潰瘍性大腸炎とDNAメチル化との関連
—one-carbon metabolism関連遺伝子多型を中心に—

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はじめに

潰瘍性大腸炎（Ulcerative Colitis: UC）は再発と緩解を繰り返す，腸管粘膜の炎症を主体とする疾患である。原因は不明であるが，免疫異常，脂肪摂取などの食事因子，生育時の環境，細菌感染および遺伝要因などが関連する多因子病と認識されている。遺伝要因としてさまざまな遺伝子多型がUCの疾患感受性遺伝子の候補として挙げられているが，一定の見解を得られていない。

また現在，癌を中心に疾患の原因として，DNAメチル化を含むエピジェネティックな異常が主要なものとして考えられている。DNAメチル化異常は腫瘍のみならず，正常部でも加齢に伴うage-related methylationが存在することが知られている。さらに炎症性腸疾患の消化管粘膜，バレット食道や肝炎の組織でもDNAメチル化が認められている。本稿ではDNAのメチル化と潰瘍性大腸炎の関連について，特にone-carbon metabolism関連遺伝子多型に焦点を合わせた当科の研究結果を踏まえ述べる。

DNAメチル化異常のしくみ

消化器癌の発生・進展におけるゲノム構造異常についてはさまざまな研究がなされてきたが，遺伝子変異や欠失とならんでエピジェネティックな異常が重要であることが明らかになりつつある。エピジェネティックな変化とは塩基配列の変化を伴わない遺伝性の遺伝子発現の変化と定義され，DNAメチル化やヒストン修飾が代表的なものである。

DNAメチル化とはシトシン塩基の5位の炭素に結合している水素がメチル基に置換されることを言い，DNA methyltransferase（DNMT）familyにより制御されている。遺伝子プロモーター領域におけるCpGアイランドにメチル化が起こると，ヒストン修飾の変化や転写因子との結合性の変化を通じて，転写を強力に抑制する[1]。CpGアイランドにおける高メチル化は癌細胞においてRetinoblastoma（*RB*），von-Hip-

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pel-Lindau（*VHL*），*P16*などの癌抑制遺伝子でみられることが報告されている[2)]。癌抑制遺伝子におけるCpGアイランドのメチル化による転写の抑制は癌の発生・進展に重要な役割を果たすと考えられている。癌におけるもう一つのDNAメチル化異常としてゲノムワイドな低メチル化が見られることも知られている。ゲノムワイドな低メチル化により正常細胞では発現が抑制されている癌遺伝子の活性化や染色体不安定性を引き起こすと考えられている[3)]。

■潰瘍性大腸炎とDNAメチル化

癌以外の消化管疾患において，DNAメチル化が認められるものには加齢，*H.pylori*感染，逆流性食道炎，潰瘍性大腸炎(UC)が挙げられる。IssaらはUC患者の大腸粘膜では*Estrogen receptor*（*ER*），*p16*などの遺伝子のCpGアイランドのメチル化が加齢により促進していると報告している[4)]。大腸癌部のCpGアイランドにおけるメチル化はcancer-restricted methylation（type C）とage-related methylation（type A)の2つのタイプに大別される[4)]。UCの大腸粘膜ではage-related methylationが多く認められたと報告されている[4)]。その原因として，炎症による細胞周期の加速や酸化ストレスなどが考えられている。メチル化は細胞増殖している組織において増加する傾向にあり，この現象は大腸癌における*ER*遺伝子で証明されている[5)]。このことより，細胞周期が加速していると考えられるUCなどの慢性炎症ではage-related methylationが増加傾向にあると考えられる。またTominagaらはUC患者において腺腫あるいは癌を発症した患者の大腸粘膜では正常粘膜でも*ER*遺伝子のメチル化が高度に検出されることを報告している[6)]。筆者らはage-related methylationが増加することはUCにおける癌化の初期に見られる現象であると推察している。また$p14^{ARF}$遺伝子においてUCにおけるcolitic cancerの患者では癌部だけでなく，非癌部の粘膜においても高メチル化が認められることが報告されている[7)]。$p14^{ARF}$は癌抑制遺伝子の一つで*p53*遺伝子のMDM2を介した活性の低下を阻害する役割がある。$p14^{ARF}$遺伝子のCpGアイランドにおいて高メチル化が起こることは，その活性を低下させると考えられる。同様にUC患者において$p14^{ARF}$遺伝子のメチル化の頻度を解析した研究で$p14^{ARF}$遺伝子の高メチル化が直腸に検出されたUC患者はdysplasiaを発症する例が多く，$p14^{ARF}$遺伝子の高メチル化がUCの発癌の予測因子となる可能性を示唆された[8)]。このようにUC患者における発癌において，特定遺伝子のメチル化が重要な役割を果たすことが示唆されている。

またTaharaらは*protease-activated receptor 2*（*PAR2*）遺伝子における解析で，直腸型より全結腸型のUC患者において*PAR2*遺伝子のプロモーター領域の高度のメチル化が認められたことを報告した[9)]。PAR2はさまざまな細胞に発現し，炎症の制御に関与する。*PAR2*遺伝子のメチル化による*PAR2*遺伝子の機能への影響は明らかではないが，この結果から筆者らは*PAR2*遺伝子のメチル化はUCの重症化と関連があるとしている。

このようにUCとDNAメチル化については研究が進んでいる。

■One-carbon metabolismとDNAメチル化

葉酸を介したone-carbon metabolismはDNAメチル化と密接な関連があることが知られている。葉酸は一炭素基を与えるドナーとしてone-carbon metabolismに寄与している[10)]。葉酸はDNAメチル化以外にDNA修復やヌクレオチドの合成にも関連している[11)]。またメチオニンはこの代謝経路内で重要な役割を果たしている[12)]。これらの代謝経路を図1に示す[10)]。この経路中で葉酸は数段階を経て，methylenetetrahydrofolate reductase（MTHFR）によ

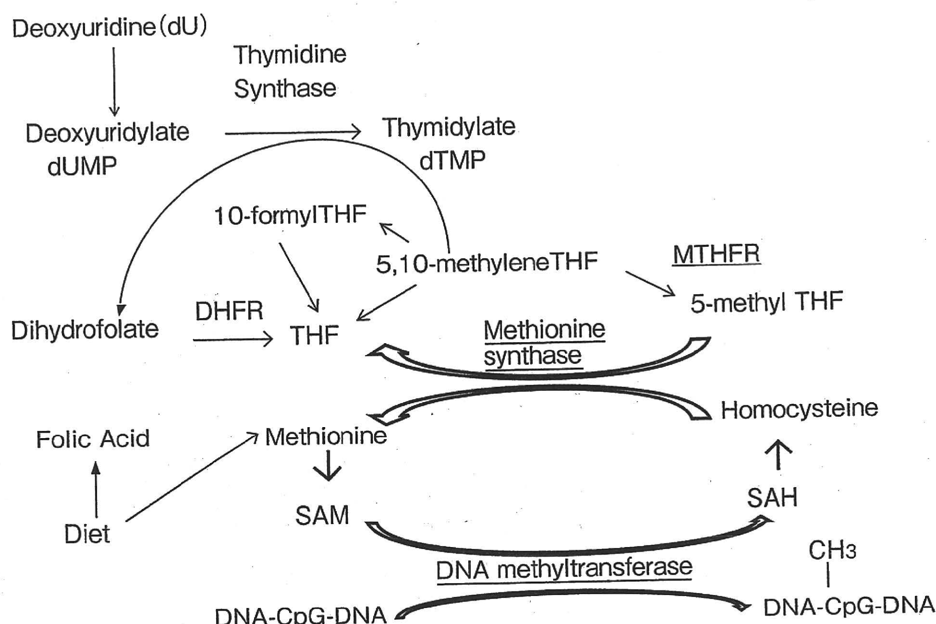
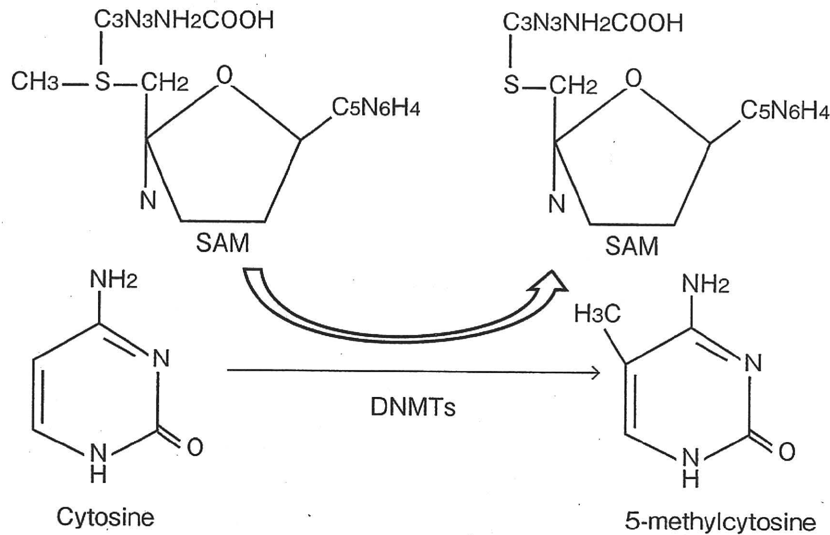


図1 one-carbon metabolismにおける代謝経路(文献10より引用，一部改変)

り5-methyl tetrahydrofolate (THF)に変換される。5-methylTHFはmethionine synthase (MS)によりTHFへと変換される。またMSはホモシステインをメチオニンに変換する。メチオニンはDNAメチル化におけるメチルドナーとして働く。メチオニンはこの代謝回路内でS-adenosylmethionine (SAM)に変換される。SAMはDNMTの作用により，S-adenosylhomocysteine (SAH)に変換される。SAMはメチル基供与体として機能する。またDNMTの反応がSAMの供給およびSAHの除去に依存しているため，SAMとSAHの比はいわゆるmethylation indexとして用いられる[11]。すなわちSAM/SAHの比により高メチル化あるいは低メチル化といった状態が示されるのである。DNMTの作用によりシトシン基の5位の炭素にメチル基が結合するというDNAメチル化が起こる[13](図2)。このようにDNAメチル化には葉酸を介した一炭素代謝が重要な役割を果たしている。

UCとone- carbon metabolism関連遺伝子多型

One- carbon metabolism関連遺伝子における主要な遺伝子多型を表1に示す。*MTHFR*遺伝子は1p36に存在し，主な遺伝子多型として*C677T*多型および*A1298C*多型が知られている。*MTHFR*遺伝子の677部位でシトシン(C)がチミン(T)に変換される変異が起こると当該部位のアミノ酸基がアラニンからバリンに変化する。この変異の存在が血中のホモシステイン値の上昇と関連があるとされる。UC患者においても*MTHFR*遺伝子*C677T*多型のTT遺伝子型を有する割合が健常者より多く，これらの患者は血中のホモシステイン値が健常者よりも高値であることが報告されている[14]。血中のホモシステイン値が高値であるといわゆる過凝固状態を招き，UC患者の合併症の一つである静脈血栓症と関連があると考えられている。



SAM: S-adenosylmethionine, SAH:S-adenosylhomocysteine
DNMT: DNA methyltransferase

図2　DNMTの作用とDNAメチル化(文献13より引用)

表1　one-carbon metabolism関連遺伝子と主な遺伝子多型

遺伝子名	遺伝子座	主な遺伝子多型	関連文献
MTHFR	1p36	*C677T* *A1298C*	Mahmud et al,1999 [15] Herrlinger KR et al, 1995 [16]
MS	1q43	*A2756G*	Chen M et al, 2008 [17]
DNMT3B	20q11.2	*-149CT* *-579GT*	Shen H et al, 2002 [18] Hong YS et al, 2007 [22]

MTHFR: Methylenetetrahydrofolate reductase
MS: Methionine Synthase
DNMT: DNA methyltransferase

