공학박사 학위논문

*Vibrio mimicus*의 collagenase의 특성 및 domain의 기능에 관한 연구

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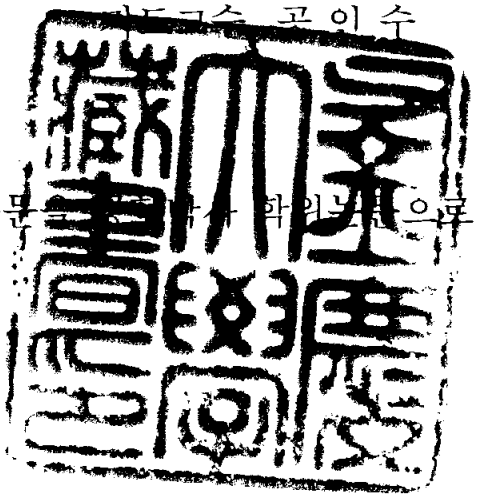

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Chapter I

Characterization of collagenase from *Vibrio mimicus* and the function of domain

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Abstract

Vibrio mimicus is a typical strain of *V. cholerae* and was regarded as non-sucrose fermenting *V. cholerae* non-O1 before 1981. The metalloprotease of this strain was reported as class II type [Lee et al. 1998]. To investigate the metalloprotease of *V. mimicus*, the recombinant metalloprotease (rVMC) of *V.mimicus* was overexpressed in *E.coli* as premature (VMC52) and extracellular (VMC62) types and purified by metal affinity chromatographies. The rVMC showed maximum activity at the temperature around 37 ℃ and pH around 8. The purified VMCs showed collagenase activity toward gelatin, type I, type II, type III and synthetic peptide Z-GPLGP, Z-GPGGPA. But it did not degrade type V collagen. The VMC also showed the cytotoxcity on the CHSE-214 cell and FHM cell. The 3´of collagenase gene (*vmc*) was digested serially with exonuclaease III and resultant 100 to 500bp truncates corresponded to 57 to 42kDa of C-terminal truncates of 61kDa rVMC were isolated and overexpressed in *E. coli*. The collagenase activity of truncates were investigated using gelatin as substrate. The gelatin degrading activities of truncates were disappeared by 301bp deletion (50kDa) from 3´deletion region but 200bp deleted truncate (53kDa) have a same activity with intact collagenase (rVMC62: 61kDa). To investigate the relation of collagenase activities and collagen binding capacities, the C-terminal truncates were incubated with type I collagen and filtered to remove the collagen

and analyzed by SDS-PAGE. The result from SDS-PAGE showed that the collagenase activity and collagen binding capacity was exactly coincided. And it also implied that the C-terminal region of VMC act as a collagen binding domain. The various lengths of VMC C-terminus were overexpressed as GST fusion peptides and investigated collagen-binding capacity of the purified fusion peptides. The collagen binding capacity was checked the GST activity of filtrates. As a result, core region for binding to collagen was ^{528}LVLSRRPGQFAQWAQTVKNLGEQYNAEFAVWL DT^{561}. The reiterated amino residues FAXWXXT were found in amino acid residues of 537 to 543 and 555 to 561. The half of this peptide (^{528}LVLSRRPGQFAQWAQ^{542}) did not bind to collagen. The K_d of this core region and VMC were calculated by scatchard plot analysis. The K_d of 33 amino acids and VMC were 4×10^{-10} M and 2.1×10^{-10} M, respectively.

Characterization of *Vibrio mimicus* metalloprotease (VMC)

Abstract

Metalloproteases were reported from mammalian to bacteria strains. The active sites of metalloproteases were composed of HEXXH and this was known for zinc binding motif. Metalloproteases of *Vibrio* sp. were classified as two distinct categories by amino acid sequence similarities, as class I and II [Lee et al. 1998]. Class I have large signal peptide region (about 200 aa). The zinc-binding domains of class I have HEXXH and have an extra glutamic acid near the zinc-binding motif. Whereas, class II only have HEXXH motif and one possible extra glutamic acid was located at the distance from zinc-binding motif. Beyond this, these two classes showed differences in their biochemical properties. In the class I metalloproteases, the polypeptide of C-terminal region was removed by autocalalytic cleavage mechanism and resulted a changes of the substrates specificity and this quite differ from class II metalloproteases. The C-terminal truncated metalloprotease did not digest the insoluble substrates and this region was suggested as substrate binding related region.

Vibrio mimicus is a typical strain of *V. cholerae* and was regarded as non-sucrose fermenting *V. cholerae* non-O1 before 1981. The metalloprotease of this strain was reported as classII type [Lee et al. 1998]

To investigate the metalloprotease of *V. mimicus*, the recombinant metalloprotease (rVMC) of *V.mimicus* was overexpressed in *E.coli* as premature (VMC52) and extracellular (VMC62) types and purified by metal affinity chromatographies. The rVMC showed maximum activity at the temperature around 37 ℃ and pH around 8. The purified VMCs showed collagenase activity toward gelatin, type I, type II, type III and synthetic peptide Z-GPLGP, Z-GPGGPA. But it did not degrade type V collagen. The VMC also showed the cytotoxcity on the CHSE-214 cell and FHM cell. The deletion of 100 amino acid in C-terminal region of rVMC62 resulted the completely loss of collagenase activity.

서론

Vivrio sp. 는 수, 해양유래의 병원성 미생물로 gram negative이며 2종을 제외하고는 oxidase negative이며 rod형의 통상혐기성의 미생물이며 한 개의 polar flagellum에 의해서 운동을 하는 미생물이다. 이 가운데 *V. cholerae*와 *V. mimicus*는 NaCl이 없는 환경에서는 자랄 수 없는 halophilic 미생물로 알려져 있다. 현재 약 35종이상의 *Vibrio* sp.가 공표되어 있으며 이중 13종의 *Vibrio*가 인체에 감염을 일으키는 것으로 보고되고 있다.(표1). 또한 이러한 *Vibrio*는 8가지의 주요 생리적 차이에 의해서 6 group으로 크게 분류되고 있으며 병리적 증상이 심하게 미치는 *Vibrio* sp.는 *V. cholerae, V. vulnificus, V. parahaemolyticus, V. mimicus, V. fluvialis, V. hollisae*의 6종류로 보고되고 있다.

대표적인 수, 해양유래의 병원성 미생물인 *V. cholerae*는 2개의 serotype으로 분류할수 있는데 *V. cholerae* Inaba, Ogawa와 2 가지의 biotype 인 classical, El Tor로 분류된다. 1992년이후 O group 1 specific antiserum에 반응하지 않는 새로운 serotype인 O139 (Bengal)가 보고되었다. *V. cholerae*로부터의 대표적 toxin으로는 cholera toxin (CTX)이 있으며 CTX는 5개의 binding (B) subunits (11kDa)과 1개의 active (A1) subunit (23kDa), bridging piece (A2) (5.5kDa)로 이루어져 있으며 소장의 표피세포에 부착하여 adenylate cyclase를 활성화시켜 cAMP의 생산을 활성화 시키고 이렇게 intracellular에 대량으로 생산된 cAMP는 intestinal lumen으로의 막

전위를 교란시켜 과다한 수분이 장내에서 빠져 나가는 심한 탈수현상을 일으키며 이러한 CTX의 활성화에는 hemagglutinin (HA)/ protease가 CTX를 A subunit을 nicking시켜 활성화 시키는 것으로 알려져 있다 [Dotevall et al. 1985].

*V.parahaemolyticus*는 어류의 섭취로 인한 장염의 원인균으로 hemolysin [Nishibuchi et al. 1992], lethal toxin [Sarkar et al. 1987], metalloprotease [Yu et al 1999], vascular permeability factor [Honda et al. 1976] 등의 toxin등이 보고되었다. 이중 hemolysin은 *V. parahaemolyticus*의 병원성을 나타내는 중요한 지표로 kanagawa phenomenon (KP) [Miyamoto et al. 1969]을 일으키는 thermostable direct hemolysin (TDH) 과 KP negative strain에서 발견되는 TDH related hemolysin (TRH)의 2가지 type의 hemolysin이 보고되고 있다. TDH는 *V. parahaemolyticus*의 대표적인 toxin으로 약 42kDa의 분자량을 가지고 100℃, 10분간의 가열에서도 실활 되지 않을 정도로 높은 안정성을 가지고 있다. KP negative 인 *V. parahaemolyticus*에서 발견되는 TRH의 경우도 높은 cytotoxcity activity를 가지고 있다.

*V. vulnificus*의 경우 만성 간질환, 알코올중독자, 그리고 면역 저하자에게서 출혈성 수포 증상인 패혈증을 일으키는 원인균이며 *V. vulnificus*로부터 pathogenic factor로 생각되어지는 여러 toxin들이 보고되어 왔다. 이러한 toxin으로 hemolysin [Kreger et al. 1981, Wright et al. 1991] , metalloprotease [Cheng et al. 1996], LPS [Moncada et al. 1991] 이 보고되었다.

또한 gram negative bacteria의 LPS는 다양한 cell에서 NO(nitric oxide)를 생산하고 이는 저혈압성의 패혈증성 shock을 유발한다고 알려져 있다 (Moncada et al. 1991). 이외 hemolysin은 56kDa의 분자량을 가지고 있으며 two step processing 을 거쳐 분비되며 CHO cell과 mouse실험에서 강력한 cytotoxicity를 보이며 mouse, sheep, pig등 17종의 erythrocyte에 대해 hemolytic activity가 보고되었다 [Kreger, Lockwod 1981, Gray et al. 1985].

Two step processing은 비슷한 유전자를 가지고 있는 *V.cholerae*, *V. mimicus*에서는 관찰이 되지만 역시 유사한 유전자를 가지고 있는 *V. fluviails*에서는 관찰되지 않는 것으로 보고되고 있다 [Kim et al. 1997, Rahman et al. 1997, Han et al. 2002].

*V. vulnificus*의 metalloprotease의 유전자는 cloning되어 염기서열 등 기본적인 특성이 밝혀져 46kDa의 mature protease를 가지고 *V. anguillarum*, *V.cholerae*의 HA/protease, *V.proteolyticus*의 protease와 높은 유사성을 가지고 있는 것으로 보고되었으며 [Chang et al. 1997], 계속된 연구를 통해 C-terminal region의 10kDa의 peptide region이 autocatalytic process를 통해 잘려진다는 것이 보고되었다 [Yamamoto et al. 1990].

*V.mimicus*는 이전의 분류동정 시에는 non-sucrose fermenting *V. cholerae* non-O1로 분류되었으나 1981년 Davis등에 의해 *V.cholerae* non-O1로부터 *V.mimicus*로 새롭게 분류되었다 [Davis et al. 1981]. Sucrose분해능이 없어 TCBS배지상에서 노란 군락을 형성하지 못하고 청록색 군집을 형성하

는 균으로 최근의 연구를 통해 여러 toxin유전자들이 *V. mimicus*로부터 분리 보고되었고 이 균으로부터의 여러 toxin역시 *V. cholerae* O1이나 non-O1에서 보고되는 toxin 유전자들과 매우 유사한 염기서열을 가지고 있어 이들 균종 간의 유사성을 더욱 뒷받침하고 있다.

병원성 *V. mimicus*로부터 보고된 여러 toxin은 CT (cholerae toxin) [Spira et al. 1984], phospholipase [Kang et al. 1998], hemolysin [Kim et al. 1997], metalloprotease [Lee, et al. 1998], protease [Chowdhury et al. 1990]등과 같은 여러 virulence factor들이 발견 보고되었으며 이들 또한 여러 *Vibrio*들과 그 gene의 염기서열에 있어 서로 많은 유사성을 보여주고 있다.

Vibrio sp.에서 보고되는 hemolysin의 경우 *Vibrio* sp.로부터의 장염의 증상중 하나인 혈변증에 관계되는 것으로 보고되고 있으며 서로의 hemolysin gene의 염기서열상에 많은 유사점을 보이고 있다. *V. cholerae* hemolysin의 염기서열과 아미노산 서열은 *V. mimicus*, *V. fluvialis*, *V. anguillarum*과 높은 homology를 보이고 있다. 이러한 유사성은 *Vibrio* sp. 유래의 phospholipase에서도 발견되며, *V. cholerae*의 phospholipase를 coding 하고 있는 *lec* gene 또한 *V. mimicus*의 phospholipase를 coding하는 *phl* gene과 높은 homology를 보이고 있다 [Kim et al. 1997, Kang et al. 1998].

Vibrio sp.로부터의 protease의 경우 HA/protease [Booth et al 1984], elastase [Kothary et al. 1987], metalloprotease [Cheng et al 1996], collagenase [Lee et al. 1998] 등 여러 다양한 형태가 보고되고 있는데 이러한 protease

의 중요한 기능으로는 host cell receptor를 파괴하여 다른 cell로 이전하는 데 중요한 역할을 하는 것으로 추정되고 있다.

*V. anguillarum*을 이용한 초기감염 연구에서도 metalloprotease가 결여된 mutant는 wild type보다 10배 낮은 감염율을 가지고 있다고 알려져 *Vibrio*로 인한 감염에 중요한 역할을 하는것으로 보고되고 있고 [Milton et al. 1992] 또한 *V. mimicus*로 부터의 분리된 31kDa의 extracellular protease의 경우 skin의 vascular permeability를 증대 시키며 rabbit의 ileal loop의 flood accumulation을 일으키는 것으로 보고되고 있다 [Chowdhury et al. 1990].

Animal model에서 enterotoxigenicity를 보이고 있는 병원성균주인 *V. mimicus* E33으로부터의 HA/protease는 *V. cholerae*의 HA/protease와 매우 높은 유사성을 보이는 2개의 다른 hemagglutinin이 발견되었는데 각각 rabbit erythrocyte와 chicken erythrocyte에 활성을 보이고 있어 R-HA와 C-HA로 명명되었으나 C-HA의 경우 rabbit 과 guinea pig의 erythrocyte에 모두 활성을 보이고 있다. R-HA의 경우 LPS인 것으로 알려져 있고 C-HA의 경우 39kDa의 outer membrane protein으로 보고되었다 [Alam et al. 1996].

최근의 연구를 통해 *V. cholerae* El Tor의 전체 genome 의 nucleotide sequence가 밝혀져 보고 되었다. *V. cholerae*의 genome 상에서는 약 2.8Mb와 1Mb의 2개의 chromosomal DNA를 가지고 있는 것으로 보고되

었고 [Heidelberg et al. 2000]이 nucleotide sequence 상에는 총 24개의 protease관련 gene이 존재하고 있는 것으로 보고되었으며 이중 1개의 유전자가 collagenase로 밝혀졌다. 이 collagenase gene (VC1650)은 *V. cholerae*의 chromosome 1에 존재 하고 있으며 *V. mimicus*의 *vmc* gene과 74%의 높은 homology를 보이고 있다. 특이한 사항은 또 다른 metalloprotease인 VCA0223가 chromosome 2에 존재하고 있다는 사실이다.

본 연구실에서는 *Vibrio* sp의 metalloprotease 의 염기서열 분석하여 유사도에 따라 2가지로 분류될수 있음을 처음으로 보고하였는데 (Fig.2) [Lee et al. 1998] 첫번째 class에 해당하는 것은 *V. proteolyticus*, *V. fluvialis*, *V. vulnificus*, *V. angullarum*의 metalloprotease에 해당하는것으로 45kDa정도의 mature protease형태를 이루고 C-terminal 의 10kDa의 polypeptide가 autocatalytic process에 의해서 잘려나가 최종적으로 35kDa 정도의 크기의 protease를 형성하는 것으로 보고되고 있다 (Table 2.). 반면에 class II에 해당하는 *V. cholerae* 569B, *V. parahaemolyticus*, *V. alginolyticus*유래의 metalloprotease의 경우 N-terminal region의 signal peptide가 잘리는 것 이외의 C-terminal region의 processing은 없으며 HEXXH의 zinc binding motif를 가지고 있으며 이 motif에서 상당히 떨어진 곳에 또 하나의 glutamic acid를 가지고 있는 것으로 조사되었다.

이들 class간의 차이는 염기서열의 차이뿐 아니라 그 생화학적 특성에

서도 현격한 차이를 나타내고 있다. Class I에서 10kDa의 C-terminal region이 제거되면 insoluble 기질의 분해능이 현격히 떨어지고 있으며 이러한 차이가 기질 결합능과 상관관계가 있는 것으로 추정되고 있는 반면 class II의 metalloprotease에서는 C-terminal region의 역할에 대한 보고가 없어 정확한 기능을 알지 못하고 있다. 본 연구에서는 대표적인 class II metalloprotease인 *V. mimicus*의 VMC를 이용하여 그 생화학적인 특성을 연구하였고 C-terminal region과 metalloprotease의 활성의 관계를 조사하였다.

재료 및 방법

균주, plasmids

Metalloprotease (VMC)를 분리하기 위한 *V. mimicus*는 공시 균주인 ATCC33653을 사용하였으며 cloning을 위한 vector는 pUC19와 pGEM4Z (promega)를 사용하여 유전자의 cloning과 subcloning에 이용하였으며 이미 본연구실에서 분리한 metalloprotease의 유전자가 함유되어 있는 plasmid인 pVMC193 [Lee et al. 1998] 또한 subcloning에 사용하였다.

단백질의 대량발현과 정제를 위한 vector는 novagen사의 pET22b(+)를 사용하였다. *E.coli* host로는 cloning을 위해서는 DH5α또는 XL1-blue를 사용하였으며 대량발현을 위한 host로는 T7 RNA polymerase를 chromosomal DNA내에 함유하고 있는 host인 BL21(DE3)를 사용하였다.

*V. mimicus*로부터 metalloprotease gene (*vmc*)가 knock out된 mutant의 제조를 위해서는 suicide vector인 pNQ705를 이용하였으며 이때 *E.coli* host로 SM10λ *pir*를 이용하였다.

배양

*V. mimicus*를 배양하기 위한 배지로는 BHI (Brain Heart Infusion)배지를 사용하였으며 plate의 제조를 위해서는 BHI배지에 약 1% (w/v)의 agar powder를 첨가하여 사용하였다. 배양을 위한 온도는 25-30℃의 온도범위에서 배양을 하였으며 incubator의 회전속도는 125rpm을 유지하여 24시간 배양을 하였다. *E. coli*의 배양을 위해서는 LB (Luria-Bertani) 배지

를 사용하였으며 plasmid를 함유한 host의 배양을 위해서는 적절한 항생제를 첨가 하여 사용하였다.

Chromosomal DNA 및 Plasmid DNA의 분리

Gene cloning을 위한 chromosomal DNA 및 plasmid의 분리는 current protocol in molecular biology의 방법을 이용하였으며 연기서열분석을 위한 plasmid의 분리는 Qiagen miniprep kit (Qiagen co.)을 이용하여 준비 하였다. 분리된 DNA의 정량은 spectrophotometer를 이용하여 260nm에서 흡광도를 측정하여 정량 하였고 적절한 제한효소를 이용하여 agarose gel상에서 분자량을 측정하였다.

염기서열결정 및 분석

염기서열의 결정은 ABI prism 377 (Perkin-Elmer) DNA sequencer를 이용하여 결정 하였다. pUC계열의 plasmid는 m13RP또는 UP의 primer를 사용하였고 pET22b(+)의 경우 T7 terminal region의 부위를 primer로 사용하였다. 염기서열로부터 얻어진 deduced amino acid sequence의 homology의 분석과 multi-alignment의 분석은 NCBI의 BLAST search program및 Clustal W multi-alignment tool을 이용하여 분석 하였다. 분석된 염기서열과 아미노산서열의 배열은 GeneDoc (ver 2.6) program을 이용하였다.

Recombinant plasmid의 제조

이전의 연구를 통하여 *V. mimicus*로부터 metalloprotease gene (*vmc*)의 염기서열을 확인 하였으며 이를 바탕으로 5′의 개시 codon인 GTG로부터 종결 codon인 TAA를 함유하고 있는 유전자 부위를 증폭할수 있는 primer와 metalloprotease의 mature region을 증폭할 수 있는 primer를 제작하였고 subcloning을 위해 primer의 5′ 부위에 *Nde*I과 *Bam*HI의 인지부위를 포함시켰다. 본 연구에서 사용한 metalloprotease는 host cell에서 발현 후 extracellular로 분비되고 있으며 분비된 metalloprotease의 N-terminal 아미노산 서열을 분석한 결과 Glu91-Ala92가 절단된 61kDa의 metalloprotease임을 확인하여 보고하였다 [Lee et al. 1998].

목적 부위의 증폭은 PCR을 이용하여 denaturing (94℃, 30sec), annealing (60℃, 30sec), extension (72℃, 2min)의 조건에서 행하였다. 사용된 primer는 premature type을 증폭하기 위해서는 primer5 (5'-GGCCCATATGGTG TACTCTCAACAATTG-3' : *Nde*I) 와 primer2(5'-GGCCGGATCCCCTGTA AAGATCGCGTGG-3': *Bam*HI)를 사용하였으며 extracellular protease type을 증폭하기 위해서는 primer6(5'-GGCCCATATGGCAGAACAAGCCCAA CGC-3': *Nde*I)와 primer2를 사용하였으며 PCR반응 후 반응물을 동량의 chloroform으로 처리하여 12,000 × g 에서 15분간 원심분리 후 상층액을 모아 다시 2배의 cold ethanol을 처리하여 침전을 시키고 다시 증류수에 녹인 후 0.8% (w/v)의 agarose gel에 전기영동을 실시하여 증폭된 크

기를 측정하고 확인한 후 다음실험 때까지 -20℃에서 보관하였다. 증폭된 부위는 제한효소 *Nde*I과 *Bam*HI을 사용하여 절단 하였으며 동일한 제한효소가 처리된 pET22b(+) vector에 연결하여 *E.coli* XL1-Blue에 형질전환하여 ampicillin이 함유된 LB배지에 도말 하였다. 제조합체의 screening은 PCR 증폭반응에 사용된 primer5와 primer2 그리고 primer6과 primer2를 사용한 colony PCR을 이용하여 선별하였다. Colony PCR을 통해 premature region과 mature region을 포함하고 있는 recombinant를 1차 선별하였으며 선별된 colony로부터 alkaline lysis법을 이용하여 plasmid를 분리한 후 cloning에 사용된 제한효소인 *Nde*I과 *Bam*HI을 사용하여 insert을 분리하여 PCR product의 크기를 확인 후 연기서열의 결정을 통하여 recombinant plasmid를 확인하였다.

확인된 plasmid를 pETMETA52(premature)와 pETMETA62 (extracellular metalloprotease)로 명명하였으며 이를 대량 발현용 균주인 *E.coli* BL21(DE3)에 형질전환 하여 metalloprotease의 분리 정제에 사용하였다.

정제

*E.coli*로부터 metalloprotease를 분리 정제 하기 위해 1 colony를 10ml의 LB 배지에 접종한후 37℃에서 하룻동안 전배양 후 이를 starter로 사용하여 다시 1L의 LB배지에 재접종후 이를 다시 37℃에서 600nm에서의

흡광도가 0.6의 값에 이를 때 까지 배양한후 IPTG (isopropyl β-D-thio-galactopyranoside)를 1mM의 농도가 되도록 첨가하여 3-4시간 동안 배양하였다. 배양이 끝난후 배양액을 7000 x g, 4℃에서 20분간 원심분리후 cell pellet을 얻은후에 다시 20mM Tris-HCl pH 8.0에 현탁하여 초음파 파쇄기 (Branson 250)을 이용하여 *E.coli*를 파쇄하였다. 파쇄한 cell extract를 다시 10,000 x g, 4℃에서 20분간 원심 분리를 실시하여 insoluble material을 제거하고 soluble한 cell 파쇄액을 분획하였다.

Soluble cell extract로부터 metalloprotease를 분리하기 위하여 recombinant metalloprotease의 C-terminal region에 존재하는 6개의 histidine tail을 이용하여 metal affinity chromatography (Ni-NTA)를 행하여 분리하였다. 즉50mM의 $NiSO_4$를 이용하여 resin을 pre-charging시킨후 binding buffer (20mM Tris-HCl pH 8.0, 10mM imidazole, 0.5M NaCl)로 평형화 시킨 column에 soluble cell extract를 loading하였다. 이후 column을 washing buffer(20mM Tris-HCl pH 8.0, 60mM imidazole, 0.5M NaCl)를 사용하여 비특이적으로 결합된 protein을 제거한 후 다시 elution buffer(20mM Tris-HCl pH 8.0, 500mM imidazole, 0.5M NaCl)를 사용하여 resin에 binding 되어있는 recombinant metalloprotease (rVMCs)를 elution 시켰다. Elution 된 protein은 SDS-PAGE를 통하여 농도와 정제도를 확인하였으며 단백질의 농도는 Bradford법을 이용하여bovine serum albumin을 표준물질로 사용하여 595nm에서 흡광도를 측정하였다. 정제된 단백질은 20mM Tris-HCl pH8.0

을 이용하여 4℃에서 투석을 한후 필요에 따라 100% glycerol을 동량 첨가한후 -20℃에서 보관한후 향후 실험에 사용하였다.

활성의 측정

Metalloprotease의 활성 측정을 위하여 gelatin, insoluble collagen type I, type II, type III, 그리고 Type V를 사용하였으며 또한 합성 기질인 GPLGP, GPGGPL을 사용하였다. Protease의 활성은 ninhydrin법의 변법을 이용하여 측정하였다. 0.09g의 ninhydrin과 hydridatin 0.018g을 30ml의 DMSO용액에 녹인후 10ml의 4M Lithium buffer와 혼합하여 ninhydrin용액을 제조한후 사용하였다. Gelatin의 분해능을 측정하기 위해 100㎕의 enzyme (10㎍) 용액과 100㎕의 substrate (0.2% gelatin)용액을 첨가한후 다시 100㎕의 reaction buffer(150mM Tris-HCl pH8.0, 12mM $Cacl_2$)를 첨가한후 37℃에서 1시간 또는 필요에 따라 더 이상의 시간에 반응을 시킨후 동량의 0.1N HCl을 처리하여 반응을 종료 시키고 ninhydrin용액을 첨가하여 100℃에서 15분간 반응시킨후 반응액의 흡광도를을 570nm에서 측정하였다. Insoluble collagen의 경우 1-10mg의 collagen을 증류수로 swelling시킨후 이를 다시 원심분리하여 상층액을 제거 하고 다시 100㎕의 metallorotease와 100㎕의 증류수, 100㎕의 reaction buffer(150mM Tris-HCl ph8.0, 12mM $Cacl_2$)를 첨가한후 37℃에서 2시간동안 반응 시킨후 0.1N NaOH를 사용하여 반응을 종료 시키고 ninhydrin법을 이용하여 활

성을 측정하였다. Protease의 활성은 1μmole의 leucine을 분해하는 enzyme의 양을 1unit으로 정의 하였다 [Sasagawa et al. 1993]. 다양한 기질의 분해능을 조사 하기 위해 BSA, lactoferrin, lysozyme, hemoglobin, fibrinogen, heparin등을 기질로 사용하여 protease의 활성을 측정하였다. Metalloprotease와 기질을 반응시킨후 SDS-PAGE를 통하여 기질이 protease에 의해서 분해되었는지를 확인하였다.

온도 및 pH의 영향

Metalloprotease의 온도에 대한 영향을 조사 하기 위하여 다양한 온도에서 metalloprotease의 활성과 안전성을 조사 하였다. Metalloprotease의 온도 안정성은 10mM Tris-HCl pH 8.0 buffer를 사용하여 온도범위 20-55℃의 범위에서 30분간 방치후 남아있는 metalloprotease활성을 다시 37℃에서 gelatin을 기질로 사용하여 측정하였다. Metalloprotease의 최적 활성온도는 위의 온도범위에서 1시간 동안 gelatin과 반응을 시킨후 gelatin 분해능을 ninhydrin방법을 이용하여 측정하였다.

pH가 metalloprotease에 미치는 영향을 조사하기 위하여 pH 4-11까지의 universal buffer(57mM Boric acid, 33mM Citric acid, 1M NaOH, various amount of 0.1N HCl)[Philippe et al. 1999]를 사용하여 metalloprotease의 활성을 측정하였다. Metalloprotease의 pH안정성은 각각의 pH buffer에서 30분간 방치후 남아 있는 효소 활성을 측정하였고 최적 pH는 각각의 pH buffer에

서 protease를 gelatin과 반응시킨 후 분해된 gelatin의 양을 ninhydrin법을 이용하여 측정하였다.

Antisera의 제조

Newzeland white rabbit을 이용하여 정제된 metalloprotease (rVMC52)를 20μg을 adjuvant 와 혼합하여 4주간 주사 하였다. Antisera의 정제는 current protocol in molecular biology의 방법에 따라 실시 하였다.

Western blotting

정제된 rVMC 52와 rVMC62를 시료로 12% slab gel의 SDS-PAGE를 이용하여120V환경에서 전기영동을 하였다. 전기영동 후 acrylamide gel을 150mA의 조건으로 nitrocellulose membrane에 electro transfer하였고 membrane에 transfer된 단백질의 위치와 양을 측정하기 위해서 ponceau S를 이용하여 membrane을 staining하고 증류수를 이용하여 destaining하였다. 정제된 recombinant와 molecular marker의 위치를 표지 후 1% skim milk를 이용하여 blocking하고 다시 primary antibody (1:5000), secondary antibody (alkaline phosphatase conjugated IgG, 1:30,000)를표지하고 발색제인 NBT/BCIP를 이용하여 membrane상에 나타난 signal을 확인하였다.

C-terminal region의 deletion

C-terminal region을 다양한 크기로 제거 하기 위해 10㎕의 plasmid pETMETA62를 *Bam*HI과 *Sac* I으로 처리하여 3′쪽을 blocking한후 1 unit의 exonuclease III를 처리 하고 37℃에서 1분 간격으로 반응을 종료 시켰다. 반응이 종료된 시료를 다시 상온에서 S1 nuclease처리 하여 말단을 blunt화 시킨후 ligation을 실시 하고 *E.coli* XL1-Blue에 transformation을 실시 하였다. Transformant로부터 plasmid를 분리하고 plasmid에서 3′이 잘려나간 크기의 순서를 기준으로 다시 재분류 한 후 염기서열결정을 통하여 잘려나간 3′의 부위를 측정하였다(Fig.9). 또한 발현 host인 *E.coli* BL21(DE3)에 transformation 한후 IPTG를 이용하여 발현을 시킨후 SDS-PAGE를 실시 하여 발현 된 단백질의 크기를 측정 하였다.

Cytoxocity의 측정

V. mimicus 유래의 metalloprotease가 cell에 미치는 cytotoxicity를 조사하기 위하여 CHSE-214 (Chinook salmon embryo : ATCC CRL 1681) cell과 FHM (Fathead minnow epithelial cells: ATCC CCL-42) cell에 대한 cytotoxcity를 측정 하였다. MEM배지에 항생제인 penicillin G와 streptomycin을 첨가하여 배양후 정제된 extracellular type의 rVMC62를 농도별로 첨가한후 17℃에서 24시간 처리하였다. Metalloprotease에 의한 cell의 cytotoxicity는 cell로부터 방출되는 LDH (lactase dehydrogenase)의 양을 측정하여 비교 하였으며 LDH의 양은 promega사의 CytoTox96

non-Radioactive Cytotoxicty assay kit을 이용하여 측정하였다.

Knock out mutant의 제조

Wild type의 *V. mimicus*와 metalloprotease가 결여된 *V. mimicus*간의 차이점을 확인하기 위하여 *V. mimicus*의 metalloprotease gene을 suicide vector인 pNQ705를 이용하여 knock out 시켰다. Metalloprotesae gene의 약 0.6b 정도를 primer 11(5´GGCCGTCGACACGGATAATGGTGG3´:*Sal*I) 과 primer 12(5´GGCCGAGCTCACAG TTGGTTGGCTGT3´: *Sac*I)을 이용하여 증폭하고 제한효소 *Sal*I과 *Sac*I을 처리한후 동일한 제한효소로 처리된 pNQ705 vector에 ligation시켜 이를 *E.coli* SM10 λ*pir*에 형질전환 하여 recombinant를 선별하고 transformant를 다시 *V. mimicus*와 conjugation 시켰다. Knock out mutant의 선별은 conjugation된 균체를 chloroamphenicol (50$\mu g/\mu \ell$)이 함유된 TCBS (Thiosulfate Citrate Bile Sucrose)배지에 streak하여 1차 선별하고 이를 다시 pNQ705 vector의 유래의 primer인 pnq1와 primer5를 이용하여 증폭이 일어난 균주를 2차 선별하고 southern blotting을 통해 0.6kb의 부위가 목적 하는 위치에 들어간 균주를 최종 선별하였다.

