それから先程出てまいりましたけれども、ファーマコゲノミクス、ファーマコジェネティクス、こういうものを利用した創薬というものもランプキン氏の理解する範囲では考えられるというようなことでございます。

以上で私の説明を終わりたいと思います。ご静聴有難うございました。

総合討論

（堀先生）

それでは、総合討論に移らせて頂きたいと思います。

時間が迫っておりますので、ご講演の内容について理解を深めるため、ご質問やコメントを頂きたいと思います。逐次フロアの方からご質問がありましたら、どうぞいつでも受け付けたいと思いますのでよろしくお願いいたします。

寒川先生はご自分のお仕事が新規のペプチドを発見して、それを生体の中でどういう働きをしているのかということを解明して、そしてそれを治療に使えないかというプロセスでやってきておられるわけです。しかもＡＮＰというのは循環器の治療に使われておりますし、ＢＮＰも心不全の診断薬として使われる。アメリカではＢＮＰが治療薬として使われています。寒川先生もご指摘になりましたように、まだわかっていないタンパク質のほうが多いわけですね。既知のものはごくわずかで、未知のもののほうがずいぶん多いということになりますと、網羅的にタンパクいわゆるプロテオームのライブラリーを片端から解析して、その中からその機能解析をしていくというのが一般的な流れになりつつあると思うのですけれども、実際に創薬に結びつける一番の早道というのは、寒川先生はどのように考えておられますか。

（寒川先生）

私自身はやはり臨床の先生方が、例えばこういった因子が関係あるのではないかといった一つの目安といいますか、ある程度焦点を絞らないと、むやみやたらにやっていって、それをコンピュータで処理するといった方法ではなかなかうまくいかないと思います。ですから、やはり臨床での情報、実際治療にあたられているドクターの情報と結びつけるということが非常に重要ではないかと思います。

（堀先生）

ということは、臨床の側からこういうタイプのものが有効なのではないかというヒントが出て来て、それに合うようなものを基礎研究の中から抽出していく、それで両者がドッキングすれば効率的に創薬が出来る。

（寒川先生）

それをもう少し拡大するような形で、解析自体をもっと効率的に出来るようにするというのが良いのではないかと思います。ですから、今までだと個人レベル或いは点と点みたいな形であったのをもう少し拡げるということが今回のプロテオームファクトリーの中でも必要ではないかと思います。ですから、単に病院或いは医療機関から試料を提供してもらうという形ではなしに、やはりその中で提供する側も単に提供するだけではなしに、やはりそれを調べてもらうことによって、何かわかるのではないかという積極性がないと、なかなか両方がうまくいかないのではないかと思います。

（堀先生）

ゲノムシークエンスの場合には、それがどういう機能を持っているかどうかは別にして、とにかく全て解読をして、その情報をオープンにして皆様に提供するということが、大変プラスになっているのですね。100％とはいえないにしても90％のゲノム情報が全部コンピュータで利

OPINION

DDS 19巻2号 平成16年

バイオ創薬の新たな展開と効果的な推進に向けて

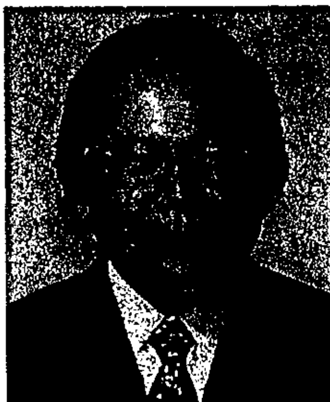
早川堯夫

現在は創薬史上に類のない時機である．ゲノム解読後のゲノム科学をベースにした創薬，幹細胞学の発展などをベースにした細胞治療や再生医療用の製品開発，その他の生命科学や先端技術の進展を背景にした創薬など，いずれも熾烈な国際的競争が展開されている．医薬品などが疾病の予防，診断，治療を通して保健衛生の向上に寄与するものであり，それゆえに人類に恩恵をもたらす共通の資産であるという本質を考えれば，創薬が国際競争により推進されることは，当然望ましいことである．わが国としては，科学技術立国を目指すということも含めて，米・欧に伍して，産・官・学あげてこの課題に取り組む必要がある．

