The Journal of Microbiology, December 1998, p.283-288

Vol. 36, No. 4

Expression of Human Protease Inhibitor Nexin-I in *Escherichia coli*

Sang-Deuk Ha, Ji-Ha Kim, Hey-Lyoun Park, Hyung-Hwan Hyun, Hyung-Min Chung[3], Yunhee Kim Kwon[1], Seong-Yum Seo[2], Jung-Jae Ko[3], and Hyune-Hwan Lee*

Department of Microbiology, College of Natural Sciences, Hankuk University of Foreign Studies, Yongin-Si, Kyunggi-Do 449-791, Korea,
[1]Department of Biology, Kyunghee University, Seoul 130-701, Korea,
[2]Department of Biology, KongJu University, KongJu 314-701, Korea,
[3]Pochon Cha University, Pochon 487-800, Korea

(Received November 5, 1998 / Accepted November 26, 1998)

Human protease inhibitor nexin-I (NX-I) cDNA (1.2 Kb) was isolated from human lung cDNA library and expressed under the control of T7 promoter as a fused protein in *Escherichia coli* BL 21 and *E. coli* GJ1158 by addition of IPTG and NaCl as inducers. For GJ1158, 300 mM NaCl was added for induction after the cell reached A_{600}=0.6. As a result, *E. coli* GJ1158 showed higher expression level than BL21 with lesser extent of inclusion bodies. The optimum concentration of NaCl exerting no induction effect but shortening the time to reach A_{600}=0.6 was 50 mM. All the results suggested that *E. coli* GJ1158 was a useful host for efficient expression of NX-I using NaCl as an inducer. The expressed NX-1 showed an inhibitory effect on thrombin activity. The expressed protein was purified by immobilized metal affinity column chromatography (IMAC) and characterized by digestion with enterokinase (EK).

Key words: Protease inhibitor, nexin-I, cloning, expression, inducer, inclusion body, IMAC, mature form

Human nexin-I (NX-1) is a 40 kDa serine protease inhibitor (serpin) which is expressed and secreted by human fibroblasts, astrocytes, glial cells, and other extravascular cells (2, 4, 7, 8, 10). It has been known that the NX-I inhibits plasmin, plasminogen activators, urokinase (14), and especially thrombin (5, 6) at physiological concentration by forming a SDS-stable covalent complex (14). Because the thrombin serves as the major component in the regulatory events of blood coagulation, proliferation, and differentiation and especially in preventing neurite outgrowth activity (19), inhibition of the thrombin by NX-1 may stimulate neurite outgrowth. The possibility of the neurite outgrowth by NX-1 was indirectly demonstrated by the fact that the nucleotide and amino acid sequences of rat glial-derived neurite outgrowth factor (GDN, glial derived nexin) is identical to those of NX-1 (15). Another possible role of NX-1 is the involvement in the development of the Alzheimer disease (AD). Wagner *et al.* (19) reported that the NX-1 activity in the brains of individuals with AD shows a dramatic decrease compared to that of healthy controls. They found that the NX-1 mRNA levels were almost same in AD and control brains. These results indicated that the decrease in NX-1 is possibly due to the formation of NX-1-protease complex.

During the study of effects of NX-1 on the growth of nerve cells and reproductive cells, it was necessary to overexpress the NX-1. In this study, we report the expression of the NX-1 cDNA in *E. coli* GJ 1158 by induction with NaCl under the control of T 7 promoter. The expression level was compared to that of IPTG, especially in the formation of inclusion body. The expressed NX-1 was characterized by immunological detection and partially purified by immobilized-metal affinity column chromatography (IMAC).