결과

정제

Metalloprotease의 대량발현을 위해 pVMC193으로부터 primer5와 primer2 그리고 primer6과 primer2를 사용하여 premature region의 metalloprotease (VMC52, 71kDa)와 extracellular type의 metalloprotease (VMC62, 61kDa)의 2가지 type을 subcloning 하였다. VMC52는 GTG start codon으로부터 시작되어 628 amino acid를 coding 하고 있는 1884bp의 염기서열을 가지고 있고 extracellular region의 경우 537 amino acid의 1611bp에 해당하는 크기를 가지고 있다. Subcloning된 vector는 각각 pETMETA52와 pETMETA62로 명명하였으며 metalloprotease를 분리하기 위해 1L의 LB 배지에 접종하여 배양후 IPTG를 이용하여 발현 시키고 *E.coli*를 파쇄후 Ni-NTA resin을 이용하여 정제 하였다(Table 3). Total cell lysate로부터 약 50mg의 soluble한 protein을 얻을 수 있었고, soluble 한 fraction은 Ni-NTA resin을 거쳐 his-tag affinity를 이용한 정제를 과정을 통해 약 0.5mg의 정제된 protease를 얻을 수 있었다. 정제된 단백질의 크기를 확인하기 위해 12% slab gel을 이용해 SDS-PAGE를 실시하였으며 각각 71kDa와 61kDa의 정제된 metalloprotease를 확인할 수 있었다(Fig. 5). Protease의 활성을 측정하기 위해 각각 10㎍의 protease를 100㎕의 0.2% gelatin solution과 반응시켜 ninhydrin반응을 이용하여 활성을 측정하였으며 VMC62에서 VMC52보다 높은 활성을 기지고 있음을 확인하였

Table 1. Association of *Vibrio* spp. with different clinical syndromes[b]

Species	Clinical Syndrome				
	Gastroenteritis	Wound Infection	Ear Infection	Primary Septicemia	Secondary Septicemia
V. cholerae O1	+++	+			
V. cholerae non O1	+++	++	+	+	+
V. mimicus	++		+		
V. fluvialis	++				
V.parahaemolyticus	+++	+	+		+
V.alginolyticus	(+)	++	++	+	
V.cincinnatiensis				+	
V.hollisae	++			+	
V.vulnificus	+	++		++	++
V.furnissii	(+)				
V.damsela		++			
V.metschnikovii	(+)			(+)	
V.carchariae		+			

a+++ = frequently reported, ++ = less common (6-100 reports); + = rare (1-100 reports); + = rare (1- 5 reports), and (+) = association is unclear. [b]Table taken from Pavia et al. (1989).

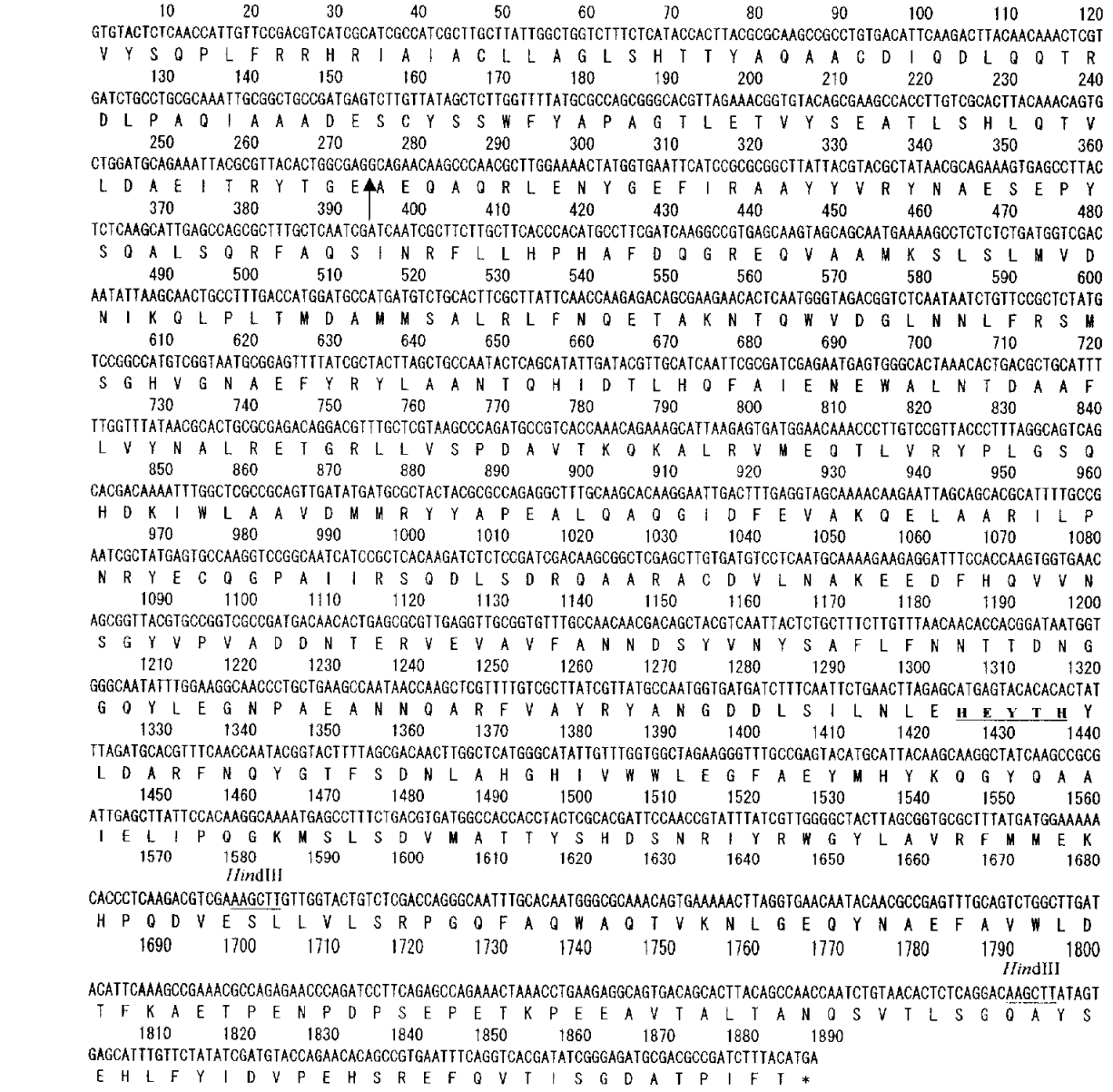

Fig 1. The nucleotide sequence of metalloprotease gene (*vmc*) and the deduced amino acid sequence.

Vertical arrow indicates the N-terminal amino acid sequence of extracellular protease.

GenBank Acession No. AF04832

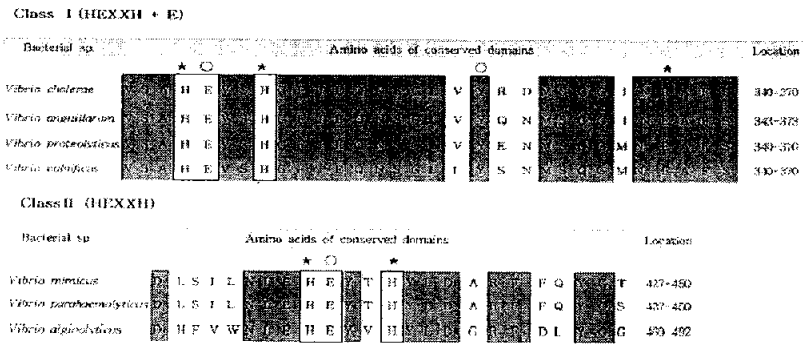

Fig 2. Classification of metalloproteases of *Vibrio* sp.

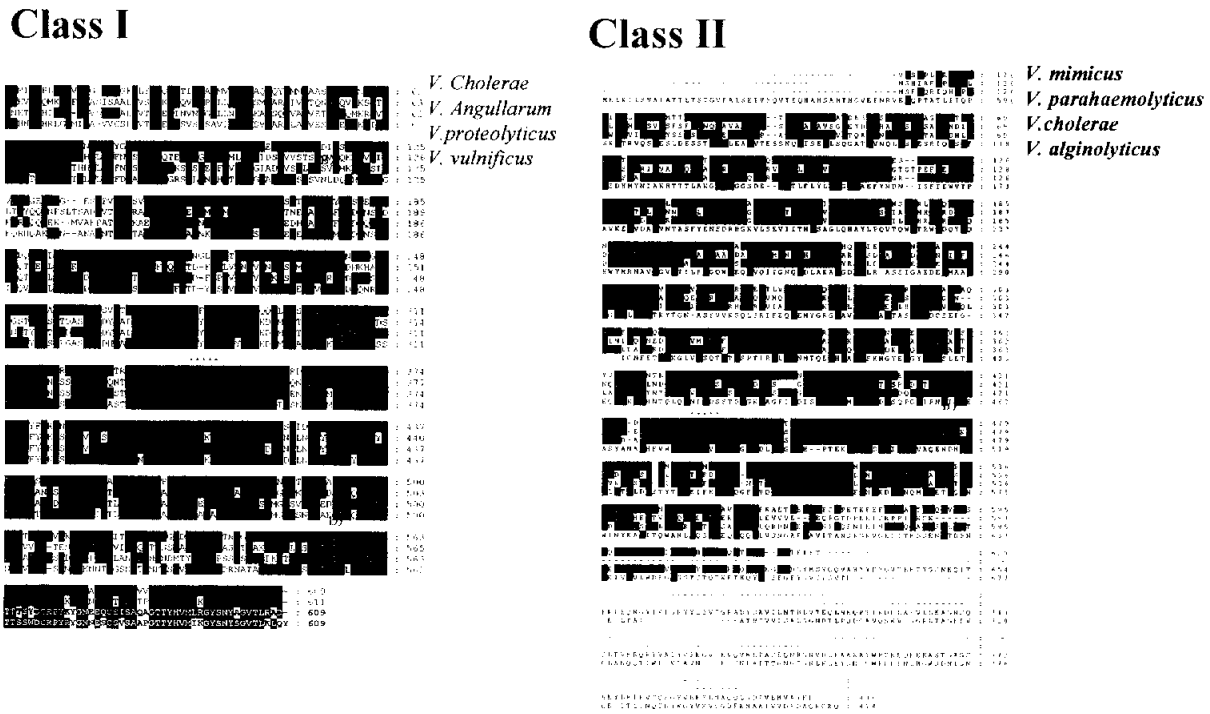

Fig 3. The amino acid sequence alignment of class I and class II metalloproteases of *Vibrio* sp.

Table 2. Comparison of signal peptide cleavage site and extracellular metalloproteases of *Vibrio* sp.

Strains	Total ORF sequence (amino acid sequence)	Signal peptide cleavage site	Calculated molecular weight of extracellular protease from deduced amino acid sequence
V. cholerae	609 aa	^{195}His↓^{196}Ala	46.7 kDa
V.anguillarum	611 aa	^{199}His↓^{200}Ala	44.6 kDa
V. proteolyticus	609aa	^{196}His↓^{197}Ala	44.8 kDa
V. vulnificus	609aa	^{196}His↓^{197}Ala	46.7 kDa
V. parahaemolyticus	587aa	^{25}Ser↓^{26}Phe	63.2 kDa
V.alginolyticus	814aa	^{75}Ser↓^{76}Thr	81.7 kDa
V. mimicus	**628aa**	**^{91}Glu↓^{92}Ala**	**61.2 kDa**

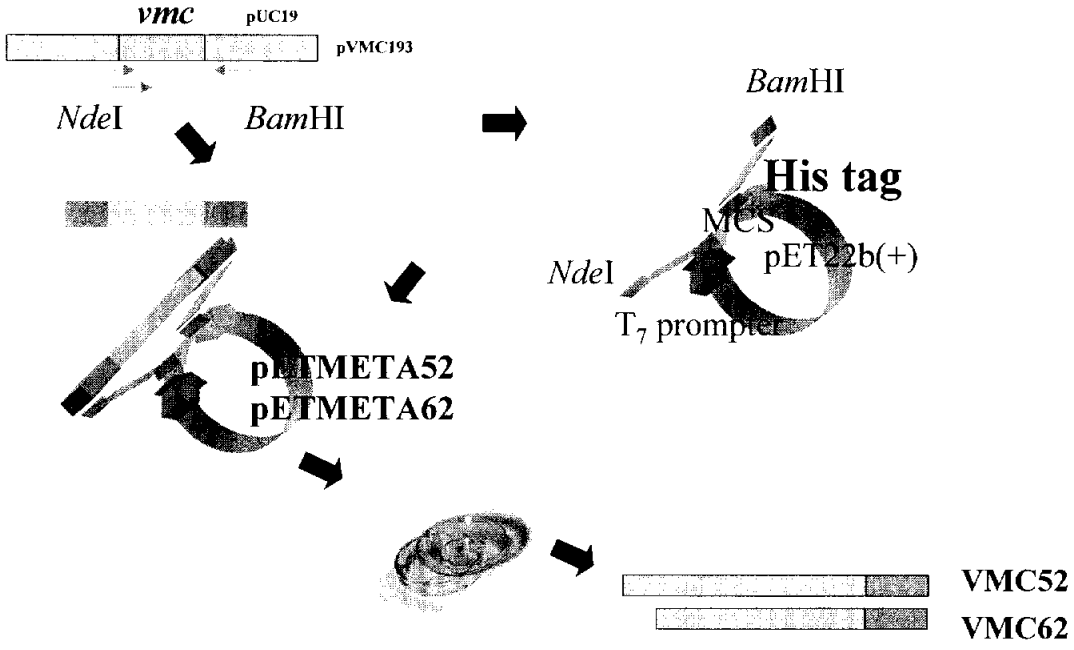

Fig 4. Scheme for construction of metalloprotease overexpression vector

Table 3. Purification steps of rVMC 52 and rVMC 62

Plasmids (Protein)	Purification Steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
pETMETA52 (VMC 52)	Total cell lysate	51	18	0.4	100	1
	Ni-NTA	0.8	0.9	1.1	5.0	3.2
pETMETA62 (VMC 62)	Total cell lysate	50.1	16.7	0.3	100	1
	Ni-NTA	0.5	0.8	1.6	4.8	4.8

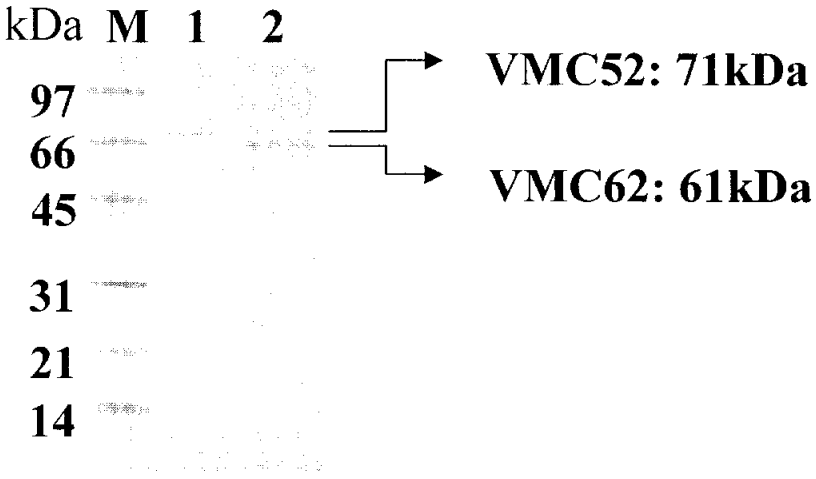

Fig 5. SDS-PAGE of purified metalloprotease (rVMCs) with his-tag

Lane1: rVMC52, Lane 2: rVMC62.

10㎍ of purified rVMCs were loaded on the 12% slab gel SDS-PAGE and stained with comassie R 250.

Western blotting

Western blotting을 실시 하기 위한 antibody를 제조하기 위해 약 20㎕의 정제된 VMC52를 adjuvant와 mixing하여 rabbit에 4주간 피하주사 한 후 rabbit으로부터 전혈을 채취하여 혈장으로부터 anti-VMC antibody를 얻을 수 있었다.

정제는 current protocol in molecular biology의 방법에 의해 실시하였으며 antibody의 활성을 측정하기 위해 rVMC52와 rVMC 62를 12% slab gel에 전기영동을 실시한후 nitrocellulose membrane에 Hoffer electroblot unit을 이용하여 150mA의 조건에서 1시간 동안 electro-transfer시켰다. Blocking agent로는 1% skim milk/TBS (Tris buffered saline)용액을 사용하였으며 정제된 anti-VMC antibody와 secondary antibody로는 alkaline phosphatase conjugated anti-rabbit IgG를 사용하여 signal을 확인하였다(Fig. 6). Western blotting을 통해 recombinant metalloprotease의 signal을 확인하였으며 wild type의 *V. mimicus*를 배양하고 상층액을 동량의 20% TCA (trichloroacetic acid)를 첨가하여 ice에 5분간 방치후에 12,000 x g에서 원심분리후 100㎕의 cold aceton을 첨가하고 speed vac을 이용하여 진공농축시킨후 이를 위의 방법으로 western blotting을 실시하였고 extracellular type인 rVMC62와 비슷한 위치에서 signal을 확인하였다 (data not shown).

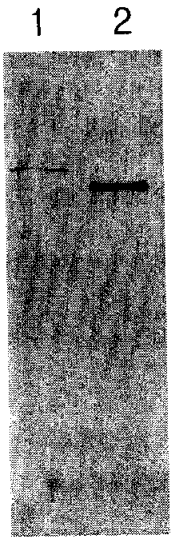

Fig 6. Immunoblotting of recombinant VMCs

Lane 1: rVMC52 (71kDa), Lane 2: rVMC62 (61kDa)

온도 및 pH의 영향

VMC의 활성에 미치는 온도영향을 측정하기 위해 20℃에서 55℃사이의 온도에서 기질과 VMC를 반응시킨 후의 protease활성을 측정하였다. 최적의 활성은 37℃근처의 온도에서 활성을 나타내었다. 온도에 대한 stability를 측정하기 위해 각각의 온도에서 30분간 반응시킨후 다시 37℃에서 protease활성을 측정하였다. 이때 37℃ 까지는 비교적 안정적이나 그 이상의 온도에서는 급격한 활성의 감소가 관찰되었다.pH가 VMC의 활성에 미치는 영향을 조사하기 위해 pH4-11까지의 pH범위에서 protease활성을 측정하였다. 최적 pH는 pH8전후인 것으로 나타났으며 pH 6-10까지의 pH범위에서 비교적 안정적으로 나타나 산성조건에서는 안정적이지 못한 것으로 나타났다 (Fig. 7-8.).

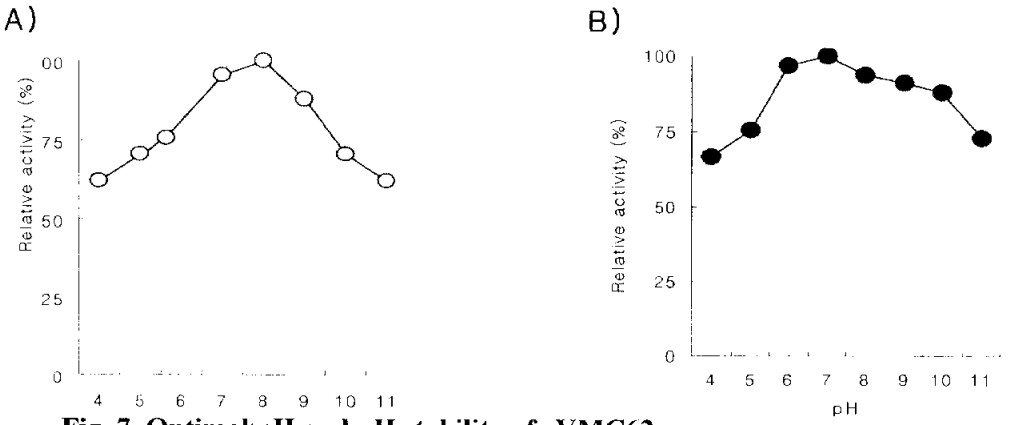

Fig. 7. Optimal pH and pH stability of rVMC62

A) Optimal pH, B) pH stability

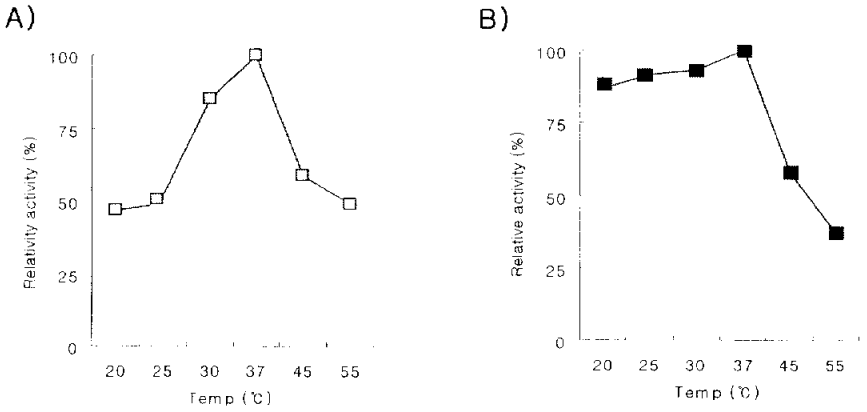

Fig. 8. Optimal temperature and thermo stability of rVMC62

A) Optimal temperature, B) Thermo stability

VMC의 기질 특이성

VMC의 기질 특이성을 조사하기 위하여 여러 단백질, heparin (human), lysozyme (human milk), cytochrome C (horse heart), alcohol dehydrogenase , ovalbumin (Egg) (yeast), glycerolaldehyde 3 phosphate dehydrogenase (Rabbit muscle), carbonic anhydroase (bovine erythrocyte), BSA (bovine), lactoferrin (bovine), hemoglobin (bovine), fibrinogen (bovine), collagen type I, II, V을 VMC와 반응시킨후 SDS-PAGE를 통하여 기질 분해능을 조사 하였다. VMC는 fibrinogen과 collagen을 제외한 기질 분해능은 없었으며 class I의 metalloprotease에서 보이는 다양한 기질 분해능은 관찰되지 않으며 collagenase과 fibrinogen과 같은 조직의 구성에 관여하는 단백질만을 을 특이적으로 분해하는것으로 조사 되었다(Table 4). 또한 여러 type의 collagen과 collagen analogue인 합성 peptide(GPGGPA, GPLGP)를 이용하여 분해능을 조사하였고 gelatin과 type III collagen을 가장 잘 분해하는 것으로 조사되었다(Table 5).

Table 4. Substrate specificity of rVMC62

Substrate		Activity
Heparin (human)		-
Lysozyme (human milk)		-
Cytochrome C (horse heart)		-
Alcohol dehydrogenase (yeast)		-
Ovalbumin (Egg)		-
Glycerolaldehyde 3 phosphate dehydrogenase (Rabbit muscle)		-
Carbonic anhydroase (bovine erythrocyte)		-
BSA (bovine)		-
Lactoferrin (bovine)		-
Hemoglobin (bovine)		-
Fibrinogen (bovine)		+
Collagen (bovine)	Type I	+
	Type II	+
	Type III	+
	Type V	-
	Gelatin	++

Table 5. Substrate kinetics of rVMC62

Type	Collagen					Synthetic peptide	
	I	II	III	Gelatin		GPGGPA	GPLGP
Vmax (ug/ml/min)	0.7	0.8	1.4	2.0	*Vmax* (umole/ml/min)	0.012	0.006
Km(mg/ml)	3.2	4.3	4.6	3	*Km*(mM)	0.16	0.1

Deletion of 3′ region of pETMETA62

C-terminal region이 VMC의 활성에 미치는 영향을 조사하기 위해 metalloprotease VMV62를 coding하고 있는 plasmid인 pETMETA62를 exonuclease III처리하여 3′으로부터 deletion된 vector를 제조하였다.

3′에서 5′으로 deletion을 시키기 위해 pETMETA62를 *Sal*I과 *Sac*I으로 처리하여 한쪽을 blocking시키고 exonuclaease III를 사용하여 5′으로의 deletion을 유도하였다. 30℃에서 반응을 시키고 1분간격으로 반응을 종료 시켜 다양한 크기의 deletion된 mutant를 얻을 수 있었다(Fig. 10). 이 mutant들을 *E.coli* BL21(DE3)에 retransformation 한후 IPTG를 이용해 발현 시킨후 SDS-PAGE를 실시해서 그 크기를 비교 하였다. 또한 염기서열 결정을 실시 해서 정확한 deletion 위치를 확인하였다 (Table 6).

Deletion작업을 통해 rVMC62의 61kDa으로부터 57kDa과 42kDa 사이의 통해 5개의 C-terminal region이 deletion된 mutant를 얻었으며 각각 57, 53, 50, 48, 42kDa의 크기를 가지고 있었다 (Fig. 11). 이와 같이 발현된 protein의 크기는 deduced amino acid sequence로부터 예상된 분자량의 크기와 잘 일치하고 있음을 잘 일치하고 있음을 보여 주었다.

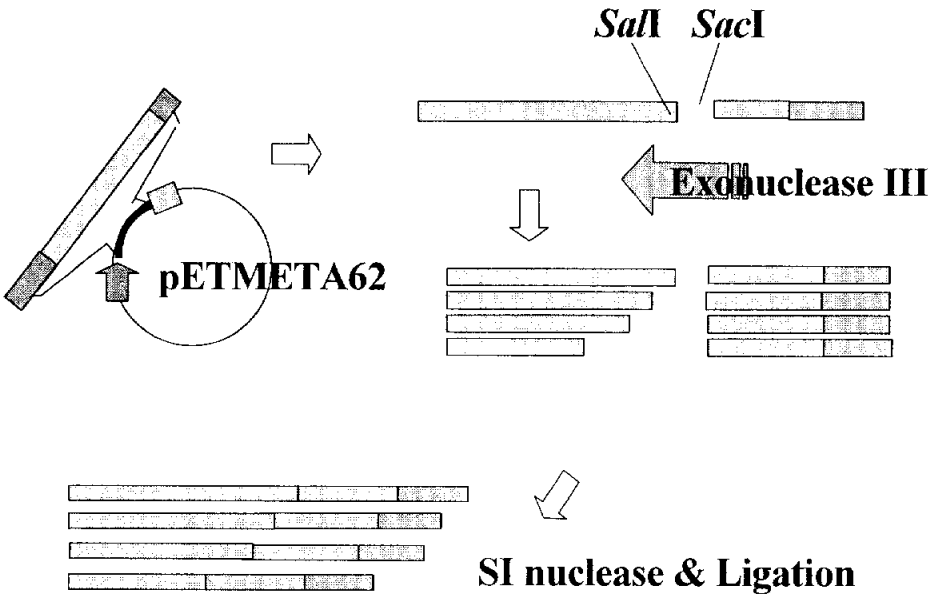

Fig. 9. Deletion of C-terminal region of *vmc* gene by Exonuclease III treatment

Table 6. Plasmids for overexpression of the C-terminal truncated metalloproteases

Plasmids / strains	Genotype or relevant characteristics	Reference or source
Plasmid		
pETMETA62	Ap^r, contains a 1611 base *NdeI*/*BamH*I fragment containing *vmc*	This study
pETMETA62-I	Ap^r, derivative of pETMETA62 truncated 102bp	"
pETMETA62-II	Ap^r, derivative of pETMETA62 truncated 201bp	"
pETMETA62-III	Ap^r, derivative of pETMETA62 truncate 300bp	"
pETMETA62-IV	Ap^r, derivative of pETMETA62 truncate 339bp	"
pETMETA62-V	Ap^r, derivative of pETMETA62 truncate 501bp	"
E. coli strains		
BL21(DE3)	F-*ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Novagen co.

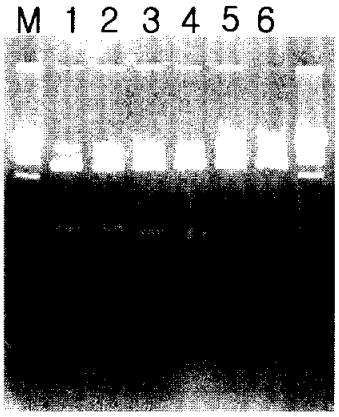

Fig. 10. Agarose gel electrophoresis of exonuclease III treated plasmids (pETMETA derivates)

M: Molecular marker (lamda *Hin*dIII), 1: pETMETA62, 2: pETMETA62-I, 3: pETMETA62-II, 4:pETMETA62-III, 5: pETMETA62-IV, 6: pETMETA62-V

Mutant의 활성비교

C-terminal region의 deletion이 활성에 미치는 영향을 조사 하기 위해 각각의 발현된 mutant와 VMC62를 LB배지에 배양하고 이를 다시 IPTG를 사용하여 induction을 시킨후 sonifier를 이용하여 *E.coli*를 파쇄하였다. 파쇄된 cell을 전기영동을 실시하여 발현양을 확인한후 이를 0.5%의 gelatin이 함유된 1.2%agar plate에 동량 처리한후 37℃에서 반응을 시킨 후 frazier solution을 이용하여 staining 시킨 결과 201bp가 deletion 된 plasmid를 가지는 mutant로부터 얻어진 protein에서는 활성을 확인할수 있었지만 300bp가 deletion된 plasmid를 가지는 mutant로부터 얻어진 정제된 50kDa의 protein에서는 활성을 확인할수 없었다 (Fig. 12). 따라서 C-terminal의 200bp부분부터 300bp까지 100bp부분이 매우 중요한 부분임을 확인할 수 있었다.

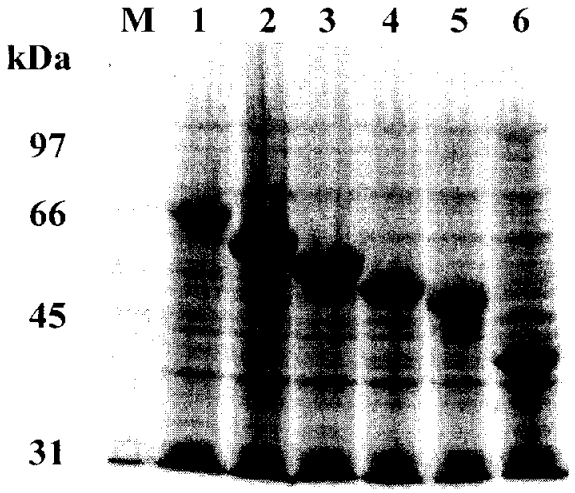

Fig. 11. SDS-PAGE of cell lysate harboring plasmids, pETMETA62 and truncates

1: Molecular marker, 2: pETMETA62 (VMC62), 3: pETMETA62-I(VMC57), 4: pETMETA62-II(VMC 53), 5: pETMETA62-III(VMC50) , 6: pETMETA62-IV (VMC 48), 7: pETMETA62-V(VMC42)

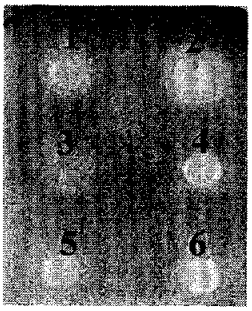

Fig. 12. Gelatinolytic activity of cell lysates, harboring the truncates.

1: rVMC 62, 2: rVMC 57, 3: rVMC 53 , 4: rVMC 50, 5: rVMC 48,6: rVMC 42

Table 7. Gelatinolytic activity of total cell lysate, harboring the truncates

Proteins	Specific activity (U/mg)
rVMC 62	0.3
rVMC 57	0.3
rVMC 53	0.3
rVMC 50	-
rVMC 48	-
rVMC 42	-

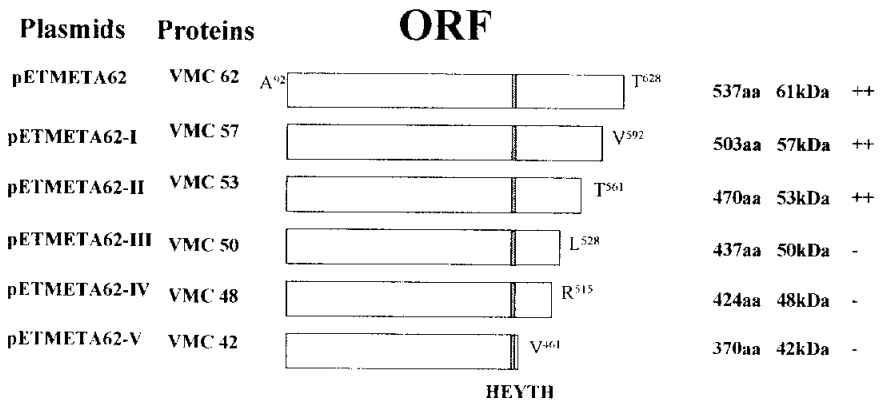

Fig. 13. Graphic representation of truncates and gelatinolytic activity

C-terminal truncates의 정제 및 활성의 측정

C-terminal region이 deletion된 mutant를 정제하고 활성을 측정하였다. C-terminal region 6 histidine을 이용한 metal affinity chromatography를 이용하여 정제 하였다 (Table 8). 1L의 배양액으로부터 약 0.5mg의 정제된 protein들을 얻을수 있었고 53kDa의 protease인 VMC 53까지는 비슷한 활성을 나타내었으나 3kDa이 더 deletion된 VMC50으로 부터는 활성을 확인할수 없었다 (Fig. 15).

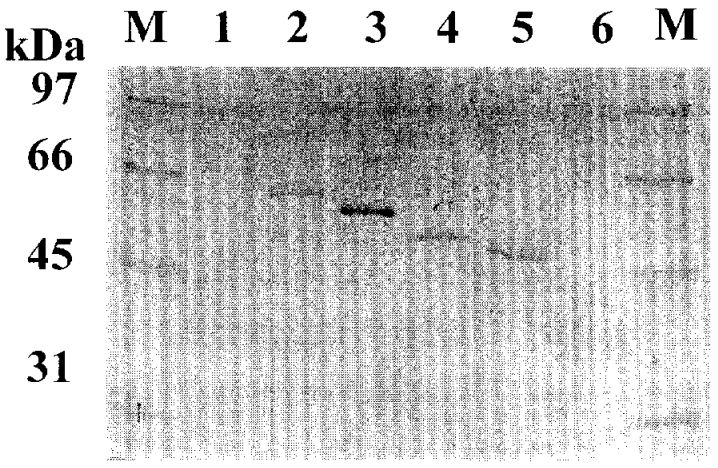

Fig. 14. SDS-PAGE of purified rVMC62 and truncates.

