J. Biochem. **135**, 577–582 (2004)
DOI: 10.1093/jb/mvh070

Preparation of Recombinant α-Thrombin: High-Level Expression of Recombinant Human Prethrombin-2 and Its Activation by Recombinant Ecarin

Hiroshi Yonemura[*,1], Takayuki Imamura[*,2], Kenji Soejima[2], Yo Nakahara[1], Wataru Morikawa[1], Yoshitaka Ushio[1], Yasuharu Kamachi[1], Hiroshi Nakatake[2], Keishin Sugawara[1], Tomohiro Nakagaki[2] and Chikateru Nozaki[†,2]

[1]Applied Research Department and [2]First Research Department, The Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN), Kawabe, Kyokushi, Kikuchi, Kumamoto 869-1298

Received February 12, 2004; accepted March 3, 2004

We have established a large-scale manufacturing system to produce recombinant human α-thrombin. In this system, a high yield of α-thrombin is prepared from prethrombin-2 activated by recombinant ecarin. We produced human prethrombin-2 using mouse myeloma cells and an expression plasmid carrying the chicken β-actin promoter and mutant dihydrofolate reductase gene for gene amplification. To increase prethrombin-2 expression further, we performed fed-batch cultivation with the addition of vegetable peptone in 50 liters of suspension culture. After five feedings of vegetable peptone, the expression level of the recombinant prethrombin-2 reached 200 μg/ml. Subsequently, the recombinant prethrombin-2 could be activated to α-thrombin by recombinant ecarin expressed in a similar manner. Finally, recombinant α-thrombin was purified to homogeneity by affinity chromatography using a benzamidine-Sepharose gel. The yield from prethrombin-2 in culture medium was approximately 70%. The activity of the purified recombinant α-thrombin, including hydrolysis of a chromogenic substrate, release of fibrinopeptide A, and activation of protein C, was indistinguishable from that of plasma-derived α-thrombin. Our system is suitable for the large-scale production of recombinant α-thrombin, which can be used in place of clinically available α-thrombin derived from human or bovine plasma.

Key words: α-thrombin, ecarin, prethrombin-2, recombinant.

Abbreviations: Gla, γ-carboxyglutamic acid; CHO, Chinese hamster ovary cell line, dhfr, dihydrofolate reductase; mdhfr, mutant dihydrofolate reductase; MTX, methotrexate.

Prothrombin, a 579–amino acid, single-chain glycoprotein (M_r 72,000), contains a Gla domain, two kringle domains, and a protease domain. Prothrombin is biosynthetically produced in hepatocytes in a vitamin K–dependent manner, and circulates in the blood at a concentration of 100 to 150 μg/ml as a precursor to thrombin, a serine protease (*1–5*).

During prothrombin activation, prothrombinase [a complex of activated factor X (Xa) and activated factor V (Va)] cleaves two peptide bonds sequentially. The Arg320-Ile321 bond is cleaved first, resulting in the formation of meizothrombin. Subsequently, the Arg271-Thr272 bond is cleaved through restricted cleavage to form α-thrombin, which is a two-chain molecule consisting of an A chain and a B chain. Prethrombin-2 (corresponding to residues from Thr272 to Glu579 in prothrombin), which is a product produced by the removal of the Gla domain and two kringle domains from prothrombin, is known to be the smallest single-chain precursor to α-thrombin (*1–5*).

α-Thrombin exerts a variety of physiological activities by restricted cleavage of a number of plasma proteins or various thrombin receptors on the cellular membrane. It participates locally in the formation of a larger thrombus by binding to a fibrinous thrombus, and is able not only to convert fibrinogen into fibrin but also to activate factor XIII to trigger fibrin cross-linking (*6*, *7*). Finally, α-thrombin accelerates coagulation by activating factor V (*8*) and factor VIII (*9*). Thus, thrombin plays an important role in haemostasis and, hence, is highly useful as a haemostatic reagent.

On the other hand, thrombin changes its substrate specificity upon binding to thrombomodulin on vascular endothelial cells, which causes it to promote dramatically the activation of protein C; in this way, thrombin is a physiologically important anticoagulant (*10*). Thus, thrombin is also useful as a processing enzyme in the preparation of recombinant activated protein C, a reagent that is clinically available for the treatment of patients with severe sepsis.

α-Thrombin activates platelets by activating thrombin receptors on the platelet surface (*11*), and also exhibits mitogenic activity, thus promoting wound healing. These activities of thrombin can be controlled by serine protease inhibitors such as antithrombin (*12*) and heparin cofactor II (*13*).