Materials and Methods

Bacterial strains, plasmids, and growth conditions

E.coli TB1[F^-, *ara*, (*lac*$^-$, *proAB*), *rpsL*, *hsdR17*), r_k^-, m_k^+], BL21(DE3) (F^-, *hsdS*, *lacUV5-T7 gene 1*) (17), and GJ1158 (3) were used as hosts for protein expression. Plasmid pGEM-T was purchased from

* To whom correspondence should be addressed.
(Tel) 0335-330-4280; (Fax) 0335-333-1696
(E-mail) hyunelee@san.hufs.ac.kr

Promega Co. pMAL-P2, pMAL-C2 (New England Biolabs), and pRSET-B (Invitrogen Co.) were used as vectors for cloning and expression of NX-I cDNA. *E.coli* TB1 and BL21(DE3) were grown in LB media, and GJ1158 were grown in the LB-ON medium containing 10 g of tryptone and 5 g of yeast extract per 1 litre.

Materials

Restriction enzymes and DNA modification enzymes were purchased from Promega. Oligonucleotides were synthesized by Bioneer Co., Korea. Rabbit Anti-MBP was purchased from NEB, and mouse Anti-Xpress antibody which binds to the Enterokinase (EK) moiety were purchasd from Invitrogen Co. Thrombin and thrombin substrate (N-p-Tosyl-Gly-Pro-Arg-p-Nitroanilide) were purchased from Sigma. PMSF (Phenyl-methyl-sulfonyl fluoride) was purchased from Boehringer mannheim Co.

DNA manipulation

Plasmid DNA and chromosomal DNA preparation, DNA ligation, bacterial transformation, agarose gel electrophoresis, and Southern blotting were carried out by the standard techniques as described elsewhere (13).

Construction of recombinant plasmids

The NX-I cDNA was cloned by PCR from human lung cDNA library (Clontech. Co.). The two oligonucleotides are S1: 5'-AGC TAG GAA TTC CAC TTC AAT CCT CTG-3' and S2: 5'-AGC TAG GAA TTC TCT TCA GGG TTT GTT TAT-3'. The amplified DNA fragment containing 1.2 Kb NX-I cDNA was cloned into pGEM-T vector to construct pGNX-1. The full length cDNA from pGNX-1 was isolated by *Eco*RI digestion and subcloned into the same site of expression vectors, pMAL-P_2/C_2 and pRSET-B, to generate the pMNX-1/pMCNX-1 and pRBNX-1, respectively.

Expression of NX-1 cDNA in *E. coli*

The recombinant plasmids pMNX-1 and pMCNX-1 were transformed into *E. coli* TB1, and the plasmid pRBNX-1 was transformed into *E. coli* BL21 or GJ 1158. To express NX-1 in *E. coli* TB1 and BL21, cells were grown overnight and reinoculated into fresh media followed by growth until A_{600}=0.6. 1 mM IPTG was added to the culture to induce expression (16, 17). To express NX-1 in *E. coli* GJ1158, cells were grown in LB-ON media until A_{600}=0.6. 300 mM NaCl was added to induce expression. Total induced cells were continously grown for 2~3 hours, harvested, and lysed to analyze the expressed NX-1 by SDS-PAGE and Western blotting.

Western blot analysis

Western blot analysis was carried out by the procedure of Sambrook *et al.* (13) using the Express-blot system (Promega). Crude extract or purified NX-1 was separated in 12.5% SDS-PAGE followed by electro-transfer to PVDF membrane (Amersham). The blotted membrane was incubated with the rabbit anti-MBP (for pMNX-1 or pMCNX-1) or mouse anti-Xpress (for pRBNX-1) (1:10,000 dilution) and then incubated with alkaline phosphatase-conjugated anti-rabbit IgG or anti-mouse IgG (1: 5000). The detection was carried out by the colorization reaction using the 4-nitro blue tetrazolium chloride (NBT) as substrate.

Immobilized metal affinity column chromatography (IMAC)

The expressed EK::NX-1 fused protein from *E. coli* (pRBNX-1) was partially purified by Ni^{++}-IMAC (12) using the Xpress™ protein purification system (Invitrogen Co). The mature form of NX-1 was obtained by EK cleavage as outlined in manuals of Invitrogen.

