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Purification of sufficiently γ-carboxylated recombinant protein C and its derivatives

Calcium-dependent affinity shift in immunoaffinity and ion-exchange chromatography

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ABSTRACT

Protein C, which is an important anti-thrombotic factor in the blood coagulation cascade, undergoes several post-translational modifications. γ-Carboxylation on nine glutamic acid residues at the N-terminal region of the light chain [γ-carboxylated glutamic acid (Gla) domain] is considered to be critical for full anti-clotting activity. It is also known that when recombinant protein C is expressed in animal cells this particular modification is often lost. We were successful in preparing a monoclonal antibody (PC01) which distinguishes the sufficiently γ-carboxylated protein from the rest by its specific affinity for the Ca^{2+}-induced conformational change of the former, and thereby developed a simple process of purifying sufficiently γ-carboxylated protein C. Culture supernatant of Chinese hamster ovary cell transformants was first applied to Q-Sepharose and recombinant protein C was partially purified. It was then loaded onto a PC01 affinity column in the presence of 5 m*M* calcium chloride. Sufficiently γ-carboxylated protein C was retained while insufficient-carboxylated protein C quickly passed through. The former was eluted with 5 m*M* EDTA efficiently and with high purity, contained eight Gla units per molecule, and had similar anti-clotting activity. The flow-through was relatively impure protein C which contained five Gla units per molecule and showed limited anti-clotting activity. We extended the application of the Ca^{2+}-induced conformational change to conventional ion-exchange chromatography. The sufficiently γ-carboxylated protein C was found to elute earlier in the salt gradient from an anion-exchange column in the presence of 5 m*M* calcium chloride being fully separated from the insufficiently carboxylated protein C. The same strategy was successfully applied to the purification of a protein C derivative, PCGFX, in which the Gla domain was replaced by that of factor X. Ca^{2+}-shift chromatography seems to have general utility in the quick and economical purification of these Gla-containing proteins.

INTRODUCTION

Protein C is a vitamin K-dependent plasma protein which plays an important role in the blood coagulation cascade [1,2]. Protein C is activated by the thrombin–thrombomodulin complex, and the activated form inactivates factors Va and VIIIa. Protein C is composed of a ligt chain and a heavy chain, which are linked by a disulphide bond, and undergoes post-translational modifications.

Nowadays, recombinant vitamin K-dependent proteins produced in microorganisms and mammalian cells by the gene technology have replaced plasma-derived proteins [3–6]. In many coagulation factors γ-carboxylation of the glutamic acid domains is essential for expression of full biological activity [1]. Protein C is one such factor, and this post-translational modification is usually not complete in the recombinant preparation [6,7]. Therefore it is essential to be able to remove insufficiently γ-carboxylat-

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ed protein C. We prepared a monoclonal antibody which recognizes Ca^{2+}-induced conformational change only of sufficiently modified protein C and, employing this antibody as an absorbent in affinity chromatography, were able te purify the biofunctional recombinant protein [8]. Furthermore, we found that ion-exchange chromatography should be as capable as affinity chromatography of separating the biofunctional protein C, by applying the same principle. Recently a similar trial was reported by another group [6].

EXPERIMENTAL

Materials

Purified natural human protein C was kindly provided by Dr. W. Kisiel (University of New Mexico, USA). Protein C activator was purified from the lyophilized venom of *Agkistrodon contortrix contortrix* (Sigma, USA) according to the method described by Kisiel *et al.* [9]. All chemicals were purchased from Sigma or Wako (Japan).

Culture

Human protein C was expressed in Chinese hamster ovary (CHO) dihydrofolate reductase (DHFR)-deficient ($dhfr^-$) cells (DUKX-B11) transfected with the plasmid pCs1 [harbouring the protein C-coding region under the simian virus 40 (SV40) early promoter] and pSV40Adhfr (mouse DHFR cDNA). PCGFX, a derivative whose pre–pro sequence and γ-carboxylated glutamic acid (Gla) domain were replaced with those of factor X, was also expressed in CHO cells [10]. The culture supenatants were obtained by feeding in minimal essential α-medium (Nissui Pharmaceutical, Japan) containing 2% foetal bovine serum and 0.58 μ*M* vitamin K_3 (Sigma).