[*]These authors contributed equally to this work.
[†]To whom correspondence should be addressed. Tel: +81-968-37-3100, Fax: +81-968-37-3616, E-mail: nozaki@kaketsuken.or.jp

Because of its various functions and activities, thrombin has been widely used as a haemostatic reagent, a processing enzyme, and a reagent for research. Also, a fibrin sealant used as a tissue adhesive is composed of thrombin together with fibrinogen and factor XIII.

However, thrombin used for such purposes is typically prepared from human or bovine plasma, raising the possibility of contamination with various infectious agents derived from the source plasma. In particular, bovine blood products may be contaminated with the bovine spongiform encephalitis agent. Furthermore, xenogenic immune responses to the bovine plasma-derived thrombin and one of its primary contaminants, factor V, have been reported (*14–20*). In some cases, the inhibitory antibodies developed against thrombin and factor V crossreact with human thrombin and factor V, leading to severe bleeding events (*14–18*). Moreover, anaphylaxis caused by the generation of immunoglobulin E has been reported (*19*, *20*).

The expression of recombinant human thrombin in microorganisms and mammalian cells has been reported (*21–25*). However, these methods are disadvantageous for several reasons. For example, thrombin expressed in *Escherichia coli* forms inclusion bodies, making it difficult to recover correctly folded and enzymatically active thrombin (*21–24*). Furthermore, an expression system using a Chinese hamster ovary (CHO) cell line has an insufficient level of expression (*25*). Therefore, we directed our efforts towards developing an efficient expression system for prethrombin-2 using mammalian cells.

Using these methods, thrombin is expressed in a precursor form and, therefore, must be activated by an activating enzyme such as prothrombinase, which consists of factor Xa, factor Va, and phosholipids; the protein components must be prepared by recombinant technology to exclude plasma contaminants. However, the catalytic efficacy of prothrombinase for prethrombin-2 is much lower than for prothrombin. Therefore, we used ecarin, an enzyme known to possess a specific and high activity for converting prethrombin-2 into α-thrombin (*26*). Ecarin is a snake venom–derived protease isolated from *Echis carinatus*. A cDNA encoding ecarin has been cloned by Nishida *et al*. (*27*). Ecarin is a metalloprotease, the mature form of which comprises 426–amino acid residues in a mosaic structure consisting of a Zn^{2+} chelating region, a disintegrin domain, and a cysteine-rich domain. We subsequently constructed an expression system for ecarin.

Here, we report the development of a safe and efficient preparation method for recombinant human α-thrombin on a large scale using recombinant ecarin.

MATERIALS AND METHODS

Construction of Expression Plasmids—For the expression plasmid for human prethrombin-2, a mutant dihydrofolate reductase (mdhfr) gene that converts leucine 22 {amino acid residue numbering described in Ref. *28*} in wild-type dihydrofolate reductase (dhfr) into arginine was used for efficient gene amplification (*28*). The mdhfr gene was prepared by the PCR-based mutation of a wild-type dhfr gene derived from the expression plasmid pSV2-dhfr (*29*). The plasmid pCAGG/mdhfr was generated by inserting the mdhfr gene into pCAGG, a chicken β-actin promoter-based expression plasmid for mammalian cells (*30*). The prethrombin-2 cDNA was cloned from human liver mRNA (Clontech) by PCR and ligated into pCAGG/mdhfr. The resulting plasmid, pCAGG/mdhfr/PreTB2 (Fig. 1), harbors the prethrombin-2 cDNA under the control of the chicken β-actin promoter.

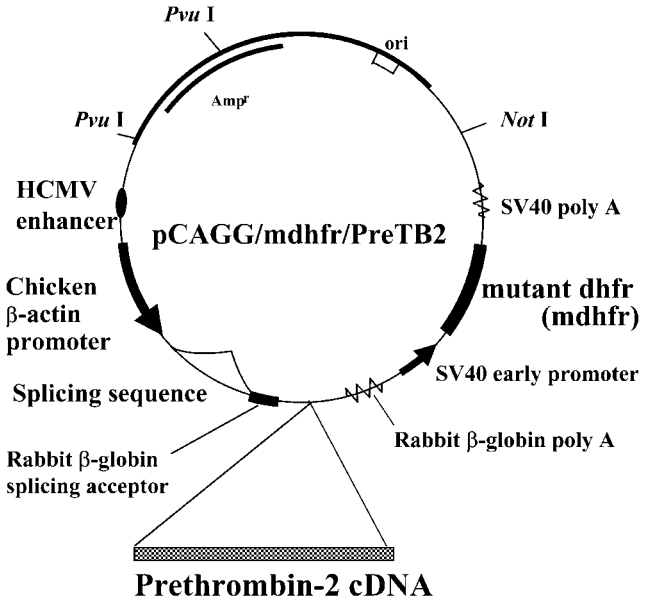

Fig. 1. **An expression vector for prethrombin-2.** A chicken β-actin promoter-based expression plasmid, pCAGG, was used as an expression vector. Prethrombin-2 cDNA was inserted into the expression plasmid, pCAGG/mdhfr, which bore a mutated dhfr gene.