Biochemical assay of NX-1

Biochemical activity of NX-1 was measured by detecting the inhibitory effects on the thrombin. Protease thrombin assay was carried out by the method of Lottenberg *et al.* (9) with minor modifications. Briefly, 10 μg of substrate for thrombin was incubated with 0.15 U of thrombin for 3 min at 25°C in a 1 ml of reaction mixture containing 50 mM sodium phosphate buffer (pH 8.0) and 0.1% PEG 8000. The reaction was terminated by addition of 1N HCl. To measure the inhibitory effect of NX-1 on thrombin activity, 6 μg of NX-1 from soluble fractions of crude extracts was added to the reaction mixture. The inhibitory activity of NX-1 was determined by the decrease in optical density at 405 nm. Control assay was conducted by the addition of 1N HCl before enzyme addition. The inhibition of thrombin activity by PMSF (1 mM) was used as a positive control.

Results and Discussion

Molecular cloning and characterization of the NX-1 cDNA.

To clone the full-length cDNA of NX-1, the DNA was amplified by polymerase chain reaction (94°C, 1 min for denaturation, 55°C, 2 min for annealing and 72°C, 1 min for elongation, 30 cycles) from human lung cDNA library. The amplified NX-1 cDNA was 1.2 Kb long and cloned into *Eco*RI site of

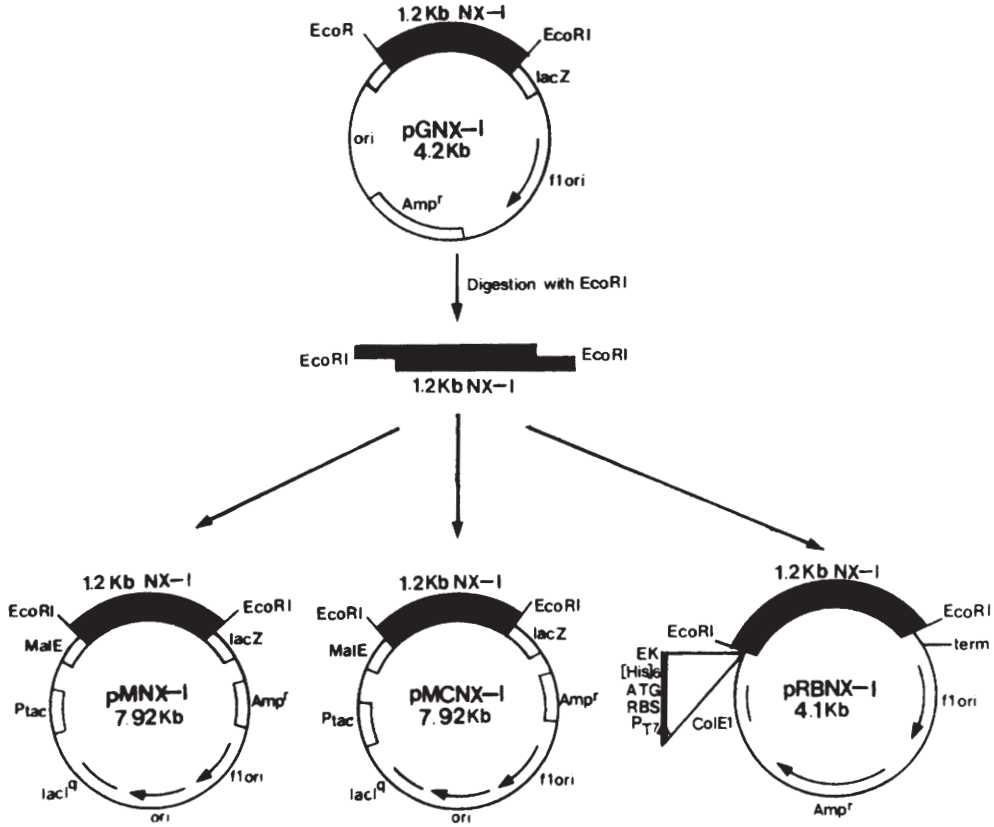

Fig. 1. Construction of expression plasmids, pMNX-1, pMCX-1, and pRBNX-1.