*MTHFR*遺伝子の*A1298C*多型が存在すると当該部位のアミノ酸基がグルタミン酸からアラニンに変化する。*A1298C*多型のCC遺伝子型を有する場合，*MTHFR*の酵素活性が低下する。ステロイド抵抗性などの理由でメトトレキセート(MTX)を使用したUC患者について*MTHFRA1298C*多型との関連を検討した報告があり[15]，*MTHFR*遺伝子の1298Cアレルを有する患者はMTXによる副作用(嘔気，皮疹など)が多かった。MTXによる副作用は5-methyl THFの値が低下することに関連があるとされ，*A1298C*多型の存在によりMTHFRの活性が低下するために副作用が増加すると考えられる。

Methionine synthase (*MS*) 遺伝子は1q43に存在し，主要な遺伝子多型として*A2756G*多型がある。*A2756G*多型の酵素活性に対する影響は明らかではないが，*A2756G*多型を有するとホモシステインが高値な傾向にある。*MS*遺伝子の*A2756G*多型とUCとの関連を調べた研究ではUC患者で*A2756G*多型のGアレルを有する例が健常者より有意に多くUC発症のリスクに関連があるとしている[16]。

*DNMT3B*遺伝子は20q11.2に存在し，主要な遺伝子多型には*-149CT*および*-579GT*多型がある。*-149CT*多型が存在するとプロモーター領域におけるDNMTの酵素活性が30%増加する。*-579GT*多型はプロモーター領域に

おいては酵素活性には影響しないとされる。*-149CT*多型は肺癌[17]および前立腺癌[18]におけるリスクの増加に関連しているとされる。また遺伝性非ポリポーシス型大腸癌(HNPCC)では年齢によるリスクとの関連が報告されている[19]。これは*DNMT3B*遺伝子の*-149CT*多型においてTアレルを有するHNPCC患者では他のアレルを有する患者よりHNPCCの発症年齢が若いというものである。*-579GT*多型は肺癌[20]および大腸癌[21]についてリスクの低下に関連があるとされる。こうした関連を受けてわれわれはUC患者136名(男性75名，女性61名)および健常者137名(男性69名，女性68名)を対象とし，*MTHFR, MS*および*DNMT*遺伝子の各多型についてUC患者の臨床因子(発症年齢，性別，罹患範囲，臨床経過による病型，重症度およびステロイド抵抗性)との関連およびUC発症のリスクとの関連を検討した。患者から文書により同意を受け，検体を得た。DNAの解析はPCR-RFLP法およびDirect sequence法を用いた。結果は現在解析中であるが，*MTHFR C677T*多型についてUC患者の臨床因子と関連がある可能性や，リスク解析についても*DNMT-579GT*多型においてUCの発症リスクとの関連が示唆されており，現在さらに解析中である。

■考　察

UCとホモシステインなどのone- carbon metabolism代謝産物との関連は以前から指摘されていた。たとえばDrzewoskiらは血中ホモシステイン値の上昇は活動期のUCにおいてより顕著に認められるとの報告しており[22]，ホモシステイン値の上昇はUCの病勢を反映するものと考えられる。IBD患者では血中のみならず大腸粘膜においてもホモシステイン値が上昇している[23]。ホモシステインの代謝が乱れることは酸化ストレスの発生と関連があり，酸化ストレスはIBDの炎症を惹起する。ホモシステイン値の上昇は*MTHFR C677T*多型が要因となっており，この多型の存在がIBDの発症と関連するものと考えられる。今回のわれわれの検討でもデータ解析中ではあるが*MTHFR C677T*多型がUCの臨床因子と関連しており，これは他の研究の結果とも合致している。

DNAメチル化はDNA methyltransferase(DNMT)により制御されている。DNMTには主要なものとしてDNMT1,3A,3Bがある。他にDNMT2というサブタイプもあるが，その機能は明らかにはなっていない。DNMT1は主にメチル化の維持に働き[24]，DNMT3A,3Bはメチル化を*de novo*に導入する働きがある[25]。*de novo*なメチル化とはDNAにおいてメチル化していない領域あるいはhemimethylationが見られる領域にメチル化を導入することである。DNMT1はDNAのメチル化におけるパターンを転写するのに必要とされる。DNMT1ノックアウトマウスを用いた研究によりDNMT1はDNA転写およびX染色体の不活化に関連することが報告されている[26]。DNMT1ノックアウトマウスにおいてもDNAの*de novo*なメチル化が生じることが報告され，これがDNMT3 familyの発見されるきっかけとなった[25]。DNMT1,3A,3Bについて大腸，腎臓，膀胱および膵の癌組織および正常組織における各酵素の活性を調べた研究があり[27]，それによると各腫瘍組織ではDNMT1,3Aは中程度の上昇に過ぎなかったのに対し，DNMT3Bは高度に発現していた。このことからDNMT3Bは腫瘍の発生に密接な関連があるとしている。前述したように*DNMT3B*遺伝子多型はさまざまな癌において関連が報告されている。肺癌に関して*-149CT*多型のTアレルを有する患者は肺癌を発症するリスクが高いとの報告がある[19]。*-149CT*多型の存在によりプロモーター領域におけるDNMT酵素活性が増加することが知られている。*-149CT*多型における肺癌のリスク増加はDNMT酵素活性の増加によるメチル化の促進が関連しているものと考えられる。こ

れに対し，*DNMT3B-579GT*多型においてGアレルを有する患者は肺癌を発症するリスクが低いとされる[21]。また*DNMT3B*遺伝子の*39179GT*（*-579GT*）多型のGTおよびGG遺伝子型を有する患者では60歳未満での大腸癌のリスクが低いとの報告がある[21]。このことから筆者らは*DNMT-579GT*多型が大腸癌の疾患感受性のマーカーとなるとしている。同様に*-579GT*多型を有する患者は健常者より有意に頻度が低く，大腸癌を発症するリスクが低いとされる[28]。*DNMT3B-579GT*多型はDNMTの酵素活性には直接の影響を与えないが，こうした関連から*DNMT3B*遺伝子の機能に何らかの影響を与えることが考えられる。

UCとDNMT遺伝子多型については今まで関連は報告されていないが，現在検討中であるが，われわれの検討により*DNMT3B -579GT*多型がUCのリスクと関連がある可能性が示唆されている。この結果からDNAメチル化とUCの発症に関連があることが考えられた。

■おわりに

これまで述べてきたように潰瘍性大腸炎とDNAメチル化について研究が進んでおり，またUCにone-carbon metabolism関連遺伝子多型が関連するといういくつかの研究が報告されている。one-carbon metabolismとDNAメチル化は密接な関連があることからUCとDNAメチル化およびone-carbon metabolismについて関連を研究することはUCの病態の解明に役立つものと考えられた。

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Induction of SCEs in CHL cells by dichlorobiphenyl derivative water pollutants, 2-phenylbenzotriazole (PBTA) congeners and river water concentrates

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ABSTRACT

We recently identified dichlorobiphenyl (DCB) derivatives and 2-phenylbenzotriazole (PBTA) congeners as major mutagenic constituents of the waters of the Waka River and the Yodo River system in Japan, respectively. In this study we examined sister chromatid exchange (SCE) induction by two dichlorobiphenyl derivatives, 3,3′-dichlorobenzidine (DCB, 4,4′-diamino-3,3′-dichlorobiphenyl) and 4,4′-diamino-3,3′-dichloro-5-nitrobiphenyl (5-nitro-DCB); three PBTA congeners, 2-[2-(acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1), 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2), and 2-[2-(acetylamino)amino]-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-6); and water concentrates from the Waka River in Chinese hamster lung (CHL) cells. Concentration-dependent induction of SCE was found for all DCBs and PBTAs examined in the presence of S9 mix, and statistically significant increases of SCEs were detected at 2 μg per ml of medium or higher concentrations. SCE induction of MeIQx was examined to compare genotoxic activities of these water pollutants. According to the results, a ranking of the SCE-inducing potency of these compounds is the following: 5-nitro-DCB ≈ MeIQx > PBTA6 > PBTA-1 ≈ PBTA-2 > DCB.

Water samples collected at a site at the Waka River showed concentration-related increases in SCEs at 6.25–18.75 ml-equivalent of river water per ml of medium with S9 mix. The concentrations of 5-nitro-DCB and DCB in the river water samples were from 2.5 to 19.4 ng/l and from 4100 to 18,900 ng/l, respectively. However, these chemicals showed only small contribution to SCE induction by the Waka River water.

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1. Introduction

Genotoxic compounds are detected in many surface waters in the world. These compounds are often released directly from industrial discharges as a result of insufficient treatment of wastewater or unintentional formation in the environment after discharge of effluents [1–5]. In the previous studies, we found two novel chemical classes, dichlorobiphenyl derivatives and 2-phenylbenzotriazole (PBTA) congeners, as major mutagenic constituents in the water of rivers flowing through several industrial areas in Japan [6–20].

Among dichlorobiphenyl derivatives, 3,3′-dichlorobenzidine (DCB, 4,4′-diamino-3,3′-dichlorobiphenyl), 4,4′-diamino-3,3′-dichloro-5-nitrobiphenyl (5-nitro-DCB), and so forth were identified as major mutagens in the water of the Waka River flowing through an industrial area in Wakayama, where a number of large chemical plants are found [6–9]. 5-Nitro-DCB is a novel chemical and is presumed to be formed unintentionally by the process of wastewater treatment of drainage water containing DCB discharged from chemical plants [6]. DCB is a raw material in the manufacture of polymers and dye intermediates, and there are large-scale chemical plants producing DCB in this industrial area. 5-Nitro-DCB is highly mutagenic in the Ames assay using *Salmonella typhimurium* YG1024, which is an *O*-acetyltransferase-overproducing derivative of TA98, with S9 mix, and its activity was ~7 times higher than that of DCB.

5-Nitro-DCB was detected in river water concentrates at the maximum level of 6.9 μg/g of blue rayon. DCB was also detected in the concentrates at 13.2–104 μg/g of blue rayon. The percent contributions of 5-nitro-DCB and DCB to the mutagenicity of the water concentrates in YG1024 with S9 mix were 11% and 28%, respectively,

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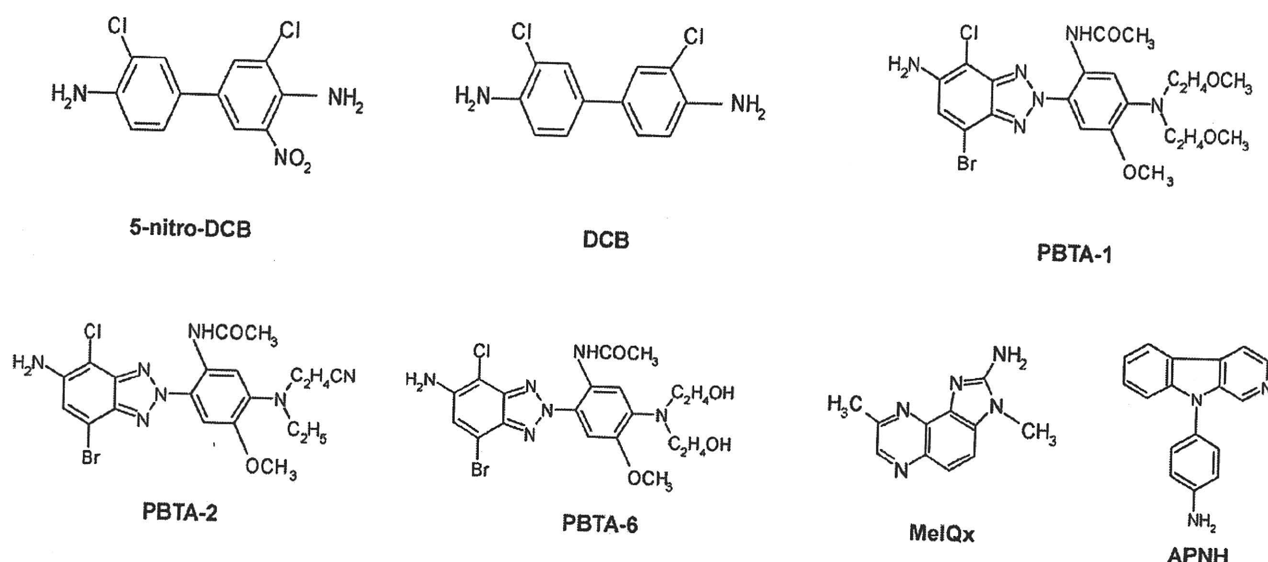

Fig. 1. Chemical structures used in the present study.

on average. 5-Nitro-DCB is a new chemical, and it has no biological activity data except for mutagenicity in the Ames assay.