The ecarin cDNA provided by Dr. S. Kawabata (Kyushu Univ.) was ligated into the pCAGG vector. The resulting plasmid, pCAGG/Ecarin, contains the ecarin cDNA under the control of the chicken β-actin promoter. Furthermore, a Kozak sequence (*31*) was introduced into each of the expression vectors pCAGG/mdhfr/PreTB2 and pCAGG /Ecarin.

Detection of Recombinant Proteins—Western-blot analysis employing a polyclonal antibody (IgG) against an ecarin peptide was performed to detect recombinant ecarin. Antiserum to the ecarin peptide (KNDYSYADENKGIVEPGTKC) was prepared by immunizing rabbits with the keyhole limpet hemocyanin (KLH)-conjugated peptide and purifying the resulting IgG fraction from the antiserum by affinity chromatography using a protein G column (Amersham Biosiences).

Detection and quantification of prethrombin-2 was carried out by Western-blot analysis and/or sandwich ELISA using anti-human thrombin antibodies (Affinity Biologicals).

Quantification of Ecarin Activity—Ten microliters of prothrombin (400 μg/ml; Hematologic Technology) was added to a sample solution (80 μl) containing ecarin, which was then incubated for 5 min at 37°C. Then, 10 mM EDTA (10 μl) and S-2238 (1 mM; 100 μl) were added to the reaction mixture. After incubation at 37°C for 5 min, the reaction was quenched with 800 μl of 0.1 M citric acid. Aliquots (200 μl) were transferred to a 96-well plate

and the OD 405 nm was measured. Ecarin derived from snake venom (Sigma) was used as a standard for quantifying ecarin activity.

Establishment of Stable Transfectants Expressing Recombinant Proteins—The pCAGG/mdhfr/PreTB2 plasmid was used for the transfection of SP2/0 Ag14 mouse myeloma cells (ATCC CRL-1581) (*32*). SP2/0 cells were washed twice with chilled phosphate-buffered saline [PBS (–); Dulbecco], and 10^7 cells suspended in 0.8 ml of PBS (–) were placed in a cuvette for electroporation (electrode width 0.4 cm; BIO-RAD). The linearized expression plasmid (20 μg) was added to the cuvette and the solution was mixed with a pipette. One pulse was applied at 0.22 kV at 975 μF using a Gene Pulser II (BIO-RAD). After the cuvette was cooled on ice for 10 min, the cell suspension was diluted with Minimum Essential Medium (MEM) alpha medium with nucleic acids containing 10% fetal calf serum (FCS) to about 5,000 cells/50 μl, plated on five 96-well plates at 50 μl/well, and cultured in a 5% CO_2 incubator overnight at 37°C. The next day, 50 μl/well of nucleic acid-free MEM alpha medium containing 10% dialyzed FCS was added, and the culture was further incubated overnight. The next day, 100 μl/well of nucleic acid-free MEM alpha medium containing 10% dialyzed FCS, 0.5 mg/ml G418, and 100 nmol/liter or 200 nmol/liter methotrexate (MTX) was added. After culture for 10 to 14 d, the transfectants that emerged in each well were assessed for the level of prethrombin-2 produced per 10^6 cells per day. Briefly, the cells were plated with nucleic acid-free MEM alpha medium containing 2% dialyzed FCS at a density of about 4×10^5 cells/ml. After culturing for 24 h, the prethrombin-2 level and the cell density in the culture medium were measured. As a result, each transfectant was found to express 1 to 2 μg of prethrombin-2/day/10^6 cells. Subsequently, transfectants with the ability to produce high levels of prethrombin-2 were selected and cultured with nucleic acid–free MEM alpha medium containing 10% dialyzed FCS and 1 μmol/l MTX for about 14 d. The obtained MTX-resistant cells were adapted to protein-free medium using YMM medium (nucleic acid-free MEM alpha medium enriched with amino acids and vitamins, containing ethanolamine and sodium selenite without insulin and transferrin) by the following steps. Briefly, cells were cultured in YMM medium with 2% dialyzed FCS, and their growth was confirmed. Thereafter, culture was continued while the added serum level was gradually reduced to 0.5%, and then further to 0.1%. Finally, the cells were cultured in completely protein-free YMM medium.