pGEM-T vector to construct the pGNX-1. The nucleotide sequence analysis of the cloned cDNA identified it as αNX-1 cDNA (11) (data not shown). To generate the expression plasmids, the cDNA fragment was subcloned into expression vector pMAL-P_2/C_2 and pRSET-B by digestion with *Eco*RI as shown in Fig. 1. The resultant plasmids pMNX-1 and pMCNX-1 contained the NX-1 cDNA fused to *malE* DNA. Another plasmid pRBNX-1 consisted of NX-1 cDNA fused to EK moiety attached to $(His)_6$-tag (Fig. 1).

Expression of NX-1 in *E. coli* by IPTG induction

To compare the expression level, the recombinant plasmids pMNX-1, pMCNX-1, and pRBNX-1 were transformed into *E. coli*. The former two plasmids were transformed into *E. coli* TB1, and the latter plasmid was transformed into *E. coli* BL21 (DE3) or *E. coli* GJ1158. Although all the cells harboring the plasmids expressed NX-1 in fused form (Fig. 2 and 3), the cells containing the plasmid pMCNX-1 expressed more of MBP::NX-1 fused protein than pMNX-1 by forming a large amount of inclusion bodies. The relative proportion of the fused protein in the cells was determined following gel electrophoresis. The distribution ratio between pMCNX-1 and pMNX-1 analysed by densitometric assay was around 3:1 (Fig. 2, compare lane 5 and 9 for 86KDa protein). Although the plasmid pMNX-1 was made for secretion of expressed protein to the periplasmic space, the cells harboring the plasmid showed more of MBP::NX-1 in the intracellular fraction than in the periplasmic fraction (data not shown). The results suggested that the MBP::NX-1 fused protein could not be secreted efficiently because of its large size (86KDa). The expression of NX-1 from the *E. coli* BL21(DE3) harboring pRBNX-1 was carried out by IPTG induction. The NX-1 was expressed in fused form with EK moiety (EK::NX-1) which was attached to $(His)_6$-tag. The resultant 45KDa protein band was detected by SDS-PAGE and Western blotting (Fig. 3). About 20% of the total protein was NX-1 fused prorein.

Expression of pRBNX-1 by NaCl induction

The expression by induction with IPTG has several disadvantages such as toxicity of IPTG, high cost, and large amount of inclusion bodies. To avoid the problems, the expression of NX-1 cDNA was conducted by NaCl as inducer using the host

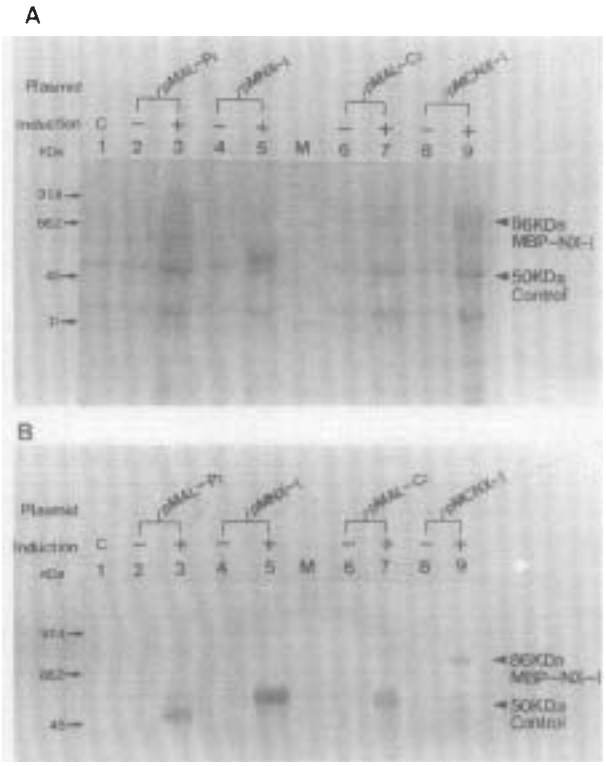

Fig. 2. Expression of human protease nexin-1. *E. coli* TB1 cells harboring each plasmid were lysed after induction by IPTG and analysed by SDS-PAGE (A) and Western blotting (B). The MBP::NX-1 fused proteins (86KDa) are shown in the figure. Lane C shows the lysate of host cells and lane M shows the protein size marker.