M: molecular marker, 1: VMC62, 2: VMC57,3: VMC53, 4: VMC50, 5: VMC 48, 6: VMC42

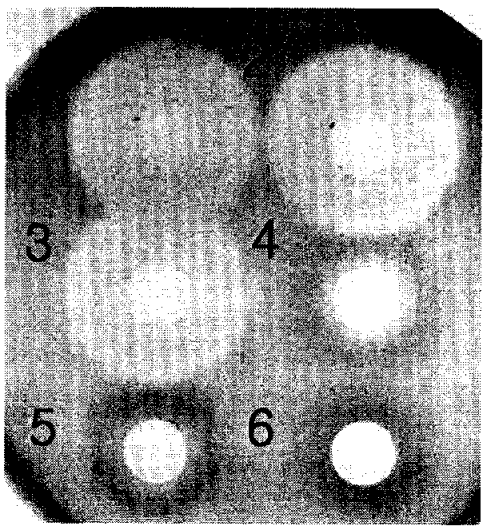

Fig. 15. Gelatinolytic activity of the purified rVMC62 and C-terminal truncates.

1: rVMC 62, 2: rVMC 57, 3: rVMC 53 , 4: rVMC 50, 5: rVMC 48,6: rVMC 42

Table 8. Purification steps of rVMC62 and C-terminal truncates

protein	Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
VMC62	Total cell lysate	50.1	16.7	0.3	100	1
	Ni-NTA	0.5	0.8	1.6	4.8	4.8
VMC57	Total cell lysate	52.5	17.3	0.3	100	1
	Ni-NTA	0.7	0.8	1.1	4.6	3.4
VMC53	Total cell lysate	50.5	15.7	0.3	100	1
	Ni-NTA	0.5	0.8	1.6	5.1	5.1
VMC50	Total cell lysate	48.2	-	-	-	-
	Ni-NTA	1.2	-	-	-	-
VMC48	Total cell lysate	47.5	-	-	-	-
	Ni-NTA	1.5	-	-	-	-
VMC42	Total cell lysate	50.8	-	-	-	-
	Ni-NTA	1.5	-	-	-	-

Cytotoxicity의 측정

VMC62의 cytotoxicity를 측정하기 위해 epithelial cell인 FHM(Fat Head Minnow)와 embryo cell 인 CHSE -214 (Chinook salmon embryo)를 사용하여 측정하였다. 10-50㎍으로 희석된 VMC를 처리하여 25℃에서 12시간 동안 반응시킨후 inverted microscope를 이용하여 cell의 morphological change를 관찰하였다. 또한 파쇄된 cell 로부터 방출되는 LDH의 양을 측정하여 비교 하였다. VMC62를 처리 하였을 때 cell로 부터 morphological change를 확인할 수 있었다 (Fig. 16-17). 이러한 결과는 prtease (collagenase) 의 활성으로 인해 cell을 지지하는 성분의 변화 가능성을 암시하고 있으며 이러한 VMC가 cell에 미치는 영향을 조사하기 위해 파쇄된 cell로부터의 LDH의 양을 측정하였다. 10㎍의 VMC 처리시에 12시간후에 CHSE 로부터 약 6%의 lysis가 발생하였으며 50㎍처리시 12시간후 24%의 lysis가 발생하였다. 100㎍ 처리시에 93% 의 lysis가 일어나는 것을 확인하였다 (Fig. 18).

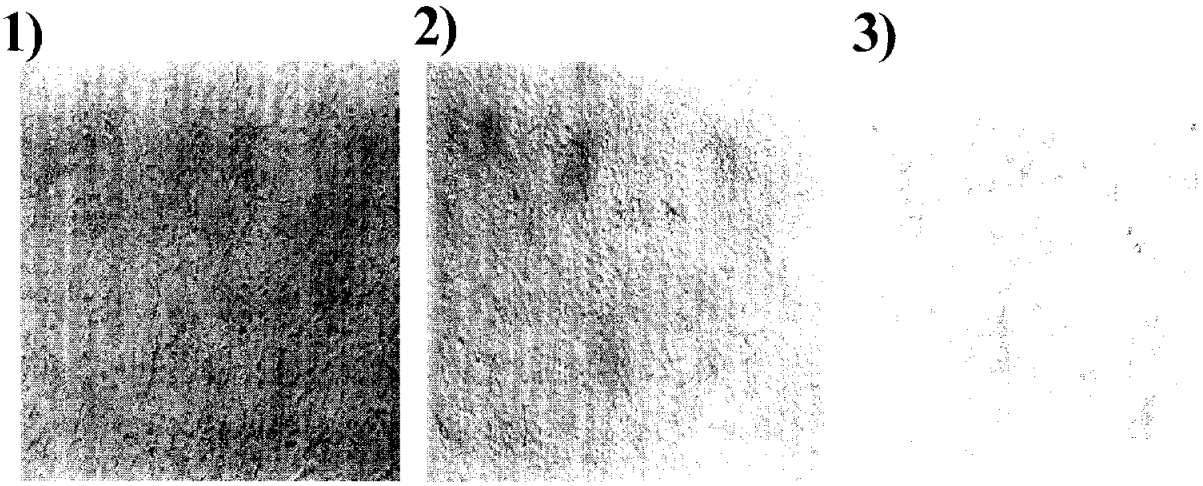

Fig. 16. Effect of rVMC62 on the FHM cell.

1) no rVMC62, 2) 50μg rVMC62, 3) 100μg rVMC62

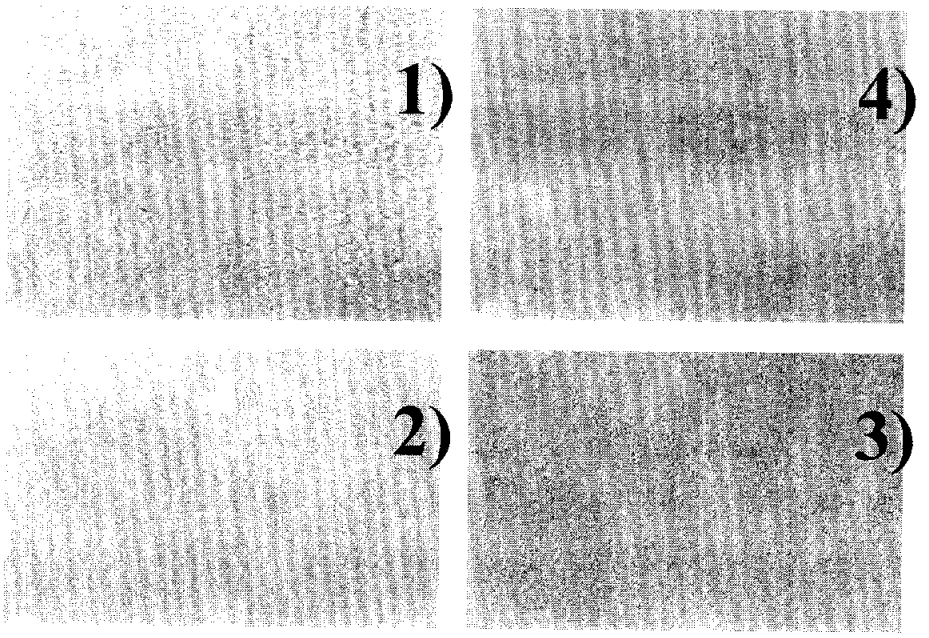

Fig. 17. Effect of rVMC62 on the CHSE-214 cell

1) no rVMC62, 2) 10μg rVMC62, 3) 50μg rVMC62, 4)100μg rVMC62

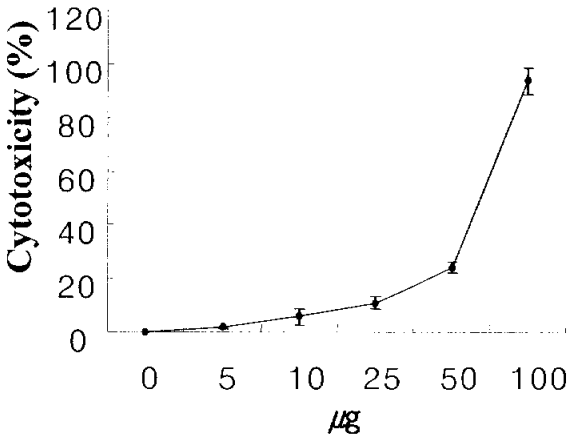

Fig. 18. Cytotoxicity of rVMC62 on the CHSE cell

The released LDH from CHSE-214 cell was detected after the treatment of rVMC62 protein.

고찰

Bacteria유래의 protease는 여러 다양한 organism에서 massive tissue damage를 일으켜 bacteria의 pathogenecity에 중요한 역할을 하는 것으로 보고되고 있다 [Grey et al 1979, Miyoshi et al. 2001].

Metalloprotease는 active site의 구성에 따라 MA(HEXXH+E)와 MB (HEXXH+H)의 두가지 clan으로 분류되고 있으며 *Vibrio*로부터 보고된 metalloprotease는 clan MA로 분류되고 있으며 이러한 active site에는 zinc 가 있는 것으로 알려져 있다 [Rao et al. 1998].

*Pseudomonas aeruginosa*로 부터의 elastase는 감염시 tissue destruction에 중요한 역할을 하는것으로 보고되고 있으며[Grey et al. 1979], *V. vulnificus* 의 elastase 역시 tissue necrosis와 관계 있는 것으로 생각되어 지고 있다. Protease의 이러한 기능이외에 *V. cholerae*의 HA/protease는 cholera toxin을 nicking시켜 activation 시키고 생체내의 중요한 방어물질인 lactoferrin, lysozyme을 분해하는 능력이 있는 것으로 알려졌다 [Fikelstein et al. 1992]. 다른 여러 *Vibrio*에서도 HA/protease와 유사한 metalloprotease가 보고되고 있으며 유전자 구조가 유사한 protease인 *V. vulnificus*의 metalloprotease는 Chang등에 의해 cloning되어 유전자의 기본적인 특성이 밝혀졌으며 46kDa의 mature protease를 가지고 *V. anguillarum*, *V. proteolyticus*의 protease와 높은 유사성을 가지고 있는 것으로 보고되었으며 [Chang et al. 1997], 이후의 연구를 통해 C-terminal region의 10kDa의 peptide region이

autocatalytic process를 통해 잘려진다는 것이 보고되었다 [Yamamoto et al. 1990]. 이렇게 10kDa이 결여된 VVP-N은 soluble protein인 casein, albumin의 분해능에는 차이를 보이지 않는 반면 insoluble protein인 collagen, elastin의 분해능은 상당히 떨어지는 결과를 보여 주고 있다.

본 연구에서 *V. mimicus*로부터의 metalloprotease의 생화학적 특성에 관해 조사 하기 위하여 이미 본 연구실에서 cloning한 유전자를 사용하여 *V. mimicus*의 metalloprotease인 VMC의 extracellular type을 *E.coli*에서 대량 발현 시킨 후 정제하였다. 약 61kDa의 extracellular protease인 rVMC62를 pET22b(+) vector를 이용하여 IPTG를 inducer로 사용하여 대량발현 시켰으며 50mg의 cell lysate로부터 약 0.5mg의 정제된 단백질을 얻을수 있었다. 정제된 rVMC62단백질의 온도 및 pH에 대한 영향을 조사 하였으며 pH7, 37℃에서 최적의 반응을 나타내었고 pH 9와 37℃까지의 조건에서 비교적 안정한 것으로 조사되었다. 이러한 metalloprotease의 cytotoxicty를 확인하기 위해 fish cell인 FHM과 CHSE-214를 이용하여 VMC를 처리하였을 때의 morphological change와 방출되는 LDH의 양을 측정하여 사멸율을 산출하였다. VMC처리시 두 cell line 모두에서 확실한 morphological change가 관찰되었으며 CHSE-214cell에 약 100㎍의 VMC를 처리 하였을 때 약 100%의 cytotoxcity가 관찰되었다.

Class I과 class II의 metalloprotease에서의 C-terminal region의 역할을 비교 하기 위하여 metalloprotease gene (*vmc*)의 3′ region을 restriction

endonuclease를 처리 하여 deletion 시켰고 결과로 약 11kDa의 C-terminal region이 deletion된 50kDa (A^{92}-L^{528})의 metalloprotease에 해당하는 mutant를 제조 하여 *E.coli*에서 발현 시킨후 정제된 50kDa의 protein에 대한 활성을 측정 하였을때 C-terminal region의 11kDa의 peptide가 제거 되었을 때 활성이 완전히 사라지는 것을 확인하였다. 이러한 결과는 class I의 metalloprotease에서 보여지는 substrate specificity의 차이 또는 hemorrhagic activity의 차이를 보이는 것과는 상반된 결과를 보이고 있다. 또한 여러 substrate를 이용하여 기질분해능을 조사하여본 결과에서도 VMC의 기질에 대한 specificity는 fibrinogen, gelatin과 collagen에 한정되어 있는 결과를 보여 주고 있으며 그 외 albumin, casein등의 여러 soluble protein은 분해하지 못하였다. 이러한 결과는 class I의 metalloprotease에서 보이는 다양한 기질분해능과는 상당한 차이를 보이는 것으로 VMC에 의해 분해되는 기질인 collagen, fibrinogen은 ECM (extracellular matrix)의 주요 성분이며 이러한 기질의 분해는 VMC가 host cell에 invasion하기위해 필수적인 요소로 추정되어 진다. ClassI의 metalloprotease인 *V. cholerae*의 HA/protease는 cholerae toxin을 activation 시키고 또한 생체내 방어 작용을 하는 lactoferrin등을 분해하는, 좀더 다양한 기질 분해능을 보이고 있으나 VMC의 경우 비교적 collagen에 한정되는 기질 분해능을 보이고 있다. 또한 C-terminal 의 deletion을 통한 실험에서는 class I의 VVP와는 달리 gelatin과 collagen분해능이 완전히

없어지는 것을 확인 하였다. 이러한 결과는 class I에서의 C-terminal region의 역할이 기질 분해능보다는 기질 결합능과 높은 상관관계를 보이는 것과는 달리 VMC의 C-terminal region이 기질분해능 또는 기질 결합능과 상당히 깊은 관계를 보여 주고 있는 결과이다.

*V. mimicus*의 경우 여러toxin들이 보고되어 있으나 metalloprotease (VMC)의 경우 *V.cholerae*를 비롯한 *V. parahaemoyticus*로 부터의 metalloprotease와 amino acid sequence상에 매우 높은 유사성을 가지고 있어 더욱 *Vibrio*로 인한 질병유발에 공통작용을 하는 것으로 추정되고 있다.

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Characterization of collagen binding domain (CBD) of *Vibrio mimicus* metalloprotease (VMC)

Abstract

Metalloproteases were reported from mammalian to bacteria strains. The active sites of metalloproteases were composed of HEXXH and this was known for zinc binding motif. Metalloproteases of *Vibrio* sp. were classified as two distinct categories by nucleotide sequence similarities, as class I and II (Lee et al. 1998). Class I have large signal peptide region (about 200 aa). The zinc-binding domains of class I have HEXXH and have an extra glutamic acid near the zinc-binding motif. Whereas, class II only have HEXXH motif and one possible extra glutamic acid was located at the distance from zinc-binding motif. Beyond this, these two classes showed differences in their biochemical properties. In the class I metalloproteases, the polypeptide of C-terminal region was removed by autocalalytic cleavage mechanism and resulted a changes of the substrates specificity and this quite differ from class II metalloproteases. The C-terminal truncated metalloprotease did not digest the insoluble substrates and this region

was suggested as substrate binding related region.

By contrast with class I metalloprotease of *Vibrio* sp., the role of C-terminal region of class II metalloprotease was not characterized yet. Recently the collagenases (ColG, ColH) from *Clostridium histolyticum* were characterized. The action of this collagenase is zinc metalloprotease and very specific for substrate as collagen and gelatin and have C-terminal collagen binding domain. In the present study, we investigated the relation between the C-terminal region of *Vibrio* class II metalloprotease and the activity involving the collagen binding. The metalloprotease (VMC) of *Vibrio mimicus* showed collagenase activity. But it lost the activity by the deletion of 100 amino acids in C-terminal region. The C-terminal deleted mutant which have additional 33amino acid in this region still showed the collagease activity. The deletion study indicated that the 33amino acids from L^{528}-T^{561} were core region for collagen binding. The collagen binding domain was overexpressed as GST fusion protein and purified by glutathion sepharose column. The fusion protein also bind with several type of collagen such as type I, typeII, and type III but it did not bind with type V collagen. The temperature also affects the collagen binding of collagen binding domain of VMC

서론

Collagen은 skin, tendon, cartilage에 풍부하게 존재하고 있으며 bone, teeth 그리고 cornea 의 주 구성성분으로 mammalian의 여러 기관 (organ)에 존재하는 단백질의 25-30%를 차지하고 있는 구성 단백질이다 [Ramachandran, 1988]. 이러한 collagen은 매우 단순한 구성성분으로 이루어져 있으며 2개의 parallel polypeptide chain으로 이루어진 rod형태의 단백질의 형태를 이루고 있다. 이러한 collagen을 이루고 있는 구성 아미노산 역시 매우 단순한 성분으로 이루어져 있으며 Glycine-X-Y (Y는 보통 Proline)의 반복서열로 이루어져 있다. 이 반복서열이 이루는 triple helix는 inter chain hydrogen bond와 inter-intra molecular cross link에 의해서 안정된 구조를 이루고 있다. 또한 collagen은 glucosaminglycan등과 결합되어 extra cellular matrix (ECM)을 싸고 있으며 integrin, fibrinonectin, vitronectin등과도 결합되어 다양한 생체 내 기능을 수행하고 있는 것으로 보고되고 있다 [Heino, 2000]. 이러한 collagen은 일반적 protease에 의해서는 분해되지 않으며 collagen을 분해하는 효소를 collagenase로 특정지어 분류하고 있다.

Collagenase는 mammalian cell에서부터 bacteria까지 다양한 source로부터 발견되고 있으며 eukaryotic cell에서의 MMP (Matrix metalloprotease)의 경우 생체 내에서 여러 고분자 물질의 생합성, 분해 또는 활성화와 생체물질의 조절 과정에 참여하고 있으며 ECM 구성성분의 분해와 de

novo synthesis를 통한 tissue remodeling 과정에서 중요한 역할을 하고 있다. 이러한 MMP의 활성을 이해하는 연구에서 중요한 역할을 하는 것이 collagen binding domain에 관한 것이며 여러 MMP에서 공통적으로 발견되는 collagen binding domain은 C-terminal region의 fibrinonectin like domain, hemopexin domain등이 보고되어 있다 [Van den Steen et al. 2001]. 이중 hemopexin domain은 propeller구조를 가지고 있는 calcium binding domain으로 MMP에서 기질특이성을 결정하는 중요한 기능을 가지고 있는 대표적인 collagen binding domain이다 [Margaret et al. 2001]. 반면, bacterial collagenase의 경우 이러한 분자내의 domain에 대한 연구는 거의 없는 실정이다. 특히 bacteria의 collagenase는 mammalian의 collagenase보다 더 넓은 기질범위를 가지고 있으며 또한 병원성세균에서 분리되는 collagenase는 효소의 기질인 collagen, gelatin등의 생체 내에서의 역할로 미루어 볼 때 감염 및 발병 기작에 중요한 역할을 하고 있는 것으로 추정된다. 대표적 병원성 미생물인 *Vibrio* sp.의 경우 병원성지표로 이용되는 cholera toxin, hemolysin [Honda et al. 1993, Kim et al. 1997] 외에도 생체막과 생체 단백질 등을 분해하며 cytotoxicity를 나타내고 있는 여러 분해효소 즉 phospholipase [Fiore et al. 1997, Kang et al. 1998, Lee et al. 2002], HA/protease [Booth et al. 1984], metalloprotease (collagenase) [Cheng et al. 1996. Lee et al. 1998] 등이 보고되었다. *V. mimicus* 는 *V. cholerae*와 생화학적으로 매우 유사하며 병원성 인자로 알려진 hemolysin, metalloprotease

(collagenase), phospholipase 유전자가 본 연구팀에서 보고 하였다. *V. mimicius* hemolysin 유전자는 *V. cholerae* 와 81.6%의 상동성을 가지고 있었으며 [Kim et al. 1997], phospholipase의 경우 74.4%의 상동성을 보이고 있고 [Kang et al. 1998] metalloprotease (collagenase)의 경우에도 76%의 상동성을 보이고 있음을 밝혔다 [Lee et al. 1998, Shin et al. 2000]. 이러한 병원성 인자들 간의 유사성은 이들 *Vibrio* sp.에 의한 감염시 발생하는 공통증상으로 미루어 그 연관성을 찾을 수 있다고 추측된다. 본 연구실은 이전의 연구를 통해 이들 *Vibrio* sp.로부터의 metalloprotease (collagenase)를 2개의 group으로 분류하여 보고하였는데 Class I에는 *V. proteolyticus*, *V. cholerae*, *V. vulnificus*의 metalloprotease가, Class II에는 *V. mimicus*, *V. cholerae* 569B, *V. parahaemolyticus* 그리고 *V. alginolyticus*의 metalloprotease의 amino acid sequence간에 매우 높은 유사도가 있음을 보고하였다 [Lee et al. 1998]. 그러나 이러한 유사성이 높은 *Vibrio* sp.의 metalloprotease (collagenase)에 대한 유전자 및 아미노산 서열에 대한 결과는 보고되고 있으나 metalloprotease (collagenase) 단백질이 가지는 단백질 자체의 domain들의 생화학적 기능에 관한 연구는 전혀 보고되고 있지 않고 있다.

최근에 미생물 유래의 collagenase를 사용하여 특이적 생화학적 특성을 지닌 domain의 규명을 시도한 첫 번째의 경우가 *Clostridium histolyticum* 유래의 collagenase이다. 상업적으로 많이 사용되고 있는 *C.*

*histolyticum*의 collagenase의 경우 여러 isotype의 collagenase를 생산하고 있는데 그 가운데 비교적 생화학적 특성에 관한 연구가 많이 되어 있는 collagenase가 ColG 단백질이다. 이 단백질은 분자량 116kDa의 collagenase로 zinc binding site인 HEXXH motif를 가지고 있는 metalloprotease인데 molecule내에 N-terminal의 active domain과 C-terminal region의 collagen binding domain이 존재하는 것으로 보고되었다 [Matsushita et al. 2001]. *C. histolyticum*의 collagenase (ColG)의 경우 C-terminal region이 결여되었을 때 soluble 형태인 denatured collagen인 gelatin은 분해하지만 insoluble protein인 collagen분해능은 결여되었으며 collagen binding 연구를 통해 collagen binding domain이 C-terminal region에 있음을 2001년에 보고하였다. *Vibrio* sp.에서의 metalloprotease관해서는 collagen binding domain에 관한 연구가 이루어져 있지는 않으나 여러 metalloprotease가 collagen 분해능을 가지고 있는 collagenase로 분류되고 있으며 Class I에 해당하는 *V. vulnificus*에서 C-terminal region이 autolytic 과정을 거쳐 deletion이 일어나면 insoluble collagen에 대한 활성이 감소되며 soluble protein인 albumin에 대한 활성에서는 차이를 보이지 않는다는 보고가 있었다 [Miyoshi et al. 1997]. 반면 Class II에 해당하는 *Vibrio* sp.의 collagenase에서는 C-terminal region과 protease 활성에 어떠한 관계가 있는지에 대한 보고는 전무한 실정이다. *V. cholerae, V. parahaemolyticus, V. alginolyticus* 그리고 *V. mimicus*는 매우 유사한 amino acid sequence를

가지고 있는 metalloprotease를 생산하고 있으며 또한 최근의 연구에서 *V. alginolyticus* collagenase의 C-terminal region의 부분이 *C. histolyticum*의 collagen binding domain과 유사하다는 보고가 있으나 amino acid sequence의 alignment 결과만의 유사성에 대해서만 언급하고 있을 뿐 실제 단백질을 이용한 연구 결과는 보여 주고 있지 않고 있다. 또한 *V. cholerae, V. mimicus, V. parahaemolyticus*의 metalloprotease는 *V. alginolyticus*의 metalloprotease에 비해 짧은 amino acid sequence를 가지고 있을 뿐만 아니라 C-terminal region이 *V. alginolyticus*와는 전혀 다른 아미노산들로 이루어져 있어 상동성을 보이지 않고 있는데도 불구하고 collagenase활성을 가지고 있었다. 이와 같은 결과는 *C. histolyticum*이나 *V. alginolyticus*의 collagenase와는 전혀 다른 mechanism에 의해서 binding과 catalytic activity를 보여 주고 있음을 강하게 암시하고 있다. 본 연구에서는 *V. mimicus*의 metalloprotease유전자를 이용하여 C-terminal region이 다양하게 제거된 mutant를 제조하고 C-terminal region의 길이와 효소활성의 상관관계를 규명하고 또한 활성의 차이를 통해 collagenase활성에 필요한 최소 길이를 확인함과 동시에 collagen binding에 필요한 최소부위를 결정하여 이 부위의 collagen binding활성을 측정 *V. mimicus* collagenase 단백질 분자내의 collagen binding domain을 결정 하였다.

본 연구에서 사용한 collagenase의 생산균인 *V. mimicus*는 uncooked marine food를 섭취하였을 때 설사, 구토, 두통, 혈변등 여러가지 증상의

직접적인 원인균으로 알려져 있는데 이균의 pathogenic process에 대해서는 알려져 있는 바가 없다. 그러나 이 균이 인체의 장관 조직을 침투하기 위해서는 조직 단백질의 주요 성분인 collagen을 분해하여야 한다는 보고가 있다. 그러므로 일반적인 collagenase가 collagen을 분해하기 위해서는 binding의 과정이 필요로 한다는 기존의 보고 [Matsushita et al. 2001]와 collagen binding domain이 제거되었을 때 soluble substrate와 insoluble substrate에 대한 분해능의 차이를 보인다는 측면에서 *Vibrio* sp.의 collagenase 역시 collagen binding을 위한 domain region을 가지고 있으며 이 domain의 분리와 생화학적인 특성분리를 통해 *Vibrio* sp.의 collagenase가 어떻게 생체내 조직을 인지하고 결합과 생체 조직을 파괴하는지에 대한 과정을 이해하는데 좋은 정보를 제공해 줄 것으로 기대되며 *Vibrio* sp.의 장내 침투 작용 기작을 규명하는데 도움을 줄 수 있을 것으로 생각한다.

재료 및 방법

Collagen binding domain의 확인

Collagen binding assay는 Matsushida [Matsushita et al. 1998]의 방법을 이용하여 측정하였다.10mg의 insoluble collagen을 10mM Tris-HCl, pH8.0을 이용하여 pre-swelling시킨후 12,000 x g에서 10분간 원심분리하여 상층액을 제거하고 다시 200㎕의 동일한 buffer를 첨가하고 다시 100㎍/ml의 metalloprotease solution을 첨가하여 상온에서 6시간에서 12시간 동안 방치후 상층액을 SDS-PAGE를 실시하여 collagen이 들어있지 않은 대조구와 collagen이 첨가된 시료에서의 collagenase의 유무를 통해 확인 하였다. Collagenase에서 collagen binding이 일어나는 최소 부위를 결정하기 위하여 exonuclease를 처리하여 얻은 다양한 길이의 C-terminal region을 가지고 있는 C-terminal truncated mutant를 이용하여 위의 방법으로 측정하였다.

Collagen biding region의 분리

최소 collagen binding region만을 분리 하기 위하여 forward primer인 primer F1(5′CCGCG TGGATCCTTGGTACTGTCTCGACCA3′: *Bam*HI)과 여러 reverse primer들인 primer RI (5′CGGCCGCTCGAGTGTAAAGATCG GCGTCGC3′: *Xho*I, LVLSRP-ATPIFT),primer RII(5′CGGCCGCTCGAGAGTT TCTGGCTCTGAAGG3′:*Xho*I, LVLSRP-PSEPET) primer RIII (5′CGGC

CGCTCGAGTGTATCAAGCCAGACTGC 3′: *Xho*I, LVLSRP-AVWLDT, 33aa) primer IV(5′CGGCCGCTCGAGTTGTTCACCTAAGTTTTT3′: *Xho*I, LVLSRP-KNLGEQ)를 사용하여 다양한 길이의 C-terminal region을 분리하고 이 분리된 단편을 GST (Glutathion S Transferase) fusion vector인 pGEX4T-1(Amersham co.) plasmid에 삽입 시킨후 *E.coli* XL1-blue에 transformation시 zu 나타나는 colony들을 위의 primer를 이용하여 colony PCR방법으로 screening하였다.

Collagen biding region의 발현 및 정제

선별된 collagen binding domain region을 발현 시키기 위하여 *E.coli* BL21에 transformation시킨 후 IPTG를 이용하여 발현을 유도 하였다. 발현된 단백질의 양과 발현된 크기를 확인하기 위하여 SDS-PAGE를 실시하였다. 다양한 크기의 CBD를 정제 하기 위해 CBD의 N-terminal region에 fusion되어 있는 GST를 이용한 glutathion affinity를 chromatography를 실시 하였다.

목적하는 plasmid를 갖는*E.coli* BL21(DE3)를 IPTG로 induction 시킨후 7000 x g, 4 ℃에서 15분간 원심 분리하여 cell을 분리하고 이를 다시 10mM의 Tris-HCl buffer pH8.0에 현탁한후 sonifier (Barnson sonifier 250)을 이용하여 파쇄하였다. Cell 파쇄액은 다시 12,000 x g, 4℃에서 15분간 원심분리 한 후 상층액을 glutathion-sepharose resin에 binding 시키고 10

volume의 washing buffer를 이용하여 비특이적인 결합을 제거하고 reduced glutathion이 함유된 elution buffer(10mM GSH, 50mM Tris-HCl,pH 8.0)를 이용하여 elution시켰다. 용출된 용액에 존재하는 단백질의 확인은 SDS-PAGE를 통하여 실시 하였다.

Binding assay

Collagenase의 collagen biding region의 fragment들의 binding activity를 측정하기 위하여 10㎍의 정제된 다양한 C-terminal fragment를 포함하는 GST fusion pepetide를 10mg의 collagen type I과 혼합 후 상온에서 12시간 방치 후 상층액을 SDS-PAGE를 실시 하여 binding유무를 확인하였고 상층액에 남아있는 GST활성을 측정하여 binding activity를 비교 하였다. GST 활성은 5㎕의 100mM GST와 5㎕의 100mM CDNB(1-chloro-2,4-dinitrobenzene)를 사용하여 340nm에서 5분간 변화하는 absorbance의 값을 standard curve에서 환산하여 측정 하였다.

Collagen binding domain (CBD)의 분리

GST fusion protein으로부터 GST fused CBD peptides를 분리 하기 위해 GST-CBD를 10U의 thrombin을 상온에서 12시간 동안 처리 하였다. 처리된 단편의 확인은 12% slab gel의 Tricine gel을 사용하여 확인하였다. 또한 CBD peptide가 collagen에 binding 하는지를 확인하기 위하여 10mg의

collagen에 thrombin을 처리한 GSTCBD-C를 혼합하여 상온에서 방치후 상층액을 Tricine SDS-PAGE를 실시하여 GST fusion protein으로부터 분리된 33개의 amino acid로 이루어진 peptide (VMCBD) 의 binding 활성을 측정 하였다.

Binding kinetics

CBD의 binding kinetics를 구하기 위해 10mg의 collagen type I과 다양한 농도의 VMCBD와 rVMC62를 반응시킨후 상층액을 Tricine SDS-PAGE를 실시하여 분석하였다. 전기영동이 끝난후 gel을 comassie R250을 이용하여 염색한후 densitometer를 이용하여 농도를 측정하여 K_d를 구하였다. K_d는 상층액에 남아있는 binding 하지 않은 CBD의 양으로부터 collagen에 binding한 CBD의 양을 역산 하여 scatchard plot을 이용하여 구하였다.

결과

Binging domain의 확인

순차적으로 C-terminal region이 deletion된 collagenase들이 기질인 collagen에 대한 결합능이 있는지를 확인하기 위하여 C-terminal이 차례로 제거된 단백질을 발현후 정제하여 기질인 collagen type I과 12시간 반응시킨후 0.22㎛ filer를 통과시키고 fliterate를 SDS-PAGE를 행하여 binding 되지 않은 단백질의 유무를 확인하였다.전기 영동결과 Fig. 20에 나타난 것 처럼 A^{92}-T^{561}까지의 53kDa의 분자량을 가지는 단백질과 그 이상의 분자량을 가지고 있는 단백질에서는 collagen에 binding하고 있는 것으로 나타나고 있으나 53kDa의 단백질보다 33개의 amino acid가 더 deletion된 50kDa의 단백질에서는 binding이 일어나고 있지 않으며 또한 50kDa이하의 분자량을 갖는 단백질은 모두 binding 하지 않는 것으로 나타나고 있다.이와 같은 결과는 33개의 amino acid가 존재하는 부위가 binding에 매우 중요하게 작용하고 있는 것으로 추측되었다.