For the selection of transfectants expressing ecarin, the same procedure was used, excluding MTX gene amplification.

Large-Scale Production of Recombinant Proteins—The transfectants producing the highest levels of ecarin or prethrombin-2, adapted to protein-free culture, were grown in suspension culture in a spinner flask. The cells were finally expanded to 50-liter fermenter cultures. For the production of ecarin, cultivation was performed at 35°C for 14 d. For the production of prethrombin-2, fed-batch cultivation with five additions of vegetable peptone (*33*, *34*) was performed for 11 d at 37°C.

Purification of Recombinant Ecarin—Recombinant ecarin was purified from its conditioned medium by a combination of two types of cation exchange chromatography and gel filtration. Briefly, the conditioned medium from the ecarin-producing cells was diluted with two volumes of distilled water, adjusted to pH 5.0 with 1 M citric acid, and filtered through a 0.45-μm filter. The sample was applied to a Macro-Prep High S Support (BIO-RAD) column equilibrated with 20 mM citrate (pH 5.0) buffer. The column was washed with the same buffer, and then the proteins were eluted with a 0 to 1,000 mM NaCl gradient. Fractions containing recombinant ecarin were pooled and dialyzed against a 20 mM sodium bicarbonate buffer (pH 9.0) containing 50 mM NaCl. The dialyzed sample was applied to a sulfate Cellulofine (SEIKAGAKU) column equilibrated with a 20 mM sodium bicarbonate buffer (pH 9.0) containing 50 mM NaCl. The column was washed with the equilibration buffer, and the protein was eluted with a 50–600 mM NaCl gradient. Fractions containing ecarin were pooled. The pooled portion was applied to a HiLoad Superdex 200 pg 16/60 gel filtration column (Amersham Bioscience) equilibrated with a 10 mM phosphate (pH 7.0) buffer containing 100 mM NaCl.

Preparation of Recombinant Human α-Thrombin—Recombinant α-thrombin was prepared as follows. Culture medium containing recombinant prethrombin-2 was adjusted to pH 6.0 with 1 M citric acid and filtered through a 0.45-μm filter. The sample was applied to an SP-TOYOPEARL 550C column (Tosoh) equilibrated with a 20 mM citrate buffer (pH 6.0) containing 0.05% Pluronic F-68. The column was washed with the same buffer, and the protein was eluted with a linear gradient of NaCl (50–1000 mM) in equilibration buffer. Fractions containing prethrombin-2 were pooled and dialyzed against 20 mM Tris-HCl (pH 8.5) buffer containing 100 mM NaCl overnight at 4°C. After dialysis, recombinant ecarin was added to the above sample at a final concentration of 6.5 U/ml, and then the mixture was incubated at 37°C for 16 h. After ecarin treatment, the reaction mixture was applied to a benzamidine column for affinity chromatography (Amersham Bioscience). The column was washed with 20 mM Tris-HCl (pH 8.5) buffer containing 500 mM NaCl, and then elution was performed with 20 mM Tris-HCl (pH 8.5) buffer containing 100 mM benzamidine chloride. The eluted fractions were pooled. Finally, the pooled fractions were dialyzed or applied to a desalting column to remove benzamidine chloride.

Assay of Various Enzymatic Activities of α-Thrombin—The enzymatic properties of recombinant human α-thrombin were compared with those of plasma-derived human α-thrombin (Hematologic Technologies) in several assays, including the hydrolysis of synthetic substrate (S-2238), release of fibrinopeptide A, activation of protein C, inhibition by antithrombin, platelet aggregation, and clotting activity, as previously described (*24*).

RESULTS AND DISCUSSION

Preparation of Recombinant Ecarin—Prethrombin-2 is one of the target substrates activated by ecarin (*26*, *27*). α-Thrombin, produced as a result of the activation, is a multifunctional serine protease capable of interacting with various substrates that plays a pivotal role in vari-