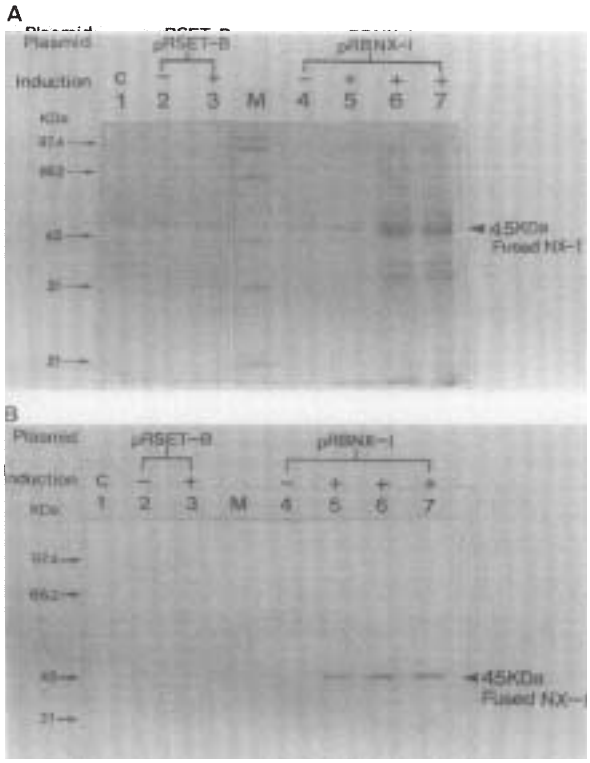

Fig. 3. Expression of human protease inhibitor nexin-1. The expression of nexin-1 in *E. coli* BL21 (pRBNX-1) was induced by IPTG. The expressed EK::NX-1 fused proteins (45 KDa) were analysed by SDS-PAGE (A) and Western blotting (B). Induction was carried out after IPTG addition for 1 hr (lane 5), 2 hrs (lane 6), and 3 hrs (lane 7). Lane C shows the lysate of host cells and lane M shows the protein size marker.

strain *E. coli* GJ1158. *E. coli* GJ1158 has the *pro-Up::T7RNAP* operon fusion in the chromosome, so that the T7 RNP can be induced by addition of NaCl into the culture medium (3). *E. coli* GJ1158 harboring the recombinant plasmid pRBNX-1 was grown overnight and reinoculated in LB-ON media until A_{600}=0.6, and 300 mM of NaCl was added to the culture medium for induction. After 2-3 hours, cells were lysed, and the crude extracts were analysed by SDS-PAGE and Western blotting (Fig. 4). The results showed that the expression was successful and furthermore, that lesser amount of inclusion bodies had formed compared to that of IPTG induction (Fig. 5). Densitometric analysis revealed that the ratio of NX-1 fused proteins between insoluble and soluble fractions (compare lane 3 to 4 and lane 7 to 8 in Fig. 5) following IPTG and NaCl induction was about 8.2:1 and 1.8:1, respectively. And approximately 4.5 times less amount of inclusion bodies formed. One disadvantage of using the system of induction by NaCl is that the cells should be grown in the medium without NaCl to prevent the induction before addition of inducer. Hence, it took relatively long time to reach to A_{600}=0.6 (about 2.5 hrs in case of LB-ON media but 1 hr in case of LB media). To overcome this problem, 0, 50, and 100 mM NaCl were added to the medium to find the optimum concentration for reducing growth time until A_{600}=0.6. The resultant expression data showed that the optimum concentration of NaCl which shorten the growth time without exerting induction effect in the cells was 50mM (Fig. 6). This data was supported by densitometric analysis of the expressed NX-1 following induction and SDS-PAGE. Although slight increase in the portion of expressed NX-1 was detected (38% and 30% of the total protein was NX-1 in 50 mM and without NaCl, respectively), no induction effects were observed in that concentration. Above the concentration, cells were induced at the beginning of the growth, so that it gave negative effects on the production of NX-1 (data not shown). These results showed that *E. coli* GJ1158 is a useful host for the production of NX-1 with less amount of inclusion bodies when 50 mM of NaCl is added to the medium for efficient cell growth.