創薬は，シーズ探索・発見と，およびそれをもとにした医薬品候補の探索・選択・最適化，製法の検討，品質・有効性・安全性評価という二つのステージに大別される．

ゲノム科学をベースにした創薬の場合，第1のステージは，疾患や薬物の作用，生体の恒常性維持に関係する新規遺伝子や蛋白質の探索とその機能解明である．このためには，各種ゲノミクス，プロテオミクス，バイオインフォマティクスなどの包括的・網羅的なアプローチや，これらにより絞り込み，推定された遺伝子や蛋白質機能の実証的な解析・確認が必要とされる．しかし，キーとなる“機能の実証的解析・確認”は，適切な技術基盤が必ずしも十分に開発，整備されておらず律速段階となっている．したがって，この点をブレークスルーすれば，米・欧に匹敵する“新規日の丸遺伝子や蛋白質”を見いだすことも可能であり，わが国独自の技術開発や研究の進展に期待したい．

第2のステージは，明らかにした遺伝子や蛋白質の機能に基づく創薬である．その際，機能が明らかにされた新たな遺伝子，蛋白質，関連機能分子自体が医薬品候補(有効成分)となるケースや，新たに機能解明された遺伝子や蛋白質を分子標的としてこれらを制御できるもの，たとえば，アンチセンスやsiRNAなどの核酸，抗体類，分子標的化学合成品，テーラーメード型製品などが医薬品候補となるケースが考えられる．第2ステージで最も重要なことは，有効性・安全性確保の観点から最終的にあるべき薬剤の姿を想定しながら開発を進めることであり，そこでDDS研究の果たす役割は大きい．



はやかわ　たかお
国立医薬品食品衛生研究所副所長

医薬品は有効成分によって第1の特性を与えられるが，DDS技術によって第2の特性を与えられる．それは，臨床目的に応じた薬物治療の最適化，究極的には，必要な場で，必要な時間，必要な濃度で有効成分が作用するという特性の賦与である．蛋白質性医薬品，核酸医薬品，遺伝子治療薬，分子標的薬などは有効成分において画期的なものであるとともに，DDS研究による適切な特性の賦与により最も有効に活用される先端的医薬品となるものが多い．また，細胞をベースにした製品のあるものは，適用された生体側とのコミュニケーションにより効能効果を示すという理想的な薬剤を目指すものである．最適なDDSは集学的に統合化して達成されるが，わが国には充実した研究基盤があり，世界をリードできる潜在力がある．

新規遺伝子・蛋白質機能解明や再生医学・細胞治療に有用な細胞の開発と，DDS研究の推進・統合により，わが国のバイオ創薬が効果的に推進され，国益に適うとともに，平和的で素晴らしい国際貢献にもなることを心から期待したい．

〔シンポジウム：プロテオミクスの新技術—電気泳動とマススペクトロメトリー—〕

LC/MS/MSを用いた糖タンパク質の糖鎖解析
—糖鎖結合位置及び結合糖鎖の解析—

伊藤さつき・原園　景・川崎ナナ・橋井則貴・松石　紫・川西　徹・早川堯夫

SUMMARY

Liquid chromatography/tandem mass spectrometry (LC/MS/MS) is a powerful tool for the analysis of glycosylation sites and of site-specific glycosylation in a glycoprotein. The glycopeptides in a complex mixture of tryptic digest can be separated and monitored by using oxonium ions produced from a carbohydrate moiety through CID-MS/MS. Based on b and y ions in the product ion mass spectra, peptides can be identified, and the structure of carbohydrates can be deduced from B ions and the molecular weight of precursor glycopeptide. Here we show the site-specific glycosylation analysis of α-fetoprotein and an SDS-PAGE gel-separated GPI-anchored protein.