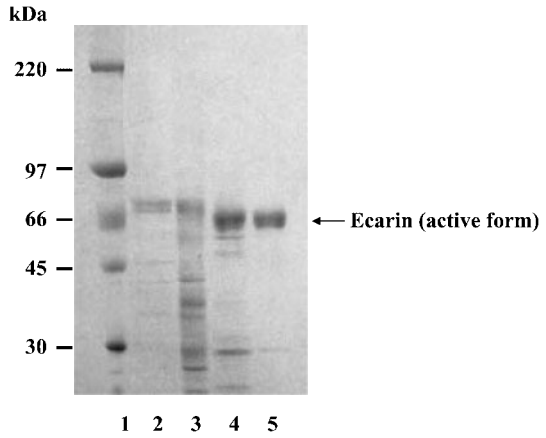

Fig. 2. **Purification of recombinant ecarin from the conditioned medium.** Fractions obtained from each purification step were subjected to SDS-PAGE in a 10% polyacrylamide gel under non-reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250. Lane 1, molecular weight markers; lane 2, concentrated culture medium; lane 3, pooled fractions eluted from a Macro-Prep High S Support column; lane 4, pooled fractions eluted from a sulfate Cellulofine column; lane 5, fraction eluted from a HiLoad Superdex 200 pg 16/60 gel filtration column.

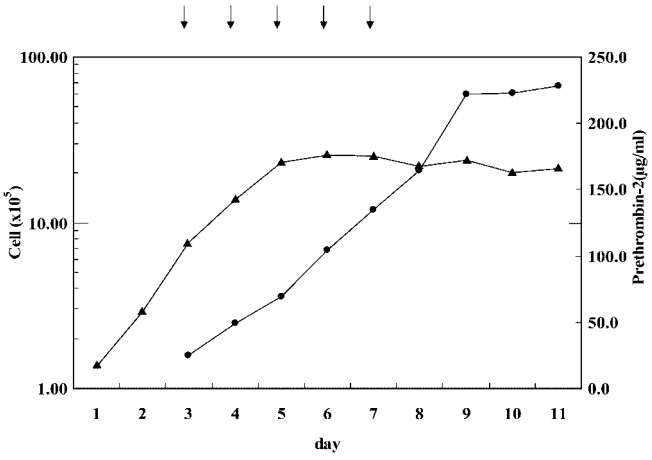

Fig. 3. **Production of prethrombin-2 using protein-free medium on a 50-liter scale.** The x-axis shows days in culture. The left y-axis shows the viable cell number (closed triangles) and the right y-axis shows the expression level of prethrombin-2 estimated by ELISA (closed circles). The arrows indicate the times for feeding with vegetable peptone.

ous biological phenomena, such as haemostasis, thrombosis, and cell differentiation (*4–11*). In human blood, prothrombin is activated by prothrombinase through restricted cleavage at two sites in prothrombin, Arg271-Ile272 and Arg320-Ile321, whereas ecarin hydrolyzes prothrombin at Arg320-Ile321 alone to produce meizo-

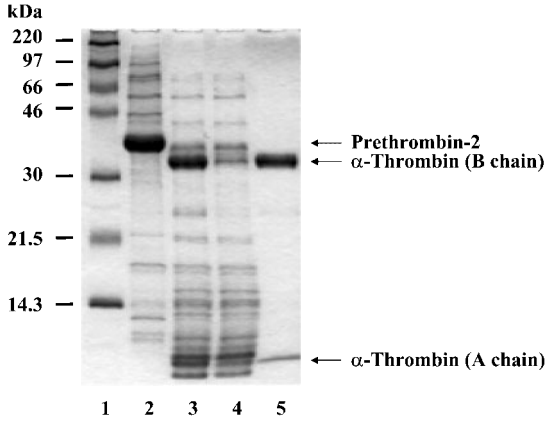

Fig. 4. **Purification of recombinant α-thrombin from the conditioned medium.** Fractions obtained from each purification step were subjected to SDS-PAGE in a 15% polyacrylamide gel under reducing conditions. The gel was stained with Coomassie Brilliant Blue R-250. Lane 1, molecular weight markers; lane 2, cation-exchange chromatography eluate; lane 3, sample after ecarin treatment; lane 4, benzamidine chromatography flow-through fraction; lane 5, benzamidine chromatography eluate.

thrombin. Due to its high substrate specificity, ecarin efficiently converts prethrombin-2, which comprises residues 272–579 of prothrombin, into α-thrombin solely through Arg320-Ile321 cleavage (*26*, *27*). Therefore, we established a line of transformant cells secreting recombinant ecarin for the activation of recombinant human prethrombin-2.