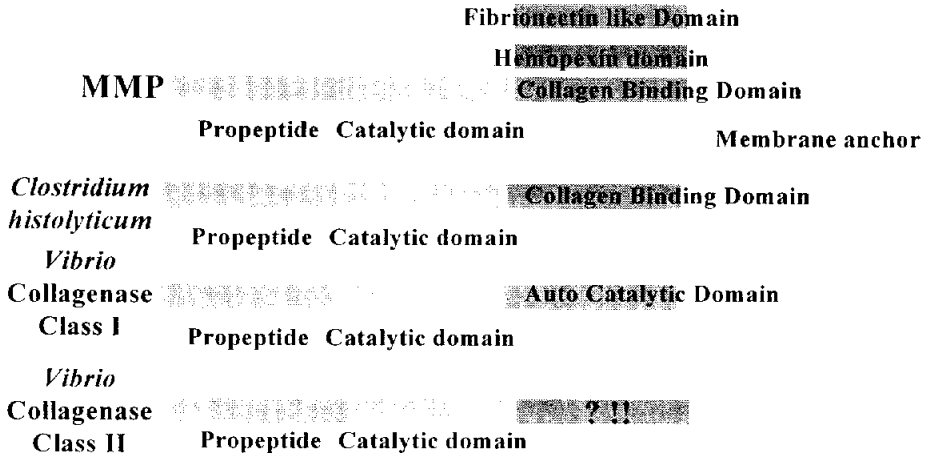

Fig. 19. Known CBD (Collagen Binding Domain) structure of collagenases

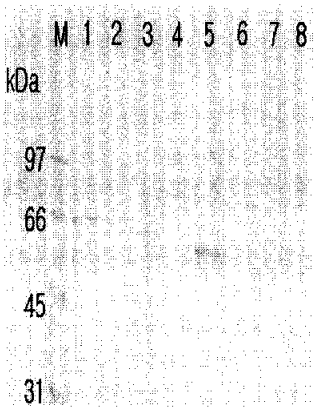

Fig. 20. SDS-PAGE of VMC62 derivates incubated with collagen.

M: molecular marker, 1: VMC62, 2: VMC62 with collagen, 3: VMC57, 4:VMC57 with collagen, 5: VMC53, 6: VMC53 with collagen, 7: VMC 50, VMC50 with collagen.

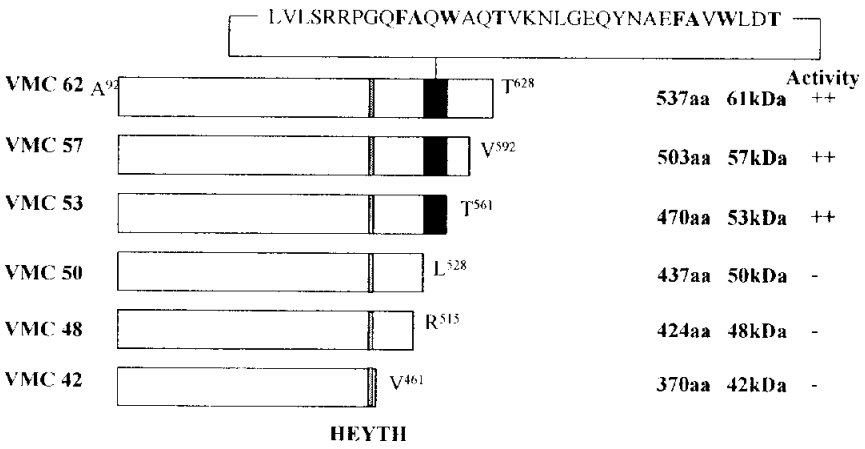

Fig. 21. Representation of domain region of metalloprotease from *V. mimicus.*

HEYTH represented the active site and black box indicated the collagen binding domain

Collagen binding domain의 분리

Collagen binding region으로 추정되는 33개의 amino acid부분을 포함하는 C-terminal region을 분리하기 위하여 Materials and Methods에 기술한것과 같이 primer를 제작하였다. 제작된 primer를 사용하여 L^{528} 부터 T^{628} (CBD-A)까지의 부분과 L^{528}부터 T^{561} (CBD-C)까지의 부위의 33개의 amino acids 부위만을 PCR방법을 이용하여 DNA를 증폭시킨후 GST fusion vector인 pGEX4T-1에 cloning하였다. 특히 33개의 amino acid부분의 amino acid서열을 분석해본결과 특이 하게도 같은 성질을 가지고 있는 매우 상동성이 높은 15개의 amino acid들이 반복되고 있음을 확인하였다. 본 연구에서는 이와 같이 반복되는 amino acid의 역할을 규명하기 위하여 15개의 amino acid만을 coding하는 DNA단편을 PCR을 이용하여 증폭시킨후 위의 방법과 동일하게 pGEX4T-1에 cloning하였다. Cloning 된 vector인 pVMCBD-A, pVMCBD-B pVMCBD-C 그리고 pVMCBD-D를 각각 발현 host인 *E.coli* BL21(DE3)에 transformation한후 IPTG를 이용하여 발현 시켰다.발현된 GST가 fusion된 collagen binding domain을 glutathion sepharose column을 통해 정제 하여 12-15%의 slab gel을 사용하여 SDS-PAGE를 실시하였다. 정제된 GST fusion protein은 GST의 크기를 포함해서 각각 37.1kDa (GSTCBD-A), 31.5kDa (GSTCBD-B), 29.7kDa (GSTCBD-C) 그리고 28.4kDa (GSTCBD-D) 의 크기를 가지고 있었으며 이는 deduced amino acid를 통해 계산되어진 크기와 일치하였다 (Fig.22-24).

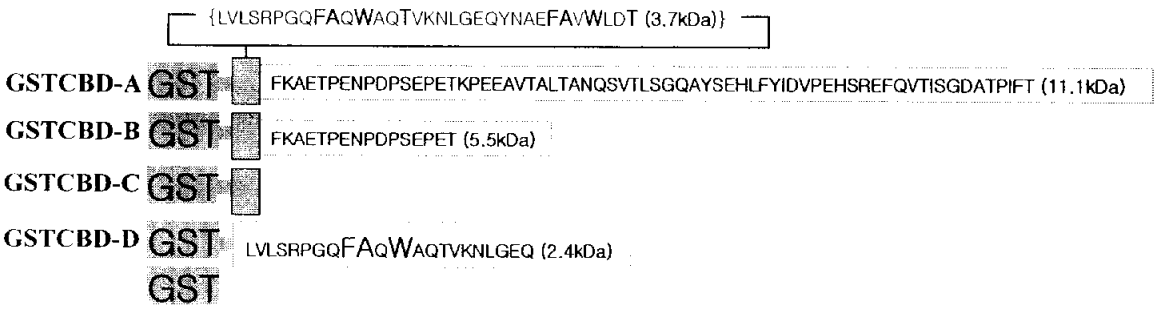

Fig. 22. Construction of GST fused peptides involving the various lengths of C-terminal region of VMC

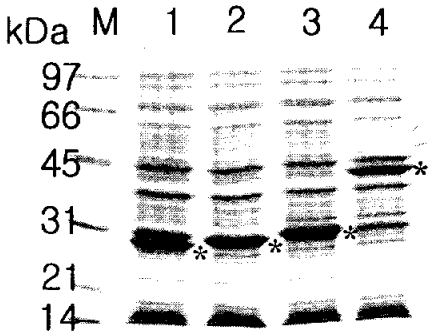

Fig. 23. SDS-PAGE of GST fused peptides involving the various lengths of C-terminal region of VMC.

M: molecular marker, 1: GSTCBD-D, 2: GSTCBD-C, 3: GSTCBD-B, 4: GSTCBD-A

Asterisks indicated the overexpressed GST fusion proteins

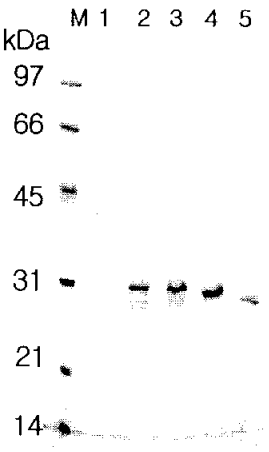

Fig. 24. SDS-PAGE of purified GST fused peptides involving the various lengths of C-terminal region of VMC.

M: molecular marker, 1: GSTCBD-A, 2: GSTCBD-B, 3: GSTCBD-C, 4: GSTCBD-D, 5: GST

Binding assay

Binding region으로 추정되는 부위가 collagen binding 능력을 가지고 있는지를 확인하기 위하여 앞에 기술한 binding assay 의 방법을 사용하였다. Collagen을 pre-swelling시킨후 Tris-HCl buffer pH 8.0과 정제된 GSTCBD-A, GSTCBD-B, GSTCBD-C,그리고 GSTCBD-D 단백질을 첨가하여 상온에서 12시간 방치시킨 후 상층액을 전기영동 하였다. 이때 대조구로 GST만을 발현시킨후 정제한 26kDa의 GST(Glutathion S Transferase)를 사용하였다. 반응후 상층액을 SDS-PAGE를 통해 전기영동하고 staining 하여 band를 관찰하여 본 결과 GSTCBD-A, GSTCBD-B, GSTCBD-C 단백질과 collagen을 반응시킨 lane에서는 첨가한 단백질이 상층액에 나타나고 있지 않았으나 collagen에 GSTCBD-D단백질과 대조구인 GST만을 첨가한 반응 상층액에서는 단백질 band가 나타나고 있었다 (Fig. 25,26). 이는 GSTCBDA~C는 collagen과 bindinggkftn 있는 능력이 있음을 보여주고 있지만 GSTCBD-D와 GST에서는 collagen과 binding할 수 있는능력이 없다는 것을 보여주고 있다. 이러한 collagen binding activity를 측정하기 위해 상층액의 GST활성을 측정하여 binding capacity를 측정 하였으며 (Table 9). GSTCBD-A~C까지는 비슷한 binding capacity를 보이고 있었고 GSTCBD-D는 전혀 binding 하지 않는 것을 알수 있었다. 기존의 CBD가 calcium ion의 영향을 받는다는 보고가 있고 또한 collagenase의 stabilizer로 이용된다는 보고가 있어 VMCBD가 calcium ion

에 대해 binding에 영향을 받는지를 확인하기 위해 calcium, EDTA등의 첨가물을 반응액에 첨가하여 영향을 조사 하였다(Fig. 27). GSTCBD-C는 calcium이나 EDTA의 영향을 받지 않고 binding 하는 것으로 조사 되었으며 여러 조건을 통해 collagen으로부터 elution시켜 보았을 때 가장 강력한 조건인 2%의 SDS에 의해서 elution되는 것으로 조사되었다 (Fig. 28). 이와 같은 결과를 바탕으로 33개의 amino acid부분이 확실히 collagen binding과 밀접한 관계를 가지고 있는 것으로 보여지고 있어 다음 연구에서는 GST와의 fusion 단백질이 아닌 33개의 amino acid로 이루어진 peptide를 얻기 위해 GST fusion protein을 thrombin처리를 하여 GST와 fusion된 peptide를 분리 시켰다. 분리된 peptide의 확인은 12% Tricine SDS-PAGE를 행하여 확인하였다 (Fig 29). 33개의 peptide의 예상되는 분자량은 약 3.7kDa으로 계산되었으며 전기영동 상에서도 비슷한 크기로 이동되어 있음을 확인하였다. 분리된 VMCBD를 collagen과 반응시킨 후 상층액을 다시 Tricine SDS-PAGE를 통하여 확인하였으며 gel 상에서 thrombin과 GST의 band는 확인하였으나 VMCBD에 해당하는 band는 보이지 않았다 (Fig. 30). 이러한 결과는 fusion protein인 GSTCBD-C를 이용하여 얻은 결과와 같은 결과를 보여주고 있으며 33개의 amino acid로 이루어진 peptide가 collagen binding region임을 나타내고 있다.

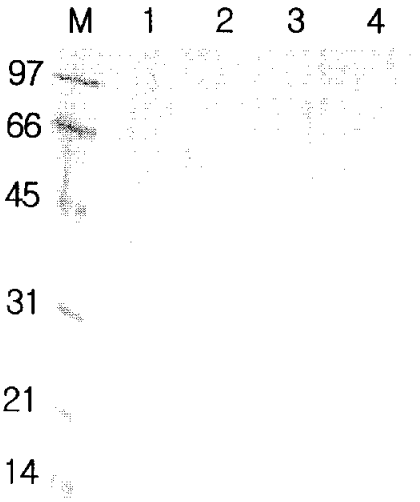

Fig. 25. SDS-PAGE of purified GST fused peptides with collagen.
M: molecular marker, 1: GSTCBD-A, 2: GSTCBD-A with collagen, 3: GSTCBD-B, 4: GSTCBD-B with collagen

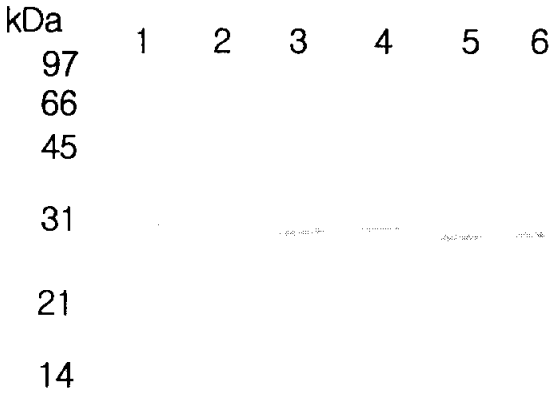

Fig. 26. SDS-PAGE of purified GST fused peptides with collagen.

M: molecular marker, 1GSTCBD-C, 2: GSTCBD-C with collagen, 3: GSTCBD-D, 4: GSTCBD-D with collagen, 5: GST, 6: GST with collagen

Table 9. Binding of GST fusion collagen binding domains with collagen

Fusion protein	Binding %
GSTCBD-A	75.5±1.2
GSTCBD-B	76.9±5.5
GSTCBD-C	75.2±3.5
GSTCBD-D	3.2±2.1
GST	3.5±1.6

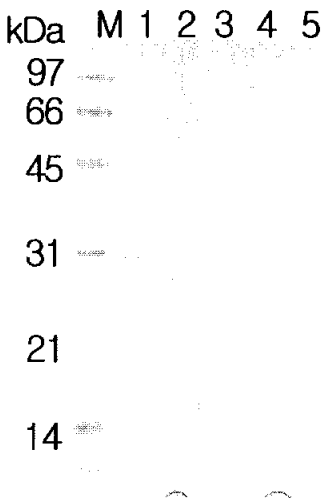

Fig. 27. SDS-PAGE of GST fused collagen binding domain (CBD) with collagen and various additives.

M: molecular marker, 1: GSTCBD-C, 2: GSTCBD-C with Ca, 3: GSTCBD-C with EDTA, 4: GSTCBD-C with EDTA and Ca

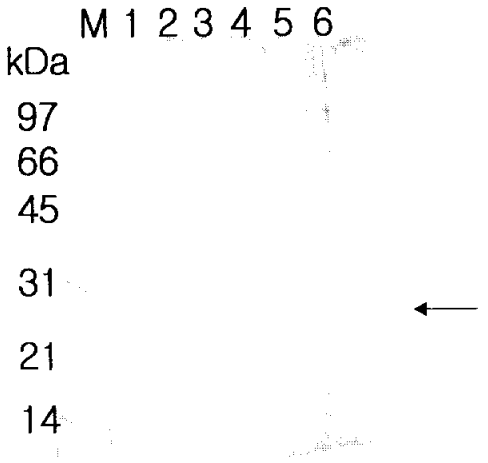

Fig. 28. Elution of collagen binding domain from collagen

M: molecular marker, 1: GSTCBD-C, 2: 2M Urea, 3: 8M Urea, 4: pH 4, 5: pH10, 6: 2% SDS

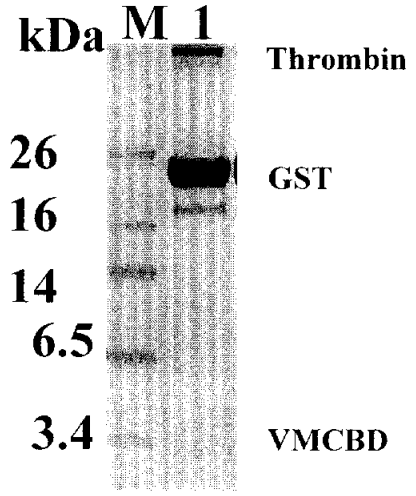

Fig. 29. SDS-PAGE of thrombin digested GST fused CBD

M: molecular marker, 1:thrombin digested GSTCBD-C

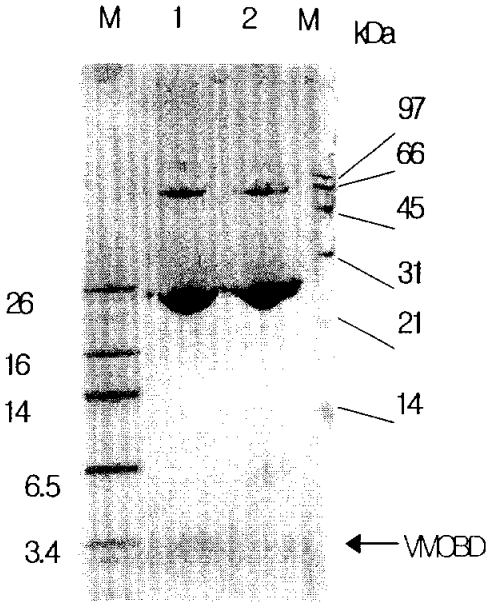

Fig. 30. Binding assay of thrombin digested GST-CBD with collagen.

M: molecular marker, 1: thrombin digested GSTCBD-C, 2: thrombin digested GSTCBD-C with collagen

Binding Kinetics

33개의 peptide로 이루어진 VMCBD peptide와 rVMC62와의 binding kinetics를 구하기 위하여 여러 농도의 정제된 VMCBD peptide또는 rVMC62를 collagen과 binding 시킨후 상층액을 전기 영동하여 binding 하지 않은 단백질의 농도를 측정하고 이와 함께 collagen에 binding한 농도를 계산하여 scatchard plot을 이용하여 해리상수K_d와$Bmax$를 구하였다 (Fig. 31). Scatchard plot을 이용하여 구한 K_d와$Bmax$를 Table 10에 나타내었다. VMC62와 VMCBD의 binding affinity인 K_d 값은 VMC62에서 좀더 낮은 수치를 나타내었고 알려진 C. histolyticum으로 부터의 collagen binding domain 보다 높은 affinity를 가지고 있었다. Total receptor를 나타내는 $Bmax$는 각각 VMC62와 VMCBD에서 2.8, 2.5 x 10^{-10} mol/mg collagen로 비슷하게 나타났다.

Modeling

Collagen binding region(VMCBD)의 molecular modeling을 위해 deduced amino acid sequence를 PBD file로 변환 후 modeling을 실시 하였다.

Comparative modeling결과 유사한 template model을 확인할 수 없어 what if program (Vriend G, 1990)을 이용하여 pocket만을 확인하였다. 확인된 sequence는 helix의 형태를 이루고 있는것으로 추정되며 helix의 뒷부분에 비교적 hydrophobic한 amino acid로 이루어진 pocket구조를 확인하

였다 (Fig32, Fig. 33).

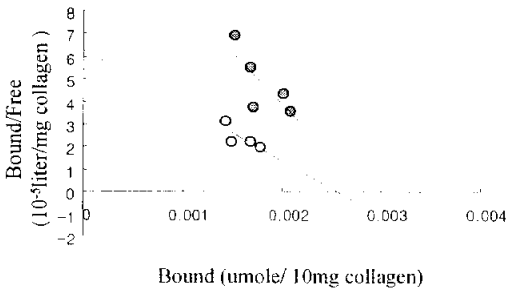

Fig. 31. Scatchard analysis of binding of collagenase (rVMC62) and collagen binding domain (VMCBD).

Table 10. Binding affinity of VMCBD and rVMC62

	VMC 62	VMCBD
Kd (10^{-6} M)	2.1	4
Bmax (10^{-10} *mole/mg collagen*)	2.8	2.5

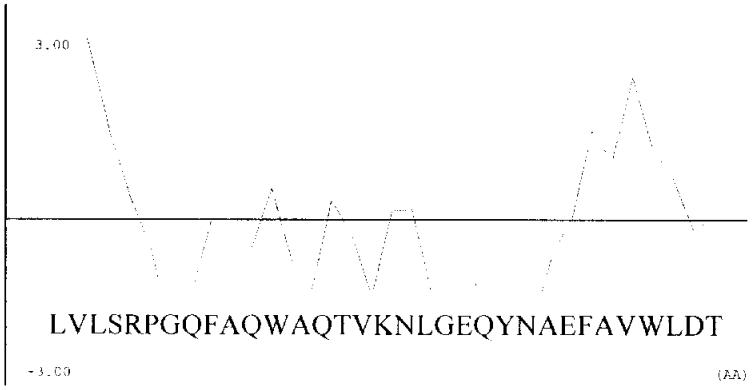

Fig. 32. Hydrophathy plot analysis of VMCBD region.

Kyte and Doolittle methods

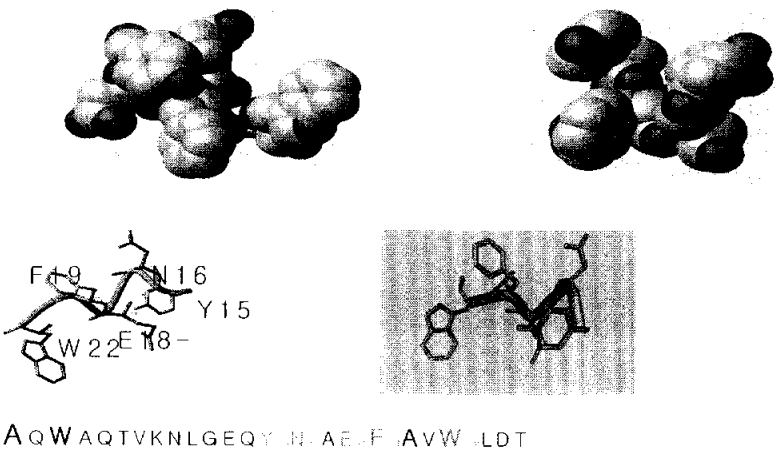

Fig. 33. Possible pocket structure in the hydrophobic region of VMCBD

고찰

Collagenase는 bacteria로부터 mammalian의 MMP까지 다양한 종으로부터 그 존재가 확인되어 왔다. Mammalian cell에서의 MMP는 cell내의 ECM (extracellular matrix)구성성분의 분해등을 통한 생체 물질의 생합성, 분해 그리고 활성화의 과정에 관여 하는 반면 bacteria로부터의 collagenase는 다른 mammalian의 collagen source로 bacteria의 nutrient를 제공하는 다른 역할을 하고 있다. 이러한 역할의 차이점은 bacteria유래의 collagenase보다 좀더 넓은 범위의 substrate specificity를 가지고 있는 것과도 상관관계를 가지고 있으리라 추정된다.

최근 MMP에서부터 bacterial collagenase까지의 연구를 토대로 binding domain에 관한 연구가 이루어 지고는 있으나 MMP에서 밝혀진 여러 binding domain (fibrinonectin like domain, hemopexin)이 발견되어 보고되고 있으나 bacterial collagenase의 경우 *C. histolyticum*으로부터 1개의 특이적 부위가 발견되었을 뿐이다. *C. histolyticum*의 collagenase 비교적 많이 연구가 이루어져 상업적(cell culture)등에 널리 쓰이는 collagenase로 6종류의 metalloprotease endopeptidase로도 알려져 있다. 이 6종의 collagenase는 sequence의 차이와 collagen분해능의 차이에 따라 classI과 classII로 나뉘어 지지만 면역학적으로 상호 교차반응이 일어나는 것으로 보고되고 있다. Class I 에는 α (68 kDa), β (115 kDa) 그리고 γ (79 kDa)의 형태가 보고되고 있고 class II 에는 δ (100 kDa), ε (110 kDa) 그리고 ϕ (125 kDa)의

것들이 보고되었다. *C. histolyticum*으로 부터 의 ColG (classI)과 ColH (classII)의 2개의 metalloprotease에서 collagen binding domain에 관한 연구가 이루어 졌다. 이들의 collagen binding domain은 MMP에서 발견되는 것과는 amino acid sequence상에서는 homology를 보이고 있지 않지만 반면에 binding domain의 형태구성에 calcium을 필요로 한다는 공통된 특징을 가지고 있다. 또한 이들 collagenase binding domain에서는 PKD (Polycystic Kidney Disease)로 분류되는 특이적인 domain구조가 존재하고 있다. 이 PKD domain의 구조는 *Vibrio*의 metalloprotease 인 *V. alginolyticus*로 부터의 metalloprotease (VAC)에서도 발견이 되는 domain 이나 비슷한 amino acid sequence를 가지고 있는 다른 *Vibrio* 유래의 metalloprotease에서는 보여지지 않는다. *V. parahaemolyticus, V. mimicus, V.cholerae*로 부터의 metalloprotease의 collagen degrading activity로 미루어 볼 때 이들의 C-terminal region 역시 collagen binding domain을 형성하리라 추정되어 지지만 C-terminal region의 amino acid의 서열에 전혀 유사성을 발견할수 없어 *V. alginolyticus*나 *C. histolyticum*의 collagenase와는 collagen binding region이 다른 형태를 취하고 있으리라 추정되어진다. 이러한 추정을 검증하기 위해 본 실험에서는 *V. mimicus*의 metalloprotease인 VMC의 C-terminal region을 deletion시켰고 최소활성을 가지는 33개의 amino acid의 부위를 결정하였다. 이전에 보고된 collagen binding domain의 크기와 비교 하여 매우 짧은 peptide가 collagen binding

에 영향을 미치는지를 확인하기 위하여 C-terminal이 deletion 된 mutant 들을 collagen과 반응 시켰고 결과로 33개의 peptide가 없는 mutant에서는 collagen에 대한 binding이 일어나지 않았다. 이러한 결과는 33개의 amino acid가 의 결여로 인한 활성의 소실이 active site의 변이에 의한 결과가 아니고 collagen binding domain의 결여로 인한 결과라는 것을 알 수 있다. 특히 흥미로운 점은 33개의 amino acid서열을 분석한 결과 FAXWXXT의 아미노산이 중복되어 존재하고 있다는 것을 확인할수 있다. 또한 33개의 peptide만으로 collagen에 binding 할 수 있는지를 확인하기 위하여 GST(26kDa)의 protein과 collagen binding region을 fusion protein으로 발현시켜 정제후 이를 collagen과 binding test를 실시 하였다. 33개의 amino acid부위(CBD-C)와 33개의 amino acid에서 발견되는 반복 amino acid (FAXWXXT)를 포함하는 부위(CBD-D) 그리고 나머지 C-terminal region(CBD-A)을 모두 포함하는 부위를 대상으로 collagen과 binding시켜본 결과 반복되는 서열이 2개가 존재하는 최소 33개의 amino acid이상이 collagen binding에 필요하다는 것을 알 수 있었다. 반면 나머지 C-terminal region은 binging에 큰 역할을 하지 않는 것으로 조사되었다. 이러한 *V. mimicus*의 metalloprotease (VMC)의collagen binding domain (VMCBD)는 기존에 보고된 collagen binding domain (CBD)과는 여러 다른 특성을 보이고 있다. 4℃의 낮은 온도에서는 binding이 일어나지 않았으며 또한 calcium이 binding에 영향을 미치지 않았다. 33개의

아미노산에 2개의 반복서열이 존재하고 있었으며 뒷부분에 hydrophobic pocket의 특이적 구조를 형성하고 있으며 이 뒷부분을 제거시에 collagen binding이 일어나지 않는 것으로 미루어 2번째 반복서열이 collagen binding에 핵심적인 역할을 수행하고 있음을 알게 되었다. *V. mimicus, V. parahaemolyticus, V. cholerae*의 경우도 VMC와 매우 유사한 sequence를 가지고 있어 이들의 경우도 이러한 종류의 collagenase binding domain을 가지고 있으리라 추정되어 진다.

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감사의 글

부족한 저에게 학문의 길을 열어 주시고 아낌없는 지도와 사랑으로 이끌어 주신 공인수 교수님께 머리 숙여 감사 드립니다. 그리고 연구 과정에 많은 관심과 애정을 보내주신 김성구 교수님, 김중균 교수님, 홍용기교수님, 박남규 교수님, 이형호 교수님, 공재열 교수님께도 감사 드립니다. 또한 연구실 생활에서 부족한 후배를 아껴주시고 끌어주신 윤수철 선배님, 박기재 선배님, 하정철 선배님, 김구택 선배님, 김영옥 선배님, 박효진 선배님, 김대경 선배님, 이동구 선배님께도 감사드리며 실험실에서 동고동락을 같이 했던 김현국, 이상봉, 최선영, 강정화, 진철호, 김형섭, 안선희, 김선회, 이은미, 조윤희, 심나영, 이지현 후배님께도 감사의 마음을 전합니다. 또 곁에서 많은 도움을 주었던 진형주, 김찬희, 장재혁군과 김한우군에게도 감사를 전합니다.

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APPENDIX

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Characterization of *Vibrio mimicus* phospholipase A (PhlA) and cytotoxicity on fish cell

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Abstract

Vibrio mimicus is a typical strain of *Vibrio cholerae* and produces a phospholipase (PhlA) which shares a highly conserved amino acid sequence with the lecithinase (Lec) of *V. cholerae*. The recombinant protein (rPhlA) produced from the *phlA* gene of *V. mimicus* was expressed in *Escherichia coli* as His-tag fused protein. The rPhlA was purified by gel filtration and Ni–metal affinity chromatographies. When the action mode was investigated by TLC and GC–MS, the purified rPhlA protein showed a phospholipase A activity, which cleaved the fatty acids at the sn-1 and sn-2 positions of phosphatidylcholine. However, it did not show lysophospholipase, sphingomyelinase, and phospholipase C activities. The rPhlA showed maximum activity at temperature of about 40 °C and pH around 8–9. Some divalent cations could affect the activity of PhlA. The addition of Co^{2+} increased the activity, whereas Mg^{2+} and Zn^{2+} did not enhance the enzyme activity. The rPhlA could lyse the erythrocytes obtained from the fish such as rainbow trout and tilapia. A significant cytotoxic activity on a fish cell line, CHSE-214, was observed after 24 h exposure to 40 μg rPhlA protein.

Keywords: *Vibrio mimicus*; Phospholipase A; Hemolytic activity; Cytotoxicity

Vibrio mimicus is a gram negative bacterium biochemically similar to *Vibrio cholerae*. The differences between the two strains are that *V. mimicus* cannot produce acid from sucrose and shows a negative response to Voges-Proskauer test [1]. *V. mimicus* is a pathogenic bacterium causing fever, diarrhea, nausea, vomiting, and abdominal cramps in human [2]. These symptoms are probably due to many kinds of virulence factors produced by *V. mimicus*. The important factors of *V. mimicus* that have been implicated in virulence and pathogenicity are known to be cholera toxin (CT) [3–5], CT-related toxin [1], *Escherichia coli* heat stable enterotoxin-like toxin [6,7], thermolabile hemolysin, and thermostable hemolysin [8]. Beside these toxins, this organism produces many proteins associated with infection, such as hemagglutinin, metalloprotease, HA/protease, lipase, and phospholipase [9,10].

Phospholipases produced by pathogenic bacteria are related with tissue destruction by causing hydrolysis of phospholipid present in the host cell membrane. They also have the potential to exert profound effects on the host indirectly by the production of lipid second messengers that modulate the signaling pathway. Phospholipase C (PLC) is the most studied phospholipase among different bacterial phospholipases [11,12]. PLC directly disrupts nucleated cell membranes as well as erythrocyte membranes. Compared with PLC, bacterial phospholipase A (PLA) has not been studied thoroughly. However, some recent studies reported that PLA plays an important role in the pathogenesis. The PLA produced by *Legionella* sp. causing severe pneumonia destroys alveolar surfactant phospholipid in the lung [13,14]. PLA of *Campylobacter coli* caused acute diarrhea in human and *Yersinia enterocolitica* PLA was known to participate not only in PLA activation but also in hemolysis [15,16].

A number of phospholipases were found in *Vibrionaceae* family. The PLA_2/lysophospholipase activity was

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shown in the thermolabile hemolysin of *V. parahaemolyticus* [17]. The lecithinase purified from *A. hydrophila* AH-3 (serogroup O:34) was known to be a crucial virulence factor to rainbow trout and mouse [18]. The egg yolk lecithin degrading enzyme (Lec) of *V. cholerae* was reported [19]. We have also reported the phospholipase (*phlA*) gene sequence from *V. mimicus* [20]. The PhlA showed 74% amino acid homology with the Lec and located adjacent to hemolysin gene as *V. cholerae*. Recently, it has been reported that *V. harveyi* hemolysin, which has a high degree of similarity with phospholipase (Lec) of *V. cholerae*, showed the hemolytic activity on the erythrocytes of rainbow trout [21]. Although the Lec and the PhlA proteins were known to degrade the egg yolk lecithin, no study was done on enzymatic characteristics.

In the present study, we purified the recombinant PhlA protein (rPhlA) from *E. coli* using an overexpression system to investigate biochemical characteristics. We report that the purified rPhlA protein demonstrates the phospholipase A (PLA). We also describe that this protein has a high hemolytic activity against fish erythrocytes and the growth inhibition effect on CHSE cell which originated from chinook salmon.

Materials and methods

Reagents. Egg yolk lecithin, L-α-phosphatidylcholine (PC, P-5394), L-α-lysophosphatidylcholine (LPC, L-4129), 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine (P-9648), 1-O-hexadecyl-2-[(*cis*)-9-octadecanoyl]-rac-glycero-3-phosphocholine (P-6159), 4-nitro-3(octanoyloxy) benzoic acid (N-1646), *p*-nitrophenylphosphorylcholine (NPPC, N-5879), and *Clostridium perfringenes* PLC were purchased from Sigma (St. Louis, MO). Silica Gel-60 TLC plates and solvents were obtained from Merck (Darmstadt, Germany). Tryptone and yeast extract were obtained from Difco (Detroit, MI). Unless specified otherwise, additional reagents were purchased from Sigma and were of the highest purity.

Purification of rPhlA from E. coli. The overexpression plasmid (pPHL13) was constructed previously [20]. The entire sequence encoding 470 amino acid residues of the *phlA* gene was cloned into expression vector pET22b(+) using PCR with two specific primers containing *Bam*HI and *Eco*RI sites at either end, respectively.