Key words: LC/MS/MS, glycopeptide, product ion scan, QqTOF-MS, gel-separated protein.

はじめに

生体内に存在する全タンパク質の半分以上が糖鎖付加を受けていると言われるように，糖鎖付加は，主要な翻訳後修飾の1つである．糖タンパク質の糖鎖部分には，結合部位ごとに不均一性が存在し，病気・発生・老化等によって変化することが知られている[1~3]．様々な生命現象における糖タンパク質の糖鎖の役割を解明するためには，結合する糖鎖構造の変化を明らかにすることはもちろんであるが，構造が変化した糖鎖がどの位置に結合していたかを明らかにすることが重要である．

液体クロマトグラフィー／タンデム質量分析法（LC/MS/MS）は，タンパク質の酵素消化によって得られたペプチドと糖ペプチドの混合物をLCで分離しながら，オンラインでMS/MS分析を行うのに用いられている．糖ペプチドのプロダクトイオンスペクトルにはペプチドだけでなく，糖鎖構造に関する多くの情報が含まれているので，LC/MS/MSは部位特異的糖鎖解析に非常に有用である．しかし，無数のペプチドピークの中から糖ペプチドピークを特定するのは難しく，いかに糖ペプチドのピークを選別するかが，糖ペプチド解析の鍵となっている．

ペプチド・糖ペプチドの中から，糖ペプチドを選別する方法として，LC上で選別する方法と，MS/MSで選別する方法がある．前者の方法として，C_{18}カラムと酢酸アンモニウム系溶離液を用いて糖ペプチドだけを選択的に溶出させる方法[4]や，レクチンカラムを用いて糖ペプチドを回収し，さらにHPLCで分離するグライコキャッチ法[5]等が報告されている．MS/MSで選別する方法としては，糖鎖に特徴的なオキソニウムイオン（$[HexNAc]^+$; *m/z* 204, $[Hex\text{-}HexNAc]^+$; *m/z* 366等）を利用して，糖ペプチドを特定する方法が知られ，インソースフラグメンテーション[6]や，プリカーサーイオンスキャン[7,8]を利用した方法が報告されている．インソースフラグメンテーションを用いた場合，クロマトグラム上でのおおよその糖ペプチドの溶出位置を特定することはできるが，プリカーサーイオンを特定することができず，溶出位置付近のマススペクトル及びプロダクトイオンスペクトルの特徴から，糖ペプチドのイオンを特定しなければならない．これに対して，プリカーサーイオンスキャンは，糖ペプチドイオンを特定することはできるが，プロダクトイオンスペクトルが得られないため，ペプチドや糖鎖に関する情報が得られないといった問題がある．

そこで，我々は，得られた無数のペプチド・糖ペプチドのプロダクトイオンスペクトルの中から，オキソニウムイオンを指標として，糖ペプチドに由来するプロダクトイオ

Glycosylation analysis of glycoproteins by LC/MS/MS: analysis of glycosylation sites and of site-specific heterogeneity.

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第54回日本電気泳動学会シンポジウム

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ンスペクトルを選択的に取り出し，ペプチドを同定すると同時に，結合糖鎖を解析する方法を用いている[9,10]．以下に四重極飛行時間型質量分析計（quadrupole time of flight-MS, QqTOF-MS）を用いて，部位特異的に糖タンパク質の糖鎖を解析した例として，α-フェトプロテイン（AFP）と，電気泳動で分離されたラット脳内 Glycosylphosphatidylinositol（GPI）アンカー型タンパク質の分析を紹介する．