The highest-yielding ecarin-producing clone adapted to protein-free culture showed an ability to produce 10 U/ml of ecarin after culture for 14 d on a 50-liter scale. The recombinant ecarin was purified by three steps of chromatography as described in "MATERIALS AND METHODS." Interestingly, the purified recombinant ecarin showed enzymatic activity without any further processing for activation, suggesting that the non-activated ecarin was activated during the purification procedure. At each purification step, the ecarin-containing fractions were subjected to SDS-PAGE analysis. The purity of the final product appeared to be more than 90% (Fig. 2). The yield of purified recombinant ecarin from the culture medium was 13% in activity, with a 12,000-fold increase in specific activity.

Preparation of Recombinant Human α-Thrombin— Using conventional methods involving CHO cell culture with the SV40 promoter and a dhfr gene amplification system, an expression level for recombinant human prothrombin-2 of 25 μg/ml has been reported (*25*). In this study, we employed a SP2/0 cell line, which is convenient for suspension culture. Furthermore, we used a mutant

Table 1. **Comparison of the enzymatic activities of recombinant and plasma-derived α-thrombins.**

	Recombinant	Plasma-derived
Hydrolysis of S-2238 [k_{cat}/K_m (μM^{-1} s^{-1})]	23.3 ± 2.5	21.2 ± 2.7
Conversion of fibrinogen into fibrin for fibrinopeptide A [k_{cat}/K_m (μM^{-1} s^{-1})]	10.9 ± 0.6	10.6 ± 0.8
Activation of protein C without thrombomodulin [k_{cat}/K_m (μM^{-1} min^{-1})]	0.58 ± 0.01	0.52 ± 0.02
Activation of protein C with thrombomodulin [k_{cat}/K_m (μM^{-1} min^{-1})]	14.0 ± 1.5	15.5 ± 0.5
Inhibition by antithrombin [Secondary reaction constant (μM^{-1} min^{-1})]	0.57 ± 0.01	0.65 ± 0.01

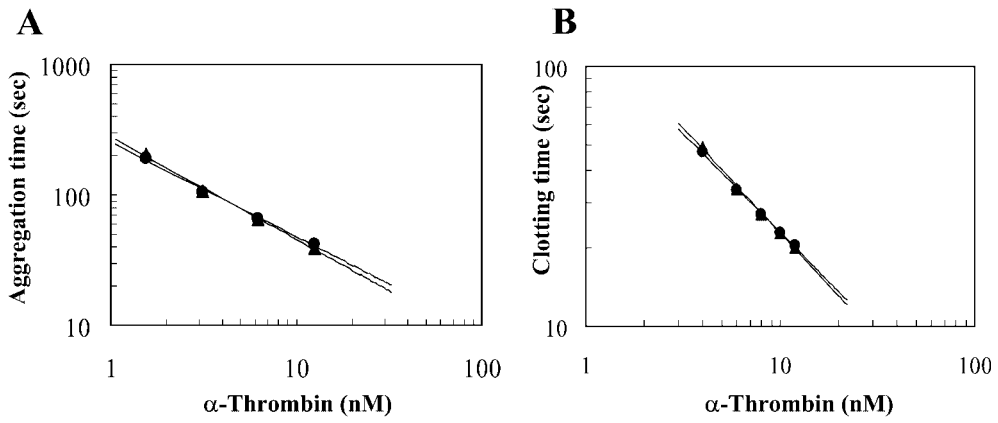

Fig. 5. **Comparison of platelet aggregation and clotting activity of recombinant and plasma-derived α-thrombins.** A: 300-μl aliquots of washed human platelets resuspended at a concentration of 1.3×10^8 platelets per ml were mixed with 100 μl of various concentrations of recombinant (closed triangles) or plasma-derived (closed circles) α-thrombin. The changes in light transmission were measured at 37°C using an aggregometer. We defined aggregation time as the time until complete aggregation of platelets, as measured by the transmission of light through the suspension. B: 200-μl aliquots of various concentrations of recombinant (closed triangles) or plasma-derived (closed circles) α-thrombin ranging from 6 nM to 18 nM in the presence of 5 mM $CaCl_2$ were added to 100 μl of factor II deficient-human plasma in cuvettes. Final concentrations of α-thrombins ranged from 4 nM to 12 nM. Clotting time was measured with a Behring Fibrintimer at 37°C.

dhfr gene amplification system (*28*) to achieve higher expression of human prethrombin-2. This altered gene can be used as a dominant selectable marker in transfectants expressing normal levels of wild-type dhfr. Furthermore, to enhance the production of recombinant prethrombin-2, fed-batch cultivation was performed. After five feedings with vegetable peptone at 37°C over 11 d in 50-liter culture, the expression level of prethrombin-2 reached 200 μg/ml (Fig. 3).