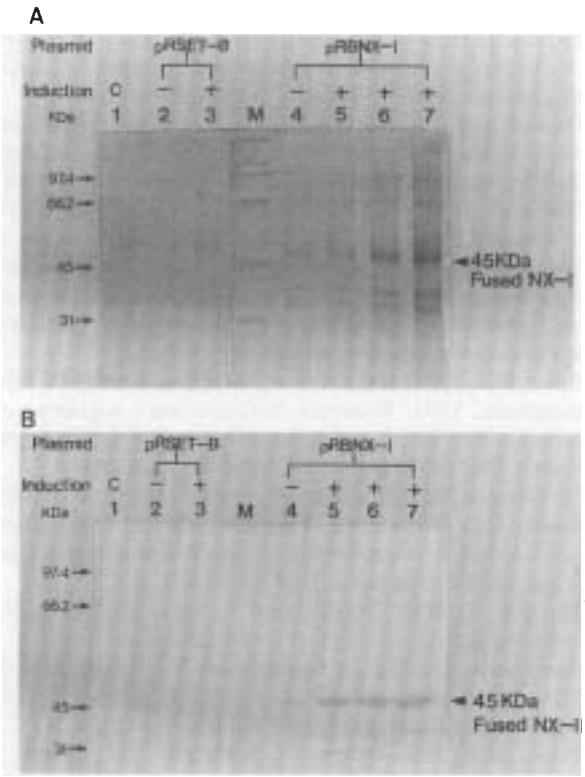

Fig. 4. Expression of nexin-1 in *E. coli* GJ1158. Cells harboring the plasmid pRBNX-1 were induced by 300 mM NaCl. The expressed EK::NX-1 fused proteins (45 KDa) were analysed by SDS-PAGE (A) and Western blotting (B). The induction was carried out for 1 hr (lane 5), 2 hrs (lane 6), and 3 hrs (lane 7) after NaCl addition. Lane C shows the lysate of host cells and lane M shows the protein size marker.

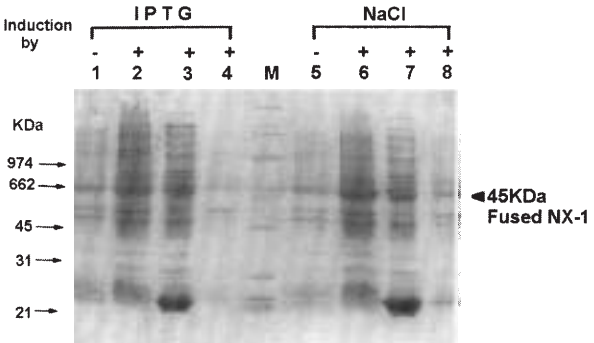

Fig. 5. Comparison of inclusion body formation after IPTG induction in *E. coli* BL21 (lane 1-4) and NaCl induction in *E. coli* GJ1158 (lane 5-8). Each cell harboring plasmid pRBNX-1 was cultured and induced at A_{600}=0.6 by addition of 1 mM IPTG or 300 mM NaCl as inducers. The lysate of the cells were separated into pellet (containing inclusion bodies) and supernatant (containing soluble proteins) fractions. Proteins were analysed by SDS-PAGE and visualized by Coomassie blue staining. Lane: 1 and 5, total lysate (unfractionated) of uninduced culture; 2 and 6, total lysate of induced culture; 3 and 7, pellet fractions of induced lysate; 4 and 8, supernatant fractions of induced lysate. Arrow indicates the expressed EK::NX-1 fused proteins. Lane M is protein size marker.