A single colony of *E. coli* strain BL21 (DE3) transformed with pPHL13 was inoculated into 50 ml Luria–Bertani medium containing 50 μg/ml ampicillin. The culture was grown at 37 °C under vigorous shaking. The preculture was inoculated to 950 ml of the same medium and allowed to grow for another 2 h attaining an OD_{600} of 0.6. The expression of the rPhlA was induced by adding IPTG (isopropyl-2-D-thiogalactopyranoside) to a final concentration of 1 mM and the culture was incubated for an additional 5 h at 25 °C under vigorous shaking. Bacterial cells were harvested by centrifugation at 7000*g* for 10 min at 4 °C and the pellet was resuspended in 100 ml of 20 mM Tris–HCl (pH 8.0). The cell suspension was sonicated for a total 20 min with a sonifier (Branson model 250). The lysed bacterial cells were centrifuged at 10,000*g* for 15 min at 4 °C. The pellet was resuspended in 50 ml of 2 M Tris–HCl (pH 12.0) and 2 M urea and dissolved for 2 h at 4 °C. The suspension was centrifuged and filtered through a 0.22 μm syringe filter unit, MSF-25 (Advantec MSF), to remove insoluble materials [22]. A Sephadex G-100 column (16 × 800 mm) was equilibrated with 1.5 column volumes of 20 mM Tris–HCl (pH 8.0) with a flow rate of 1 ml/min. The solubilized inclusion body was loaded onto a column and the eluted fractions were monitored at 280 nm. Pooled protein fractions were applied onto a metal chelating affinity chromatography column packed with 5 ml Ni-NTA resin (Qiagen) that was precharged with 50 mM $NiSO_4$. Before purification, the resin was equilibrated with 20 mM Tris–HCl (pH 8.0), 10 mM imidazole, and 0.5 M NaCl. The protein samples were loaded onto a column and washed with 10 volumes of 20 mM Tris–HCl (pH 8.0), 50 mM imidazole, and 0.5 M NaCl. Finally, the rPhlA protein was eluted with 20 mM Tris–HCl (pH 8.0), 500 mM imidazole, and 0.5 M NaCl, and the final elutant was dialyzed in 20 mM Tris–HCl (pH 8.0), containing 10% glycerol at 4 °C for 12 h. The purification steps were monitored by SDS–PAGE to determine the purity and the concentration of proteins was determined by Bradford method [23,24].

Thin layer chromatography (TLC) and gas chromatogrphy–mass spectrometry (GC–MS). To determine the specific site of deacylation by the rPhlA, L-α-PC, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine, 1-O-hexadecyl-2-[(*cis*)-9-octadecanoyl]-rac-glycero-3-phosphocholine, and L-α-LPC were used as the substrates. Twenty microliters of the substrates in ethanol (10 mg/ml) was mixed vigorously with 100 μl reaction buffer (10 mM Tris–HCl, pH 8.0; 2.7 mM sodium deoxycholate) containing 1 mg rPhlA. The reaction mixtures were incubated at 37 °C for 14 h and stopped by adding 650 μl chloroform–methanol solution (2:1, v/v). After vortexing, the mixtures were centrifuged and then the chloroform layers were collected. The chloroform layers were evaporated under vacuum and redissolved in 20 μl chloroform, respectively. The dissolved solutions were spotted on a Silica Gel-60 plate. The plate was developed with chloroform–methanol–acetic acid–water (55:17:6.5:2.5, v/v/v/v) to detect PC or LPC. Hexane–diethyl ether–acetic acid (85:15:1, v/v/v) was used to detect free fatty acids. After development, the plate was sprayed with 50% sulfuric acid followed by heating at 115 °C for 30 min to visualize PC, LPC, and free fatty acid (FFA) spots [25–27].

To investigate the positional specificity of phospholipase A, 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine was dissolved in ethanol (10 mg/ml). The substrate solution was mixed with the reaction buffer containing 1 mg rPhlA. The mixture was incubated at 37 °C for 14 h. The released FFAs were separated using preparative TLC with a developing solvent of hexane–diethyl ether–acetic acid (85:15:1, v/v/v) [25], the fatty acid spots were scraped out separately, and methylation was performed by the method of Ohta et al. [28]. The methylesters corresponding to each reaction products were subjected to gas chromatography–mass spectrometry (GC–MS) [29–31]. GC–MS analysis was performed on a Shimadzu QP-5050A (Shimadzu, Japan) using a polydimethylsiloxane capillary column HP-5MS (30 m × 0.32 mm ID, 0.25 μm film thickness) with helium (24.4 kPa) as a carrier gas at the column flow rate of 10 ml/min. The column temperature program was as follows. Injector temperature was 250 °C. The column was held for 1 min at 80 °C, then the temperature of 180 °C at a rate of 10 °C/min and next to 260 °C at a rate of 5 °C/min. The interface temperature was kept at 260 °C. Electron energy was set at 70 eV.

Measurement of enzyme activity. PLA activity was assayed as follows. Fifty microliters of the substrate [4-nitro-3-(octanoyloxy) benzoic acid, 3.1 mM in acetonitrile] was combined with 3 ml buffer (10 mM Tris–HCl, pH 8.0; 100 mM NaCl) containing 20 μg rPhlA. Each tube was vigorously stirred and placed in a water-bath at 37 °C for 1 h. After the reactions were placed on ice for 5 min, the PLA activity was determined. The value for activity was calculated by the change of absorbance at 410 nm over time per mg of total rPhlA enzyme. The change in absorbance of 0.2 AU at 410 nm was equivalent to 155 nmol of chromophore release [16,32].

Assay of hemolytic activity. Hemolytic activity was investigated with erythrocytes from rainbow trout, tilapia, rabbit, mouse, sheep, and human. The erythrocytes were washed three to four times with 10 mM Tris-buffered saline (TBS: pH 7.5) and adjusted to a final concentration of 4% (v/v) in TBS. Two hundred microliters of

erythrocytes was mixed with the same volume of rPhlA (10 μg) and incubated at 37 °C for 1 h. Reaction mixtures were centrifuged at 1000*g* for 5 min and the amount of hemoglobin released from the disrupted erythrocytes was determined spectrophotometrically. Complete hemolysis was defined as the optical density at 540 nm of hemoglobin released from the disrupted erythrocytes treated with 0.1% Triton X-100. One hemolysin unit (HU) was defined as the amount of hemolysin eliciting 50% hemoglobin release [33].

Cytotoxicity assay of the rPhlA on CHSE-214 cell line. Cytotoxicity of the rPhlA was investigated by measuring the amount of lactate dehydrogenase (LDH) released from the CHSE cells. The chinook salmon embryo cell line (CHSE-214 cell line) was inoculated in MEM containing penicillin G and streptomycin and cultivated at 17 °C for 24 h. One hundred microliters of the enzymes with various concentrations (0–50 μg) was added and incubated at the same temperature. Cytotoxicity was determined by the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega). Cytotoxicity calculations were based on manufacturer's instruction.

Results

Purification of the rPhlA from E. coli

Cells from 1 liter culture were disrupted by sonication and 78.3 mg total protein was obtained. After inclusion body was refolded, the specific activity increased by 7.1-fold while recovery was 76%. Finally, the rPhlA was purified by the metal affinity chromatography and 4.4 mg enzyme was obtained. The specific activity of purified rPhlA was 21.8 U/mg (Table 1). The purified enzyme showed a single band on SDS–PAGE and its estimated molecular weight was about 53 kDa (Fig. 1). This value was in good agreement with the molecular mass calculated from the deduced amino acid sequence.

Effects of pH and temperature

The optimal pH for enzyme activity was determined using different pH values ranging from 5 to 11. The PhlA had maximal activity in pH 8.0 and more than 80% of the activity was retained at pH 9.0 (Fig. 2A). However, the activity decreased significantly at pH higher than 10. The optimal pH observed was slightly alkalic and this result was similar to the optimal pH of acylesterase from *V. mimicus* as reported by Shaw et al. [34]. The pH stability was also estimated the residual activity after preincubating the PhlA in the pH ranges of 4–11 at 4 °C for 24 h. The enzyme was relatively stable around pH 7–9 than other pH ranges (Fig. 2B).

For the optimal temperature test, the rPhlA was incubated at 10, 20, 30, 40, and 50 °C and the PLA activity was determined. To determine the thermostability, the enzyme was preincubated at 20, 40, 50, 60, and 70 °C for 2 h, and the residual activity was assayed. As shown in Fig. 3, the optimal temperature for activity was around 40 °C. The enzyme was relatively stable at around 20–40 °C, but its stability drastically decreased at 50 °C. The fact that this enzyme shows the maximal activity at temperatures lower than 40 °C seems to be closely related with the habitat of this microorganism showing good accommodations with the aquatic environment.

Effects of metal ions

The effects of metal ions on the activity of rPhlA were investigated by adding the divalent ions (Table 2). At 1 mM of Co^{2+} ion concentration, a significant increase of 270% in the enzyme activity was shown as compared with control. And 150% increase of the enzyme activity was detected with 0.1 mM Co^{2+} ion concentration (data not shown). By contrast, the effect of Ca^{2+} ion showed that 125% increase of the enzyme activity appeared by the addition of 1 mM Ca^{2+} ion, but it did not change when the concentration of Ca^{2+} ion was reduced to 0.1 mM. The data obtained here indicated that the *V. mimicus* PhlA protein is not an enzyme activated by Ca^{2+} ion. The addition of Zn^{2+} ion rather decreased the enzyme activity. Mg^{2+} ion did not affect the enzyme activity.

To find out whether the PhlA protein belongs to metallo-enzyme, the specific inhibitors of metalloprotease, such as EDTA and 2,2′-bipyridine, were added to the standard reaction mixtures. The inhibitors did not significantly affect the enzymatic activity.

Determination of action site in phospholipid

To determine the action mode of rPhlA protein to phospholipid, the enzymatic products of phospholipid were analyzed by TLC and GC–MS. We investigated whether the PhlA protein has a PLC activity using the specific substrate, *p*-NPPC. The PLC of *C. perfringens* was used as the control. The result showed that there was no PLC activity in the PhlA protein. However, we previously showed that the PhlA could lyse egg yolk

Table 1
Purification steps of the rPhlA from *E. coli*

Purification step	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Purification fold
Cell homogenate	78.3	2.2	172.9	100	1.0
Sephadex G-100	8.3	15.7	132.0	76	7.1
Ni-NTA affinity chromatography	4.4	21.8	96.6	56	9.9

Fig. 1. SDS–PAGE of the purified rPhlA from *E. coli*. Lane 1: purified rPhlA. Lane 2: molecular marker.

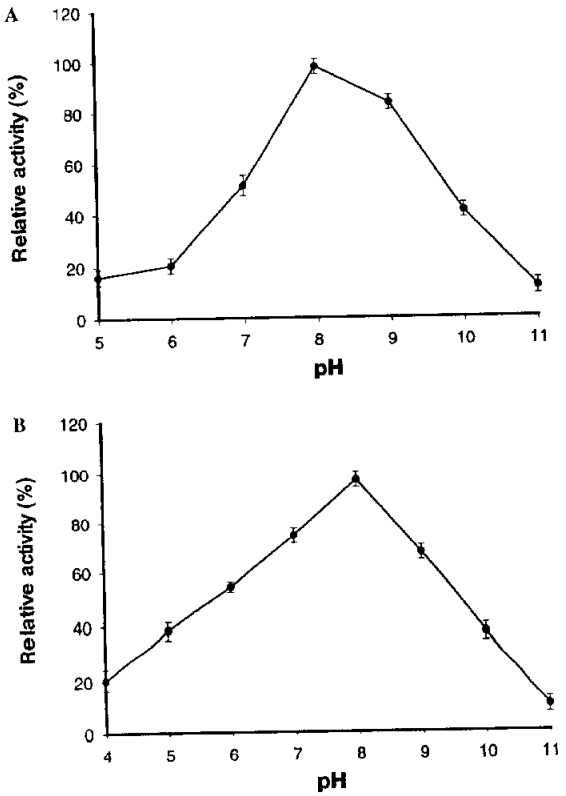

Fig. 2. Effects of pH on the rPhlA activity and stability. The optimal pH for the rPhlA activity was determined using different pHs from 5 to 11. To investigated the pH stability of the rPhlA, the rPhlA was preincubated in the different pHs at 4 °C for 24 h and then residual activity was measured under standard condition. The buffers used were 100 mM citric acid–sodium citrate from pH 4 to 6, 100 mM Tris–HCl from pH 7 to 9, and 100 mM sodium carbonate–NaOH from pH 10 to 11. (A) Optimal pH. (B) pH stability.

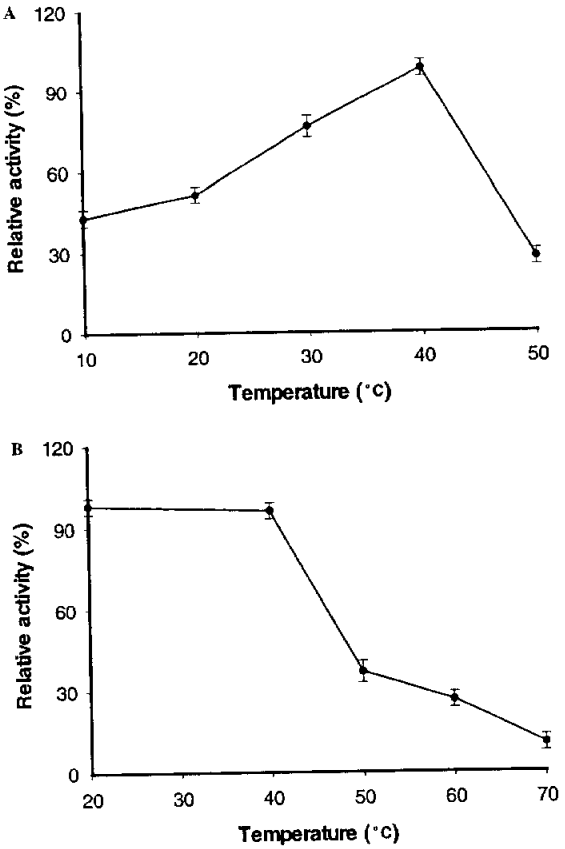

Fig. 3. Effects of temperature on the rPhlA activity and stability. The optimal temperature of the rPhlA activity was assayed under various temperatures for 1 h. To investigate the thermostability of the rPhlA, the rPhlA was preincubated at the temperatures of 20, 40, 50, 60, and 70 °C for 2 h and residual activity was assayed under standard condition. (A) Optimal temperature. (B) Thermostability.

Table 2
Effects of metal ions on the PLA activity of rPhlA[a]

Metal ion (1 mM)	Relative activity (%)
None	100
$CaCl_2$	125
$CoCl_2$	270
$MgCl_2$	100
$ZnCl_2$	70
EDTA	85
2,2'-Bipyridine	100

[a] rPhl (20 μg) and 4-nitro-3-benzoic acid stock solution 50 μl (3.1 mM) were incubated at 37 °C for 1 h in the presence of the cation. Thereafter, the activity was quantified by measuring absorbance at 410 nm. The data are means of triplicates.

lecithin whose major component is PC [20]. Therefore, PC was used for enzyme reaction. After 10 h reaction, the reaction products were analyzed by TLC as

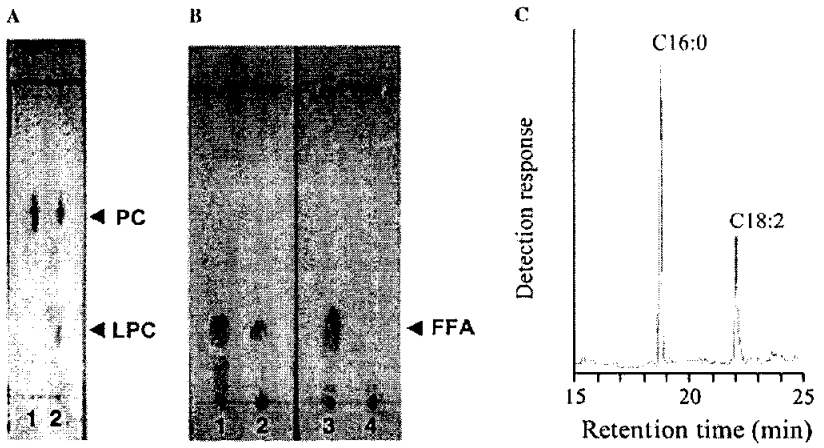

Fig. 4. Thin-layer chromatogram and GC–MS. The reaction products, after 14 h incubation of various substrates with 1 mg rPhlA, were eluted in the chloroform. Each was separated on TLC and sprayed with 50% sulfuric acid followed by heating at 115 °C for 30 min. (A) The reaction products of PC without (control) or with the rPhlA. Lane 1: PC, Lane 2: PC with the rPhlA. The R_f of PC and LPC were 0.57 and 0.2, respectively. (B) Products of the enzymatic hydrolysis with the rPhlA and synthetic phospholipids. Lane 1: 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine hydrolysis product. Lane 2: 1-O-hexadecyl-2-[(*cis*)-9-octadecenoyl]-rac-glycero-3-phosphocholine hydrolysis products. Lane 3: L-α-PC hydrolysis products. Lane 4: L-α-LPC hydrolysis products. The FFA spot (R_f 0.25) was detected at lanes 1–3 and not detected at lane 4. (C) Reaction product of 1-palmitoyl-2-linoleoyl-sn-glycero-3-phosphocholine with 1 mg rPhlA was separated on TLC plate and then analyzed with GC–MS. Palmitic acid peak, C16:0. Linoleic acid peak, C18:2.

described in Materials and methods. As shown in Fig. 4A, we identified a LPC spot at R_f 0.2, indicating that the PhlA may belong to PLA enzyme family. LPC is a degradation product of PC from which a fatty acid is removed. We further investigated whether the PhlA could produce FFAs. When 1-palmitoyl-2-linoleoyl-sn-glycero-3-PC was used as a substrate, the FFA spot at R_f 0.25 was detected (Fig. 4B, Lane 1). To examine the cleavage site, the FFA spot around R_f 0.25 was scraped from TLC plate and extracted with chloroform. The fatty acid sample was methylated and analyzed with GC–MS. As shown in Fig. 4C, a palmitic acid and a linoleic acid peaks appeared at the retention time of 18 and 22 min, respectively. The molecular masses of peaks were 270 and 294, respectively. When the mass of methyl group was subtracted from these values, the resulting values were well matched with the exact molecular weights of palmitic acid and linoleic acid. These results suggested that the PhlA could cleave both ester linkages of the sn-1 and sn-2 sites in PC.

It has been known that the acyl chain of the sn-2 site can spontaneously migrate to the sn-1 site under physiological condition [35]. Thus, it is possible that the linoleic acid at the sn-2 site transferred to the sn-1 site after the palmitic acid at the sn-1 site was cleaved from PC. To confirm that the fatty acid at the sn-2 site was not generated by this transition, 1-O-hexadecyl-2-[(*cis*)-9-octadecanoyl]-rac-glycero-3-PC, which contains a alkyl ether linkage at the sn-1 site, was used as a substrate instead of the normal PC. As shown in Fig. 4B, Lane 2, the FFA was detected on TLC. This result indicated that the PhlA protein is able to cleave the ester linkage of the sn-2 site. Taken together, the fatty acids at the sn-1 and sn-2 sites could be released at the same time by the PhlA protein. Furthermore, no fatty acid was produced when the PhlA reacted with LPC as a substrate.

Assay of hemolytic activity and cytotoxicity

The PhlA exhibited the highest activities on rainbow trout and tilapia erythrocytes. Rabbit, mouse, sheep, and human erythrocytes appeared lower sensitive or insensitive to the PhlA (Table 3). The cytotoxic effect of PhlA was also investigated using the CHSE-214 cell line originated from the chinook salmon embryo cell. As shown in Fig. 5A, cytotoxicity increased only by about

Table 3
Hemolytic activities of the rPhlA on the erythrocytes from various sources

Erythrocyte source	Hemolytic activity (HU[a])
Rainbow trout	1.70 ± 0.02
Tilapia	1.64 ± 0.03
Rabbit	0.22 ± 0.04
Mouse	ND[b]
Sheep	ND
Human	ND

Values represent means and standard deviations of triplicate.
[a] To detect hemolytic activities, 10 μg rPhlA was used. HU was defined as the amount of hemolysin eliciting 50% hemoglobin release.
[b] Not detected.

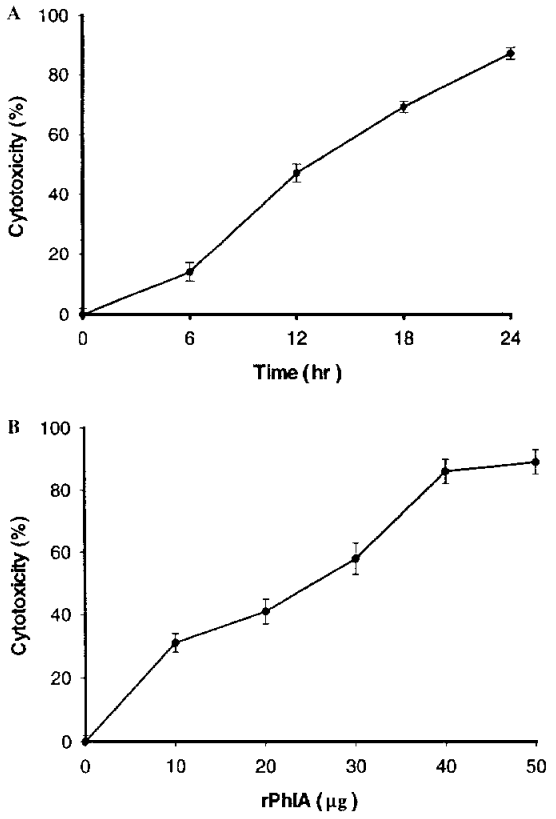

Fig. 5. Cytotoxic effect of the rPhlA on CHSE-214. Cells were cultured as described in Materials and methods, and cytotoxicity of CHSE-214 cell line was assayed by measuring the LDH amount released from the lysed cells. The released LDH was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay kit. Results represent mean ± SD of triplicates. (A) Cytotoxicity of the rPhlA on CHSE-214 over 24 h. (B) Cytotoxicity at each concentration of the rPhlA (0–50 μg) on CHSE-214.

10% after 6 h treatment, but the relative cytotoxicity was increased to 90% after 24 h following exposure to 40 μg PhlA. To examine the effect of concentration, we added the PhlA protein with 0–50 μg and incubated for 24 h. Compared to the control, the level of cytotoxicity increased by 30% at 10 μg and 90% at higher than 40 μg (Fig. 5B).

Discussion

Phospholipases produced by pathogenic microorganisms have been revealed as an important virulence factor. Recently, two phospholipase genes were isolated from *V. cholerae* [19] and *V. mimicus* [20]. The *lec* gene of *V. cholerae* and the *phlA* gene of *V. mimicus* show a high homology in their amino acid sequences, and have adjacent hemolysin genes transcribing in opposite direction on the chromosomes. From high homology in their genes, it is believed that the Lec and the PhlA would be very similar in their enzymatic activity. However, no study has been reported on the biochemical characteristics of two proteins. To determine the biochemical properties of *V. mimicus* PhlA, the cloned *phlA* gene was overexpressed using *E. coli* and the PhlA was purified as His-tag fused recombinant protein. The inclusion body was dissolved in urea and refolded to recover the activity. It has been reported that the induction at a lower temperature is effective for the overexpression of recombinant proteins in *E. coli* [36]. When the PhlA protein was induced with IPTG, the protein production yield was markedly higher at 25 °C compared with 37 °C (data not shown).

Phospholipases are classified as the esterase enzyme group dissociating glycerol–phospholipid and are divided into phospholipases A (A_1 and A_2), C, and D according to the positional specificity on phospholipids. Of these, PLC is the most studied enzyme and acts on phospholipids to produce the phospho-head group and diacylglycerol (DAG). PLA can hydrolyze the ester bond to release fatty acid located on the sn-1 or sn-2 site of phospholipids. TLC and GC analyses showed that the PhlA was a PLA enzyme cleaving both the sn-1 and sn-2 sites of phospholipid. Furthermore, according to the GC–MS data incubated with 1-palmitoyl-2-linoleoyl-sn-glycero-3-PC, two peaks of palmitic acid and linoleic acid with each area being 68% and 32%, respectively, were identified. This result suggested that the sn-1 site might be a favorite to the PhlA protein. However, the PhlA protein did not have a LPC degrading activity.

It has been reported that Ca^{2+} ion functions as a cofactor for enzyme activation in many bacterial phospholipases. In the outer membrane PLA (OMPLA) proteins of gram negative bacteria, Ca^{2+} ion function as a cofactor for the activation process and dimerization of proteins [37]. In eukaryotic cells, PLA_2 proteins are also divided into two types, Ca^{2+}-dependent and Ca^{2+}-independent, and these enzymes are different in substrate specificity as well as tissue distribution [38]. In contrast, the extracellular phospholipase of *Cryptococcus neoformans* was not influenced by divalent ions (Ca^{2+}, Mg^{2+}, or Zn^{2+}) and EDTA [39]. In this study, there was no significant effect on the enzyme activity with Ca^{2+} but enzyme activity was increased with Co^{2+}. Anne-Brit reported that a sphingomyelinase activity could be induced by the addition of Co^{2+} ion to the PLC protein of *B. cereus* [40]. However, we could not detect the enzyme activity other than PLA under the existence of Co^{2+} ion in the reaction mixtures with the PhlA protein.

Phospholipases originated from pathogenic microorganisms induce tissue destruction in the host cells or inflammation by producing a second messenger that

affects the signal transduction pathway of the host cells by working on the cell membrane. It is known that PLA either leads to tissue destruction by directly affecting phospholipids of the cell membranes or hemolysis on the host erythrocytes. Zhang et al. [21] recently reported that the amino acid sequence of *V. harveyi* hemolysin (VHH) has 86% identity with the thermolabile hemolysin of *V. parahaemolyticus* having PLA_2/lysophospholipase activity. Furthermore, the VHH showed a high sequence similarity to the Lec protein of *V. cholerae* (64% identity) and the PhlA protein of *V. mimicus* (65% identity). Interestingly, the hemolytic activity of VHH was limited to the erythrocytes of rainbow trout. This evidence led us to assess the hemolytic activity with the PhlA. Comparisons of the erythrocytes specificity for hemolysis by PhlA indicated that there was strong hemolytic activity against only fish erythrocytes such as rainbow trout and tilapia. These observations are consistent with the results reported in VHH [21]. Erythrocytes contain phospholipids of various kinds, such as PC, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol. It has been known that PC contents of total erythrocyte phospholipids from rabbit, human, and sheep were 34%, 17%, and 4%, respectively [41]. And it has also been reported that PC is the most abundant compound in fish erythrocytes [42]. A lower sensitivity was detected on rabbit erythrocytes with the PhlA but no hemolysis was observed from other erythrocytes, such as sheep, mouse, and human. The elevated percent of this PC content in erythrocytes tended to have high sensitivity. This may be useful explanation for the differences in the spectrum of erythrocyte sensitivity by the PhlA. Sphingomyelin is also known to be an important component of the outer leaflet of the phospholipid bilayer in erythrocyte. The PhlA did not show any activity to degrade sphingomyelin.

We have shown in this study that the purified rPhlA has not only the phospholipase A activity but hemolytic and cytotoxic activities against fish cell. Although the PhlA is regarded as an important virulence factor, we did not identify the role of *V. mimicus* phospholipase for infection to fish. Therefore, further studies are needed to determine the intracellular pathogenic process with the PhlA.

Acknowledgments

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BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Short sequence-paper

Isolation and sequence analysis of metalloprotease gene from *Vibrio mimicus*

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Abstract

The *vmc* gene encoding a metalloprotease of *Vibrio mimicus* (ATCC 33653) was cloned in *Escherichia coli* and sequenced. The *vmc* gene contained 1884 nt sequence which codes a polypeptide of 628 amino acids with a predicted molecular mass of 71,275 Da. The deduced amino acid sequence had the similarity of 68.5% with *V. parahaemolyticus* metalloprotease. The consensus sequence of a zinc binding motif (HEXXH) was identified to be HEYTH. The zymography analysis showed a gelatinolytic protein band around molecular mass of 61 kDa, and this result suggested that the cloned metalloprotease may undergo processing during secretion. © 1998 Elsevier Science B.V.

Keywords: Pathogenic factor; Metalloprotease; Zinc binding motif

In 1981, *Vibrio mimicus* was isolated and identified as atypical non-O1 *V. cholerae* [1]. *V. mimicus* is an enteropathogenic bacterium which inhabits aquatic environments and apparently causes diarrhea, usually after the consumption of uncooked seafoods. *V. mimicus* can produce an enterotoxin that is closely related to the cholera toxin (CT) of classical *V. cholerae* [2]. Several pathogenic factors of *V. mimicus*, including cholera toxin (CT) [3], CT-related enterotoxin [4], *Escherichia coli* heat-stable enterotoxin (ST)-like toxin [5] and protease [6] have been reported. Hemolysin is also involved in the bloody diarrhea which is one particular clinical symptom of Vm gastroenteritis [7].

Most investigations have been carried out on the function of vibrio toxins in enteric infections. However, many factors associated with pathogenicity in extra intestinal infections have recently been shown to be important for virulence in *Vibrio* sp. In general, vibrio proteases have been thought to be important virulence factors for host invasion. The extracellular metalloprotease of *V. mimicus* has been shown to be important for the enhancement of vascular permeability in skin and fluid accumulation in rabbit ileal loops [8]. Although the metalloprotease of *V. mimicus* might be related to the pathogenic process, the molecular information of this protease is unclear. As part of a molecular study for the pathogenic factor of *V. mimi-*

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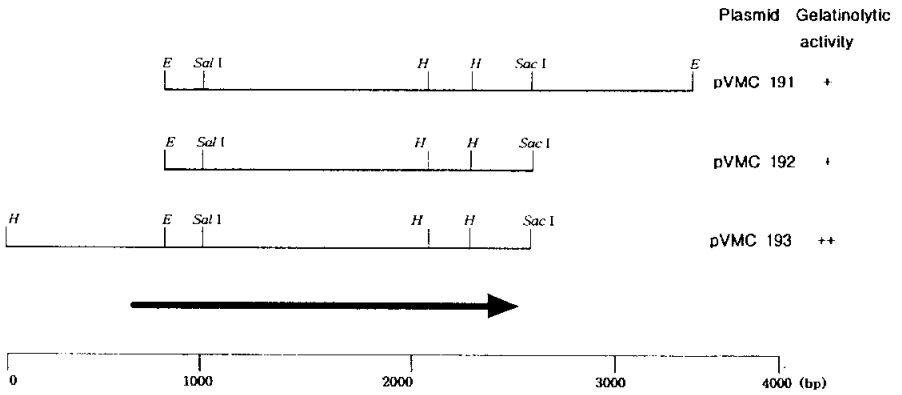


Fig. 1. Construction of a series of deletion derivatives of pVMC191 and gelatinolytic activity of transformant that bearing derived plasmid. Plasmids pVMC191 and pVMC 192 have only a part of *vmc* under the control of *lac* promoter. pVMC193 has the entire metalloprotease gene. *E*, *H* and arrow represent *Eco*RI, *Hin*dIII and ORF, respectively. The gelatinolytic activity was identified on agar(gelatin 0.5%) plate with frazier solution [10].

cus, the hemolysin DNA sequence was previously reported in our laboratory [9]. In this study, we describe the cloning and the whole DNA sequence of a metalloprotease gene of *V. mimicus*.

To identify the metalloprotease gene from the *V. mimicus*, chromosomal DNA was digested using the restriction enzyme *Eco*RI, ligated with pUC19 and introduced into *E. coli* JM83. One positive clone was obtained that formed a clear zone around colony on a gelatin containing agar plate after overlaying the frazier solutions reported by Norqvist et al. [10]. This clone harbored the insert of 2.4 kb *Eco*RI fragment. This plasmid was designated pVMC191 and the restriction map of the insert is shown in Fig. 1. Deletion analysis indicated that the 1.7 kb *Eco*RI–*Sac*I fragment was the essential region for the gelatinolytic activity. This fragment was subcloned to construct pVMC192. The 1.7 kb DNA fragment (pVMC192) was sequenced and a 1747 bp sequence which corresponds to 307–2053 bp in pVMC193 was determined. The deduced amino acid sequence of the inserted fragment was compared with a published metalloprotease genes in the NCBI with BLAST program. The metalloprotease amino acid sequence of *V. parahaemolyticus* showed high similarity with that of *V. mimicus*. However, upon determining the DNA sequence of pVMC192 and comparing this sequence with the metalloprotease sequence of *V. parahaemolyticus*, 102 amino acids of the N-terminus could not be found in pVMC192 and this observation implied that the isolated fragment is likely to be the truncated gene of the entire metalloprotease. To verify the existence of an additional upstream sequence, PCR was performed using the plasmid DNA library of *V. mimicus*. Primers were designed based on the exact sequence of pVMC192 and pUC19, respectively. The amplified DNA appeared to be 1 kb (Fig. 2). The DNA sequence of PCR product had primer sequence and overlapped with DNA sequence of pVMC192. The numbers of amino acids which

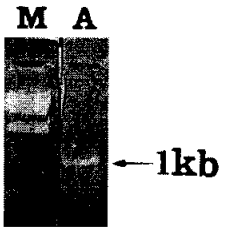


Fig. 2. The agarose gel electrophoresis of DNA molecular size marker: M (λ/*Hin*dIII) and the amplified DNA fragment which was detected by PCR: A. PCR was done with #247 primer (5′-GCAGACATCATGGCATCCATG-3′), pUC19 reverse primer (5′-AGCGGATAACAATTTCACACAGG-3′) and plasmid DNA library was used as template.

were not shown in pVMC192 are also well matched with N-terminus of *V. parahaemolyticus* gene. The confirmed DNA sequence from PCR was connected to pVMC192 in frame and designated pVMC193 (Fig. 1). We examined the gelatinolytic activity of pVMC193 by plating assay. The gelatinolytic activity of pVMC193 was higher than that of pVMC191 and pVMC192. The entire open reading frame had 1884 nucleotides encoding 628 amino acids. The putative promoter sequence is ATGAGA for the −35 and CATATT for the −10 region, respectively, and a putative SD sequence (ATAGG) is located directly upstream from the potential start codon GTG. It is very interesting that GTG (Val) might be responsible for initiation codon in *V. mimicus*. However, more studies should be necessary to support it. We found the inverted repeat sequence upstream of promoter region and there was no continuity of amino acid sequence from its DNA sequence (Fig. 3).