The recombinant products were purified and activated according to the protocols described in "MATERIALS AND METHODS." α-Thrombin produced in our large-scale manufacturing system was highly purified by benzamidine affinity chromatography (Fig. 4). Purified recombinant α-thrombin was obtained with a high final yield of approximately 70% from culture medium containing recombinant prethrombin-2 (data not shown). An expression level of 150 μg/ml prethrombin-2 was achieved even in a 2,000-liter suspension culture in a fermenter, from which α-thrombin was successfully purified (data not shown).

Comparison of the Enzymatic Properties between Recombinant and Plasma-Derived α-Thrombins—The following enzymatic properties of recombinant and plasma-derived human α-thrombins were compared: hydrolysis of a chromogenic substrate (S-2238), conversion of fibrinogen into fibrin, activation of protein C in the presence or absence of thrombomodulin, and inhibition by antithrombin. As shown in Table 1, the enzymatic parameters of the purified recombinant human α-thrombin were similar to those of the plasma-derived human α-thrombin. Furthermore, their respective platelet aggregation and plasma clotting times were also in good agreement (Fig. 5, A and B).

Thus, the present report indicates that our system for manufacturing recombinant human α-thrombin on a large scale makes it possible to obtain highly purified α-thrombin free of plasma-derived components that is thus suitable for clinical and research purposes.

We would like to thank Professor Emeritus Sadaaki Iwanaga and Dr. Shun-ichiro Kawabata (Kyushu University) for supplying the ecarin cDNA and their many helpful discussions. We would also like to thank Yuko Matsumoto, Ikuyo Yatsunami, Tomomi Sumikawa, Hitomi Shirahama, Takako Ogata, Noriko Mimura, and Sawako Fujikawa for technical assistance.

REFERENCES

1. Jackson, C.M. and Nemerson, Y. (1980) Blood coagulation. *Annu. Rev. Biochem.* **49**, 765–811
2. Mann, K.G., Jenny, R.J., and Krishnaswamy, S. (1988) Cofactor proteins in the assembly and expression of blood clotting enzyme complexes. *Annu. Rev. Biochem.* **57**, 915–956
3. Davie, E.W., Fujikawa, K., and Kisiel, W. (1991) The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry* **30**, 10363–10370
4. Mann, K.G. (1994) Prothrombin and thrombin in *Hemostasis and Thrombosis*, 3rd ed., (Colman, R.W., Hirsh, J., Marder, V.J., and Salzman, E.W.H.R., eds.) pp. 184–199, Lippincott Company, Philadelphia
5. Mann, K.G. (1976) Prothrombin. *Methods Enzymol.* **45**, 123–156
6. Takagi, T. and Doolittle, R.F. (1974) Amino acid sequence studies on factor XIII and the peptide released during its activation by thrombin. *Biochemistry* **13**, 750–756
7. Lorand, L. and Konishi, K. (1964) Activation of the fibrin stabilizing factor of plasma by thrombin. *Arch. Biochem. Biophys.* **105**, 58–67
8. Nesheim, M.E. and Mann, K.G. (1979) Thrombin-catalyzed activation of single chain bovine factor V. *J. Biol. Chem.* **254**, 1326–1334
9. Hoyer, L.W. and Trabold, N.C. (1981) The effect of thrombin on human factor VIII. Cleavage of the factor VIII procoagulant protein during activation. *J. Lab. Clin. Med.* **97**, 50–64
10. Esmon, C.T. (1995) Thrombomodulin as a model of molecular mechanisms that modulate protease specificity and function at the vessel surface. *FASEB J.* **9**, 946–955
11. Vu, T.-K.H., Hung, D.T., Wheaton, V.I., and Coughlin, S.R. (1991) Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* **64**, 1057–1068
12. Rosenberg, R.D. and Damus, P.S. (1973) The purification and mechanism of action of human antithrombin-heparin cofactor. *J. Biol. Chem.* **248**, 6490–6505
13. Tollefsen, D.M., Majerus, D.W., and Blank, M.K. (1982) Heparin cofactor II. Purification and properties of a heparin-dependent inhibitor of thrombin in human plasma. *J. Biol. Chem.* **257**, 2162–2169
14. Stricker, R.B., Lane, P.K., Leffert, J.D., Rodgers, G.M., Shuman, M.A., and Corash, L. (1988) Development of anti-