PBTA congeners were identified as major indirect-acting river water mutagens, and seven kinds of such compounds were detected in highly mutagenic river waters, e.g., the Yodo River system, the Asuwa River, the Nikko River and so on, flowing through areas of textile dyeing industries [10–20]. PBTA congeners were suggested to be formed from corresponding dinitrophenylazo dyes via reduction with sodium hydrosulfide during the industrial dyeing process and following chlorination in the disinfection process at sewage plants. PBTA congeners show potent mutagenicity in *S. typhimurium* YG1024 in the presence of S9 mix. 2-[2-(Acetylamino)-4-[bis(2-methoxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-1), 2-[2-(acetylamino)-4-[*N*-(2-cyanoethyl)ethylamino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzotriazole (PBTA-2), and 2-[2-(acetylamino)amino]-4-[bis(2-hydroxyethyl)amino]-5-methoxyphenyl]-5-amino-7-bromo-4-chloro-2*H*-benzo-triazole (PBTA-6) were detected in many river water concentrates [10–15]. However, there are few reports on the genotoxicity of PBTA congeners in mammalian cells.

In this study, we investigated the induction of sister chromatid exchanges (SCEs) by DCB, 5-nitro-DCB, PBTA-1, PBTA-2, PBTA-6, and water concentrates from the Waka River in Chinese hamster lung (CHL) cells to evaluate the genotoxic effect of water pollutants and environmental samples contaminated with DCB and 5-nitro-DCBs. DCB and 5-nitro-DCB in the river water concentrates were quantitatively analyzed, the mutagenicity of the water concentrates were evaluated in YG1024, and the contribution of 5-nitro-DCB and DCB to the mutagenicity of the river water concentrates are also estimated.

2. Materials and methods

2.1. Materials

5-Nitro-DCB (CAS 1073239-90-3) was synthesized according to the method reported previously [6]. Dichlorobenzidine dihydrochloride (CAS 612-83-9) was purchased from Sigma–Aldrich Co. Ltd. (MO, USA). PBTA-1 (CAS 194590-84-6), PBTA-2 (CAS 215245-16-2), and PBTA-6 (CAS 392274-07-6) were synthesized as described previously [11,13,16]. 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx, CAS 77500-04-0) was obtained from Wako Pure Chemical Industries (Osaka, Japan). The chemical structures of six compounds used in the present study are shown in Fig. 1. All other chemicals and reagents were of analytical grade.

2.2. Preparation of river water concentrates and analysis of DCBs

Each 10 l water sample was collected at a site where wastewater was discharged from chemical plants and a sewage treatment plant into the Waka River in Wakayama, Japan from September 2006 to March 2007. Collected water samples were passed through Supelpak2 columns (SUPELCO, PA, USA, 20 mm i.d. × 800 mm), and adsorbed materials were then extracted with methanol (300 ml) according to our previous paper [8]. Each extract was used for SCE assay, the Ames assay, and quantification of 5-nitro-DCB and DCB by HPLC. Quantification of 5-nitro-DCB and DCB was performed according to the method reported in the previous paper [6].

2.3. Chemical treatment

All chemicals tested for SCE assay were dissolved in dimethyl sulfoxide (DMSO), and freshly prepared solutions were added to cultures in appropriate final concentrations. The final concentration of DMSO in all cultures was 0.5% (v/v). Aminophenyl-norharman (APNH, Fig. 1) was used as the positive control [22] and was dissolved in DMSO before use.

2.4. SCE assay

CHL cells, obtained from Health Science Research Bank (HSRRB), Japan, were subcultured at a cell density of 1.5×10^5 cells per 60-mm dish and cultivated in 4 ml of Eagle's minimum medium (MEM, Gibco BRL) supplemented with 10% heat-inactivated fetal bovine serum (Trace Scientific Ltd., Melbourne, Australia). In the system with metabolic activation, cells were treated with each chemical for 6 h in the presence of S9 mix. The S9 mix was prepared immediately before use by mixing 1 ml of phenobarbital- and 5,6-benzoflavone-induced rat liver S9 (Oriental Yeast Co. Ltd., Tokyo, Japan) with 0.1 ml of 0.5 M glucose-6-phosphate, 0.4 ml of 0.1 M NADP, 0.2 ml of 1.65 M KCl solution, 0.2 ml of 0.4 M $MgCl_2$ solution, 4 ml of 0.25 M phosphate buffer (pH 7.4), and 4.1 ml of distilled water. The final concentration of S9 used was 1.25% (v/v). After treatment, cells were washed with phosphate buffered saline (PBS) and cultured in fresh medium for a recovery period of 24 h. In an experiment designed to detect SCEs, 5-bromodeoxyuridine (BrdU; final concentration 5 μg/ml) was added to the cultures just after addition of the test chemical.

Colcemid (final concentration 0.2 μg/ml; Gibco) was added to each culture 2 h prior to harvest. Harvested cells were then treated with a hypotonic solution of 75 mM KCl and fixed with cold methanol:acetic acid (3:1, v/v). Solvent-treated cells served as the negative control. Air-dried chromosome preparations prepared for SCEs were stained with the fluorescence plus Giemsa method described by Perry and Wolff [23]. SCEs were scored in 25 well-spread metaphases for each treatment. The results were expressed as the frequency of SCEs per metaphase. The significance between mean SCE in treated versus control groups were determined using the Student's *t*-test.

2.5. Ames assay

Ames assay was performed for DMSO solutions of chemicals and water concentrates described above using *S. typhimurium* YG1024 according to the method reported previously [24–26]. *S. typhimurium* YG1024 were kindly provided by Dr. T. Nohmi from the National Institute of Health Sciences, Tokyo. The S9 mix contained

Table 1
SCEs induced by 5-nitro-DCB, DCB, PBTA-1, PBTA-2 and PBTA-6 in CHL cells in the presence of S9 mix.

Sample	Dose (μg/ml)	SCEs per metaphase		
		MI (%)[c]	Mean ± S.D.	Range
5-Nitro-DCB[a]	1.25	2.0	13.32 ± 6.27**	4–29
	2.5	2.5	15.92 ± 7.43**	6–31
	5	2.1	18.58 ± 6.01**	7–29
	10	2.2	25.04 ± 9.50**	11–48
DCB[a]	1	2.9	11.16 ± 4.14*	4–20
	2	2.2	14.76 ± 4.35**	7–23
	10	2.9	16.96 ± 5.95**	8–35
	20	2.5	19.16 ± 7.69**	7–37
PBTA-1[b]	1.25	2.6	17.88 ± 5.21**	11–29
	2.5	2.6	18.60 ± 8.29**	9–34
	5	2.0	19.06 ± 6.27**	7–29
	10	1.7	20.04 ± 7.37**	10–43
PBTA-2[b]	1.25	2.8	14.28 ± 3.60*	8–23
	2.5	2.9	14.56 ± 6.58**	6–38
	5	2.7	17.84 ± 5.74**	8–28
	10	2.0	20.96 ± 7.81**	9–34
PBTA-6[b]	1.25	2.9	15.08 ± 4.95	8–26
	2.5	2.7	16.28 ± 6.13**	8–26
	5	2.9	19.40 ± 5.22**	12–31
	10	1.8	22.76 ± 7.04**	10–36
MeIQx[b]	1.25	3.5	15.97 ± 8.20**	6–39
	2.5	3.9	18.20 ± 5.12**	9–35
	5	3.7	23.96 ± 9.20**	9–43
	10	2.7	26.68 ± 9.02**	13–53

SCE frequency for positive control (APNH, 0.005 μg/ml) was 21.44 ± 5.04 (mean ± S.D.).

[a] SCE frequency for Control (DMSO) was 8.68 ± 4.28 (mean ± S.D.).

[b] SCE frequency for Control (DMSO) was 10.16 ± 2.65 (mean ± S.D.).

[c] MI; mitotic index. MI (%) was calculated by counting the number of mitotic cells among 1000 round nuclei.

* Significantly different from control, $p < 0.05$.

** Significantly different from control, $p < 0.01$.

25 μl of S9 (25 mg of protein/ml) at a total volume of 500 μl. Mutagenic activities of test samples were calculated from the linear portions of the dose-response curves obtained with four doses with duplicate plates in two independent experiments, and the results were the average of two independent experiments. The positive controls were 2-aminoanthracene (0.1 μg/plate) and Trp-P-1 (0.01 μg/plate) with S9 mix. The mutagenic potencies were expressed as revertants/l of river water.

3. Results

3.1. SCE induction by DCBs and PBTAs

We evaluated the genotoxic effect of 5-nitro-DCB, DCB, three PBTA congeners, and MeIQx using SCEs in cultured CHL cells in the presence of S9 mix. These chemicals are indirect-acting mutagens for bacteria and show potent mutagenicity with S9 mix [6,7,10,11,13,27]. As shown in Table 1, statistically significant increases in SCEs were found for DCBs and PBTAs at doses used in this study (up to 10 or 20 μg/ml). Among DCBs and PBTAs, the highest SCE frequency, 25.04 ± 9.50 (mean ± S.D.), was detected for 5-nitro-DCB at the dose of 10 μg/ml. MeIQx is a well-known mutagenic and carcinogenic heterocyclic amine [28], and it shares structural features common to those of DCBs and PBTAs. Dose-related increases in the frequencies of SCEs were found for MeIQx between the concentration of 1.25 and 10 μg/ml. The highest SCE frequency, 26.68 ± 9.02, was detected at 10 μg/ml. Concentrations of DCBs, PBTAs, and MeIQx leading to two-fold increases of SCE frequencies relative to that of control were shown as SCE-inducing activity in Table 2, with mutagenicity data by Ames test. SCE-inducing activities of DCBs and PBTAs were from 4.5 to 13.9 μg/ml. The SCE-inducing activity of 5-nitro-DCB, 4.5 μg/ml was as high as that of MeIQx, 4.6 μg/ml. APNH, which was used as a positive control, significantly increased SCE induction at a dose of 0.005 μg/ml in the presence of S9 mix, and this result was consistent with that of our previous report [22].

Table 2
SCE-inducing activity and mutagenicity data of 5-nitro-DCB, DCB, PBTA-1, PBTA-2, PBTA-6 and MeIQx in the presence of S9 mix.

Sample	SCE-inducing activity (μg/ml)[a]	Mutagenicity (revertants/μg)[b]	
		TA98	YG1024
5-Nitro-DCB	4.5	8,700	24,200
DCB	13.9	100	3,400
PBTA-1	8.4	88,000	3,000,000
PBTA-2	8.4	93,000	3,200,000
PBTA-6	6.9	17,900	485,000
MeIQx	4.6	117,000	1,400,000

[a] Concentration leading to a two-fold increase relative to control level.

[b] Data from reference [6,9,12,17,38].

3.2. SCE induction by the water concentrates from the Waka River

The dose-response effects of SCE induction for three water concentrates obtained from the Waka River are shown in Table 3. For all water concentrates, dose-related increases in the frequencies were found between the concentration of 6.25 and 18.75 ml eq/ml of medium in the presence of S9 mix. The highest SCE frequency, 20.84 ± 5.08, was detected for river water concentrate No. 2 at the dose of 18.75 ml/ml, but this concentrate showed toxicity at a higher dose.