Preparation of monoclonal antibodies against human protein C and PCGFX

Monoclonal antibodies (MoAbs) to human protein C, PC01 and PC02, were prepared according to the method described by Kurosama-Ohsawa *et al.* [8]. PC01 recognized the Gla domain of protein C with calcium dependency, while PC02 recognized another site of protein C and is less calcium dependent than PC01 [8]. MoAb to human factor X, PX01, was prepared in the same way, apart from immunizing with 100 μg of human factor X and boosting three times. PX01 recognized the Gla domain of human factor X calcium dependently. MoAb affinity columns were prepared by coupling the antibodies to activated CH-Sepharose 4B (Pharmacia, Sweden) as described in the manufacturer's manual. A 10-mg aliquot of antibody was coupled to 1 ml of the resin.

Enzyme-linked immunosorbent assay (ELISA)

Microtitre plates (Sanko Junyaku, Japan) for ELISA were coated with rabbit anti-human protein C antibody (Dakopatts, Denmark). The purchased antibody was 1000-fold diluted with Tris-buffered saline (TBS) (50 m*M* Tris–HCl, pH 7.2, 0.15 *M* sodium chloride). 50 μl of the solution was added to each well, and incubated for 1 h. The wells were then washed with TBS and blocked with 1% gelatin–TBS solution. The gelatin solution was removed and 50 μl of samples or standard plasma protein C, both of which were diluted with TBS, were then added to the wells and incubated for another 1 h. After washing with TBS containing 0.1% Tween 20 (TBST), 50 μl of peroxidase-conjugated rabbit anti-human protein C antibody in TBS [polyclonal antibody (PoAb) ELISA] or peroxidase-conjugated PC01 MoAb in TBS containing 5 m*M* calcium chloride (PC01 ELISA) were added and the plates incubated for 1 h. The wells were washed with TBST containing 5 m*M* EDTA (for PoAb ELISA) or TBST containing 5 m*M* calcium chloride (for PC01 ELISA). Then, peroxidase activity was measured by the common colorimetric reaction. All steps were performed a room temperature.

Purification of recombinant protein C and PCGFX using a MoAb affinity column

Purification of the recombinant proteins was carried out in two steps, batch purification and immunoaffinity chromatography.

Batch purification. CHO cell culture supernatant was mixed with a 1/20 volume of 1 *M* imidazole–hydrochloric acid buffer (pH 6.5) and diluted with distilled water to give the same conductivity given by 50 m*M* imidazole–hydrochloric acid buffer (pH 6.5) containing 0.11 *M* sodium chloride. The sample was then applied to a Q-Sepharose Fast Flow (Pharmacia) column previously equilibrated with the same buffer. The recombinant proteins were

eluted with the buffer containing 0.5 *M* sodium chloride.

MoAb immunoaffinity chromatography. Calcium chloride solution was added to the batch-purified fraction to give a concentration of 5 m*M*, and then the sample was applied to a MoAb affinity column previously equilibrated with TBS containing 5 m*M* calcium chloride. The recombinant protein was eluted with TBS containing 5 m*M* EDTA.

DEAE chromatography of recombinant protein C in the presence of calcium ions

The recombinant protein C purified by PC02 affinity column was dialysed against 50 m*M* Tris–HCl (pH 8.0) containing 5 m*M* calcium chloride. The dialysed sample was then applied to a Protein Pak G-DEAE column (Waters, USA). Recombinant protein C was then eluted with a linear gradient from 0 to 0.3 *M* sodium chloride in the same buffer (90 ml). The flow-rate was 1ml/min and each 1 ml was separately collected.

Amino acid composition analysis

Proteins were hydrolysed in 6 *M* hydrochloric acid or 3.75 *M* sodium hydroxide for Gla analysis under reduced pressure at 110°C for 21 h. The hydrolysates were analysed by the phenylthiocalbamyl method in the PICO TAG system (Waters).

Assay for enzymatic activity of recombinant protein C and PCGFX

The amidolytic activity of recombinant protein C and PCGFX was measured with the substrate H-D-Lys-Z-Pro-Arg-pNA (Boehringer Mannheim, Germany) in 25 m*M* Tris–HCl (pH 8.0) and 0.1 *M* sodium chloride. A 40-μl aliquot of human citrate plasma or either of the proteins in diethylbarbiturate acetate (DBA) buffer (20 m*M* barbital buffer, pH 7.6, 34 m*M* sodium acetate and 30 μ*M* bovine serum albumin) containing 10 m*M* trisodium citrate was mixed with the equal volume of 1 U/ml protein C activator in DBA buffer and then incubated for 10 min at 37°C. Then 400 μl of the substrate solution were added. After another incubation for 5 min, the reaction was stopped by adding 400 μl of 50% acetic acid, and the absorbance of the final solution at 405 nm was measured.