thrombin antibodies following surgery in patients with prosthetic cardiac valves. *Blood* **72**, 1375–1380
15. Zehnder, J.L. and Leung, L.L. (1990) Development of antibodies to thrombin and factor V with recurrent bleeding in a patient exposed to topical bovine thrombin. *Blood* **76**, 2011–2016
16. Streiff, M.B. and Ness, P.M. (2002) Acquired FV inhibitors: a needless iatrogenic complication of bovine thrombin exposure. *Transfusion* **42**, 18–26
17. Ortel, T.L., Mercer, M.C., Thames, E.H., Moore, K.D., and Lawson, J.H. (2001) Immunologic impact and clinical outcomes after surgical exposure to bovine thrombin. *Ann. Surg.* **233**, 88–96
18. Dorion, R.P., Hamati, H.F., Landis, B., Frey, C., Heydt, D., and Carey, D. (1998) Risk and clinical significance of developing antibodies induced by topical thrombin preparations. *Arch. Pathol. Lab. Med.* **122**, 887–894
19. Tadokoro, K., Ohtoshi, T., Takafuji, S., Nakajima, K., Suzuki, S., Yamamoto, K., Ito, K., Miyamoto, T., and Muranaka, M. (1991) Topical thrombin-induced IgE-mediated anaphylaxis: RAST analysis and skin test studies. *J. Allergy Clin. Immunol.* **88**, 620–629
20. Wai, Y., Tsui, V., Peng, Z., Richardson, R., Oreopoulos, D., and Tarlo, S.M. (2003) Anaphylaxis from topical bovine thrombin (Thrombostat) during haemodialysis and evaluation of sensitization among a dialysis population. *Clin. Exp. Allergy* **33**, 1730–1734
21. Choi, E.-H., Kim, Y.J., Kim, J.-M., Hong, H.J., Han, M.H., and Kim, J. (1989) Cloning and expression of human prethrombin 2 cDNA in *Escherichia coli*. *Korean Biochem. J.* **22**, 154–160
22. So, I.S., Lee, S., Kim, S.W., Hahm, K.S., and Kim, J. (1992) Purification and activation of recombinant human prethrombin-2 produced in *E. coli*. *Korean Biochem. J.* **25**, 60–65
23. DiBella, E.E., Maurer, M.C., and Scheraga, H.A. (1995) Expression and folding of recombinant bovine prethrombin-2 and its activation to thrombin. *J. Biol. Chem.* **270**, 163–169
24. Soejima, K., Mimura, N., Yonemura, H., Nakatake, H., Imamura, T., and Nozaki, C. (2001) An efficient refolding method for the preparation of recombinant human prethrombin-2 and characterization of the recombinant-derived alpha-thrombin. *J. Biochem. (Tokyo)* **130**, 269–277
25. Russo, G., Gast, A., Schlaeger, E.J., Angiolollo, A., and Pietropaolo, C. (1997) Stable expression and purification of a secreted human recombinant prethrombin-2 and its activation to thrombin. *Protein Exp. Purif.* **10**, 214–225
26. Morita, T. and Iwanaga, S. (1981) Prothrombin activator from *Echis carinatus* venom. *Methods Enzymol.* **80**, 303–311
27. Nishida, S., Fujita, T., Kohno, N., Atoda, H., Morita, T., Takeya, H., Kido, I., Paine, M.J.I., Kawabata, S., and Iwanaga, S. (1995) cDNA cloning and deduced amino acid sequence of prothrombin activator (Ecarin) from Kenyan *Echis carinatus* venom. *Biochemistry* **34**, 1771–1778
28. Simonsen, C.C. and Levinson, A.D. (1983) Isolation and expression of an altered mouse dihydrofolate reductase cDNA. *Proc. Natl Acad. Sci. USA* **80**, 2495–2499
29. Subramani, S., Mulligan, R., and Berg, P. (1981) Expression of the mouse dihydrofolate reductase complementary deoxyribonucleic acid in simian virus 40 vectors. *Mol. Cell. Biol.* **9**, 854–864
30. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199
31. Kozak, M. (1984) Compilation and analysis of sequences upstream from the translational start site in eukaryotic mRNAs. *Nucleic Acids Res.* **12**, 857–872
32. Shulman, M., Wilde, C.D., and Kohler, G. (1978) A better cell line for making hybridomas secreting specific antibodies. *Nature* **276**, 269–270
33. Jan, D.C., Jones, S.J., Emery, A.N., and al-Rubeai, M. (1994) Peptone, a low-cost growth-promoting nutrient for intensive animal cell culture. *Cytotechnology* **16**, 17–26
34. Zhang, Y., Zhou, Y., and Yu, J. (1994) Effects of peptone on hybridoma growth and monoclonal antibody formation. *Cytotechnology* **16**, 147–150