3.3. Concentrations of DCBs in the river water and mutagenicity in Salmonella

Table 4 shows amounts of 5-nitro-DCB and DCB in the three water samples from the Waka River and mutagenicity of water concentrates toward *S. typhimurium* YG1024 in the presence of S9 mix. 5-Nitro-DCB and DCB were detected in all the water samples. The concentrations of 5-nitro-DCB and DCB in the river water samples were from 2.5 to 19.4 ng/l and from 4100 to 18,900 ng/l, respectively. The three water concentrates showed potent mutagenicity in YG1024, and the highest activities was detected for the water concentrate No.2. The percent contributions of DCB to the mutagenicity of the river water concentrates were from 8% to 26%, but those of 5-nitro-DCB were less than 1%.

4. Discussion

In the present study, we evaluated the genotoxic effect of the water pollutants 5-nitro-DCB, DCB, PBTA-1, PBTA-2, and PBTA-6, and river water samples, which included 5-nitro-DCB and DCB as constituents, using SCEs in CHL cells. In addition, SCE induction of MeIQx was examined to compare genotoxic activities of these water pollutants. MeIQx was deduced to be possibly carcinogenic to human (Group 2B) by the International Agency for Research on Cancer (IARC) [28], and it has the structural features of an aromatic amine similar to those of DCBs and PBTAs. MeIQx was reported to induce SCEs in human lymphocyte cultures and to show mutagenicity in CHL cells for diphtheria toxin resistance in the presence of S9 mix [29,30]. As shown in Table 1, the dose-response effects of SCE induction were detected for DCBs, PBTAs, and MeIQx at doses from 1.25 to 10 μg/ml or from 1 to 20 μg/ml with S9 mix, and the increases of SCEs were statistically significant at almost all doses tested. All chemicals tested in the present study induced SCE induction in cultured mammalian CHL cells in the presence of S9 mix. Among the composites tested, 5-nitro-DCB was found to have

Table 3
SCEs induced by river water concentrates in CHL cells in the presence of S9 mix.

Sample	Sampling date	Dose (ml/ml)[a]	SCEs per metaphase		
			MI (%)[b]	Mean ± S.D.	Range
Concentrate No. 1	14 September 2006	6.25	2.4	10.12 ± 3.50	3–18
		12.5	2.9	12.12 ± 3.94**	5–22
		18.75	2.5	16.16 ± 6.08**	6–28
		25	1.2	16.12 ± 6.35**	4–31
Concentrate No. 2	19 December 2006	6.25	3.2	12.44 ± 5.08**	5–25
		12.5	2.2	18.72 ± 5.95**	9–35
		18.75	1.5	20.84 ± 5.08**	13–37
		25	0	Toxic	Toxic
Concentrate No. 3	29 March 2007	6.25	2.8	10.64 ± 2.90	6–16
		12.5	2.4	14.00 ± 5.42**	6–28
		18.75	2.6	17.44 ± 6.87**	9–38
		25	2.1	16.36 ± 5.18**	10–29

SCE frequency for Control (DMSO) was 8.68 ± 4.28 (mean ± S.D.). SCE frequency for positive control: APNH (0.005 μg/ml) 21.44 ± 5.04 (mean ± S.D.).
[a] Dose is expressed as ml eq of river water per 1 ml of medium.
[b] MI; mitotic index. MI (%) was calculated by counting the number of mitotic cells among 1000 round nuclei.
** Significantly different from control, $p < 0.01$.

the most pronounced frequency of SCEs, showing the same level of MeIQx, although mutagenicity with *Salmonella* TA 98 and YG1024 of 5-nitro-DCB was relatively low compared with those of PBTA congeners and MeIQx as shown in Table 2. Moreover, SCE-inducing activity of 5-nitro-DCB was 1.5–2 times higher than those of PBTA congeners and was 3 times higher than that of DCB. A ranking of the SCE-inducing potency of these compounds is the following: 5-nitro-DCB ≈ MeIQx > PBTA6 > PBTA-1 ≈ PBTA-2 > DCB.

All water concentrates from the Waka River also showed dose-related increases in SCEs between the concentration of 6.25 and 18.75 ml eq/ml of medium in CHL cells with S9 mix (Table 3). SCE-inducing activities of water concentrates were from 13 to 24 ml eq/ml and a ranking of the water concentrates for SCE induction was concentrate No. 2 > 3 > 1. Both 5-nitro-DCB and DCB were detected in all water concentrates, but amounts of DCB in the water samples were about 1000-fold or higher than those of 5-nitro-DCB. Since DCB and 5-nitro-DCB showed similar SCE-inducing activity, and the amounts of DCB in the water samples were much higher than those of 5-nitro-DCB, the contribution of DCB to SCE-induction of river water concentrates may be larger than that of 5-nitro-DCB. However, contribution ratios of SCE activities based on the concentration of DCB and 5-nitro-DCB, respectively, to the total SCE activities by the river water concentrates were <3% and <0.01%, respectively. Some unknown compounds may be affecting SCE induction of the river water.

Mutagenicity of these river water concentrates was also examined by the Ames assay using YG1024 with S9 mix (Table 4). These concentrate showed potent activities, and percent contributions of DCB, i.e., 8–20%, was much higher than those of 5-nitro-DCB, which was <1%. These high percent contributions of DCB were caused by an abundance of DCB in the river water examined in this study. In a previous study, we quantified 5-nitro-DCB and DCB in blue rayon extracts from the Waka River water and detected relatively high amounts of 5-nitro-DCB [6]. The concentration of 5-nitro-DCB in the river water likely differs on sampling days. More quantitative investigations are necessary to estimate the effect of DCB and 5-nitro-DCB to the genotoxicity of the Waka River water.

In a previous study, we reported that blue rayon extracts from the water of the Yodo River system, Japan, collected in October and December, 1991, showed SCE induction in CHL cells with S9 mix [21]. PBTA-1, PBTA-2, and PBTA-6 were detected in the water samples collected from the same river system in 1994, 1995, and 1999, respectively [10,11,13]. Besides these three PBTAs, other PBTA congeners were continually detected in the water samples collected from this river system from 1994 to 2005 [15]. From synthesis

Table 4
Mutagenicity of water concentrates from the Waka River and amounts of 5-nitro-DCB and DCB.

Sample	Mutagenicity (revertants/l)[a]	Amount (ng/l)		Contribution ratio (%)[b]	
		5-Nitro-DCB	DCB	5-Nitro-DCB	DCB
Concentrate No. 1	246,200	4.8	18,900	<1	26.1
Concentrate No. 2	374,800	19.4	18,200	<1	16.5
Concentrate No. 3	179,300	2.5	4,100	<1	7.8

[a] Mutagenicity was examined in *S.typhimurium* YG1024 with S9 mix.
[b] The mutagenic potencies of 5-nitro-DCB and DCB used to calculate the contribution ratios were 24,200 and 3400 revertants/μg, respectively [6,7].

studies, PBTAs are thought to be formed from corresponding dinitrophenylazo dyes used in textile dyeing factories and released into the river system. The SCE induction by the blue rayon extracts from the Yodo River system might be due to PBTA congers.

Besides 5-nitro-DCB and DCB, three dichlorobiphenyl derivatives, i.e., 4-amino-3,3′-dichloro-5,4′-dinitrobiphenyl (ADDB), 3,3′-dichloro-4,4′-dinitrobiphenyl, and 4-amino-3,3′-dichloro-4′-nitrobiphenyl, which were mutagenic in YG1024, were detected in water samples collected from the Waka River in 2003–2004 [8,9]. These DCB derivatives are thought to be formed from DCB, like 5-nitro-DCB. DCB was positive in some *in vivo* genotoxicity assays, e.g., the chromosomal aberration test [31], the micronucleus assay [32], and the alkaline single cell gel electrophoresis assay (comet assay) [33]. Moreover, DCB is carcinogenic in mice, rats, hamsters, and dogs [34], and it has been designated a probable human carcinogen (Group 2B) by IARC [35]. PBTA-1 and PBTA-2 induced micronuclei in Chinese hamster cell line V79-NZ [36]. PBTA-6 and ADDB induced micronuclei in gill cells by i.p. injection into goldfish [37]. Furthermore, DNA damaging activity was detected for PBTA-6 and ADDB in peripheral erythrocytes of goldfish *in vivo* by the comet assay [37].

Our results indicate that various dichlorobiphenyl derivatives and PBTA congeners were detected in the water of the Waka River and the Yodo River system, respectively. Except for DCB, biological activities of these water pollutants have been evaluated mostly by the Ames assay, and data on biological effects of these compounds, including genotoxicity in mammalian cells and *in vivo*, are quite limited. To estimate risks of these compounds to aquatic biota and human health, further investigations on their biological activities to aquatic organisms and experimental animals are necessary. In addition, quantitative studies on these compounds in these rivers are important, and exposure levels of aquatic organisms and human

to those compounds need to be determined. Because aquatic organisms inhabiting in these rivers may be exposed chronically to these genotoxic chemicals, ecological studies, including the incidence of cancer in fish and aquatic animals, are also needed.

Conflicts of interest

None.

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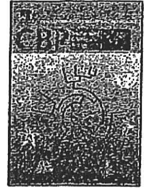

Molecular cloning of apoptosis-inducing Pierisin-like proteins, from two species of white butterfly, *Pieris melete* and *Aporia crataegi*☆

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ABSTRACT

Pierisin-1, present in cabbage butterfly, *Pieris rapae*, induces apoptosis against various kinds of cancer cell lines. Another cabbage butterfly, *Pieris brassicae*, also has an apoptosis-inducing protein, Pierisin-2. These proteins exhibit DNA ADP-ribosylating activity. Pierisin-like proteins are found to be distributed in subtribes Pierina, Aporiina and Appiadina. In this study, we performed the cDNA cloning of Pierisin-like proteins designated Pierisin-3 from gray-veined white, *Pieris melete*, and Pierisin-4 from black-veined white, *Aporia crataegi*. The nucleotide sequences of Pierisin-3 and -4 encode an 850 and an 858 amino acid protein, respectively. The partial peptide sequences of Pierisin-3 and -4 purified from pupae were identical to the deduced amino acid sequence of ORF. The deduced amino acid sequence revealed that Pierisin-3 is 93% similar to Pierisin-1 and Pierisin-4 is 64%. Pierisin-3 and -4 synthesized *in vitro* with the rabbit reticulocyte lysate exhibited apoptosis-inducing activity against human cervical carcinoma HeLa and human gastric carcinoma TMK-1 cells. Site-directed mutagenesis at a glutamic acid residue comprising the NAD-binding site resulted in a significant decrease in cytotoxicity of both proteins. Moreover, the proteins incubated with calf thymus DNA and β-NAD resulted in the formation of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine, as in the case of Pierisin-1 and -2. These findings could provide useful information for understanding the importance of apoptosis-inducing ability and molecular evolution of Pierisin-like proteins in family Pieridae.

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1. Introduction

Pierisin-1, an apoptosis-inducing protein, has been identified from the cabbage butterfly, *Pieris rapae* (Koyama et al., 1996; Watanabe et al., 1998). Pierisin-1 has potent cytotoxicity against various human cancer cell lines, with 50% inhibitory concentration values ranging from 0.043–150 ng/ml (Kono et al., 1999). The amino acid sequence of this protein deduced from the cDNA contains 850 amino acids with a calculated molecular weight of 98,000. Amino acid alignment indicates that Pierisin-1 is 32% similar to the mosquitocidal toxin (MTX), a kind of ADP-ribosyltransferase from *Bacillus sphaericus* SSII-1 (Watanabe et al., 1999; Carpusca et al., 2006).