The amino acid sequence was observed to be homologous with the metalloprotease from *V. parahaemolyticus* [11]. The alignment of two sequences is shown in Fig. 4. The overall amino acid sequence homology was 68.5%. However, the identity increased to 70.2% within the region without C-terminus (1–501 amino acid).

In these sequences, the conserved zinc-binding motif (His-Glu-Tyr-Thr-His) was identified. This motif (HEXXH) is important to transfer electron with zinc for the hydrolysis of peptide bonds. Following the general classification of zinc proteases, five groups are present [12]. Three groups out of five have the HEXXH motif for the zinc binding site. With regard to the structural similarity around this HEXXH region, we proposed that the *Vibrio* metalloproteases could be specified in two classes (Fig. 5). The metalloproteases from *V. cholerae*, *V. anguillarum* and *V. proteolyticus* have a glutamate residue besides two

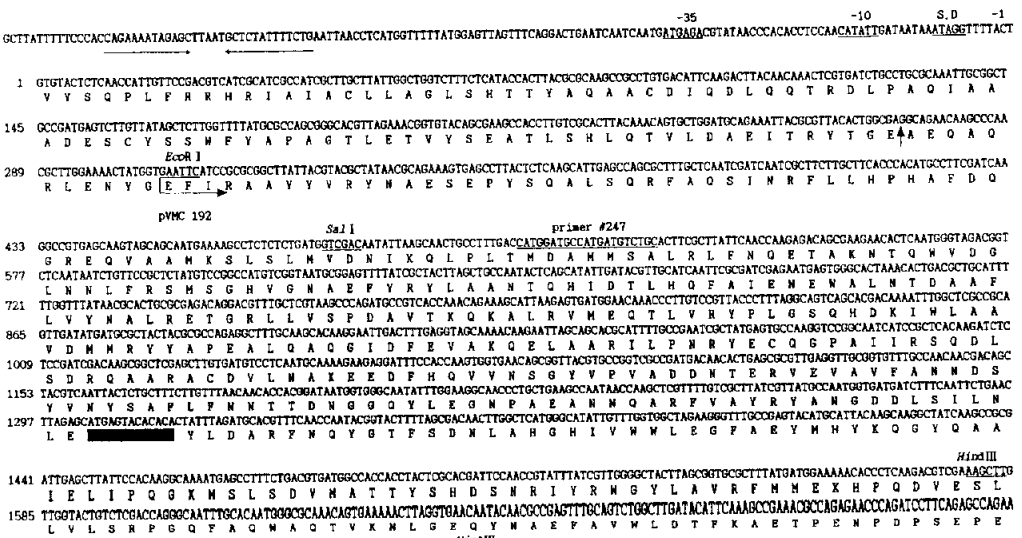


Fig. 3. The nucleotide sequence of metalloprotease gene and its deduced amino acid sequence from *V. mimicus*. The open reading frame is composed of 1884 nt and 628 amino acids. Putative promoter region is ATGAGA as −35 region and CATATT as −10 region. Putative ribosome binding sequence is ATAGG, black box indicated HEXXH motif. Inverted repeat sequence, N-terminal amino acid sequence of the secreted protein and stop codon are represented by horizontal arrow, vertical arrow and asterisks, respectively. (The GenBank accession number: AF004832)

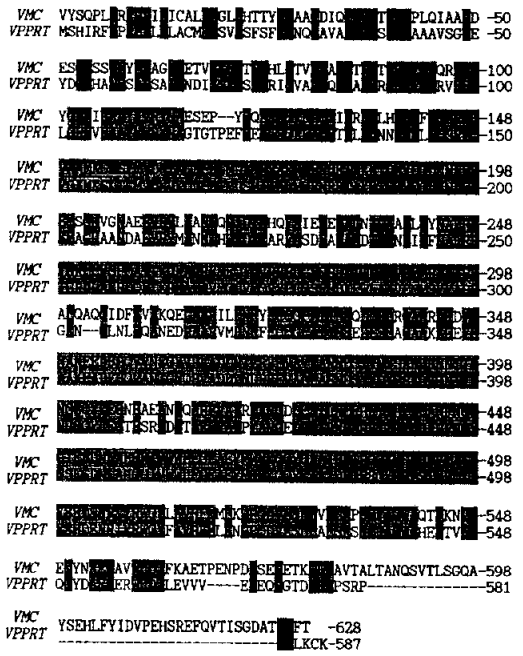
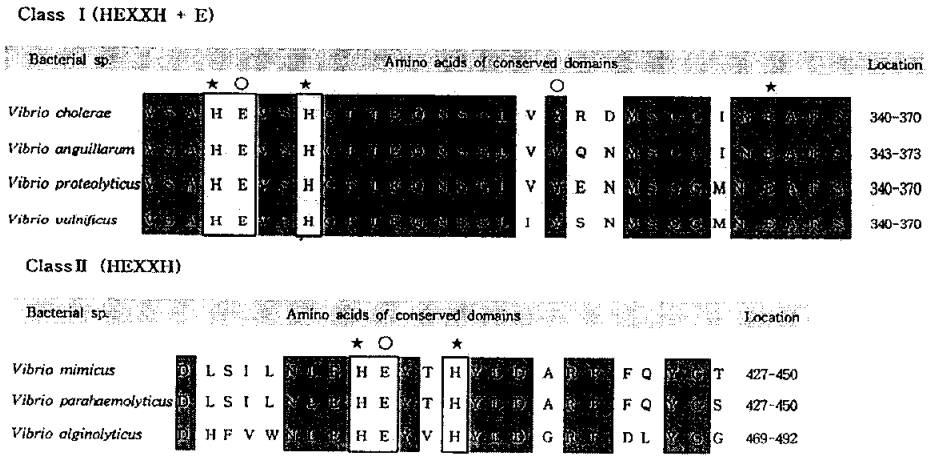


Fig. 4. The amino acid alignment of *vmc* and *vpprt* (*V. parahaemolyticus* metalloprotease gene) showed high degree of similarity. Numerals indicate the amino acid position beginning at the initiation Val and Met, respectively. The identical amino acid was represented by black box. From the amino acid sequence of 1–628 shared 68.5% identity and 70.2% identity showed from amino acid sequence from 1–561.

histidines of the HEXXH motif as metal ligands (class I, HEXXH + E) and this glutamate residue plays an important catalytic role. Class II metalloprotease (HEXXH) does not involve an extra glutamate residue and metal ligand of this class II protease in addition to HEXXH motif is still unclear. Vallee and Auld [13] demonstrated that three ligands need to play the role of catalytic zinc sites by using the computer analysis. According to this investigation, the possible three ligands of the metalloprotease (*vmc*) are two histidine residues of HEXXH motif and another histidine which is separated from HEXXH by a long space of 19 amino acids.

SDS-PAGE and zymography were performed to investigate that the cloned DNA encoded gelatinolytic protein. The extracellular proteases were prepared from culture supernatants followed by the fractionation with ammonium sulfate. The zymogram showed that one band is reactive with gelatin. And this gelatinolytic protein migrated to the position of about 61 kDa on SDS-PAGE (Fig. 6). From this result, we suggest that the mature metalloprotease (*vmc*) was considered to receive the processing during secretion since estimated molecular mass from the deduced amino acid sequence was 71 kDa. To determine the N-terminal amino acid sequence of extracellular protease, the protein band corresponding to gelatinolytic activity was transferred onto PVDF membrane. The N-terminal 6 amino acid sequence



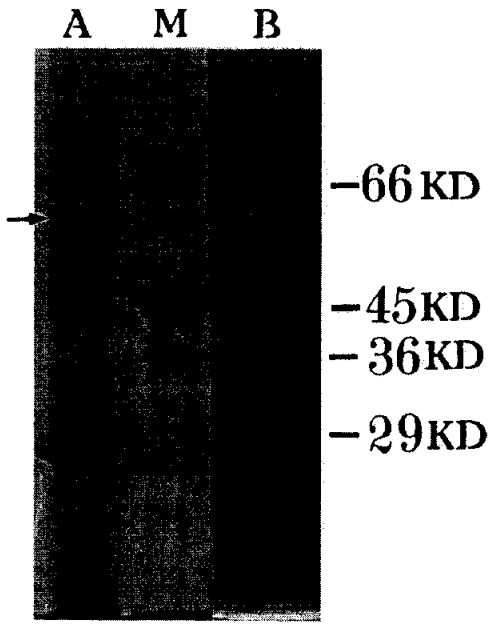


Fig. 6. SDS-PAGE and zymography were done to detect the extracellular metalloprotease which was expressed in *E. coli* [24,25]. M is protein molecular marker (66 kDa: Bovine serum albumin, 45 kDa: Ovalbumin, 36 kDa: Glyceraldehyde-3-phosphate dehydrogenase, 29 kDa: Carbonic anhydrase). Electrophoresis pattern from SDS-PAGE and zymogram were shown in A and B lane, respectively. Zymogram of *vmc* showed a major gelatinolytic band located at 61 kDa. *E. coli* was cultivated at 37°C for 14 h.

was identified with Ala^{92}-Glu-Gln-Ala-Gln-Arg^{97}. The molecular mass from the Ala^{92} was calculated to be 61,182. This molecular mass coincided well with that of 61 kDa measured by SDS-PAGE. Many extracellular bacterial proteases are synthesized as inactive precursors with an additional polypeptide segment to keep the protease inactive inside the cell, and undergo several stages of processing, including cleavage of signal peptide to form mature protein [14,15]. In this work, we didn't decide the substrate specificity of this cloned metalloprotease of *V. mimicus*. So the further work is necessary to characterize the biochemical property of metalloprotease (*vmc*) and clarify the relationship between other protease in virulence mechanism of *V. mimicus*.

Many toxins and proteases have been considered to be potential virulence factors for pathogenicity. A number of metalloproteases from pathogenic microorganisms were suggested to be involved in invasive mechanism. *V. cholerae* secretes a metalloprotease which is the causative agent of epidemic cholera. The HA/protease of *V. cholerae* has been shown to nick and activate the A subunit of the cholera toxin [16]. The HA/protease gene was cloned and characterized [17]. The deduced amino acid sequence of the HA/protease showed high homology with *Pseudomonas aeruginosa* elastase. The elastase from *P. aeruginosa* has been shown to be responsible for the tissue destruction during host infection [18]. The halophilic bacterium *V. vulnificus* produced a metalloprotease with elastolytic activity and this enzyme contributed to edema formation during infections by enhancing vascular permeability [19]. *V. anguillarum* which has been known as an important pathogenic microorganism infecting fresh water fish as well as marine fish secreted a metalloprotease associated with invasion to the fish [10]. The corresponding gene of *V. anguillarum* was determined [20]. The collagenase of *V. alginolyticus* was identified to be an effective enzyme to promote healing of abnormal skin scars and its gene was identified [21]. The extracellular protease gene was characterized in *V. parahaemolyticus* which is one of a major causes of acute gastroenteritis [11].

V. mimicus was classified as atypical strain of *V. cholerae*. The high similarity of hemolysin gene between *V. cholerae* and *V. mimicus* was shown in our previous report [9] and this fact suggested that both strains have evolved from a common ancestor. However, the metalloprotease found in *V. cholerae* was 31 kDa protease, HA/protease, which has the activity of hemagglutination. Therefore, it is possible that

Fig. 5. Conserved domains around HEXXH motif divided the metalloprotease of *Vibrio* sp. into two classes. One is HEXXH + E class which involved *V. cholerae* [17], *V. anguillarum* [20], *V. proteolyticus* [22] and *V. vulnificus* [23], another is HEXXH class. *V. mimicus* [this study], *V. parahaemolyticus* [11] and *V. alginolyticus* [21] were involved in this class. In each class, the location site of HEXXH motif is very similar and amino acids immediate to HEXXH motif share high degree of identity. Putative zinc-binding residue, active site residues, identical amino acids and HEXXH motif are indicated by asterisks, open circles, black box and box, respectively.

another metalloprotease which is very close to the metalloprotease described in this report may exist in *V. cholerae*.

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BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Short sequence-paper

Nucleotide sequence of the *vmhA* gene encoding hemolysin from *Vibrio mimicus*

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Abstract

The structural gene (*vmhA*) of hemolysin from *Vibrio mimicus* (ATCC33653) was cloned and sequenced. The *vmhA* gene contains an open reading frame consisting of 2232 nucleotides which can code for a protein of 744 amino acids with a predicted molecular mass of 83 059. The similarity of amino acid sequence shows 81.6% identity with *Vibrio cholerae* El Tor hemolysin.

Keywords: *Vibrio*; Hemolysis; Enteropathogenic bacterium; Vm-hemolysin

V. mimicus is an enteropathogenic bacterium which inhabits aquatic environments and apparently causes diarrhea, usually after the consumption of uncooked seafood [1]. Several pathogenic factors of *V. mimicus*, including cholera toxin (CT) [2], CT-related enterotoxin [3], *Escherichia coli* heat-stable enterotoxin (ST)-like toxins [4,5] or protease [6], have been reported. Many *V. mimicus* strains isolated from environment are capable of causing diarrhea, even though they cannot produce these enterotoxins. Therefore, it was postulated that another toxin is involved in the bloody diarrhea which is one particular clinical symptom of *V. mimicus* gastroenteritis. In addition to the above toxins, hemolysins are suspected to be the pathogenic factor of the vibrio. Two kinds of hemolysin produced by *V. mimicus*, Vm-hemolysin (M_r 58 000), Vm-rTDH (M_r 22 000), have been reported [7]. The former is immunologically cross-reactive with *V. cholerae* El Tor hemolysin and the latter is cross-reactive with *V. parahaemolyticus* thermostable direct hemolysin (TDH). The nucleotide sequence comparison of Vm-rTDH and Vp-TDH revealed that they were very homologous and had only minor variations but the flanking sequences of the hemolysin genes were dissimilar, indicating that they have a common ancestor and suggesting that they may have been transferred between vibrio species as a descrete genetic unit [8]. In this communication, we report the nucleotide sequence of gene encoding hemolysin similar to *V. cholerae* El Tor hemolysin.

A gene bank of the *V. mimicus* (ATCC33653) chromosomal DNA (partially digested *Pst* I/*Sal* I fragments) was prepared in the pUC19. One clone, pVMH194, was isolated by β-hemolysis on TSAII medium containing 5% sheep blood and the nucleotide sequence, containing an ORF of 2232 bp,

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was determined. Fig. 1 shows the nucleotide sequence and deduced amino acid sequence of the hemolysin, consisting of 744 aa with a predicted molecular mass of 83 kDa. Typical GAGGT, RBS or SD, sequence exists in 5 bp upstream from the ATG hemolysin initiation codon. However, the protein size (83 kDa) is larger than that of previously purified Vm-hemolysin (58 kDa) [7]. In *V. cholerae* El Tor hemolysin, the 82 kDa preprotoxin synthesized in the cytoplasm is secreted through the membrane into the culture medium as the 79 kDa inactive protoxin after cleavage of the signal peptide and is then further processed into the 65 kDa active hemolysin by release of the N-terminal 15 kDa fragment [9]. Thus,

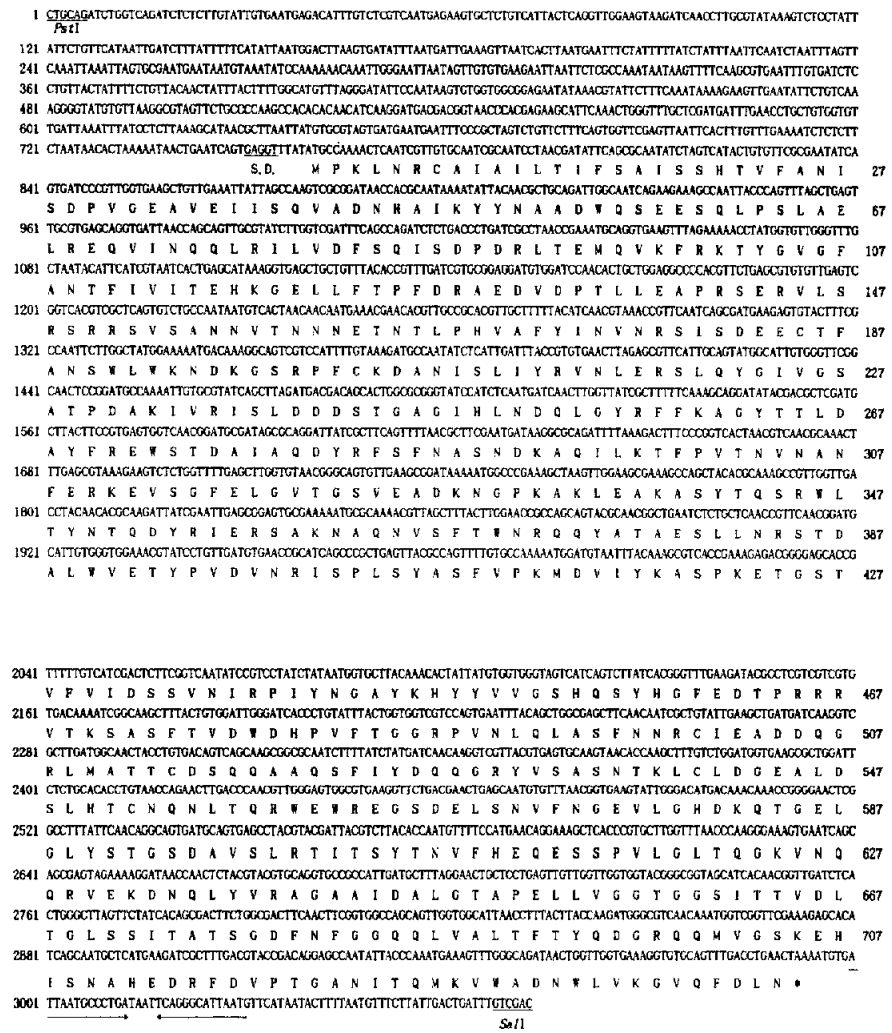

Fig. 1. The complete nt and deduced aa sequence of the *vmhA* gene. The nt sequence is numbered on the left of the sequence and the deduced aa sequence on the right. Asterisk represents the stop codon. Rho-independent stop region of mRNA is indicated by arrows. The GenBank accession number is U68271.

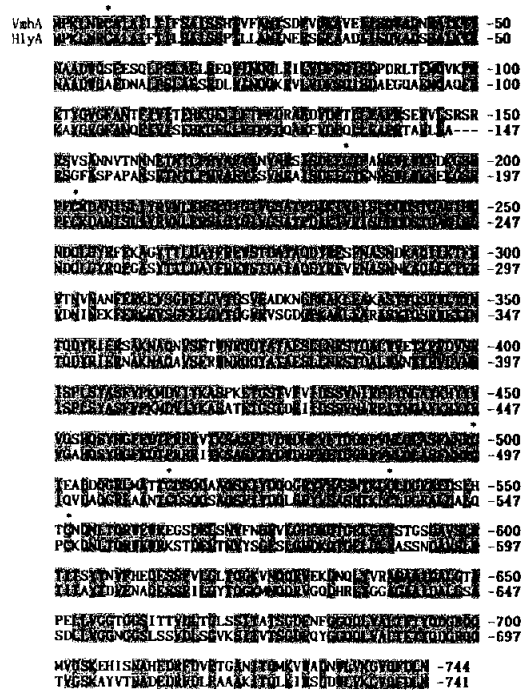

Fig. 2. Alignment of predicted amino acid sequences of *VmhA* from *V. mimicus* and HlyA from *V. cholerae* O1 Biotype El Tor. Identical amino acid residues in the two hemolysins are boxed. Conserved cysteine residues are marked by asterisks.

we assume that Vm-hemolysin may also have very high possibility of the same two-step processing.

The hemolysin shared 81.6% identity with *V. cholerae* El Tor hemolysin, consisting of 741 aa with a predicted molecular mass of 82 kDa, having only a major variation of three amino acid deletion from 148 to 150 [9–11] (Fig. 2). However, no sequence homology with other hemolysins/cytolysins was found. The placements and numbers of cystine residues for Vm-hemolysin and El Tor hemolysin were identical, reflecting the similarity of their secondary structures. According to the comparison of cysteine residues, Vm-hemolysin might be divided into three regions; the N-terminal region (Met-1 to Cys-185), the central region (Cys-185 to Cys-552) and the C-terminal region (Cys-552 to Asn-744). The N-terminal region, showing relatively lower homology and having the variation with El Tor hemolysin, seemed to be less important for hemolytic activity. It was supported by the fact that *V. cholerae* El Tor hemolysin is processed twice at Asn-26 and Asn-158, and the mature hemolysin has higher activity than the precursors by removing the N-terminal region [9]. The central region (Cys-185 to Cys-552) with six cystines had higher homology than other regions. The suggestion had been reported that the C-terminal region of El Tor hemolysin may be involved in the proper configuration of the protein for maximal hemolysin activity [12,13]. Thus, it might have an important role for the hemolysin activity although the C-terminal region of Vm-hemolysin has less homology than the central region. In addition, it will be required to explain the biochemical differences between Vm-hemolysin and El Tor hemolysin. The function of the central and C-terminal region of Vm-hemolysin will be elucidated by further study.

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Biochimica et Biophysica Acta 1394 (1998) 85–89

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Short sequence-paper

Cloning and identification of a phospholipase gene from *Vibrio mimicus*

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Abstract

The phospholipase gene *phl* was identified from *Vibrio mimicus* (ATCC33653) and sequenced. The entire open reading frame (ORF) was composed of 1410 nucleotides and encoding 470 amino acids. The *phl* was placed upstream of hemolysin gene (*vmh*A) with opposite direction of transcription. From the BLAST search program, the deduced amino acids sequence showed 74.4% identity with phospholipase gene (*lec*) from *V. cholerae* El Tor. The entire ORF of phospholipase gene was amplified by PCR and inserted into an *Escherichia coli* expression vector, pET22b(+) and introduced *E. coli* BL21(DE3). SDS–PAGE demonstrated that a protein corresponding to the phospholipase was overexpressed and migrated at a molecular mass of 53 kDa.

Keywords: Hemolysin; Lecithin degradation; Phospholipase; (*Vibrio mimicus*)

In *Vibrio* spp., virulence factors are associated with hemorrhagic septicemia and diarrhea [1]. Many virulence factors including hemolysin, cytotoxin, protease, hemagglutinin, cell adhesion factor and lipase are suggested to play a important role in the host infection and intestinal disease [2]. In some cases, bacterial phospholipases were related with intestinal secretion in the pathogenesis of disease [3]. *V. mimicus* was identified as atypical non-01 *V. cholerae* and inhabits an aquatic environment [4]. Several pathogenic factors including cholera toxin-like toxin, *Escherichia coli* enterotoxin-like toxin, protease, hemagglutinin, and hemolysin have been characterized in *V. mimicus* [5]. Recently, phospholipase (lecithinase) gene (*lec* gene) of *V. cholerae* was identified, and this gene lies upstream of the *hlyA* gene which encodes a hemolysin [6]. Both genes are transcribed with opposite direction. We have previously reported the hemolysin gene (*vmhA*) of *V. mimicus* and compared the amino acid sequence homology with *V. cholerae* El Tor hemolysin [7]. The amino acid homology was shown to be 81.6%. Based on this similarity, we assumed that the phospholipase gene can be observed around the hemolysin gene in *V. mimicus*. In the present paper, we have identified the phospholipase gene located upstream of the hemolysin gene and have determined the nucleotide sequence of this gene.

To isolate the flanking region of the hemolysin gene (*vmhA*), *V. mimicus* (ATCC 33653) chromosomal DNA was digested with *Eco*RI and ligated with pUC19. The 8.0 kb insert containing the hemolysin gene was isolated by the hemolytic assay on blood agar plate and designated as plasmid pVMH191. To investigate whether transformant harboring

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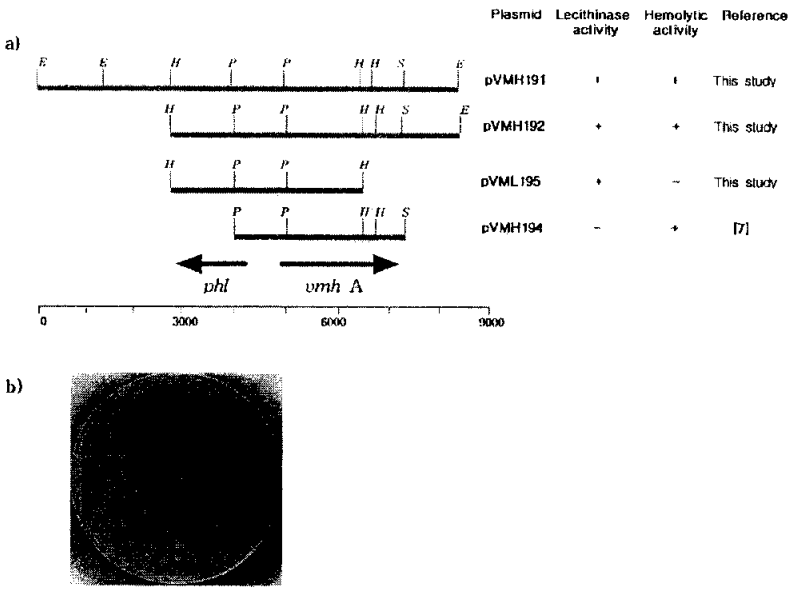

Fig. 1. The restriction map of derivatives from pVMH191 and its lecithin degrading activity on egg-yolk plate. (a) Restriction map of pVMH191 and its derivatives. Arrows indicate the open reading frame of *phl* and *vmh*A. E, H, P and S represent restriction enzyme *Eco*RI, *Hin*dIII, *Pst*I and *Sal*I, respectively. (b) Lecithin degrading activity of *E. coli* with plasmid. A, pVMH191; B, pVMH192; C, pVML195; D, pVMH194; E, pUC19.

pVMH191 plasmid has lecithin degrading activity, *E. coli* (pVMH191) was transferred on egg-yolk agar plate. A clear zone around the colony could be detected. According to the restriction map of pVMH191, the 3.1 kb fragment (*Pst*I–*Sal*I fragment) encoding the hemolysin gene (*vmhA* gene) in pVMH194 was involved [7] and an additional segment was found upstream of *vmhA* gene. This result suggested that the upstream region of the hemolysin gene may involve the gene contributing lecithin degradation. To confirm that the upstream sequence was related to lecithin degradation, the derivatives of pVMH191 were constructed and the enzyme activity examined (Fig. 1a). pVMH192 and pVML195 showed lecithin hydrolytic activity on egg-yolk agar plate (Fig. 1b). However, *E. coli* harboring pVML195 could not produce the hemolysin, because this plasmid contains the truncated hemolysin gene. From the DNA sequence of pVMH192, we could find the *vmhA* gene sequence as previously reported [7] and another open reading frame transcribed in opposite direction with *vmhA* gene (Fig. 2). The open reading frame (*phl* gene) was composed of 1410 bp encoding 470 amino acids with a predicted molecular mass of 53 kDa. AAGAT, located 5 bp upstream from initiation ATG, was assumed to be the ribosome binding site. In *V. mimicus*, the potential lipid binding motif was found at position 200 and 209, KVIVFGDSLS. The lipid binding motif of *V. cholerae* was identified to be KVIAFGDSLS [6]. A comparison of amino acid sequence with various lipid hydrolytic enzymes indicates that the pentapeptide, GDSLS, was conserved in the putative lipid binding domain. The deduced amino acid sequence of *phl* gene was 74.4% identical with that of *V. cholerae* (Fig. 3). However, the similarity increased to 84% in a region containing 55–470 amino acids in the *phl* gene. Interestingly, the additional 54 amino acids at the N-terminal of *phl* gene could not be observed in the phospholipase gene (*lec*) of *V. cholerae*. Rhaman et al. reported the hemolysin gene of *V. mimicus* E-33 isolated from regional environment [8]. This hemolysin gene shows 96.6% homology with that of *V. mimicus* ATCC 33653 used in this study. Also, the partial sequence of phospholipase gene appeared at the 5′ flanking region of the

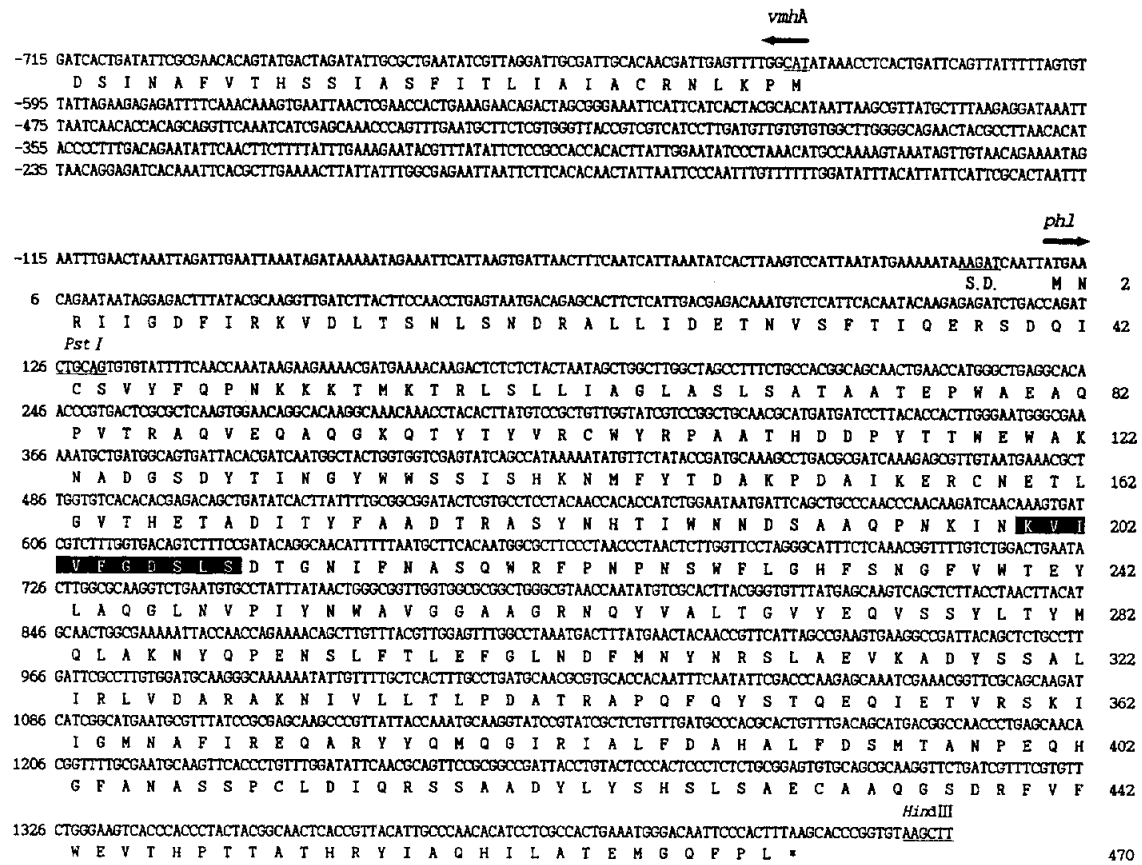

Fig. 2. The nucleotide sequence of *phl* gene (a) and the partial sequence of *vmh*A gene from *V. mimicus* (ATCC33653) (b). The open reading frame (ORF) of *phl* is composed of 1410 nucleotides and 470 amino acids. The putative S.D. sequence is underlined. Arrow indicates translational directions of *phl* and *vmh*A, respectively. Black box is the conserved motif of the lipid binding domain. (GenBank accession number: AF035162.)

hemolysin gene in *V. mimicus* E-33. When DNA sequence homology of the 5′ region of the *phl* gene was compared with the 5′ flanking sequence of *V. mimicus* E-33 hemolysin gene, a high level of DNA sequence identity (97.5%) between both *V. mimicus* strains was confirmed except for one base (A, at position 147 base from the translation start site of *phl* gene) deletion in *V. mimicus* E-33. This result suggests that the phospholipase from *V. mimicus* E-33 may not contain the extra 54 amino acids which can be found in this study. Since the phospholipase DNA sequence of *V. mimicus* E-33 has not been reported, we could not identify this further. To confirm that the entire DNA sequence of the *phl* gene can be transcribed through another promoter, sequences encoding residues 1 to 470 amino acids of the *phl* gene without its own promoter were cloned into the expression vector, pET22b(+) (Novagen), using PCR, and the phospholipase activity was examined. A 1410 bp fragment was amplified from pVML195 by using two specific primers containing *Bam*HI and *Eco*RI sites at either end, respectively (5′-GGCCGGATCCTATGAACAGAATAATAGGAG-3′ and 5′-GGCCGAATTCAGTGGGAATTGTCCC-3′; *Bam*HI and *Eco*RI sites are underlined). The PCR product was digested with *Bam*HI, *Eco*RI and this fragment was ligated with pET22b(+) plasmid to create plasmid pPHL13. *E. coli* BL21(DE3)

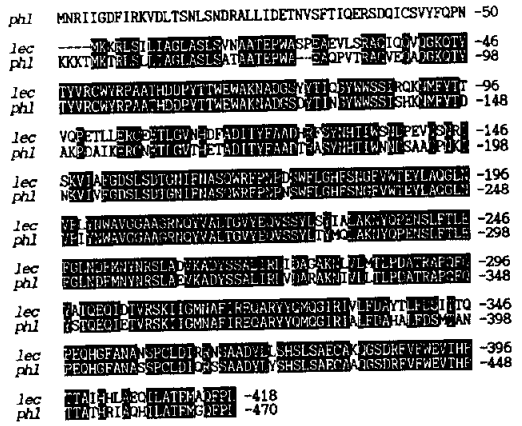

Fig. 3. Alignment of the amino acid sequence between *phl* (from *V. mimicus*) and *lec* (from *V. cholerae*) gene. The deduced amino acids show 74.4% similarity between *phl* and *lec*. The identical amino acids are represented by black boxes.