The anti-clotting activity of protein C in plasma was determined by measuring the activated partial thromboplastin time (APTT). A 50-μl aliquot of protein C in DBA buffer containing 10 m*M* sodium citrate, 50 μl of protein C-depleted plasma (American Diagnostica, USA) and 50 μl of protein C activator was mixed with 50 μl of Pathromtin (Behringwerke, Germany). After having incubated the mixture for 10 min at 37°C, the clotting reaction was started by adding 50 μl of 25 m*M* calcium chloride. The clotting time was measured by "Fibrin timer" (Behringwerke).

RESULTS AND DISCUSSION

Elution profile of recombinant protein C in PC01 immunoaffinity chromatography

At the preliminary stage, we employed 35% ammonium sulphate fractionation instead of batch purification to quickly obtain a crude recombinant protein C specimen to evaluate the PC01 immunoaffinity column. The specimen was applied to the column and then the protein C was eluted by the method described in the Experimental section. Fig. 1 shows the elution profile monitored by UV absorption, antigenicity and activity. About 30–50% of recombinant protein C was found in the PC01-unretained fraction and the rest was eluted with 5 m*M* EDTA afterwards as the PC01-retained fraction. The ratio between the fractions varied with the culture conditions [7]. The PC01-unretained fraction contained protein C which was recognized by PoAb ELISA and amidolytic enzyme assay but not by PC01 ELISA.

Characterization of PC01-bound and -unbound protein C

PC01-unbound protein C was further purified using the PC02 affinity column under the same elution conditions employed for the PC01 counterpart. We then analysed the amino acid composition and the amidolytic and anti-clotting activities of the purified PC01-unbound protein C as well as the bound protein C. Table I shows the contents of Gla and the activities of both the bound and the unbound protein C. The number of Gla residues in the bound protein C was consistent with the theoretical value, whereas that of the unbound protein C was significantly less. The rest of the amino acid composition of both bound and the unbound protein C agreed with the theoretical estimation. The bound had

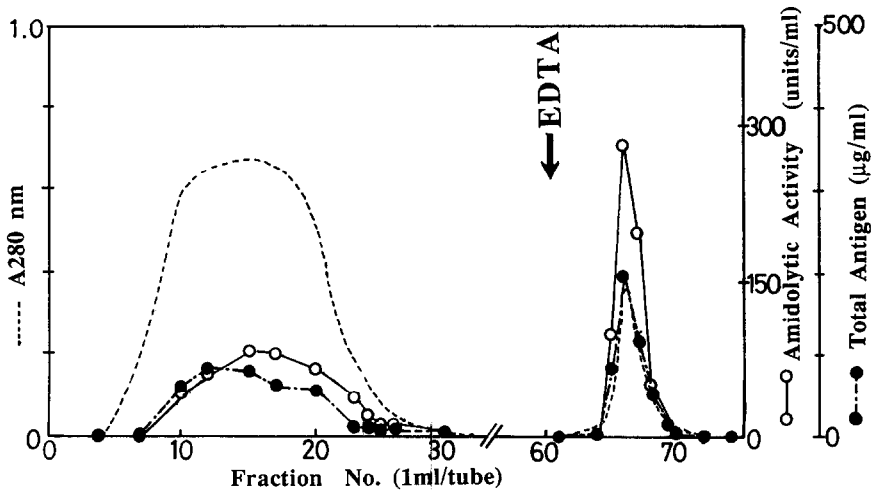

Fig. 1. PC01 immunoaffinity chromatography of recombinant protein C. The dotted line indicates the absorbance at 280 nm, the closed circles indicate the amount of antigen estimated by ELISA using rabbit polyclonal antibody against human protein C and the open circles indicate the amidolytic activity.

amidolytic and anti-clotting activities similar to those of plasma-derived protein C. The unbound had similar amidolytic acitvity but significantly less anti-clotting activity. These findings confirm that sufficient γ-carboxylation in the Gla domain is essential for the biological activity of protein C. The insufficiently γ-carboxylated protein has been considered to undergo insufficient interaction with phospholipids on the cell surface [11].