ADP-ribosylating toxins, such as pertussis and cholera toxins, target α-subunits of G-proteins (Zhang et al., 1995; Locht and Keith, 1986) while diphtheria toxin ADP-ribosylates a diphthamide residue on elongation factor 2 (Bell and Eisenberg, 1996). MTX ADP-ribosylates proteins with molecular masses of 42 and 38 kDa in lysates of mosquito *Culex quinquefasciatus* G7 cells (Thanabalu et al., 1993) and *Escherichia coli* elongation factor Tu (Schirmer et al., 2002). ADP-ribosyltransferases also exist in mammals. For example, mammalian ectoenzyme ART2-catalyzed ADP-ribosylation of cell membrane proteins induces formation of cytolytic membrane pores by activating the P2X7 purinoceptor (Koch-Nolte et al., 2008). On the other hand, Pierisin-1 modifies N^2 amino groups of guanine residues in DNA to yield N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine (Takamura-Enya et al., 2001). The 27-kDa N-terminal region of Pierisin-1 has DNA ADP-ribosylating activity, and the 71-kDa C-terminal region binds to glycosphingolipid receptors such as globotriaosylceramide (Gb3) and globotetraosylceramide (Gb4) on the surfaces of mammalian cells and incorporates Pierisin-1 into cells (Kanazawa et al., 2001; Matsushima-Hibiya et al., 2003). The mRNA of pierisin-1 is highly expressed in the fifth instar larvae, and the protein is accumulated in the fat bodies during the fifth instar larvae to early pupae (Watanabe et al., 2004). Therefore, Pierisin-1 may act to remove the unnecessary cells of larval tissues in the pupation. Moreover, Pierisin-1 may have the ability to protect from natural enemies, such as parasitic wasps, by exhibiting potent cytotoxicity in *P. rapae*.

The other cabbage butterfly, *Pieris brassicae*, also contains an apoptosis-inducing protein named Pierisin-2. cDNA of Pierisin-2 encodes 850 amino acids. The amino acid sequence deduced from the cDNA shows that Pierisin-2 is 91% similar to Pierisin-1. Pierisin-2 exhibits cytotoxicity similar to Pierisin-1 (Matsushima-Hibiya et al., 2000). Pierisin-2 catalyzes

Abbreviations: Gb3, globotriaosylceramide; Gb4, globotetraosylceramide.

☆ Database: The nucleotide sequences of pierisin-3 from *Pieris melete* and pierisin-4 from *Aporia crataegi* have been submitted to the DDBJ/EMBL/GenBank database under the accession numbers AB477051 (pierisin-3) and AB477052 (pierisin-4).

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ADP-ribosylation of dG in DNA to give the same reaction product as demonstrated for Pierisin-1 (Takamura-Enya et al., 2004).

Recently, we reported the distribution of Pierisin-like proteins in the subfamily Pierinae, family Pieridae (Matsumoto et al., 2008). Protein extracts from 13 species of butterflies in this subfamily exhibited DNA ADP-ribosylating activity and cytotoxicity against the human cervical carcinoma HeLa and gastric carcinoma TMK-1 cells. All of these extracts contained substances recognized by anti-Pierisin-1 antibodies. Moreover, sequences containing NAD-binding sites, conserved in ADP-ribosyltransferases, were amplified from genomic DNA from 13 species of butterflies by PCR. However, three species of butterflies in the subfamily Pierinae and four species of butterflies in the subfamily Coliadinae, the family Pieridae showed neither cytotoxicity nor ADP-ribosylating activity, and did not contain substances recognized by anti-Pierisin-1 antibodies. Sequences containing NAD-binding sites were not amplified from the genomic DNA from these seven species (Matsumoto et al., 2008). Thus, Pierisin-like proteins are distributed in butterflies not only of the subtribe Pierina, including the genus *Pieris*, but also of the subtribes Aporiina and Appiadina. Interestingly, the subfamily Pierinae is divided into two types by the morphology of the pupal stage (Braby et al., 2006), and this classification of pupal morphology corresponds with the distribution of Pierisin-like protein, except for that in *Appias lyncida* (Matsumoto et al., 2008).

In this study, we report the cDNA cloning of apoptosis-inducing proteins named Pierisin-3 from the gray-veined white, *Pieris melete*, and named Pierisin-4 from the black-veined white, *Aporia crataegi*. These two species belong to subtribes Pierina and Aporiina respectively, and these extracts had been shown to have Pierisin-like activity (Matsumoto et al., 2008). The deduced amino acid sequence from the cDNA indicated that Pierisin-3 and -4 have a close amino acid similarity to Pierisin-1 and -2. Both *in vitro* expressed proteins exhibited cytotoxicity and DNA ADP-ribosylating activity similar to Pierisin-1 and -2. The evolutionary conservation for amino acid sequences of Pierisin-3 and -4 to compare with Pierisin-1 and -2 is discussed.

2. Materials and methods

2.1. Insects and RNA isolation

The fifth instar larvae of *P. melete* were collected in the Tochigi Prefecture, Japan, and were reared on natural host plants, cabbage, *Brassica oleracea*, at room temperature until pupation in our laboratory and stored at −80 °C until use. The fifth instar larvae of *A. crataegi* were purchased from the Eikoh Science Corp. (Osaka, Japan), and were reared on natural host plants, Yoshino cherry, *Prunus yedoensis*, at room temperature until pupation in our laboratory and stored at −80 °C until use. Some fifth instar larvae of *P. melete* and *A. crataegi* were frozen at day 1 and stored at −80 °C for the preparation of total RNA. Total RNA was prepared from the whole body of a day 1 fifth instar larvae by using Micro-to-Midi Total RNA Purification System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The 5′ RACE and 3′ RACE were performed by using GeneRacer™ Kit (Invitrogen) according to the manufacturer's instructions and 1 µg of total RNA as starting material. The 5′- and 3′- phosphate groups of RNA were dephosphorylated, the 5′-cap structures of full length mRNA were replaced by GeneRacer™ RNA Oligo by oligo-capping method (Maruyama and Sugano, 1994). Then, first-strand cDNA synthesis was carried out with SuperScript® III Reverse Transcriptase using GeneRacer™ Oligo dT Primer.

2.2. cDNA cloning of Pierisin-3 and -4

For cDNA cloning of Pierisin-3 and Pierisin-4 from *P. melete* and *A. crataegi*, respectively, the sequences containing NAD-binding sites, which are conserved in ADP-ribosyltransferase were cloned. Then, the full length of cDNAs was obtained by PCR, as described below.

The sequences containing NAD-binding sites were amplified using degenerate primers PierisinDP_NF1 and PierisinDP_NR1 deduced from the Pierisin-1 and -2 amino acid sequences (DDBJ accession numbers AB030305 and AB037676) (Watanabe et al., 1999; Matsushima-Hibiya et al., 2000). The PCR was performed for 2 min at 98 °C followed by 35 cycles of 10 s at 98 °C, 20 s at 58 °C, 1 min at 72 °C, and a final extension for 7 min at 72 °C by using Phusion™ High-Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) in iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The PCR products were purified by Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). The purified PCR products were cloned into pCR®-Blunt II-TOPO® vector (Invitrogen), and were then sequenced in both directions, using ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with DYEnamic ET Terminator Cycle Sequencing Kit (GE Healthcare, Amersham Place, England).

In Pierisin-3, Pm_F5 primer and GeneRacer™ 3′ Nested Primer were used for 3′ RACE and Pm_R2 primer and GeneRacer™ 5′ Nested Primer were used for 5′ RACE. The PCR was performed for 2 min at 98 °C followed by 35 cycles of 30 s at 98 °C, 30 s at 58 °C, 1–4 min at 72 °C, and a final extension for 7 min at 72 °C. The PCR products were excised from the agarose gel and purified by using Wizard® SV Gel and the PCR Clean-Up System, and then the PCR fragments were cloned into the pCR®-Blunt II-TOPO® vector or StrataClone™ PCR Cloning Vector pSC-B (STRATAGENE, La Jolla, CA, USA) and sequenced. The full length of cDNA was obtained by PCR using forward primer PmF_F and reverse primer PmF_R. The amplified PCR product was purified by Wizard® SV Gel and PCR Clean-Up System and subjected to the PCR direct sequencing to determine the full length cDNA sequence.

In the case of Pierisin-4, Ac_F1 primer and GeneRacer™ 3′ Primer were used for 3′ RACE and Ac_R3 primer and GeneRacer™ 5′ Nested Primer for 5′ RACE were used. Moreover, the full length of cDNA was obtained by the PCR using forward primer AcF_F and reverse primer AcF_R. The amplified PCR product was purified and sequenced in both directions as in Pierisin-3.

2.3. Construction of clones of the coding region of the Pierisin-3 and -4 genes

The coding region of the *Pierisin-3* gene, with 442 bp of 3′-flanking region, was amplified, and *Sal*I and *Eco*RI restriction sites were introduced into the 5′ and 3′ ends, using the primer pair Pm_Sal and Pm_Eco. The amplified fragment was digested with *Sal*I and *Eco*RI and inserted into the *Sal*I-*Eco*RI site of pMAL-p2x (New England Biolabs, Ipswich, MA, USA) in the opposite direction to the *tac* promoter. In the same way, the coding region of the *Pierisin-4* gene, with 355 bp of 3′-flanking regions, was amplified, and *Nhe*I and *Sac*I restriction sites were introduced into the 5′ and 3′ ends, using the primer pair Ac_Nhe and Ac_Sac. These clones were confirmed by sequencing the insert in both directions.

2.4. Purification and amino acid sequence determination of Pierisin-3 and -4

Because the chromatographic characteristics of *Pierisin-3* from *P. melete* were expected to be similar to *Pierisin-1*, purification of *Pierisin-3* from pupae of *P. melete* was carried out using the same method for *Pierisin-1* (Watanabe et al., 1998, 1999). The crude extracts from 112 pupae were added with ammonium sulfate to give 35% saturation and then centrifuged. The precipitates were dialyzed and applied to an anion-exchange DEAE-cellulose DE52 column (2.5×7 cm; Whatman, Kent, England). The fractions containing the cytotoxicity were separated by SDS-PAGE. About 98-kDa bands of these fractions were excised and digested in gel with trypsin. The digested peptides were separated by reverse-phase-HPLC with a Symmetry® C18 3.5 µm column (1.0×150 mm; Waters, Milford, MA, USA). Amino acid sequences of the isolated peptides were determined by a Procise 494 cLC Protein Sequencing System (Applied Biosystems).

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Pierisin-1                              MADRQPYMTNGIQAAVVEWIRALDLEIISLLLSRAWPMALLATSELRWRPT   51
Pierisin-2                              MSNNPPYMTNGIQAAVVEWIRALDLEIISLLLSRAWPLALLTTTELRWRPT   51
Pierisin-3                              MADRPPYMTNGIQAAVVEWIRALDLEIISLLLSRAWPMAILGISELRWRPT   51
Pierisin-4                        MPKPDDGRAPPPEITNGVLAAVVAWIRFVNLEVINLYLTRNWPQSLLGVSEPRWRPI   57
MTX-1        MAIKKVLKIILAIIIIISCQLPLNQKTVYASPNSPKDNTWIQAASLTWLMDMSSLLYQLISTRIP..SFASPNGLYMREQ   78

Pierisin-1   VLTDTDNVVRLDRRQRLVRWDRRPPNEIFLDGFVPIVTREN.PDWEETDLYGFAKNNHPSIFVSTTKTQ.RN..KKKYVW  127
Pierisin-2   VLTDTDNVVRLDRRQRLVRWDRRPPNEIFLEGFVPIVTRED.PDWEETDLYGFAKNNHPSIFVSTTKTQ.RN..KKKYVW  127
Pierisin-3   VLTDTDNVVRLDRRQRLVRWDRRPPNEIFLNGFVPIVTRED.PDWEETDLYGFAKNNHPSIFVSTTKTQ.RN..KKKYVW  127
Pierisin-4   EVRDTDNVVRLDRRQRLVRWDRRPPNEIFLNGFVPIVTNEN.PDWSQTDLYNFAKSNVPSIFVSTTKTQFKK..NGKYVW  134
MTX-1        TIDSNTGQIQIDNEHRLLRWDRRPPNDIFLNGFIPRVTNQNLSPVEDTHLLNYLRTNSPSIFVSTTRARYNNLGLEITPW  158
                               *                                           ***