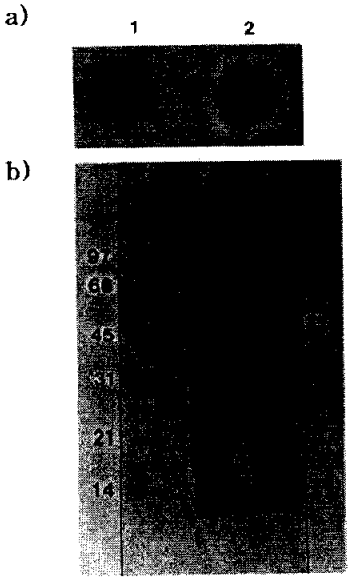

Fig. 4. Lecithinase activity of pPHL13 which was expressed by T7 promoter (a) and the expression of phospholipase in *E. coli* BL21 (DE3) (b). (a) 1, pET22b(+)/BL21(DE3); 2, pPHL13/BL21(DE3) in egg-yolk plate supplied with 1 mM IPTG. (b) Cells harboring pPHL13 were harvested after induction (4 h). 1, molecular marker (low range marker; BioRad, USA); 2, pET22b (+)/BL21 (DE3); 3, pPHL13/BL21 (DE3). An arrow on the right indicates the position of the *E. coli* expressed phospholipase.

harboring pPHL13 plasmid appeared to have lecithin degrading activity on egg-yolk plate (Fig. 4a). As shown in Fig. 4b, the expressed protein induced by addition of 1 mM IPTG was observed at a molecular mass of 53 kDa. However, the analysis of purified enzyme remains to be investigated, and the biological role of the presence of this extra amino acid residue is not clear. To identify whether the *phl* gene (*Pst*I–*Hin*dIII fragment of pVML195) hybridizes to a specific region of *V. mimicus* chromosome, Southern blot analysis was performed. When the chromosomal DNA was completely digested with *Eco*RI or *Hin*dIII, specific bands appeared at 6.9 kb or 3.5 kb, respectively (Fig. 5). These results could be expected based on the restriction patterns of pVMH191 plasmid.

The various functions of phospholipases involve the disease process, with the promotion of intracellular spread of infected microorganisms. Phospholipases from pathogenic bacteria attached to host cell membrane and produced some mediators which affect normal cellular physiology. Several types of phospholipases have been identified, including not only those enzymes defined as phospholipase A_1, A_2, C, D and lysophospholipase, but also other enzymes such as sphingomyelinase, lecithin-cholesterol acyltransferase, platelet activating factor acetyl hy-

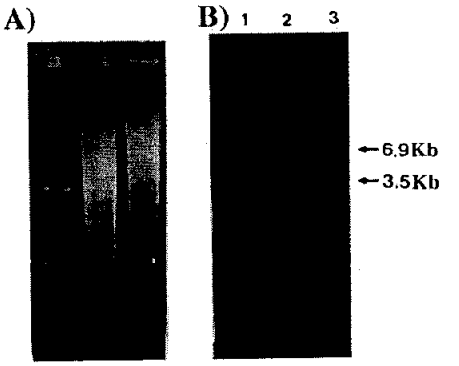

Fig. 5. Southern hybridization of *V. mimicus* chromosomal DNA. The restriction enzyme digestion of chromosomal DNA was analyzed on 0.8% agarose gel (A), and hybridized with *Hin*dIII–*Pst*I fragment of pVML195 as a DNA probe (B). (A) Lane 1, size marker (1 kb ladders were purchased from Promega). Chromosomal DNA was digested with *Eco*RI (lane 2) and *Hin*dIII (lane 3). (B) Southern hybridization analysis of *V. mimicus* chromosomal DNA digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), and molecular marker (lane 3) as control.

drolase and lysoplasmalogenase. In *Vibrio* spp., the lecithin-dependent hemolysin was found from *V. parahaemolyticus* [9]. This lecithin-dependent hemolysin showed hemolytic activity containing phospholipase A_2/lysophospholipase which could disrupt the erythrocyte membrane. In this report, we identified that the *phl* gene product does not have hemolytic activity. However, the *phl* gene was located upstream of the *vmhA* gene which encodes hemolysin, and this gene arrangement seems feasible as the hemolysis may closely associate with the disruption of erythrocyte membrane by the action of phospholipase. Further studies are needed to clarify the mode of action and the role of this enzyme.

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Purification, characterization and molecular cloning of *Vibrio fluvialis* hemolysin

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Abstract

Hemolysin of *Vibrio fluvialis* (VFH) was purified from culture supernatants by ammonium sulfate precipitation and successive column chromatographies on DEAE-cellulose and Mono-Q. N-terminal amino acid sequences of the purified VFH were determined. The purified protein exhibited hemolytic activity on many mammalian erythrocytes with rabbit erythrocytes being the most sensitive to VFH. Activity of the native VFH was inhibited by the addition of Zn^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+} ions at low concentrations. Pores formed on rabbit erythrocytes were approximately 2.8–3.7 nm in diameter, as demonstrated by osmotic protection assay. Nucleotide sequence analysis of the *vfh* gene revealed an open reading frame (ORF) consisting of 2200 bp which encodes a protein of 740 amino acids with a molecular weight of 82 kDa. Molecular weight of the purified VFH was estimated to be 79 kDa by SDS-PAGE and N-terminal amino acid sequence revealed that the 82 kDa prehemolysin is synthesized in the cytoplasm and is then secreted into the extracellular environment as the 79 kDa mature hemolysin after cleavage of 25 N-terminal amino acids. Deletion of 70 amino acids from the C-terminus exhibited a smaller hemolytic activity, while deletion of 148 C-terminal amino acids prevented hemolytic activity.

Keywords: *Vibrio fluvialis*; Hemolysin; *vfh* gene

1. Introduction

Vibrio fluvialis was originally described by Lee et al. [1] and its association with human disease was heightened after isolation from many patients with apparently inflammatory diarrhea in Bangladesh [2]. The symptoms of enteric disease attributed to *V. fluvialis* are similar to those caused by *Vibrio cholerae*. Patients typically have watery diarrhea with vomiting, abdominal pain, moderate to severe dehydration and often fever. A notable difference from cholera is the frequent occurrence of bloody stools in infections due to *V. fluvialis* [2]. From the enzyme-linked immunosorbent assay, Chikahira and Hamada [3] have reported that several *V. fluvialis* strains isolated from environmental and human sources produced an enterotoxin which is immunologically indistinguishable from cholera toxin (CT).

V. fluvialis produces several toxins that may be important in pathogenesis including an enterotoxin-like substance, protease, cytotoxin, and hemolysin [4]. Endotoxin activity of *V. fluvialis* has been demonstrated in vitro using Chinese hamster ovary (CHO) cells. Lockwood et al. [5] reported that at least four biologically active substances could be found in culture supernatants of *V. fluvialis* strain 5489. CHO cell elongation factor, CHO cell killing factor (CKF), and cytolysin active against rabbit erythrocytes were identified when the bacterium was grown without lincomycin. Finally, CHO cell rounding toxin, which is known to be a protease, was found. CKF was internalized and cell death was induced by disruption of cellular function [4]. These four active substances were heat-labile and each crude concentrate caused fluid accumulation in the small intestines of infant mice. Of many virulence factors produced from *V. fluvialis*, hemolysin was thought to be most important.

Hemolysin has been known to be an important virulence factor in the pathogenic processes of many clinical micro-

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organisms, causing hemorrhagic septicemia and diarrhea [6,7]. It can lyse erythrocytes and a variety of other cells including mast cells, neutrophiles, and polymorphonuclear cells as well as enhance virulence by causing tissue damage or by dissolving material that would prevent spreading of the pathogen throughout the tissue. Several extracellular hemolysins have been characterized from *Vibrio* spp. The thermostable hemolysin from *Vibrio parahaemolyticus* exhibited enterotoxic effects on human and rat cell monolayers [8,9]. To date, *Vibrio* hemolysin genes have been isolated from *V. cholerae* [10], *V. parahaemolyticus* [11], *V. anguillarum* [12] and *V. mimicus* [13]. However, the role and biological properties of hemolysin from *V. fluvialis* have not been studied.

In this paper, we describe the purification and some of the biological properties of the extracellular hemolysin of *V. fluvialis* and we also report the nucleotide sequence of the gene encoding this toxin.

2. Materials and methods

2.1. Bacterial strain, and growth conditions

V. fluvialis (ATCC 33809) strain was obtained from Korean Collection for Type Cultures (KCTC). Cells were grown at 37 °C on solid or in liquid brain heart infusion (BHI) medium (Difco). *Escherichia coli* XL1-Blue [*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*F′ *proAB*$^+$ *lacI*^{q} *lac*ZΔM15 Tn*10* (Tet^r)] was used as a host to propagate plasmid DNA and was grown in Luria–Bertani broth (LB) medium. Transformation of plasmid DNA into *E. coli* was performed with $CaCl_2$-treated cells.

2.2. Purification of VFH

The bacterium were cultivated at 37 °C for 24 h in 1 l of BHI broth with shaking (140 cycles/min), and the culture supernatant was collected by centrifugation at 7000 × *g* for 30 min. Solid ammonium sulfate was added to 80% saturation (591 g/l). After 8–10 h, the resulting precipitate was collected by centrifugation at 7000 × *g* for 30 min at 4 °C, and dissolved in 60 ml of 10 mM Tris–HCl buffer (pH 7.5). The suspension was centrifuged at 7000 × *g* for 20 min to remove insoluble residue. Dialysis was performed overnight against 2 l of 10 mM Tris–HCl buffer (pH 7.5) for two times at 4 °C, and about 85 ml of dialyzed material was applied to a DEAE-cellulose column (2 × 5 cm, Sigma) equilibrated with 100 ml of 10 mM Tris–HCl buffer (pH 7.5), and eluted with 50 ml of 10 mM Tris–HCl buffer (pH 7.5) containing 0.5 M NaCl with stepwise elution at a flow rate of 1 ml/min. The fractions containing VFH were collected, dialyzed and concentrated by freeze-drying. The concentrated fractions were finally applied to a Mono-Q HR 10/10 column (Pharmacia) which was equilibrated with 10 mM Tris–HCl buffer (pH 7.5), and eluted with a linear gradient from 0 to 0.5 M NaCl in 10 mM Tris–HCl buffer (pH 7.5) at a flow rate of 0.5 ml/min. Each step of purification was monitored with activity assay on trypticase soy agar containing 5% sheep red blood cells (BBL). Elutant (50 μl) was transferred onto a paper disk and incubated at 37 °C for 12 h and elutants that had certain hemolytic activity was selected. To examine the purity, SDS-PAGE was carried out as described by Laemmlie [14] using a 12% acrylamide gel. The amount of purified enzyme was determined by the Bradford method [15] using bovine serum albumin as a protein standard. N-terminal amino acid sequences were determined by standard Edman degradation on a model ABI 491 microsequencer (Applied Biosystems).

2.3. Assay of hemolytic activity

Hemolytic activity was determined with rabbit erythrocytes. Briefly, rabbit erythrocytes were washed with 10 mM Tris-buffered saline (TBS: pH 7.5) three to four times and adjusted to a final concentration of 4% (v/v) in TBS. A diluted VFH solution (0.2 ml) was mixed with rabbit erythrocytes (0.2 ml) and incubated at 37 °C for 1 h. Reaction mixtures were centrifuged at 1000 × *g* for 5 min and the amount of hemoglobin released from disrupted erythrocytes was determined spectrophotometrically. One hundred percent hemolysis was defined as the optical density at 540 nm of hemoglobin released from erythrocytes treated with 0.1% Triton X-100. One hemolysin unit (HU) was defined as the amount of hemolysin eliciting 50% hemoglobin release.

2.4. Osmotic protection experiments

For these experiments, 0.2 ml of 4% rabbit erythrocyte suspensions containing an osmotic protectant was mixed with 0.2 ml of VFH solution (2 HU). Glucose, sucrose, maltotriose, inulin and PEG 4000 were used as the osmotic protectants at a final concentration of 30 mM. Dextran 10 and PEG 6000 were used at a final concentration of 15 mM. The mean hydrated diameters of glucose, sucrose, maltotriose, inulin, PEG 4000, dextran 10, PEG 6000 were 0.72, 0.9, 1.2, 2.8, 3.66, 4.7, and 5.66 nm, respectively [16]. Protection from hemolysis was calculated as follows: % protection=(1 − hemolytic rate in the presence of saccharide/hemolytic rate without osmotic protectant) × 100.

2.5. Effect of divalent cations on hemolytic activity

Inhibitory activity of cations on hemolysis was determined by addition of divalent cations as the chloride forms in TBS. The cation solution (0.1 ml) was mixed with 0.1 ml of VFH (2 HU) and 0.2 ml of the rabbit erythrocyte suspensions. The mixture was incubated at 37 °C for 1 h, and immediately subjected to the hemolytic activity assay.

Table 1
Purification of extracellular hemolysin produced by *V. fluvialis*

Purification stage	Volume of purifying solution (ml)	Total amount of protein recovered (mg)	Total hemolytic activity (HU)	Specific activity (HU/mg)	Relative activity	Yield of activity (%)
Collection of culture supernatant	1000	170	33,660	198	1	100
Ammonium sulfate precipitation (80%)	85	62	19,310	311	1.6	57.4
DEAE-cellulose chromatography	50	2	1910	955	4.8	5.7
Mono-Q column chromatography	1	0.02	200	10,000	49.3	0.6

2.6. Effect of temperature on hemolytic activity of VFH

Hemolytic activity of VFH was assayed at 37 °C with 2 HU, routinely. To determine the effect of temperature on hemolytic activity of VFH, a suspension of the washed 4% rabbit erythrocytes (0.2 ml) was incubated with VFH (2 HU in 0.2 ml of TBS) for 5–60 min at different temperatures, and measured the absorbance of supernatant at 540 nm after incubation.

2.7. Cloning and characterization of V. fluvialis VFH gene

V. fluvialis chromosomal DNA was digested with *Hin*dIII and ligated into *Hin*dIII site of pGEM-4Z (Promega) plasmid vector treated with the alkaline phosphatase. The recombinant plasmids were transformed into competent *E. coli* XL1-Blue. White colonies were analyzed on blood agar plates and one positive clone containing a 4.6 kb insert was obtained. The resultant plasmid was named pVFH460.

The nucleotide sequence of the 4.6 kb fragment was first determined with the universal forward and reverse primers followed by primer walking. Sequencing was performed with ABI prism 377 (Applied Biosystems), and the nucleotide sequence of hemolysin gene (*vfh*) was deposited in the GenBank database under accession no. AF348455.

2.8. Construction of C-terminal deletion mutant

The two C-terminal deletion mutants were constructed in the following manner. The 2.1 kb and 2.4 kb fragments containing the partial *vfh* gene were amplified from pVFH460 by PCR with pUC/M13 forward primer (Promega) and designed reverse primers. The sequences of the reverse primers involving *Eco*RI site were 5′-GGCCGAATTCATGACCACCGATTGCACCTG-3′(position 1759 to 1776) and 5′-GGCCGAATTCATGCTTTCACTGGCGGAGGC-3′ (position 1993 to 2010). The amplication conditions were set at one cycle of 94 °C for 5 min, followed by 25 cycles of amplification consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, and then followed by one final extension cycle of 72 °C for 7 min. The PCR product was subcloned into the plasmid vector pGEM-4Z (Promega) as a *Hin*dIII and *Eco*RI restriction fragment.

3. Results

3.1. Purification of VFH

VFH was purified from culture supernatants as indicated in Materials and methods. The purification results are shown in Table 1. Ammonium sulfate precipitation followed by dialysis resulted in 1.6-fold increase of specific activity. The protein was then adsorbed onto DEAE-cellulose, and the hemolytic activity was recovered with the majority at 0.5 M NaCl. Subsequently, the pooled active fractions were applied to a Mono-Q FPLC column. The specific activity from the Mono-Q fraction was about 10,000 U/mg; VFH was purified approximately 49-fold with 0.6% yield. The SDS-PAGE analysis of the purified VFH revealed a single band of 79 kDa (Fig. 1). The N-terminal amino acid sequences of the purified protein was determined to be Asp-Ile-His-Asp-Pro-Val.

3.2. Susceptibilities of mammalian erythrocytes to VFH

The purified VFH exhibited high hemolytic activity in the order of rabbit, chicken, and mouse erythrocytes. As shown in Table 2, lower hemolytic activity was observed with sheep and rat erythrocytes when it was compared to that of rabbit. Consequently, rabbit erythrocytes were used

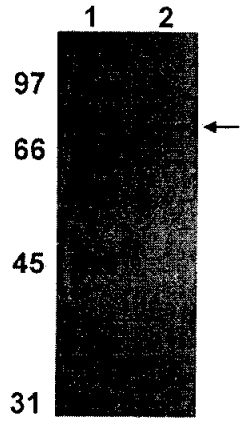

Fig. 1. SDS-PAGE of the purified hemolysin from culture supernatant of *V. fluvialis*. Lane 1, molecular weight markers; lane 2, purified VFH (5 μg).

Table 2
Hemolytic activities of the VFH on the erythrocytes from various sources

Erythrocytes source	Specific activity (HU/mg protein)	Relative hemolysis (%)
Rabbit	9770	100
Chicken	7700	77
Mouse	7320	73
Rat	4200	42
Sheep	730	7

to assay hemolytic activity because of their documented sensitivity.

3.3. Effect of temperature on hemolytic activity of VFH

As shown in Fig. 2, there were significant differences in hemolytic activity when VFH was incubated at various temperatures (15–42 °C). The lysis of erythrocytes by the purified VFH was temperature-dependent and the optimum temperature was 37 °C. Hemolysis began after a few minutes and was completed by 60 min at 37 °C, whereas no hemolysis occurred at 4 °C. The observation that VFH did not lyse rabbit erythrocytes at 4 °C prompted a series of experiments to determine whether VFH binds to rabbit erythrocytes at 4 °C. To further demonstrate that the binding of VFH to erythrocytes was temperature-dependent, rabbit erythrocytes were incubated with 2 HU VFH at 4 °C for 60 min, subsequently washed with cold TBS to remove unbound VFH and resuspended in TBS. When the resuspended erythrocytes were incubated at 37 °C for 1 h, hemolysis could be detected (data not shown). This result indicated that VFH had bound to erythrocytes at 4 °C. There is also strong evidence in *Vibrio* spp. hemolysins showing that the binding step of hemolysin appeared to be temperature-independent, but the lysis was temperature-dependent [17–19].

3.4. Inhibitory effect of divalent cations

Cations possibly function for protection from the increase of the intracellular osmotic pressure through blockage of the influx of extracellular water via pores [20]. The effect of several cations on hemolytic activity of the purified VFH was tested. Hemolytic activity was assayed after divalent cation, such as Zn^{2+}, Ni^{2+}, Cu^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, Mg^{2+}, and Ca^{2+} were added to the reaction mixture at various concentrations. Erythrocyte suspensions including cation were incubated with VFH at 37 °C for 1 h. The concentration of VFH was adjusted to result in approximately 50% lysis of erythrocytes. With increasing concentration of cation, progressive inhibition of hemolysis was observed. Hemolysis by VFH was prevented by divalent cations in decreasing order, $Zn^{2+}>Ni^{2+}>Cd^{2+}>Cu^{2+}$ and collective results are depicted in Table 3. It is noteworthy that monovalent cation such as Cs^{+} or Li^{+} had no effect on hemolysis (data not shown). Evidence

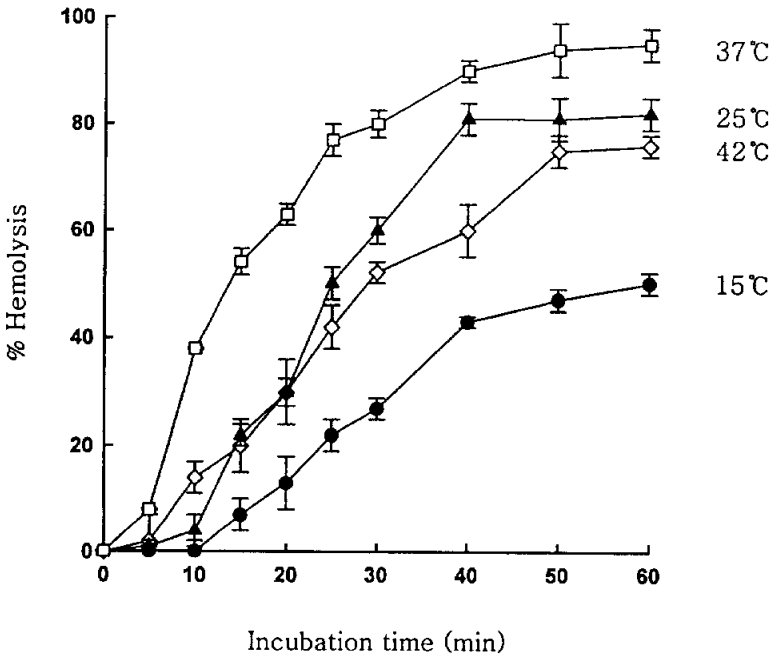

Fig. 2. Kinetics of erythrocyte lysis by VFH as a function of temperature. Washed rabbit erythrocytes were incubated with VFH (2 HU) for 5–60 min and hemolysis was determined by absorbance at 540 nm.

Table 3
Inhibitory effect of divalent cations on the hemolytic activity[a]

Divalent cation[b]	ID_{50} (mM)[c]
Zn^{2+}	0.18
Ni^{2+}	0.2
Cd^{2+}	0.7
Cu^{2+}	1
Mn^{2+}	>25
Co^{2+}	>25
Mg^{2+}	>25
Ca^{2+}	>25

[a] VFH (2 HU) and rabbit erythrocytes (4%) were incubated at 37 °C for 1 h in the presence of the cation. Thereafter, hemolysis was quantified by measuring absorbance at 540 nm.
[b] The chloride salt of each cation was used.
[c] ID_{50} is defined as the concentration of divalent cations that inhibit 50% of hemolytic activity.

that these divalent cations inhibit membrane destruction was obtained from the following result. Erythrocytes were incubated with VFH and each divalent cation at 4 °C for 1 h, followed by centrifugation. The sedimented erythrocytes were washed twice and further incubation was performed at 37 °C for 1 h in fresh TBS. Remarkable hemolysis was observed, indicating that the divalent cations did not inhibit the binding of hemolysin to erythrocytes.

3.5. Inhibitory effect of osmotic protectants on hemolysis

To assess whether hemolysis is differentially affected by osmotic protectants, we estimated hemolytic activity in the presence of saccharides, PEG solutes and dextran. When rabbit erythrocytes were mixed with VFH in the presence of 30 mM of glucose, sucrose, maltotriose and inulin, hemolysis was appeared. However, there was a significant inhibition of hemolysis (>90% protection from hemolysis) by PEG 4000 and complete osmotic protection was afforded by dextran 10 and PEG 6000, respectively (Fig. 3). The inhibitory effects were dependent on the molecular diameter of the protectant colloids and hemolysis was osmotically protected by mean hydrated diameters of 2.8–3.7 nm.

3.6. Cloning of V. fluvialis hemolysin gene

Approximately 3000 *E. coli* transformants from the genomic library of *V. fluvialis* were screened on blood agar plates and one colony showing clear halo formation was selected. Plasmid DNA was isolated from this colony. Restriction enzyme analysis revealed that the plasmid, named pVFH460, contained an insert of 4.6 kb. The complete nucleotide sequence of this insert was determined. Translation of the nucleotide sequence revealed a single open reading frame (ORF) of 2220 bp, encoding a polypeptide of 740 amino acid residues, with a calculated molecular mass of 81,508 Da. The proposed translational ATG start codon is proceed by a probable ribosome binding site, AGGAC. An inverted repeat, which could function as a transcription termination signal, was found 57 bp downstream from the stop codon. The G+C content of the *vfh* gene was 54%, which is slightly higher than those of hemolysin genes reported previously from *V. cholerae* (48%), *V. mimicus* (47%) and *V. anguillarum* (44%). The

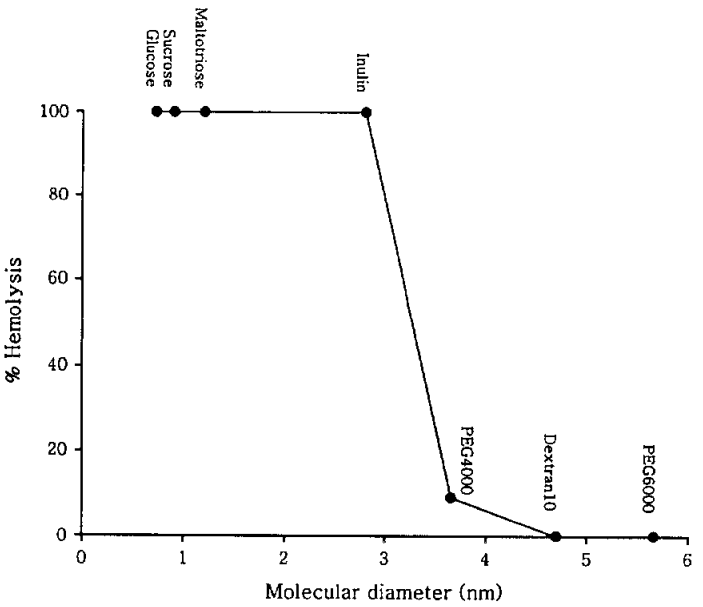

Fig. 3. Inhibitory effect of osmotic protectants on hemolysis. Rabbit erythrocyte (4%, v/v) suspensions containing 30 mM glucose, 30 mM maltotriose, 30 mM inulin, 30 mM PEG 4000, 15 mM dextran 10, and 15 mM PEG 6000 were incubated with VFH (2 HU) at 37 °C.

deduced amino acid sequences starting from Asp^{26} were identical to the N-terminal amino acid sequences determined by using the purified VFH. This indicates that cleavage at the Ala^{25}–Asp^{26} occurred during the secretion of mature VFH into culture medium. The derived molecular weight from DNA sequences, which was predicted to be 78,979 Da, is in good agreement with the molecular weight obtained by SDS-PAGE analysis.

To identify whether the *vfh* gene hybridizes to a specific region of *V. fluvialis* chromosome, Southern blot analysis was performed. Southern hybridization of *V. fluvialis* genomic DNA digested with *Hin*dIII revealed that the internal region of pVFH460 hybridized with a single 4.6 kb genomic fragment under high stringency conditions (data not shown).

3.7. Construction of C-terminal deletion mutant

In order to understand the deletion effect of the C-terminal region on the hemolytic activity, a series of C-terminal deletion derivatives of pVFH460 were constructed by PCR. Two different plasmids containing the deleted *vfh* gene fragments were identified and designated as pVFH460C1 and pVFH460C2 (Fig. 4A). pVFH460C1 and pVFH460C2 were truncated 210 and 444 bp, respectively, from the *vfh* gene in pVFH460. As shown in Fig. 4B, the cell lysates of *E. coli* containing each plasmid were examined for hemolytic activity on the blood agar plate. pVFH460C1 that deleted 70 amino acid residues had smaller areas of clear zone on blood agar plate when compared to pVFH460. This result implicated that the deletion of 70 amino acid residues exerted an important effect on complete hemolysis. The role of 148 amino acids in the C-terminal region of VFH was not certain. However, deletion beyond 148 amino acids resulted in a complete loss of hemolytic activity. This result suggested that C-terminal 78 amino acids, which were absent in pVFH460C2, were critical region for hemolytic activity or stability of VFH.

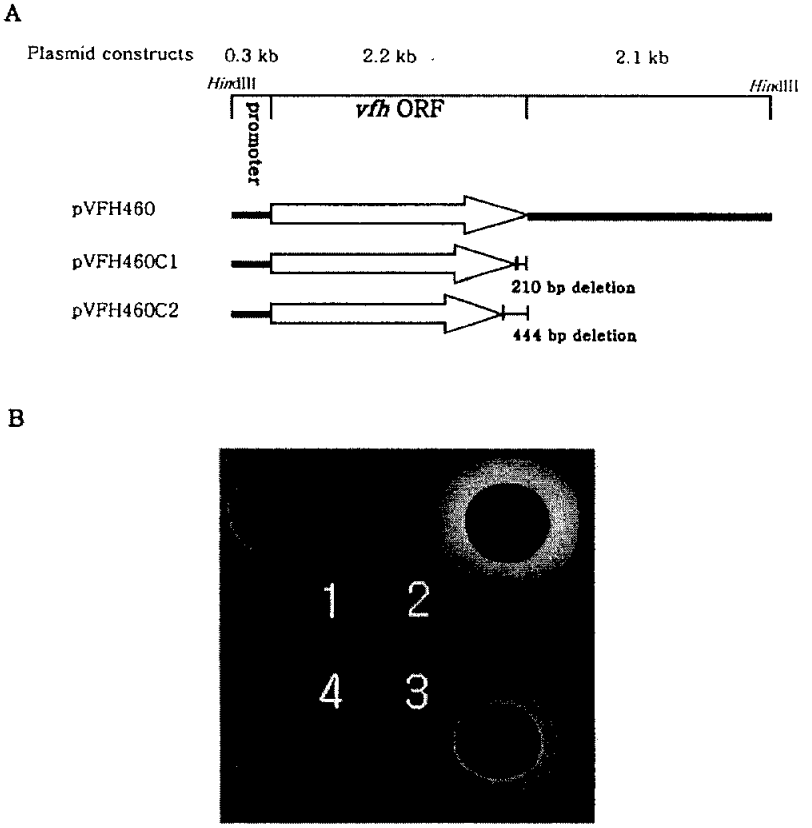

Fig. 4. (A) Construction of a series of the deletion plasmids. (B) Hemolytic activity of *E. coli* XL1-Blue containing pVFH460 and the deletion plasmids. The cells were cultivated in LB broth at 37 °C for 12 h and harvested by centrifugation at 7000 × *g* for 30 min. The cell pellets were disrupted by sonication on ice and centrifuged to remove insoluble fraction. About 0.1 mg of protein in clear supernatant was used as crude VFH solution for hemolytic activity assay. (1) *E. coli* XL1-Blue; (2) pVFH460; (3) pVFH460C1; (4) pVFH460C2.

4. Discussion

Hemolysin production has been shown to be associated with virulence for many bacterial species. Hemolysin can also play a multifunctional role in disease. Production of hemolysin by *V. fluvialis* can be identified by β type hemolysis on blood agar. In spite of the importance of hemolysin as a potential virulence factor of *V. fluvialis*, virtually nothing is known about its role and biological properties associated with virulent activities.