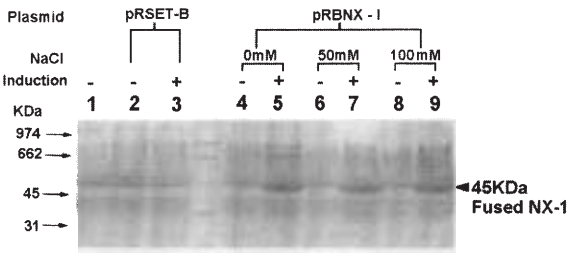

Fig. 6. Determination of NaCl concentration for efficient growth of *E. coli* GJ1158 before induction. 0, 50, and 100 mM NaCl were added to the medium. After the cell reached A_{600}=0.6, 300 mM NaCl was added for induction. Arrow indicates the EK::NX-1 fused band.

Partial purification and characterization of recombinant NX-1

NX-1 from *E. coli* GJ1158 was partially purified by one step Ni^{++}-IMAC. The purified NX-1::EK was identified and characterized by SDS-PAGE and Western blot analysis. The expressed protein was identified as 45KDa by SDS-PAGE. The purified protein was digested with EK. The resulting mature NX-1 showed an almost single band in SDS-PAGE with a molecular weight of 40 KDa (data not shown). The mature form disappeared in the Western blot with the anti-Xpress antibody due to the loss of EK moiety. (Fig. 7). The expressed NX-1 protein showed inhibitory activity on the thrombin by decreasing optical density (Fig. 8). The inhibitory activity would be due to the binding of recombinant NX-1 to thrombin as reported by Scott *et al.*(14). This result suggested that the NX-1 expressed in *E. coli* would have a biological activity regardless of glycosylation. For furth-

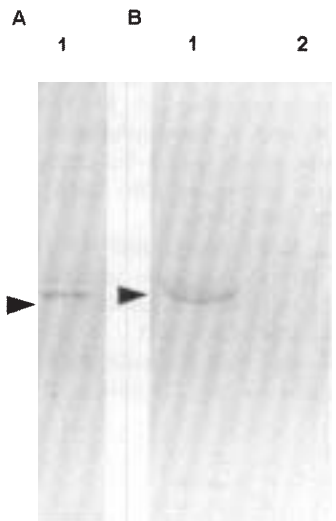

Fig. 7. Partial purification and characterization of EK::NX-1. The proteins were analysed by SDS-PAGE (A) and Western blotting (B). Lane: 1, partially purified EK::NX-1; 2, Detection of EK::NX-1 by anti-Xpress antibody.

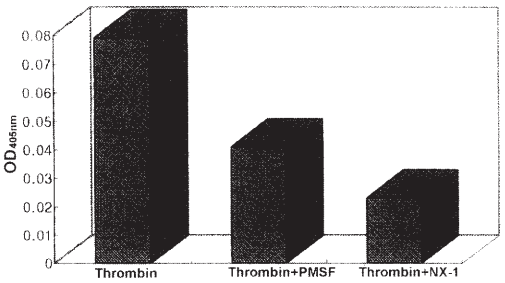

Fig. 8. The inhibitory effects of recombinant NX-1 on thrombin activity. 150 ml of cultured cells were harvested, resolved in 15ml of thrombin assay buffer, and lysed by French Press followed by centrifugation for preparing crude extracts. The soluble fractions containing NX-1 were used for assay.

er charactorization of the recombinant NX-1, other biochemical and biological studies will need to be carried out with the expressed protein after proper folding steps.

Acknowledgments

We thank Dr. Joel A. Jesse (Director of Research, Life Technologies, USA) for the kind donation of the strain *E. coli* GJ1158. Part of the present study is supported by the grants of Ministry of Health and Human Services of Korea (No: HMP-96-D-1-1032).