Pierisin-1   TPRNANRGIVYQYEIYAPGGVDVNDSFS.DASPWPNQMEVAFPGGIQNIYIRSARELHNGRIQRIWINPNELDPGDLEPI  206
Pierisin-2   TPRNANRGIVYQYEIYAPGGVDVNDSFS.DASPWPNQMEVAFPGGIQNIYIRSARELHNGRVQRIWINPNFLDPGDLEPI  206
Pierisin-3   TPRNANRGIVYQYEIYAPGGVDVNDSFS.DASPWPNQMEVAFPGGIQNIYIRSARELHNGRVQRIWINPNFLDPGDLEPI  206
Pierisin-4   TPRSANRGIVYQYEIYAPGGLDVNESLS.EQSPWPNQMEVAFPGGIQNVYIRSARELHDGRVQRIWINPNFLDPNELAPI  213
MTX-1        TPHSANNNIIYRYEIFAPGGIDINASLSRNHNPFPNEDEITFPGGIRPEFIRSTYEYHNGEIVRIWINPNFINPSTLNDV  238
                                                   *

Pierisin-1   VSSSRTPQVIWRMNHPDGGHRDQRSERSASSY..DDLMYGGTGNVQEDTFGDEPNNPKPIAAGEFMIESIKDKNSFLDLS  284
Pierisin-2   VSSSRTLQVIWRVNHPDGGNKDGRSERSTSSY..DDLMYGGTGNVQEDTFGDESNNPKPIADGEFMIESIKDKNSFLDLS  284
Pierisin-3   VSSSRTLQVIWRIDHPDGGNKDERSERSTSSY..DDLMYGGTGNVQEDTFGDESDNPKPIAAGEFMIESIKDKNSFLDLS  284
Pierisin-4   ACSSRTPQVMWRENHPDGGNKD..SGRAQISP..DELMYGGDGDVVEDPFDNETDNAQPFPNGQFMIESVLNDNYFLDLA  289
MTX-1        SGPSNISKVFWHENHSEGNNMDSKGFILDLDYNQDFDMFAPNGEIPNNNLLNN.NSLNVIQNSEYQIKNKKDRNIVVTLD  317

Pierisin-1   KNVNGGVIHSNLYSGG.DNQIWVFSYDDNKKAYRIQSYQNSYLYLSWDSNASSKEMILRGYTNSGSNNQYWQIEQTGKN.  362
Pierisin-2   KNVNGGIIHSNVYSGG.DNQIWVFSYDDNKKAYKIKSYQNSSLYLSWDSNASSKEIILRGYTNSGSNNQYWQIEQTGKN.  362
Pierisin-3   KNVNGGIIHSNVYSGG.NSQIWVFSYDDNKKAYKIKSYQNSSLYLSWDSNASSKEMILRGYTNSGSNNQYWKIDQTGRN.  362
Pierisin-4   QNKQGGIVHSHAYNGAWLNQRWEFIYDSSKKAYKIKSKLNANLLLSWDSNANPKEMILRGYTVSGSNNQYWRIEHVNDEC  369
MTX-1        SDYGGSPVESYKNFGF.ENQKWNIKYDSKKNAYKIYNRETPTLLLSWNSNSSNGEQVIRGYTESGSNNQYWTIEKNVNGF  396
                                +   +                                              +   +

Pierisin-1   YRLRNLLNLDMLITAQDKPSAFGGKEVIVNTEISNSNTKISQEWKMIPFDERPIIDGDYNIENVDLSNQVVDFSNQPDLL  442
Pierisin-2   YRLRNLLNLNMLITAQDKSSAFGGKEVIVNTEISNSNTKSSQEWNIIPFDERPIMDGDYNIENLDLPNQVVDFSNQPDLL  442
Pierisin-3   YRLRNLLNLDMLITAQDKSSAFGGKEVIVNTEITNSNTKISQEWKMIPFDERPIMDGDYNIENLDLSNQVVDFSNQPDLL  442
Pierisin-4   YRLRNLENLNMIVTVQNNFNLLGGKEVLVESGISTSGKDNNQKWRMRPVSEQVIPDGNYRIENCKEPDIVVDYSNQNDHL  449
MTX-1        YKFRNLSDPSKILDLKD.GNTLNKTPLVVSSE....NSSSSQEWLIEKTNYQTVKDGTYQVSSKLNENKVIEQISTNKIH  471
                                                      +   +

Pierisin-1   VHGHIFCDNENQTWHFTYNSTYHAYKIWSGRKSNLLLTWDSNAASKEMVVRAYTESRSKNQYWRIEQTGSKSYKVRNLEN  522
Pierisin-2   VHGHEFCDNENQTWHFTYNSTYHAYKIWSGRKSNLLLTWDSNATSKEMVVRAYTESRSKNQYWRIEQTGSKSYKLRNLEN  522
Pierisin-3   VHGHEFCDNENQTWHFTYSSTHHAYQIWSGRKSNLLLSWDSNAASKEMVVRAYTESGSKNQYWRIEPTGSKSCKLRNLEN  522
Pierisin-4   IHGHSFLDSNSQEWRFTYDDKKESYKIISGLNSSLFLSWDSNANPKEVILRAYTNSGSNNQYWRIEQNEDGSYKLRNMAD  529
MTX-1        IFSN..SDKENQVWNLIYNPILKAYKIKSLKYPNYSLAWDSN..NTRTIVAA.TGD.YNDQYWLIERNEDNTYIIRNYEN  545
                        +   +                                          +   +

Pierisin-1   SSMILGLT.RVSTPYGGLNLMVEDDSDGHSDLHSDWDIKPIFYQDIPDGDYNIENDNFPNIAIDFTNQEGSLIHGHNFCS  601
Pierisin-2   SNMILGLT.NVSTSYGGLNLMVQDDSNGNSNLHSDWDIKPIIYQYVPDGDYNIENDNFPNIAVDYTNQEGALVHGHNFCS  601
Pierisin-3   SSMILGLS.KVSTSYGGLNLMVEDDSDGHSNLHSDWDIKLIFYQYIPDGDYNIENDNFPNIVVDYTNQEGALIHGHNFCS  601
Pierisin-4   LSLIMQLDYQKNSPHGGLNLIVHNDSKDYPNLYPNWKIVLVSYKCIPDGNYNIENRHMPNIVIDSSNQKDALIHGHAYCA  609
MTX-1        RKIVLDLS.NGSTT.DGNGLL...GFEFHGGINQRWIIKPFSFNSIQDGIYQFMTVINQDLIADLTTNNYTIATKTNNYS  620
                                                   +

Pierisin-1   NNNQKWSFVFDGKRKAYRIKSGVRSNLWLSWDSNASSKEMVLRAYTESGSSNQYWRLDEANDGSYRIRNLQDYYKLIALT  681
Pierisin-2   NNNQKWSFVYDGKKKAYKIKSGVNSKLWLTWDSNASSKEMVLRAYTESGNWNQYWRLYQANDGSYIIRNLKEFKMLIALT  681
Pierisin-3   NNNQKWSFVYDGKRKAYRIKSGVRSNLWLSWDSNASSKEMVLRAYTESGSSNQYWRLDPANDGSYGIRNLQDFKKLIALT  681
Pierisin-4   NNNQKWSFVYDENKKAYKIKSGINSALWLSWDSNAASKEILLRAYTESGSLNQYWRIELTNDGSYKLRNLLNLEKIIAVV  689
MTX-1        .SNQKWTVTYNDKKRAYKIRNLQHAHLSLAWDSNHSDK..IFGA..TGDYDDQYWIPILQTDGSFIFRNYKNPNKIFG..  693
                +   +                                          +   +

Pierisin-1   NKNTPYGGKELIVSD.NKESGNTWYLKKLGEVPLPNRKFRIATKLNYKKVID.SSTSYNLIITHDLNFASSIWELVYDSS  759
Pierisin-2   NIDTPYGGKQLIVTD.TKESGNHWYLKKLGEVPLPNRKFRIATKLNYKKVID.SSTAYNLIITHDLNFASSIWELVYDSN  759
Pierisin-3   NKNTPYGGKELIVSA.NKESGNTWYLKNLGEVPLPNRKFRIATKLNYKKVID.SSTSYNLIITHDLNFASSIWELVYDSS  759
Pierisin-4   NKNSPYGGKELIVSD.SNEGWSNWYLNRIGEVVIPDGKYRIATKVNYKKVID.YNRDRNLVIMDNINLASSEWEIKYDST  767
MTX-1        TNGQPINDIPLKAQDVTGQNNQKWYLRHLNSSNNFTGYFNISSKKNFNKIITMNSNKTQAVIFDNIGINNQSWKLKYNDN  773
                                   +                                               +

Pierisin-1   KKAYNIYSSDINNLGWIYQNKNFFVKLGNIDGPDHGDLRYFWTIEYSMQTGCYLIRSLHDPANA.....VGYTDSESVIT  834
Pierisin-2   KKAYNIYSADINNLGWVYQNKNFFVKLDNIDGPDHGDLRYFWTIEYSMQTGCYLIRSLYDPAHA.....VGYTDKDSVIT  834
Pierisin-3   KKAYNIYSSDINNLGWIYQNKNFFVKLGNIDGPDHGDLRYFWTIEYSMQTGCYLIRSLYDPAHA.....VGYTDNDSIIA  834
Pierisin-4   KKAYNIYSGQYFNIGWIYQNKNEYVKVDNIDGTDHGDSRYFWIIEYSIQYGCYMIRSLYDPSHA.....VGYSNNNFVVT  842
MTX-1        KNAYQIH..ILDNFLYFQGGHNIVATMQNV...TNDDLRSYWYVEYNFNKDGFIIRNAFDTSYVLDVFQGNFANNTPIIT  848
                                                 +