DEAE chromatography to separate sufficiently and insufficiently γ-carboxylated protein Cs

Our recombinant protein C preparation was, as already mentioned, comprised of sufficiently and insufficiently γ-carboxylated protein C. We found that calcium-dependent retention could also be used to separate sufficiently and insufficiently γ-carboxylated protein C by DEAE chromatography. Fig. 2 shows DEAE chromatogram of the recombinant protein C preparation in the presence of calcium ions. Chromatography gave two peaks in the presence of calcium ion and, in the absence, only one at a higher salt concentration. The first of the two peaks (peak I) in the presence of calcium ion was, therefore, more calcium-sensitive in elution than the second peak (peak II). The ratio between the first and the second peak was consistent with that between the PC01 immunoaffinity column-bound and -unbound protein C. PC01 recognized only protein C in the first peak. These results indicate that sufficiently and insufficiently γ-carboxylated protein C can be separated by the conventional method as well as by immunoaffinity chromatography.

TABLE I

NUMBERS OF γ-CARBOXYLATED GLUTAMIC ACIDS AND ACTIVITIES OF PC01-BOUND AND -UNBOUND PROTEIN C

Protein C	Gla[a] (residues per molecule)	Activity (% of plasma protein C)	
		Amidolytic	Anti-clotting
Bound	8.1	90	67
Unbound	5.2	60	8

[a] The number of Gla in plasma-derived protein C in nine.

Purification of recombinant protein C and PCGFX

Table II shows the purification profile of recombinant protein C using PC01 immunoaffinity chromatography. The yield was 71%. Table III shows the corresponding profile of PCGFX using PX01 immunoaffinity chromatography. The yield in this case was 47%. PX01 was able to distinguish the sufficiently γ-carboxylated factor X in the presence of calcium ion. The apparent low yield of PCGFX

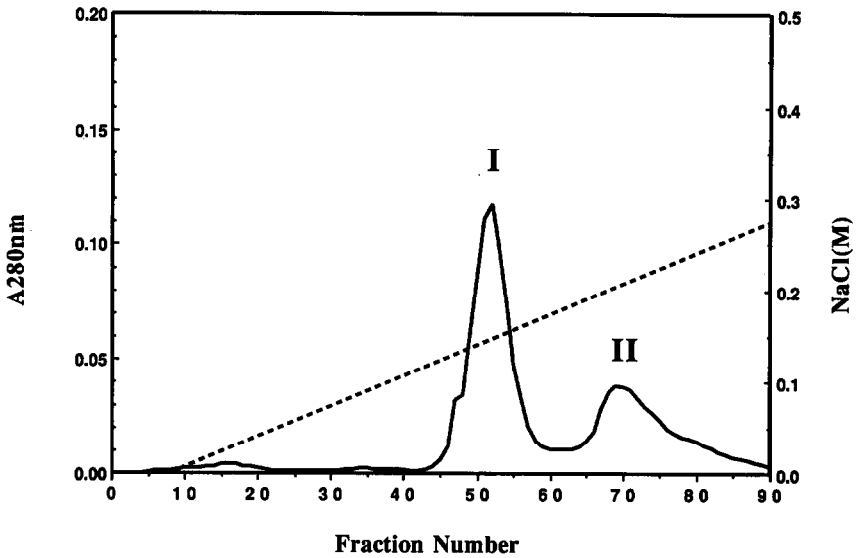

Fig. 2. DEAE chromatography of recombinant protein C in the presence of calcium ion. The solid line indicates the absorbance at 280 nm, and the dotted line indicates the concentration of the sodium chloride gradient in the buffer described in the Experimental section.

may be ascribed to the small ratio of sufficiently γ-carboxylated PCGFX in the culture supernatant.

The amidolytic and anti-clotting activities of PCGFX were 104% and 59%, respectively, of those of the plasma-derived protein C. These results indicate that fully biofunctional recombinant protein C and PCGFX can be purified by a simple method using a MoAb which recognizes calcium-induced conformational change. Fig. 3 shows the sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) pattersn of both the purified recombinant protein C and the plasma-derived protein C under reduced conditions.

The plasma-derived protein C preparation had the two-chain form and contained a small amount of β-heavy chain and no γ-heavy chain. On the other hand, recombinant protein C comprised of single- and two-chain form and contained more β- and γ-heavy chains. The purified PCGFX showed practically the same pattern as recombinant protein C, except for the molecular weight of the light chain. The ratio of single- and two-chain forms of the recombinant proteins varied with the culture and purification conditions.

We demonstrated here that calcium-sensitive retention in affinity chromatography is an efficient

TABLE II

PURIFICATION OF RECOMBINANT PROTEIN C

	Volume (ml)	Protein (mg)	Activity[a] (U)	Yield (%)
Culture supernatant	790	717	668	100
Q-Sepharose Fast Flow	83	38	709	106
PC01 affinity column	11.5	1.7	475	71

[a] The amidolytic activity is standardized with the activity of protein C in 1 ml of normal citrate plasma (1 U).