In this report, we purified hemolysin from *V. fluvialis* and cloned the corresponding gene. Sequence analysis of the cloned gene revealed that the 4.6 kb DNA fragment contained an ORF of 2220 bp, coding a protein of 740 amino acids. Alignment of the deduced VFH amino acid sequence to three other hemolysins from *V. cholerae* [21], *V. mimicus* [13], and *V. anguillarum* [12] showed 71%, 70%, and 59% identities, respectively (Fig. 5). The DNA sequence identity was 69%, 67%, and 60%, respectively. The amino acid sequence identity in the N-terminal region from Met[1] to

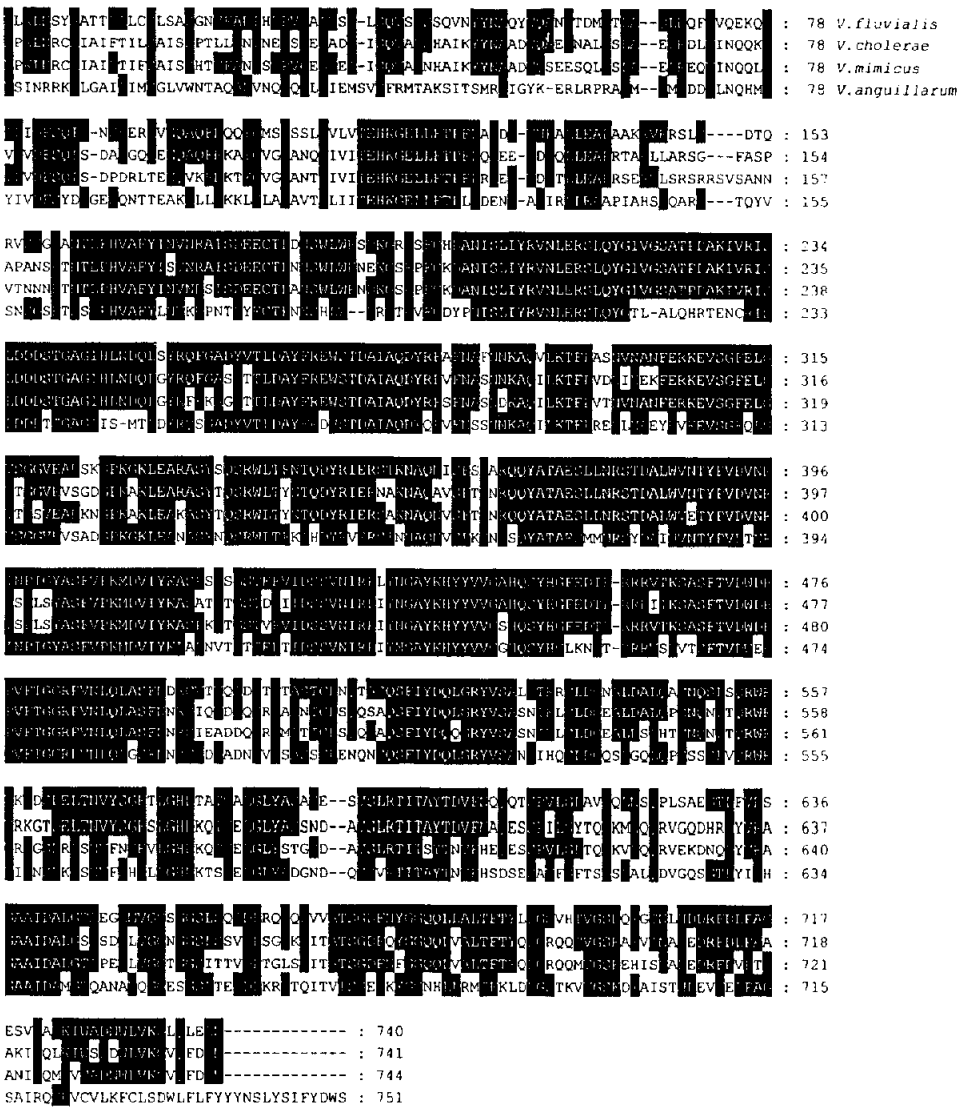

Fig. 5. Sequence comparison of VFH with *V. cholerae* (X51746), *V. mimicus* (U68271), and *V. anguillarum* (S83534) hemolysins. Conserved amino acids are indicated by black box. The homologous proteins are initially found with BLAST searches of the database at NCBI. The alignments of the amino acid sequence were performed with Clustal W.

Ala^{160} amino acid was very low and ranged from 25% to 47%. In contrast, the central region from Asn^{161} to Asn^{493} was highly conserved. (74–88%). The sequence identity in C-terminal regions, from Asp^{494} to Asn^{740}, of VFH with hemolysins from *V. cholerae*, *V. mimicus*, and *V. anguillarum* was shown to be 66%, 61% and 51%, respectively. Based on sequence alignment of these proteins, a highly conserved segment is believed to be of putative functional or structural importance in the mechanism of hemolysis.

The molecular weight by SDS-PAGE and N-terminal sequences of the purified VFH revealed that the cleavage of the signal peptide in VFH is different from that of other hemolysins from *Vibrio* spp. In *V. cholerae*, the hemolysin was initially synthesized as an 82 kDa protein and processed to 79 kDa protoxin by cleavage of the signal peptide during secretion through the inner membrane. The protoxin is then processed into 65 kDa active mature hemolysin during the transport to culture medium. Specifically, the preprotoxin synthesized in the cytoplasm is secreted into the periplasm, and the protoxin is secreted into the culture medium by two-step processing [21]. Rahman et al. [22] suggested that the strong probability of the two-step processing could exist in the production of mature VMH from *V. mimicus*, as found in *V. cholerae* hemolysin. Unlike the two-step processing found in *V. cholerae*, VFH is secreted into the extracellular environment as the 79 kDa protein after cleavage of 25 residues in N-terminal region. Molecular weight of the secreted mature VFH hemolysin is larger than that of *V. cholerae* and *V. mimicus*.

We found that VFH was temperature-independent for binding to erythrocytes, but lysis step showed temperature dependency. This result is similar to the mechanism of *V. cholerae* cytolysin that does bind to the erythrocytes at 4 °C and induces lysis of erythrocytes in a temperature-independent manner [19]. The temperature dependence of the erythrocytes disruption step seems to be due to the requirement of high temperature for increasing the membrane fluidity to allow the pore formation.

The hemolytic activity of VFH was inhibited by addition of Zn^{2+}, Ni^{2+}, Cd^{2+} and Cu^{2+} at low concentration of ID_{50}. Of the eight divalent cations we examined, a fairly high concentration (>25 mM) of ID_{50} was required in four cations (Mn^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+}). Miyoshi et al. [20] reported that the inhibitory effect of divalent cations on hemolysis by VMH was divided into two categories on the basis of their inhibitory potentials. Cu^{2+}, Zn^{2+}, and Ni^{2+} showed inhibitory effect on both hemoglobin release and K^+ efflux, but Ca^{2+} inhibit only hemoglobin release at a comparatively high concentration. Hemolysins from *Vibrio metschnikovii* and *V. cholerae* El Tor also showed a similarity in the inhibitory pattern by divalent cations, though there were different susceptibilities to mammalian erythrocytes [19,23]. In this study, we also obtained a similar result with VMF that hemolysis was influenced by divalent cations. Hemolytic activity was recovered by dialyzing to remove the cation and this result also coincided with other known *Vibrio* hemolysins.

Rabbit erythrocytes were very susceptible to VFH. This is in contrast to a study with TDH from *V. parahaemolyticus* where mouse erythrocytes were the most sensitive, and chicken and sheep erythrocytes were the least sensitive [24]. Shinoda et al. [25] reported that horse erythrocytes were the most sensitive to hemolysin (VMH) from *V. mimicus*. In case of *V. cholerae* hemolysin, rabbit erythrocytes were more sensitive, while horse erythrocytes were less sensitive [26].

In this study, we found that VFH forms pores in erythrocyte membrane and by using osmotic protectants. We estimate the diameter of the pores to be 2.8–3.7 nm. This size seems larger than those formed by other *Vibrio* hemolysins such as *V. cholerae* [19,27], *V. parahaemolyticus* [17], *V. metschnikovii* [23], *V. mimicus* [25], and *V. vulnificus* [28]. Our results strongly suggest that VFH, a major hemolysin of *V. fluvialis*, is a pore forming toxin and induces osmotic lysis in erythrocytes.

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〈NOTE〉

Cloning of the *Vibrio mimicus* Hemolysin (Vm-hemolysin) Gene and Expression in *Escherichia coli.*

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Key words : *Vibrio mimicus, Hemolysin, Recombinant DNA*

Vibrio mimicus is an enteropathogenic bacterium which apparently causes diarrhea, usually after the consumption of uncooked seafood (Shandera *et al.*, 1983). *V.mimicus* produced several pathogenic factors such as cholera toxin (CT) (Chowdhury *et al.*, 1991), CT-related enterotoxin (Davis *et al.*, 1981) and *Escherichia coli* heat-stable enterotoxin (ST)-like toxins (Gyobu *et al.*, 1988; Nishibuchi *et al.*, 1983). The hemolysin is involved in the bloody diarrhea which is one particular clinical symptom of *V.mimicus* gastroenteritis (Shandera *et al.*, 1983). *V.mimicus* produced two kinds of hemolysin, Vm-hemolysin (M.W 58,000) and Vm-rTDH (MW22,000) (Honda *et al.*, 1987). Vm-hemolysin is immunologically cross-reactive with *V.cholerae* biovar El Tor hemolysin while Vm-rTDH is cross-reactive with *V.parahaemolyticus* thermostable direct hemolysin (TDH). In the present study, we have cloned the gene encoding Vm-hemolysin of *V.mimicus* and expressed in *E.coli* JM83.

For the construction of genomic library, chromosomal DNA of *Vibrio mimicus* (ATCC 33653) cultured in BHI broth was partially digested with *Eco*RI (1unit) at 37℃ for 30 min and ligated into pUC 19, digested with *Eco*RI. This ligation mixture transformed into *E.coli* JM83. To find out hemolysis positive clone, the screening was performed by observing β-hemolysis activity on TSAII medium (BBL Co., USA) containing 5% sheep blood supplemented with ampicillin (100 μg/mℓ). Among about 7,000 recombinants, a single clone was detected for hemolysis after incubation for 40 hr at 37℃ (Fig. 1). Plasmid DNA was isolated from this hemolysis-positive (hly+) clone when the isolated DNA was retransformed to *E.coli* cell, each colony produced 100% hemolysis, indicating that the isolated plasmid contains hemolysin gene. The recombinant plasmid was designated as pVMH191 and was mapped by several restriction endonucleases.

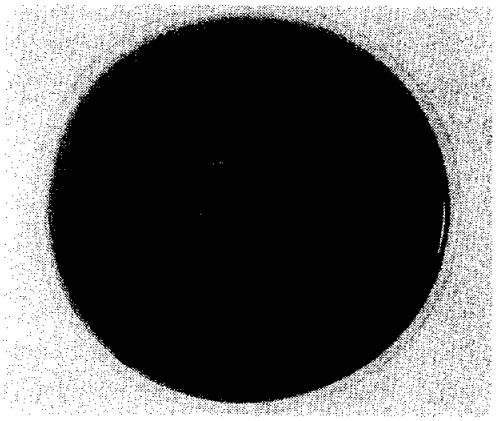

Fig. 1. Screening of *E.coli* JM83 (pVMH191) containing recombinant hemolysin gene on TSA II medium.

*Eco*RI digestion of pVMH191 yielded 2.7kb of vector and 8 kb of insert. For the subcloning, restriction endonuclease sites within the pVMH191 insert were decided. Plasmids termed as pVMH 192 and pVMH193 had the 5.3 kb-*Hin*dIII/*Eco*RI and 4 kb-*Pst*I/*Eco*RI inserts, respectively, in the same orientation of the pUC19 vector (Fig. 2). Plate assay for extracellular hemolysin production showed that these subclones have the strong hemolytic activity than that of *E. coli* JM83 (pVMH191) (Fig. 3). To further characterize pVMH193, pVMH193 insert was subcloned using other restriction endonucleases. However, as shwon in Fig 2, no hemolysis was detected in pVMH193-1 and the pVMH193-2. These result showed that 4 kb-*Pst*I/*Eco*RI insert contains entire ORF encoding Vm-hemolysin. (Fig. 2).

Expression of Vm-hemolysin gene in *E.coli* JM83 containing pVMH193 was measured by the activity against

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Overexpression and Characterization of *Vibrio mimicus* Metalloprotease

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Abstract To investigate the biochemical properties of *V. mimicus* metalloprotease, whose gene was isolated previously from *Vibrio mimicus* ATCC33653, overexpression and purification were attempted. The 1.9 kb of open reading frame was amplified by PCR from pVMC193 plasmid which ligated the *vmc* gene with pUC19 and introduced into *Escherichia coli* BL21 (DE3) using the overexpression vector, pET22b (+). The overexpressed metalloprotease (VMC) was purified with Ni-NTA column chromatography and characterized with various protease inhibitors, pHs, temperatures, and substrates. The purified VMC showed the proteolytic activity against gelatin, soluble and insoluble collagens, and synthetic peptides. Unlike the observations made with all metalloproteases originated from other *Vibrio* sp., the VMC did not hydrolyze the casein. The proteolytic activity was critically decreased when the VMC was treated with metal chelating reagents, such as EDTA, 2,2-bipyridine, and 1,10-phenanthroline. In particular, the 71 kDa VMC exhibited the hemagglutinating activity against human erythrocyte. As the purified VMC was treated with $CuCl_2$ and $NiCl_2$ for the chemical modification of metal binding, the proteolytic activity and hemagglutinating activity were profoundly influenced. The multialignment analysis made on the reported *Vibrio* metalloproteases showed the difference of amino acid sequence similarity between the two distinctive classes of *Vibrio* metalloproteases.

Key words: *Vibrio mimicus*, metalloprotease, hemagglutination, zinc-binding motif

Vibrio mimicus is a causative agent of human diarrhea. Clinical studies on *V. mimicus* infection to human revealed that diarrhea was accompanied by vomiting and abdominal cramps [11, 29]. In a minority of patients, fever and bloody diarrhea can also be seen. *V. mimicus* is mainly isolated from cases of gastroenteritis, but different strains have also been isolated. Gastroenteritis due to *V. mimicus* has been caused by ingestion of seafoods because the reservoir of this strain is an aquatic environment [8].

Several pathogenic factors including cholera toxin [30], cholera toxin-related enterotoxin [12], heat-stable enterotoxin [26], and two types of hemolysins (a thermolabile hemolysin and a thermostable hemolysin) [17, 25] have been reported from *V. mimicus*. It has been previously observed that *V. mimicus* has the ability to colonize rabbit intestinal mucosa [3, 31] and to produce some other extracellular factors, such as protease [6], hemagglutinin, and siderophore [27]. Although adherence to the intestinal mucosa is an important step in a diarrheal disease, there is little information on potential colonization factors of *V. mimicus*. In *V. cholerae*, cholera toxin has been shown to act as a powerful mucosal adjuvant [22] and a metalloprotease has been considered to be a causative agent to enhance the activity of cholera enterotoxin in intestinal loops by changing the protective layer of epithelial mucus [9]. Hemagglutinin (HA)/protease of *V. cholerae* has been shown to activate the cholera toxin A subunit [4] and to destroy the host cell receptors for a putative *V. cholerae* adhesin [13]. Moreover, HA/protease has been demonstrated to degrade the host defending proteins against cholera, such as mucin, fibronectin, lactoferrin, and secretory immunoglobulin A [14, 32]. These degrading activities have been suggested to be important for the evasion of the host immune response and the penetration of mucous layers to colonize the lower intestine.

Hemagglutinating and protease activities were suspected as the key factor for the colonization and invasion of host cell in pathogenic bacteria. Some of these metalloproteases were shown to be a bifunctional molecule capable of

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Vibrio fluvialis 유래의 hemolysin 정제와 생화학적 특성

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Purification and characterization of biochemical properties of hemolysin from *Vibrio fluvialis*

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Abstract

Hemolysin (VFH) of *V. fluvialis*, which is a pathogenic bacteria, causing watery diarrhea with vomiting, abdominal croup, was purified. *V. fluvialis* was cultivated in BHI medium and the culture supernatant was precipitated by ammonium sulfate. The protein was purified by chromatographies on columns of DEAE-cellulose and Mono-Q. Molecular weight of the purified VFH was estimated as 79kDa by SDS-PAGE. The optimal temperature for a maximum hemolytic activity was at around 35℃ and the activity was decreased at 40℃. Cytotoxicity of VFH was also investigated using RTG-2 cell line. LDH assay study showed that 50μg/ml of VFH release 80% of total cellular LDH (lactate dehydrogenase) from RTG-2 cell and microscopic observation also showed the morphological change of cell.

Key words – *Vibrio fluvialis*, Hemolysin, VFH, Cytotoxicity, RTG-2 cell

서 론

Vibrio sp.는 해수 및 담수에서 발견되는 대표적 병원성 미생물로 어패류, 인체의 장에서 발견되는 gram 음성 세균이다. 인체에 감염되는 것으로 알려진 12종의 *Vibrio* sp. 가운데 잘 알려진 것이 *Vibrio cholerae* 이며[1,14], 장염, 식중독 및 패혈증의 원인균으로 알려져 있는 *V. parahaemolyticus* [9], *V. mimicus*[6], *V. vulnificus*[2,19], *V. fluvialis*[17] 등이 잘 알려져 있다. 이들 *Vibrio*로부터 여러 병원성 인자들이 발견 보고 되어 있고, 특히 심한 탈수가 주요 증상인 콜레라를 일으키는 *V. cholerae*와 *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*에 관한 연구에 집중되어 왔다. 탈수와 설사 등의 공통성을 가지고 있는 이들 병원균에서 여러 공통적인 pathogenic factor가 발견되어 왔으며, phospholipase[8,13, 20], protease[16], hemagglutinin[3,7], TDH (Thermostable direct hemolysin)[10], TRH (TDH related hemolysin)[11], Cholera Toxin (CT)[14] 등의 세포 상해성 인자들이 보고되었다.

*V. fluvialis*는 1978년 Lee 등[17]에 의해 보고된 병원성 세균으로 감염시의 증상이 열, 탈수, 복통, 설사 등의 *V. cholerae*와 유사한 증상을 일으키며, 면역학적으로 *V. cholerae*의 CT와 동일한 독성이 보고된 바 있다[5]. *V. fluvialis*도 pathogenic에 관여하는 것으로 생각되는 여러 독성물질을

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Expression of Gilthead Seabream (*Sparus aurata*) Growth Hormone in *Escherichia coli* Using Alginate Lyase Gene Promoter of *Pseudomonas* sp.

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The promoter region of alginate lyase gene (*aly*) from *Pseudomonas* sp. W7 was used for the high expression of gilthead seabream (*Sparus aurata*) growth hormone (GH) gene in *Esherichia coli*. PCR product encoding the premature segment of the growth hormone was cloned to the downstream of *aly* promoter. GH was overexpressed with 46 amino acid of alginate lyase as fusion protein. GH was immunoreactive and production of GH was repressed with supplementation of 0.4% glucose into culture media.

Key words: gilthead seabream, growth hormone, alginate lyase promoter, overexpression

Introduction

Growth hormone (GH) is one of polypeptide hormones secreted by somatotrophs in anterior portions of pituitary glands of vertebrates and plays an important role in the regulation of somatic growth and maintenance of protein, lipid, carbohydrate and mineral metabolism (Ganong et al., 1983). The cDNA of preGH is consisted of 612 nucleotides and 204 amino acids, which had a predicted molecular weight of 22kDa (Funkenstein et al., 1991). There have been a number of studies demonstrating the growth promoting effect of recombinant growth hormone on fish via several delivery routes. Injection (Agellon et al., 1988; Tsai et al., 1994; Tsai et al., 1995), immersion (Schulte et al., 1989; Moriyama et al., 1990), constant infusion (Down et al., 1989) and oral administration (McLean et al., 1990; Moriyama et al., 1993) of GH showed the increase of body length and body weight of fishes. Among the various routes of growth hormone delivery to fish oral administration appears to be practical method so far (Jeh et al., 1998).

The choice of an expression system for production of recombinant proteins depends on many factors such as growth characteristics, expression levels, orientation of expression and the cost of production including purification yield. The essential component for production of protein is promoter. The most applicable promoters for large-scale protein production used thermal induction or chemical inducers in *E.coli* system. However, not only these chemical inducers are so expensive and toxic (Makrides, 1996), and also conditions for preventing repression is quite cumbersome. So it needs a system for producing of protein with inexpensive and effective way. We already reported the promoter region of alginate lyase gene (Kim et al., 1996; Lee et al., 1998). From previous report, *aly* promoter has strong ability for the expression of alginate lyase in *E.coli* (Kim et al., 1996; Lee et al.,1998). In this study, we have constructed an expression system for fish growth hormone (gilthead seabream) connected with *aly* promoter. The expression system was constructed by assembling the DNA fragments containing the promoter region of alginate lyase gene.

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Materials and Methods

Plasmids

pGSBGH which containing the cDNA of gilthead seabream GH was obtained from Dr B. Cavari

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해양의 *Pseudomonas* sp. 로부터 분리한 alginate lyase 유전자의 promoter에 의한 대장균 내에서의 β-agarase 유전자의 발현과 catabolite repression의 변화

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Expression of β-agarase Gene and Catabolite Repression in *Escherichia coli* by the Promoter of Alginate Lyase Gene Isolated from Marine *Pseudomonas* sp. Jin, Cheal-Ho, Je-Hyeon Park, Jeong-Hyun Han, Yoon-Hyeok Chae, Jong-Hee Lee, Jung-Kee Lee[1], and In-Soo Kong*. *Faculty of Food Science and Biotechnology, Pukyong National University, Pusan 608-737, Korea, [1]InBioNet Co. 1690-3 Taejon 306-230, Korea* – Promoter is a key factor for expression of the recombinant protein. There are many promoters for overexpression of protein in various organisms. The *aly* promoter of *Pseudomonas* sp. W7 isolated from marine environment was known to be a constitutive expression promoter of the alginate lyase gene, and it's promoter activity is repressed by glucose in *Escherichia coli*. To investigate the catabolite repression of the *aly* promoter and association between the promoter mutants, β-agarase gene, which was also cloned from *Pseudomonas* sp. W7 was connected to the *aly* promoter with the sequence the coding 46 N-terminal amino acids of the alginate lyase gene. The constructed plasmid was introduced into *E. coli* and the agarase activity was measured. Fourty six amino acids of the alginate lyase gene was serially deleted using PCR to the direction of 5' upstream region and subcloned. The agarase was overexpressed by the *aly* promoter and the production of agarase was repressed by the addition of glucose into culture media. Fourty six amino acids of alginate lyase did not affect the production of agarase at all. The deletion of a putative stem-loop structure in the *aly* promoter induced the decrease of β-agarase productivity.

Key words: β-agarase gene, alginate lyase gene promoter, *Pseudomonas* sp., overexpression promoter, catabolite repression

유전자 재조합기술에 의한 장점 중의 하나는 연구용 또는 의약품 및 산업용 단백질을 대량생산할 수 있다는 점이다. 외래 단백질을 대량으로 생체내에서 생산하는데 사용하는 host로는 현재 *E. coli*, *Bacillus*, 효모, 동물세포 등이 있으며 이 가운데 *E. coli*가 가장 많이 사용되고 있다. *E. coli* 내에서 외래 단백질을 생산하기 위한 vector로는 heat-shock induced expression vector, IPTG induction vector 등이 개발되어 사용되고 있는 중이다. IPTG에 의한 induction은 식품 첨가물 및 의약용 단백질을 생산하는데 부적합하다. 왜냐하면 인간 및 동물의 체내에 IPTG가 흡수시 문제점을 일으키기 때문이다. 따라서 이러한 용도로 사용할 때 정제 과정이 복잡해지게 된다[9]. Heat-shock induction의 경우 발효시 미생물 발효 reactor 전체의 온도를 변화시켜 주어야 하는 어려움과 온도 상승에 따른 경비 상승의 단점이 있다[13]. 이러한 어려움을 극복하기 위하여 배양하는 기질의 농도에 따른 autoregulation방법이 산업용으로는 바람직하다.

Autoregulation은 배양하는 기질 성분의 농도에 따라서 생체내에서 repressor-operator interaction이나 activator interaction에 의해 인위적으로 조절 가능하기 때문에 비교적 간단한 조작으로 발현량을 조절해 줄 수 있기 때문에 이의 개발이 절실히 요구되고 있다. 탄소원 가운데에서도 glucose에 의한 조절이 대표적이라 할 수 있다. 기존 알려진 glucose에 의해 조절되는 대표적인 발현 vector들은 대부분 IPTG에 의한 inducible vector이기 때문에 이와는 다른 작용기작을 갖는 glucose 조절의 다른 강력한 promoter를 지닌 vector의 개발이 필요하다.

본 연구실에서는 해양의 *Pseudomonas* sp. W7으로부터 분리한 alginate lyase유전자(pKAL24)가 *E. coli*내에서 효율좋게 발현하고 있으며 특히 glucose의 첨가에 의해서 catabolite repression을 강하게 받고 있는 특성을 보고한 바 있으며 외래 유전자의 발현에 우수한 promoter임을 밝힌바 있다[4,6,7]. 본 연구는 *Pseudomonas* sp. W7으로부터 분리한 alginate lyase 유전자의 promoter와 β-agarase 유전자의 open reading frame(ORF)만을 연결시켰을 때 효과적으로 *E. coli*내에서 대량발현시킬 수 있는가를 검토하고 promoter

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The High Production of Multimeric Angiotensin -converting-enzyme-inhibitor in *E. coli*

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Multimeric angiotensin-converting-enzyme-inhibitor (ACEI) containing a trypsin cleavable linker peptide between ACEI was constructed. We made synthetic DNA coding for the ACEI peptide with asymmetric and complementary cohesive ends of linker nucleotides. A tandemly repeated DNA cassette for the expression of concatameric short peptide multimers was constructed by ligating the basic units. The resultant multimeric peptide expressed as soluble and trypsin treated peptide was shown at the same retention time with chemically synthetic ACEI by HPLC. The present results showed that the technique developed for the production of the ACEI multimers with trypsin cleavable linker peptides can be generally applicable to the production of short peptide.

Key words: Multimeric peptide, High production, Angiotensin-converting-enzyme-inhibitor, *E. coli*

Introduction

The angiotensin-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) plays an important physiological role in the regulation of blood pressure. It cleaves angiotensin I to a powerful vasoconstrictor and saltretaining octapeptide, angiotensin II, and inactivates the casodilator and natriluretic nonapeptide, bardykinin (Soffer et al., 1976; Peach et al., 1977). Small molecules with strongly antihypertensive activity are synthesized and used as angiotensin-converting-enzyme-inhibitor (ACEI). Food derived peptide ACEI have recently received attention because of the development of functional foods contributing to homeostasis (Ondetti et al., 1977; Patchett et al., 1980; Ariyoshi et al., 1993). For functional foods or pharmaceutical applications, a large quantity of ACEI needs to be produced economically. Chemical synthesis is not practical because of its high cost and safety issues. Therefore, a biological expression system would be the most cost-effective method for the mass production of the ACEI. In this study, we produced ACEI from the concatameric short peptide multimers that were produced in *E. coli*.

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Materials and Methods

Bacterial strains and vectors

E. coli strain XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn10 (Tet^{r})]^{c}) was used as a host for subcloning and *E. coli* BL21 (DE3) (F^{-} *ompT hsdS*$_{B}$ (r_{B}^{-} m_{B}^{-}) *gal dcm* (DE3)) was used for gene expression. pGEM4Z (Promega Co.) and pET22b (+) (Novagen Co.) were used as vectors for the multimerization and expression of peptide, respectively.

Oligonucleotides for tandem multimerization of a gene encoding ACEI

For the left adaptor, primer III (5'-CATATGCCC-

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Effect of Temperature Shock on Cultured Olive Flounder (*Paralichthys olivaceus*) and Black Rockfish (*Sebastes schlegeli*)

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Aim of this research is to investigate the effect of temperature shocks on the physiological responses of cultured olive flounder (*Paralichthys olivaceus*) and black rockfish (*Sebastes schlegeli*). Olive flounder and black rockfish were suffered with high and low temperature shocks for 4 and 8 h, respectively, in laboratory conditions and then the changes in glucose, lactate, total protein, uric acid, and triglycerides-glycerol in blood plasma were analyzed. We observed that lactate and uric acid increased for up to 4 h and then decreased for up to 8 h by the high and low temperature shocks, and total protein decreased for up to 4 h and then recovered for up to 8 h by the high temperature shock in both fishes. Glucose by the high and low temperature shocks and triglycerides-glycerol by the low temperature shock increased for up to 4 h, and then decreased in olive flounder, but increased for up to 8 h in black rockfish. From the result, we speculated that the two fishes have an interspecific variation in the regulatory systems of glucose and triglycerides-glycerol. Glucose would play important role as an energy source during the temperature shocks and for an intermediate substance for low temperature tolerance, and glycerol of triglycerides-glycerol would play an important role for low temperature tolerance. In olive flounder, the turnover of chemical change by temperature shock took more than 4 h, all chemicals returned almost to the initial level for up to 8 h, but fish death followed only in 8 h with the high temperature shocked group within two days. Therefore, we suggested that fish would be damaged severely by the longer time exposure of high temperature and mortality would occur after a certain time later than the shocked time as a post-effect.

Key words: Acute temperature fluctuation, High temperature shock, Low temperature shock, Aquaculturing marine fishes, Blood plasma

Introduction

Olive flounder (*Paralichthys olivaceus*) and black rockfish (*Sebastes schlegeli*) are very important aquaculturing fishes in Korea, comprising up to 85 % of the total annual fish product in aquaculture. During the summer, there have been a few cases of the fish death in aquaculture, which have been suspected by the sudden exchanges between the high and low temperature seawater currents since Korea is located in the temperate-zone, but there is no clear evidence on it. In summer, it has been reported that the highest sea water temperature goes up to 25~28℃, and the lowest temperature goes down to 10℃ during the current fluctuation in Korea. Therefore, it is presumptive that the acute temperature fluctuation of seawater tide would affect the physiology of fish. On physiological responses of fish to the high or/and low temperatures, several investigations have been made on fresh water fishes including rainbow trout (Connors et al., 1978; Schneider et al., 1981; Wagner et al., 1997), tilapia

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〈단보〉

Immuno Gold 표지법을 이용한 대장균내 *Vibrio fluvialis* MotX 단백질의 존재 부위 결정

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Detection of the Recombinant MotX Protein of *Vibrio fluvialis* in *Escherichia coli* with Immuno-Gold Labeling Method

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The rotation of the flagellar motor is powered by the electrochemical gradient of specific ions across the cytoplasmic membrane. Recently, the genes of the Na^+-driven motor have been cloned from marine bacterium of *Vibrio* sp. and some of the motor proteins have been purified and characterized. Also, *motX* gene encoding a channel component of the sodium type flagellar motor was identified from *Vibrio fluvialis* (KTCC 2473). The amino acid sequence of MotX protein from *V. fluvialis* shared 90, 85, 85% identity with *V. cholerae, V. alginolyticus, V. parahaemolyticus,* respectively. We have studied the localization of the expressed MotX protein in *Escherichia coli* by immuno-gold labeling of ultra-thin frozen section. Our observation of the expressed protein indicated that MotX protein could be existed as attachment to inner membrane in *E. coli*.

Key words: *Vibrio fluvialis*, MotX, Sodium channel, Immuno-gold labeling

*Vibrio fluvialis*는 호염성 미생물로 위장염과 관련된 병원성 미생물로 주로 해산물의 섭취를 통해 인간에게 감염을 일으키며 이로 인한 증상은 열을 동반한 설사, 구토, 복통을 일으키는 것으로 보고되고 있다 (Lee et al., 1981). *V. fluvialis*를 비롯한 여러 *Vibrio* sp.는 그람 음성 박테리아로서 일반적으로 다른 생물의 생체 내에 침투하여 증식을 위한 에너지원을 찾아 이동하기 위한 수단으로 편모 운동성을 가지고 있다 (Yomohiro and Michio, 2001). *V. cholerae*는 극성 편모를 회전시키기 위해 Na^+ 이온을 동력으로 사용하는 반면에 *V. alginolyticus*와 *V. parahaemolyticus*는 Na^+ 이온과 H^+ 이온 둘 다를 사용하여 편모 운동을 하는 것으로 보고되고 있다 (Atsumi et al., 1992; Kawagishi et al., 1995; McCarter and Silverman, 1990).

여러 *Vibrio* sp.로부터 편모 모터와 관련된 여러 유전자가 보고되어 있으며 (Yomohiro and Michio, 2001), *V. parahaemolyticus*에서는 편모 모터에 관한 유전자로 *motX, motY, motA, motB* (Jaques et al., 1999; McCarter, 1994a; 1994b), *V. alginolyticus*의 경우에는 *motX, motY, pomA, pomB, motA, motB* (Asai et al., 1997; Yorimitsu et al., 1999)의 유전자가 보고되어 있다. *V. alginolyticus*의 *motX* 유전자의 경우 *V. parahaemolyticus*의 *motX* 유전자와 80%의 유사성을 가지고 있다 (Okunishi et al., 1996). *V. cholerae*에서 보고된 유전자로 *pomA, pomB, motX, motY*가 밝혀져 있는데, 이 유전자들은 *V. parahaemolyticus*의 유전자들과 높은 유사성을 가지고 있다 (Yomohiro et al., 2001).

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V. fluvialis MotX 단백질의 211개의 아미노산 서열을 *V. cholerae, V. alginolyticus, V. parahaemolyticus*의 아미노산 서열들과 유사도를 조사해 본 결과, *V. cholerae*와는 90%, *V. alginolyticus*와는 85%, *V. parahaemolyticus*와는 85%의 높은 유사성을 가지고 있었다 (Fig. 1). 이들 네 가지 MotX 단백질의 아미노산 서열들은 전반적으로 높은 유사성을 가지지만, 반면 같은 *Vibrio* sp.에서도 *V. fluvialis*의 MotX 단백질은 *V. cholerea*와 좀 더 높은 유사성을 보였으며 *V. alginolyticus*의 경우 *V. parahaemolyticus*와 좀 더 높은 유사성을 보였다. MotX 단백질의 N말단 부분에 29개의 아미노산으로 이루어진 membrane spanning region이 있는 것으로 알려져 있으며 multi-alignment 결과, 이 부분에서의 유사도는 상당히 낮게 나타났다. 기존의 보고에 의하면 MotX 단백질은 세포 내막을 통과하여 존재하면서 1개의 막 통과 부위를 가지고 있고 N말단이 세포 내막 안쪽에, C말단이 세포 내막 바깥쪽에 위치하여 전하를 띠면서 channel 구성요소나 회전기의 구성요소를 형성하는 것으로 알려져 있다 (McCarter, 1994a). Multi-alignment 결과에서 보듯이 전반적으로 높은 아미노산 서열의 유사성에도 불구하고 이 막 통과 부위로 추정되는 곳에서 높은 아미노산 변이정도를 보이는데 이는 내막에 파묻혀서 통로의 기능만 하는 putative spanning region을 구성하는 아미노산 서열은 약간의 변이가 있어도 미생물 편모 운동성의 에너지를 제공하는 역할을 수행하는 데는 별다른 지장이 없는 것으로 생각된다.

Park et al. (2001)에서 *V. fluvialis*의 *motX* 유전자를 클로닝하여 형질전환 후에 IPTG로 유도하여 과발현시킨 *E. coli*는 유도 30분 이후부터 성장이 현저히 저해를 받았으며 이때 생육은 100 mM의