Pierisin-1   DTSTYSDNQLFHFILM                                                                  850
Pierisin-2   DTSTYSDNQLFHFILM                                                                  850
Pierisin-3   DTSTYSDNQLFHFILM                                                                  850
Pierisin-4   DTSTYSDNQLFHFIPI                                                                  858
MTX-1        YQNYLNDNQLWNFIPSLGVEPR                                                            870
                     +
```

Pierisin-4 was isolated from the 19 pupae of *A. crataegi* using a method similar to that for *Pierisin-3*, except for the purification with Phenyl–Sepharose CL-4B hydrophobic interaction column (1.5×6 cm; GE Healthcare) after purification with DEAE-cellulose DE52 column.

2.5. *In vitro transcription and translation of Pierisin-3 and -4*

To express *Pierisin-3* and *-4* genes, *in vitro* transcription and translation were carried out as previously described (Watanabe et al., 1999). To obtain cDNA fragments containing a T7 promoter sequence at the 5′ end of the coding region, the primer pairs Pm_T7 and Pm_R3 for *Pierisin-3*, Ac_T7 and Ac_R2 for *Pierisin-4*, were used for the PCR. The amplified PCR products were transcribed using MEGAscript® T7 Kit (Ambion, Austin, TX, USA) and translated using rabbit reticulocyte lysate (Retic Lysate IVT™, Ambion). The translation efficiencies were confirmed by autoradiography of SDS-PAGE gels of [^{35}S]methionine incorporated products.

2.6. *Site-directed mutagenesis*

A DNA fragment containing a sequence alteration at the desired position was amplified from an intact cDNA subclone of Pierisin-3 and -4 with overlap PCR technique described previously (Matsushima-Hibiya et al., 2003; Nakano et al., 2006) To obtain overlapped 5′- and 3′- fragments of Pierisin-3, two separate PCR reactions were carried out using a 5′ primer Pm_T7 and 3′ primers PmD_R or PmQ_R for 5′-fragment, and 5′ primers PmD_F or PmQ_F and 3′ primer Pm_R3 for 3′-fragment. These mutations are that the glutamic acid residue at position 165 was replaced with aspartic acid or glutamine. The 5′ and 3′ fragments were mixed together and used as the template for a second round PCR to obtain full length mutated DNA fragments.

In the case of Pierisin-4, two separate PCR reactions were carried out using a 5′ primer Ac_T7 and 3′ primers AcD_R or AcQ_R for 5′-fragment, and 5′ primers AcD_F or AcQ_F and 3′ primer Ac_R2 for 3′-fragment. These mutations are that the glutamic acid residue at position 172 was replaced with aspartic acid or glutamine. The 5′ and 3′ fragments were mixed together and used as the template for a second round PCR to obtain full length mutated DNA fragments. The appropriate recombination was confirmed by DNA sequencing, and the resultant DNA was used as the template for the *in vitro* expression system described here.

2.7. *Analysis of cytotoxicity and DNA ADP-ribosylating activity of in vitro expressed proteins*

The cytotoxicity of the *in vitro* expressed proteins against HeLa cells and TMK-1 cells was examined by using WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (Dojindo Laboratory, Kumamoto, Japan)] cell proliferation assay as described previously (Watanabe et al., 1998; Kono et al., 1999). Apoptotic cells were stained with TdT-mediated dUTP-biotin nick-end labeling (TUNEL) assay using *in situ* Apoptosis Detection Kit (Takara Bio, Otsu, Japan) and Hoechst 33342 after the formalin fixation, and analyzed with a fluorescence microscope. DNA ADP-ribosylating activity was determined by HPLC analysis as previously described (Matsumoto et al., 2008; Nakano et al., 2006).

To confirm the structure of the ADP-ribosylated DNA, the reaction of the *in vitro* expressed proteins, DNA and β-NAD was performed and the detection of the reaction products was carried out as described previously (Nakano et al., 2006). Fifty microliters of the *in vitro* expressed proteins were incubated with 0.1 mg of calf thymus DNA, 2 µmol β-NAD in 1 ml of reaction buffer for 2 h at 37 °C. The DNA was recovered, digested by treatment with micrococcal nuclease and phosphodiesterase II, and treated with bacterial alkaline phosphatase. The reaction products were analyzed by HPLC with a LC-10A system (Shimadzu, Kyoto, Japan) armed with a SPD 10Avp photodiode array detector (Shimadzu) and a Develosil RPAQUEOUS column (4.6×250 mm; Nomura Chemical, Seto, Japan). These products were also analyzed by electrospray ionization-mass spectrometry using ZQ 2000 instrument (Micromass, Manchester, England) armed with an HP 1000 HPLC system (Hewlett-Packard, Palo Alto, USA).

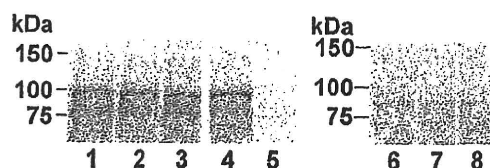

Fig. 2. Detection of *in vitro* expressed [^{35}S]methionine-labeled Pierisin-3 and -4, and their mutated proteins by SDS-PAGE. Five-microliters each of *in vitro* expressed lysates were loaded; lane 1, Pierisin-3 wild type; lane 2, Pierisin-3 E165D; lane 3, Pierisin-3 E165Q; lane 4, Pierisin-1 wild type; lane 5, lysate without template RNA; lane 6, Pierisin-4 wild type; lane 7, Pierisin-4 E172D; lane 8, Pierisin-4 E172Q.

3. Results

3.1. *Identification of Pierisin-3 in P. melete*

To obtain partial cDNA fragments of Pierisin-3, degenerate primers were designed from the conserved catalytic site motif of ADP-ribosylating protein. Total RNA was extracted from the fifth instar larva of *P. melete*. About 300-bp PCR products were cloned and sequenced to design the primer for 5′ and 3′ RACE. In addition, the PCR amplified Pierisin-3 coding sequence was inserted into the pMAL-p2x vector. The basal-level expression of the *Pierisin-3* gene would be highly toxic to *E. coli*, as expected from our studies of Pierisin-1 and -2, and we inserted the PCR product in the vector in an opposite direction to the *tac* promoter. The positive clones were sequenced and confirmed that the two of three clones had no nonsynonymous substitution. One of these two clones had less synonymous substitutions (G1516A, T2137C, T2176A). Thus, we concluded that the clone is an intact clone at the amino acid level, and it was employed for the Pierisin-3 expression *in vitro*.

The complete cDNA sequence of Pierisin-3 consists of 3347 bp associated with a putative initiator codon ATG at position 95–97, a stop codon at position 2645–2647 and a polyadenylational signal at position 3328–3333 close to the poly A sequence (Suppl. Fig. 1A). The ORF encodes 850 amino acids with a calculated molecular weight of 97,598. From the deduced amino acid sequence, Pierisin-3 is 93% similar to Pierisin-1 and 91% to Pierisin-2 (Fig. 1). Furthermore, the partial peptide sequences of Pierisin-3 purified from pupae in the same process as Pierisin-1 were identical to the deduced amino acid sequence of ORF. The identified three internal amino acid sequences, Glu91-Gly102, Gly135-Ala144 and Ser816-Gly825, are shown in Suppl. Fig. 1A (bold type with underlines). The essential regions for ADP-ribosyltransferase activity (Masignani et al., 2000; Domenighini

Fig. 1. Alignment of the deduced amino acid sequences of Pierisin-3 and -4. These sequences were aligned together with that of Pierisin-1 (DDBJ accession number AB030305), Pierisin-2 (DDBJ accession number AB037676) and MTX (DDBJ accession number M60446). Identical amino acid residues in Pierisins are highlighted in gray. The conserved arginine (Region I), Ser–Thr–Thr motif (Region II), and glutamic acid residues (Region III) for ADP-ribosylating activity and the QXW motifs for receptor binding are boxed in black with asterisks (*) or plus signs (+). Sequence alignment between Pierisins and MTX was decided referring to Watanabe et al. (1999) and Carpusca et al. (2006).

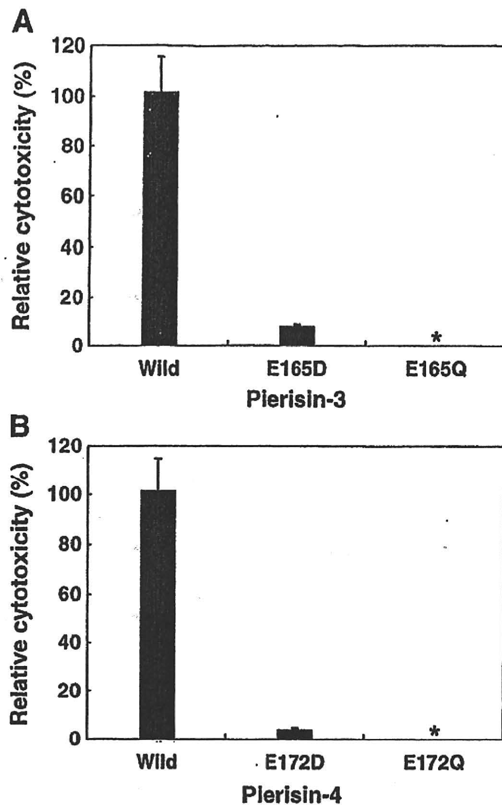

Fig. 3. Cytotoxicities of wild type and mutated Pierisin-3 or -4 against HeLa cells. Cells were incubated with a final concentration of 1% of rabbit reticulocyte lysate containing *in vitro* expressed Pierisin-3 (A) and Pierisin-4 (B) for 48 h at 37 °C and subjected to WST-1 cell proliferation assay. The cytotoxicity of mutated proteins relative to the wild type proteins is indicated. Asterisks show cytotoxicity not detected in this assay. Data are means of three independent experiments. Error bars represent standard deviations.

and Rappuoli, 1996), and ricin B chain-like QXW motif that binds glycosphingolipid receptors, such as Gb3 and Gb4, are conserved, as shown in Fig. 1 (Matsushima-Hibiya et al., 2003).

3.2. Identification of Pierisin-4 in *A. crataegi*

cDNA sequence and subcloning of Pierisin-4 were determined using the same method as that for the Pierisin-3. Three positive clones were sequenced and confirmed that all the clones had no synonymous nor nonsynonymous substitution. Thus, we concluded that these clones are intact clones, and one of the clones was employed for the Pierisin-4 expression *in vitro*. Pierisin-4 consists of 3319 bp associated with a putative initiator codon at position 77–79, a stop codon at position 2651–2653 and a polyadenylational signal at position 3293–3298 close to the poly A sequence I (Suppl. Fig. 1B). The ORF encodes 858 amino acids with a calculated molecular weight of 99,204. Amino acid alignment showed that Pierisin-4 is 64% similar to Pierisin-1, -2 and -3 (Fig. 1). Deduced amino acid sequence entirely encodes the partial peptide sequences of purified Pierisin-4 from pupae of *A. crataegi*. The essential regions for ADP-ribosyltransferase activity (Masignani et al., 2000; Domenighini and Rappuoli, 1996), and ricin B chain-like QXW motif are also conserved, as shown in Fig. 1 (Domenighini and Rappuoli, 1996).