TABLE III

PURIFICATION OF PCGFX

	Volume (ml)	Protein (mg)	Activity[a] (U)	Yield (%)
Culture supernatant	25 000	27 830	6550	100
Q-Sepharose Fast Flow	620	1937	6665	102
PX01 affinity column	36	8.84	3072	47

[a] The amidolytic activity is standardized with the activity of protein C in 1 ml of normal citrate plasma (1 U).

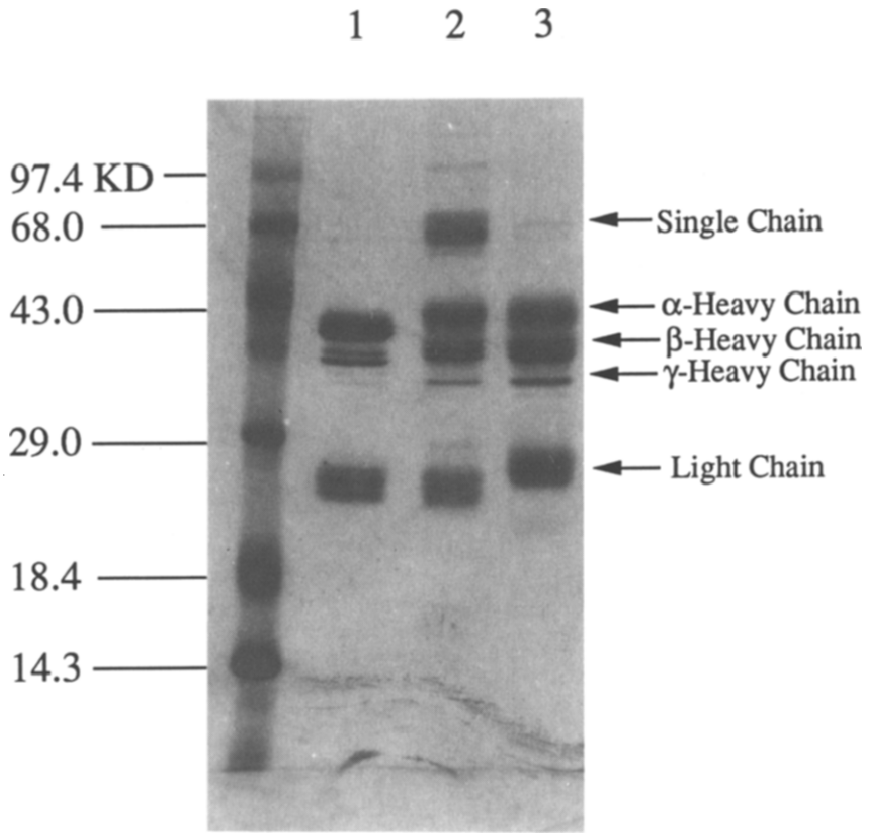

Fig. 3. SDS-PAGE of purified recombinant protein C and PCGFX. Electrophoresis was carried out in 10–20% gradient polyacrylamide gel under reduced conditions and the gel was stained with Coomassie brilliant blue. Lanes: 1 = plasma-derived protein C; 2 = purified recombinant protein C; 3 = purified PCGFX. KD = kilodalton.

method of highly purifying sifficiently γ-carboxylated protein C and PCGFX with more than 90% recovery, although the yield varies with the degree of post-translational modification in the expression system. Moreover, we also successfully applied calcium-sensitive retention to conventional ion-exchange chromatography to perform the same purification. This undoubtedly indicates that the separation obtained should be ascribed solely to the calcium-induced change in the ligand molecule. Quite often purification of a recombinant protein demands development of a new method. In particular, when some post-translational modification is essential for the protein's physiological function, the technique must be able to separate the sufficiently modified protein from that which is insufficiently modified; the separation is generally more difficult than normal protein isolation. Immunoaffinity purification, in this regard, is an excellent tool for such purification by virtue of the selective binding of antibodies. However, an antibody column is in general inferior to a conventional one in terms of stability and cost. Therefore the calcium-dependent affinity shift in DEAE chromatography described above seems to have great potential for large-scale commercial production. We also believe that one can make general use of the combination of conventional ion-exchange chromatography and calcium-sensitive retention to purify any Gla-rich recombinant protein in a large-scale purification.

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