constant. The major peak remaining at 60°C was isolated and the eluent removed under vacuum. The material was dissolved in 0.1% TFA and injected into a column at room temperature. The peak profile observed (Fig. 4) was comparable to that obtained from unfractionated PDGF.

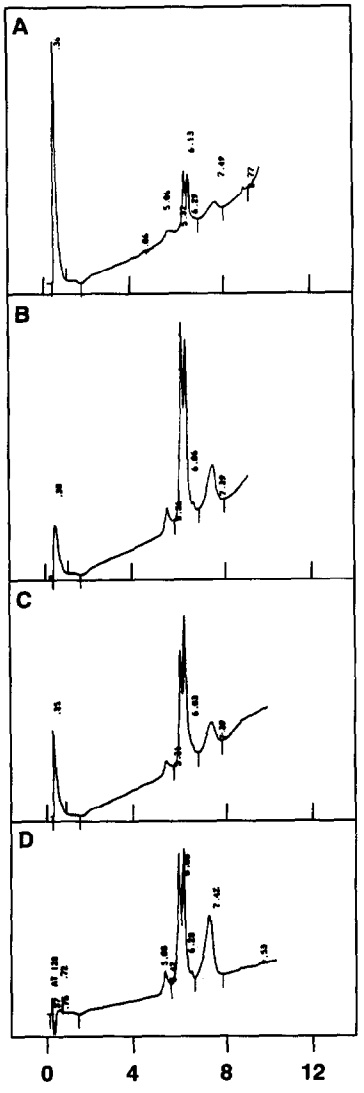

Fig. 5. Elution profile of isolated four peaks present in Fig. 2A. Elution conditions as in Fig. 2A. (A) Peak at 6.72 min; (B) peak at 7.40 min; (C) peak at 7.62 min; (D) peak at 8.88 min.

TABLE I

RELATIONSHIP OF BIOACTIVITY TO PEAK AREA FOR PDGF FRACTIONATED BY RPLC

Peak No.	t_R[a] (min)	Units bioactivity	Peak area	Bioactivity/area
1	6.72	44	1000	0.044
2	7.40	124	3550	0.035
3	7.62	116	3010	0.039
4	8.88	126	3660	0.034

[a] Retention times.

Four individual peaks obtained from RPLC of PDGF at room temperature were isolated, dried under vacuum, and redissolved in 0.1% TFA. Reinjection resulted in essentially the same elution profile as present in unfractionated PDGF (Fig. 5). From SDS-PAGE, it was determined that all four fractions had the same molecular mass as each other and with PDGF. The mitogenic bioassay showed that all fractions were active and when these results were expressed on an area basis, as determined from the RPLC chromatogram, the activity per unit area was essentially equal in all fractions (Table I).

DISCUSSION

In the majority of instances involving RPLC of proteins, a single peak is present which is accepted as complementary evidence for product purity. In recent years, it has been shown that a seemingly pure protein can give rise to multiple peaks for a variety of reasons, a quite common one being due to the existence of conformational changes caused by the hydrophobic column matrix disrupting the native protein structure. This effect has been demon-

stated to occur with papain [14], ribonuclease [15] and lysozyme [16] amongst others.

When PDGF was examined by SDS-PAGE, hydrophobic-interaction and size-exclusion chromatography the data showed that a single component was present. RPLC, on the other hand, consistently showed that at least four species were present with a wide range of hydrophobicities. Similar results were obtained from two quite dissimilar columns, Vydac C_4 and Toso Haas NPR C_{18} indicating that the effect was caused by the reversed-phase process and was not specifically related to a single column source. Increased column contact time favored a shift in peak profiles toward the later eluting peak, peak 4. Results from incubation studies and magnitude of the peak shift indicated that these were not the major cause leading to the formation of multiple peaks. Raising and lowering the temperature produced more dramatic changes in elution profiles. When the temperature was raised, the number of peaks decreased until a single peak existed. Isolation and reinjection of this peak resulted in the appearance of the same initial four peaks when RPLC was performed at ambient temperature. Lowering the temperature has the opposite effect on peak profiles favoring the presence of early eluting peaks. Isolation of the four peaks present at room temperature followed by their reinjection resulted in the reappearance of the same four initial peaks from unfractionated PDGF. In addition, SDS-PAGE demonstrated that all four peaks had the same migration as each other and with intact PDGF. The activity data indicated that all four fractions had essentially the same activity when corrected for relative amounts present (Table I). Taken together, these results suggest that PDGF exists in a variety of conformational states which are separable during the time course of chromatography.