References

1. **Ausubel, F.M., R.Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhi.** 1991. Current protocols in Molecular Biology Vol. 1. John Wiley and Sons, New York.
2. **Baker, J.B., D.A. Low, R.L. Suniner, and D.D. Cunningham.** 1980. Protease-nexin: a cellular component that links thrombin and plasminogen activator and mediates their binding to cells. *Cell.* **21**, 37-45.
3. **Bhandari, P. and J. Gowrishankar.** 1997. An *Escherichia coli* Host strain useful for efficient overproduction of cloned gene production with NaCl as the Inducer. *J. Bacteriol.* **179**, 4403-4406.
4. **Eaton, D.L., R.W. Scott, and J.B. Baker.** 1984. Purification of human fibroblast urokinase proenzyme and analysis of its regulation by protease and protease nexin. *J. Biol. Chem.* **259**, 6241-6247.
5. **Farrel, D.H. and D.D. Cunningham.** 1986. Human fibroblasts accelerate the Inhibition of thrombin by protease nexin. *Proc. Natl. Acad. Sci. USA* **83**, 6858-6862.
6. **Farrel, D.H. and D.D. Cunningham.** 1987. Glycosaminoglycans on fibroblasts accelerate thrombin inhibition by protease nexin-1. *Biochem. J.* **245**, 543-550.
7. **Howard, E.W. and D.J. Knauer.** 1986a. Human protease nexin-1. Further characterization using a highly specific polyclonal antibody. *J. Biol. Chem.* **261**, 684-689.
8. **Howard. E.W. and D.J. Knauer.** 1986b. Biosynthesis of protease nexin-1. *J. Biol. Chem.* **261**, 14184-14190.
9. **Lottenberg, R., U. Christensen, C.M. Jackson, and P.L. Coleman.** 1982. Assay of coagulation proteases using peptide chromogenic and fluorogenic substrates. *Methods. Enzymol.* **80**, 341-361
10. **Low. D.A., J.B. Baker, W.C. Koonce, and D.D. Cunningham.** 1981. Released protease-nexin regulates cellular binding internalization, and degration of serine proteases. *Proc. Natl. Acad. Sci. USA* **78**, 2340-2344.
11. **McGropan, M., J. Kennedy, M.P. Ling, C. Hsu, R.W. Scott, C.C. Simonsen, and J.B. Baker.** 1988. Molecular cloning and expression of two forms of human protease nexin-I. *Bio/technology* **6**, 172-177.
12. **Porath, J.** 1992. Immobilized metal ion affinity chromatography. *Protein Expr. Purif.* **3**, 263-281.
13. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor laboratory Press. Cold Spring Harbor, N. Y.
14. **Scott. R.W., B.L. Bergman, A. Bajpai, R.T. Hersh, H. Rodriguez, B.N. Jones, C. Barreda, S. Watts, and J. B. Barker.** 1985. Protease nexin. Properties and a modified purification procedure. *J. Biol. Chem.* **260**, 7029-7034.
15. **Sommer, J., S.M. Gloor, G.F. Rovelli, J. Hofsteenge, H. Nick, R. Meier, and D. Monard.** 1987. cDNA sequence coding for a rat glia-derived nexin and its homology to member of the serpin superfamily. *Biochemistry* **26**, 6407-6410.
16. **Studier, F.W. and B.A. Moffatt.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113-130.
17. **Studier, F.W., A.H. Rosenberg, J.J. Dunn, and J.W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol.* **185**, 60-89.
18. **Towbin, H., T. Staehlin, and J. Gordon, J.** 1987. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheet: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350.
19. **Wagner, S.L., J.W. Geddes, C.W. Cotman, A.L. Lau, D. Gruw, P. J. Isackson, and D.D. Cunningham.** 1989. Protease nexin-1, an antithrombin with neurite outgrowth activity, is reduced in Alzheimer disease. *Proc. Natl. Acad. Sci. USA* **86**: 8284-8288.