3.3. Cytotoxicity and apoptosis-inducing activity of in vitro expressed Pierisin-3 and -4

Pierisin-3 and -4 were expressed by rabbit reticulocyte lysate, since it is impossible to express Pierisin-3 and -4 in *E. coli*, probably due to the high toxicity of the Pierisins. The translation efficiency and molecular mass of the *in vitro* expressed Pierisin-3 and their mutated products were confirmed by SDS-PAGE of [^{35}S]methionine-labeled proteins, and those of the *in vitro* expressed Pierisin-4 and their mutated products were also confirmed in the similar way (Fig. 2). To examine whether Pierisin-3 and -4 have cytotoxicity and apoptosis-inducing activity against mammalian cells, HeLa and TMK-1 cells were treated with *in vitro* expressed Pierisin-3 and -4. Both expressed proteins showed cytotoxicity (Fig. 3), and similar cytotoxicity of both expressed proteins was observed in TMK-1 cells (data not shown). Furthermore, the cytotoxicity levels of the *in vitro* expressed Pierisin-3 and -4 were almost equivalent to the *in vitro* expressed Pierisin-1. Both expressed proteins induced chromatin condensation and nuclear fragmentation indicating apoptotic cell death, in HeLa cells, as observed by fluorescence microscopy (Fig. 4). Glutamic acid residues, Glu165 in Pierisin-3 and Glu172 in Pierisin-4 (Fig. 1, boxed in black),

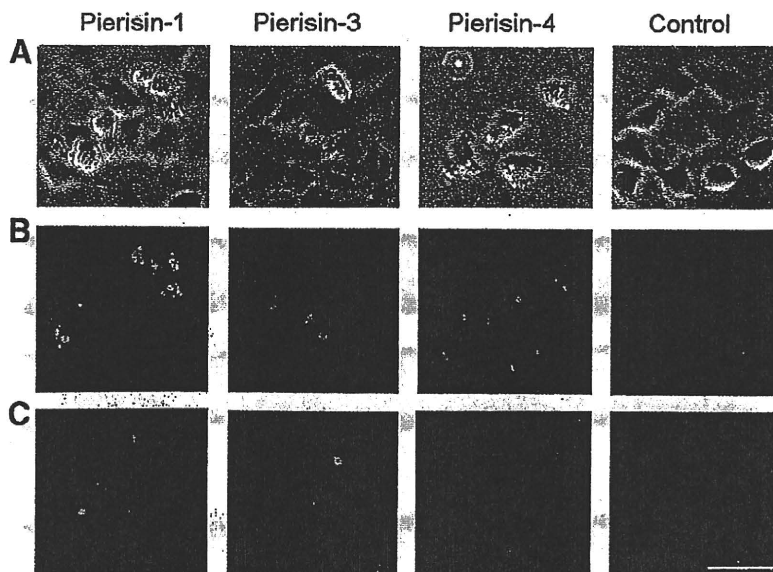

Fig. 4. Morphological analysis of HeLa cells treated with the *in vitro* expressed Pierisin-3 and -4 proteins. The cells were treated for 24 h with 1% of *in vitro* expressed proteins or 1 ng/ml purified Pierisin-1. (A) Phase-contrast micrographs for morphological changes analysis. (B) Fluorescence micrograph of TUNEL assays for detection of DNA fragmentation undergoing apoptotic cells. The TUNEL-positive cells were labeled by FITC. (C) Fluorescence micrograph of Hoechst 33342-stained cells. Bar = 50 μm.

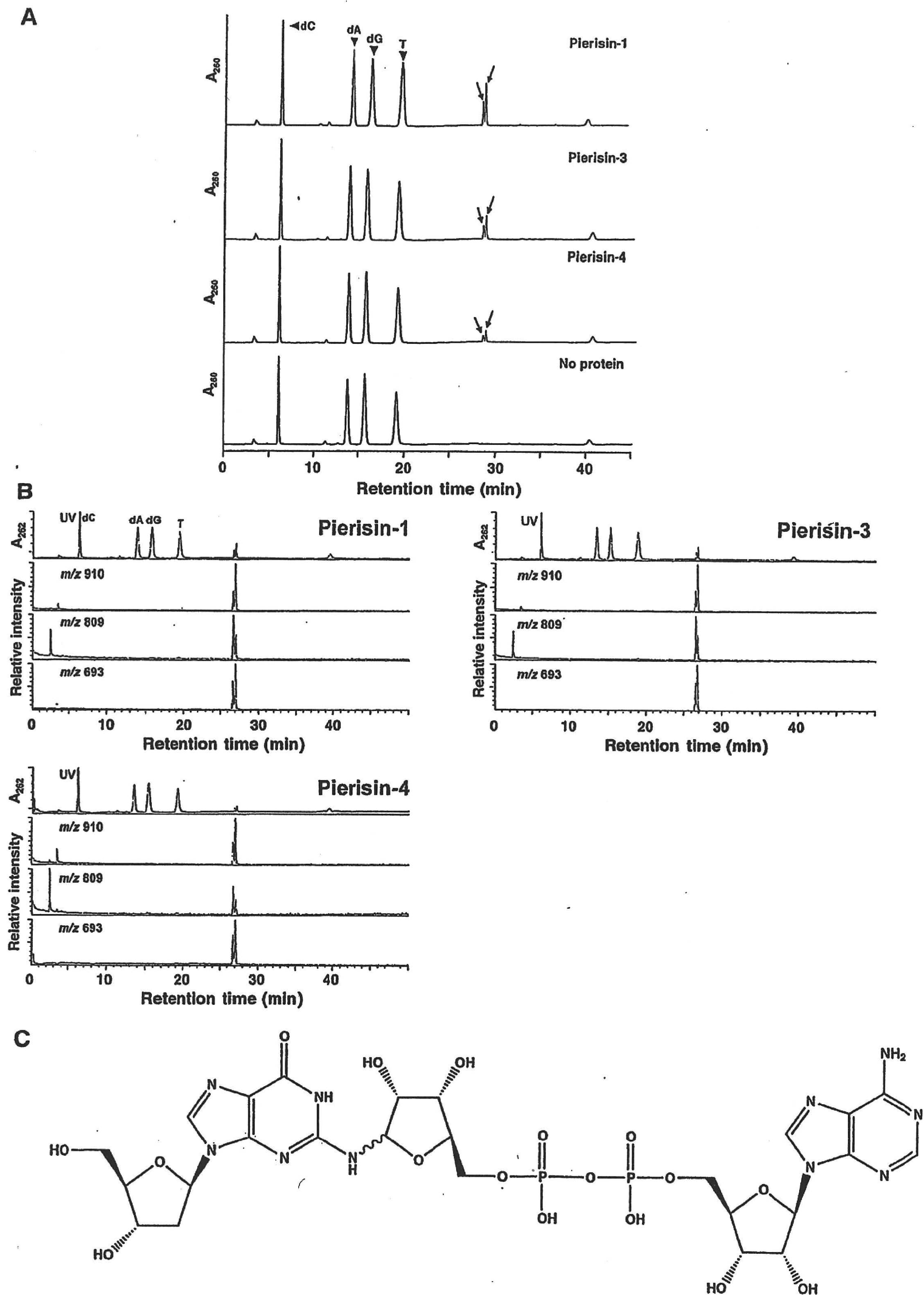

Fig. 5. Analysis of reaction products formed from DNA treated with Pierisins and β-NAD. HPLC elution patterns of hydrolysate of DNA incubated with purified Pierisin-1, *in vitro* expressed Pierisin-3, -4, or no protein. These DNA samples were enzymatically hydrolyzed to deoxyribonucleosides (dA, dC, dG and T) and injected into a Develosil RPAQUEOUS column. The UV absorbance of the eluate was monitored at 260 nm. Arrows indicate the peaks coincident with N^2-(ADP-ribos-1-yl)-2'-deoxyguanosine. Arrowheads indicate deoxycytidine (dC), deoxyadenosine (dA), deoxyguanosine (dG) and thymidine (T). (B) Liquid chromatography-electrospray ionization-MS analysis of reaction products formed from DNA and Pierisins. The HPLC profiles (UV) and the ion chromatograms (*m/z* 910, 809 and 693) of DNA hydrolysate are shown. (C) Chemical structure of N^2-(ADP-ribos-1-yl)-2'-deoxyguanosine.

were predicted to be the catalytic center for the ADP-ribosylating activity. As in the cases of Pierisin-1 and -2, site-directed mutation of Pierisin-3 and -4 by replacements of these glutamic acid by aspartic acid and glutamine resulted in markedly reduced or entirely lost cytotoxicity to HeLa cells, respectively (Fig. 3). Induction of nuclear condensation and chromatin fragmentation by mutated proteins were also reduced concurrently (data not shown).

3.4. Formation of N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine by in vitro expressed Pierisin-3 and -4

In order to investigate DNA ADP-ribosylating activity of the *in vitro* expressed Pierisin-3, this protein was incubated with calf thymus DNA and β-NAD, then the DNA was enzymatically digested, and the formation of the ADP-ribosylated DNA adducts was analyzed by HPLC. The chromatograms for the Pierisin-3 showed that the area of the dG peak decreased approximately 10%, while the area of dC, dA, and T peaks remained unchanged. In addition, two peaks corresponding to the ADP-ribosylated dG were newly detected at retention times of 28.5 and 29.0 min, as in Pierisin-1 (Fig. 5A). The λ_{max} of UV spectra of the newly detected peak fractions were 256 nm, as in the case for Pierisin-1. The UV spectra of these two peaks were exactly the same as those for the N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine (data not shown). The area of these two peaks was reduced with site-directed mutation of Pierisin-3 (data not shown). In addition, the LC-ESI-MS analysis showed the reaction products in this peak fraction to have a molecular ion peak at *m/z* 809, an ion peak at *m/z* 693 arising from the loss of a deoxyribose moiety, and an ion peak at *m/z* 910 corresponding to a triethylamine addition, derived from HPLC eluent, to the parent mass at *m/z* 809 (Fig. 5B,C). The results of these analyses showed that N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine was the reaction product generated through the reaction of the calf thymus DNA and the β-NAD with *in vitro* expressed Pierisin-3. Similarly, in the case of the Pierisin-4, the area of the dG peak decreased approximately 8%, and the newly detected two peaks at retention times of 28.5 and 29.0 min were shown to be N^2-(ADP-ribos-1-yl)-2′-deoxyguanosine by the UV spectra and the LC-ESI-MS analysis. (Fig. 5A,B).

4. Discussion

The protein extract from *P. melete* and *A. crataegi* exhibited cytotoxicity against HeLa cells and DNA ADP-ribosylating activity (Matsumoto et al., 2008). In the present study, we cloned the cDNA of apoptosis-inducing protein from *P. melete*, named Pierisin-3, and *A. crataegi*, named Pierisin-4. Deduced amino acid sequence of Pierisin-3 encodes 850 amino acids, and the *in vitro* expressed Pierisin-3 exhibited cytotoxicity against HeLa and TMK-1 cells. In addition, Pierisin-3 catalyzed ADP-ribosylation of 2′-deoxyguanosine residue in DNA to form N^2-(ADP-ribos-1-ly)-2′-deoxyguanosine. Pierisin-4 encodes an 858 amino acid, and Pierisin-4 protein also exhibited cytotoxicity and DNA ADP-ribosylating activity similar to the Pierisin-3 from *P. melete*.

In this study, the *in vitro* expressed Pierisin-3 and -4 were shown to have cytotoxicity and DNA ADP-ribosylating activity similar to Pierisin-1. The three highly conserved regions in the ADP-ribosyltranferase (Masignani et al., 2000; Domenighini and Rappuoli, 1996) and their surrounding amino acid sequence hold high homology in Pierisin-3 and -4 (Fig. 1). The arginine residue (Region I) is thought to maintain the structure of the reaction pocket, the Ser–Thr–Thr/Ser motif (Region II) is considered to construct a β-strand-α-helix structure, and the glutamic acid residue (Region III) serves as NAD-binding site.

Moreover, the C-terminal regions of Pierisin-3 and -4 consist of a ricin B chain-like domains including QXW motif (Fig. 1). These domains possess receptor-binding ability, responsible for incorporating Pierisin-1 into cells (Matsushima-Hibiya et al., 2003). Thus, Pierisin-3 and -4 would have the same abilities of receptor recognition and incorporation in mammalian cancer cell lines.

Pierisin-1, -2, -3 and -4 have similar cytotoxicity and DNA ADP-ribosylating activity. However, Pierisin-1, -2 and -3 slightly differ from Pierisin-4 in their amino acid sequence. Pierisin-1, -2 and -3 have 91% to 93% amino acid identity, while Pierisin-4 shares 64% amino acid identity not only with Pierisin-1 but also with Pierisin-2 and -3. Differences are also observed in chromatographic patterns of these proteins. On the DEAE-cellulose anion-exchange column chromatography, Pierisin-1, -2 and -3 were eluted with 40–70 mM NaCl, while Pierisin-4 was eluted with 12–35 mM NaCl (data not shown). Furthermore, on the Phenyl-Sepharose hydrophobic interaction column chromatography, Pierisin-4 was eluted with higher concentrations of ammonium sulfate (9–10%) compared to Pierisin-1 and -2 (≤1%). These results suggested that the isoelectric point and hydrophobicity of Pierisin-4 are different to some extent from those of Pierisin-1, -2 and -3, and these differences may reflect the genetic distance between the genera *Pieris* and the *Aporia*. Interestingly, MTX shares 32% amino acid identity not only with Pierisin-1, -2 and -3, but also with Pierisin-4. This suggests that the conserved regions among MTX and those four Pierisins should be the most important regions for the ADP-ribosylation/receptor binding, such as the ADP-ribosyltransferase to construct the NAD-binding core, and ricin B chain-like QXW motif for the receptor binding (Fig. 1, boxed in black). The regions with low homology between the Pierisins and the MTX might contain motifs for the recognition and targeting of DNA or protein.

In this study, we cloned cDNAs of Pierisin-3 and -4 from the total RNA of a day 1 fifth instar larva in *P. melete* and *A. crataegi*, respectively, because the mRNA of Pierisin-1 is highly expressed in *P. rapae* at this stage. Since the amounts of Pierisin-1 are at maximum from the fifth instar larva to early pupa, Pierisin-1 might play an important role in pupal metamorphosis (Watanabe et al., 2004). Pierisin-2, -3 and -4 have similar properties to Pierisin-1, and these proteins are also considered to play some role in the developmental stages, because of the larvae and the pupae of *P. brassicae*, *P. melete* and *A. crataegi* exhibiting higher cytotoxicity than the adults (Matsumoto et al., 2008). It is also possible that these proteins are protective against invading organisms, such as the parasitic wasps. Understanding the role of the Pierisins and investigation of the origin of these genes should provide information about the biological significance of the Pierisins in Pierina butterflies.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cbpb.2009.07.007.

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