What might give rise to these conformational states? While PDGF contains a number of proline residues, a Pro–Pro sequence at positions 41–42 [1] stands out as a possible site for *cis–trans* isomerization (Fig. 1). The presence of a Pro–Pro sequence is more likely than any single Pro containing sequence to elicit *cis–trans* isomerization. In PDGF, these residues are located in a hydrophobic region of the primary structure (...Ala–Asn–Phe–Leu–Val–Trp–Pro–Pro–Cys–Val...) and are bounded by a Trp residue and a Cys residue involved in a disulfide linkage. In such an environment, *cis–trans* isomerization of Pro residues might be expected to be relatively slow and the various forms could be observed in real time, *e.g.*, during the RPLC process. The multiple Pro residues in PDGF together with the results from heating and cooling chromatographic processes provide possible explanation for the presence of the multiple forms of PDGFduring reversed phase chromatography. The ...Trp–Pro–Pro-Cys... sequence is also found in human angiogenin [17]. This protein gives rise to a doublet peak on RPLC on a C_{18} column presumably a result of a reversible conformational change in the protein (see footnote in ref. 17). A tryptic fragment containing this sequence also gives rise to two peaks on reversed phase chromatography, however, the interpretation for this duplex is complicated in part by the presence of two peptides joined by a disulfide linkage.

Jacobsen *et al.* [18] has shown that the *cis* isomer of Pro has a more hydrophobic surface area than the *trans* isomer and can interact to a much greater extent with hydrophobic ligands on columns. As a result, *cis–trans* conformers of proline containing peptides can be separated by RPLC [19]. The existence of multiple peaks from proline containing peptides has been explained as being directly related to the slow kinetics of isomerization of *cis–trans* conformers that occurred during the time course of the chromatographic separation. Peptide bonds that include proline are characterized by their relative rigidity as compared with other amino acids. Melander *et al.* [20] showed that temperature, pH and flow velocity were involved in affecting the peak shape of these proline containing peptides upon RPLC.

Many studies have suggested that proline *cis–trans* isomerism plays a central role in the folding of proteins. As a result, proline *cis–trans* isomerism has been directly implicated in the formation of multiple conformations of protein in solution [21–25]. Using conformational energy calculations, Levitt [26] demonstrated the existence of three types of proline residues in the folded, native state of bovine pancreatic trypsin inhibitor. In the first type, proline residues can isomerize freely; in the second type, proline isomerization destabilizes the native conformation, but not enough to disrupt the overall conformation; in the third type, isomerization is so disruptive as to prevent the proper protein confir-

mation without the proper proline isomer. Definitive evidence for proline *cis–trans* isomerism is readily achieved with small peptides, however, for larger proteins such information is much more difficult to obtain. *cis–trans* isomerism of proline in staphylococcal nuclease and in calbindin D_{9k} have been recently shown by two-dimensional ^1H NMR to be the source of conformational heterogeneity in the folded form of these proteins [27]. The use of NMR for demonstrating distinct conformations is not a realistic option of DGF at the present time. PDGF contains multiple prolines of which Pro–Pro at positions 41–42 is proposed to be a likely site for *cis–trans* isomerization. Replacement of these proline residues, either individually or together could provide indirect evidence for the presence of proline *cis–trans* isomerization should the resulting RPLC profile be simplified. While it is not presently possible to prove definitively that the existence of Pro–Pro bonds are a direct cause of the multiple peak formation observed with PDGF, the experimental evidence, nevertheless, provides some indication that such a possibility exists.

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