I. LC/MS/MS を用いた糖タンパク質の糖鎖構造解析 —α-フェトプロテイン（AFP）の解析—

血液中に存在する糖タンパク質の中には，疾患等により結合糖鎖構造が変化することから，診断マーカーとして用いられているものがあり[11]，その代表的なタンパク質として AFP が知られている．AFP は，N-結合型糖鎖付加部位（Asn233）が一箇所存在する分子量約 68,000 の血清糖タンパク質で，肝細胞癌において，還元末端 GlcNAc のフコシル化率が増加することから，レクチンアフィニティー電気泳動とイムノブロットを用いた早期診断に利用されている[12,13]．LC/MS/MS を用いて，AFP の糖鎖を詳細に解析できるようになれば，今後，より簡便に，且つ微量のサンプルで，早期診断が可能になることが期待される．ここでは，臍帯血由来 AFP について解析を行った例を示す．

Fig. 1（A）は，AFP を還元カルボキシメチル化し，脱塩後，トリプシン消化を行い，LC/MS 分析を行ったものである．ペプチド・糖ペプチド混合物のため多くのイオンが検出されているが，データ依存的 MS/MS データ（Fig. 1（B））の中の *m/z* 204（$[HexNAc]^+$）をモニターすることによって（Fig. 1（C）），糖ペプチドに由来するプロダクトイオンスペクトルを取り出すことができる．

例えば，Fig. 2 は，24 分に溶出された糖ペプチド（*m/z* 1061.8^{+3}）のプロダクトイオンスペクトルである．低分子量側に，*m/z* 204（$[HexNAc]^+$）に加えて，糖鎖に由来する *m/z* 168（$[HexNAc\text{-}2H_2O]^+$），*m/z* 186（$[HexNAc\text{-}H_2O]^+$）及び *m/z* 366（$[Hex\text{-}HexNAc]^+$）や，シアロ糖鎖に由来する *m/z* 292（$[NeuAc]^+$）及び *m/z* 274（$[NeuAc\text{-}H_2O]^+$）の B イオンが検出されている．高分子量側には，ペプチド VNFTEIQK に由来するイオン（*m/z* 978.5）と，b 及び y イオンが検出されている．Fig. 2 中の表はペプチド VNFTEIQK から生じる b 及び y イオンの理論 *m/z* 値を示し，その中で太字で示された値は，実際，プロダクトイオンスペクトル上で検出されたイオンを示している．さらに，ペプチドに HexNAc が 1 または 2 分子，さらに Hex が 1～3 分子結合したイオンが検出され，N-結合型糖鎖のコア部分を確認することができる．糖鎖構造は，プロダクトイオンスペクトル上の B イオンと，TOF-MS で得られた糖ペプチドの分子量（3182.3 Da）からペプチドの理論分子量（977.5 Da）を差し引くことにより得られた分子量（2222.8 Da）から，シアル酸が 2 分子結合した 2 本鎖糖鎖であると推定される．このように，*m/z* 204 が検出されたプロダクトイオンスペクトルとそのプリカーサーイオンを解析することによって，AFP に結合する糖鎖を推定することが可能である（Table 1）．

臍帯血由来 AFP の結合糖鎖は，これまでにメチル化分析やレクチン分画等によって分析されており，主にジシアロ 2 本鎖型糖鎖であることが報告されている[14]．今回の LC/MS/MS 分析の結果で，その他に，アシアロ糖鎖，3 本鎖糖鎖や混成型糖鎖等が結合していることが示唆され，簡単な操作で，より詳細に糖鎖構造が解析できることがわかる．

以上のように，オキソニウムイオンを指標として，糖ペプチドのプロダクトイオンスペクトルを選び出す方法は，ペプチドの b, y イオン及び糖鎖の B イオンをもとにペプチドの同定と結合糖鎖の構造推定を行うことが可能であることから，部位特異的糖鎖構造解析に有用である．次に，LC/MS/MS による糖ペプチド解析法を，電気泳動法で分離された糖タンパク質の解析に応用した例を示す．

II. ゲル内糖タンパク質の糖鎖構造解析 —ラット脳内 GPI アンカー型タンパク質の解析—

脳・神経系の細胞膜上には，細胞間認識やシグナル伝達に関与する多数の糖タンパク質が存在し，糖鎖はこれらの

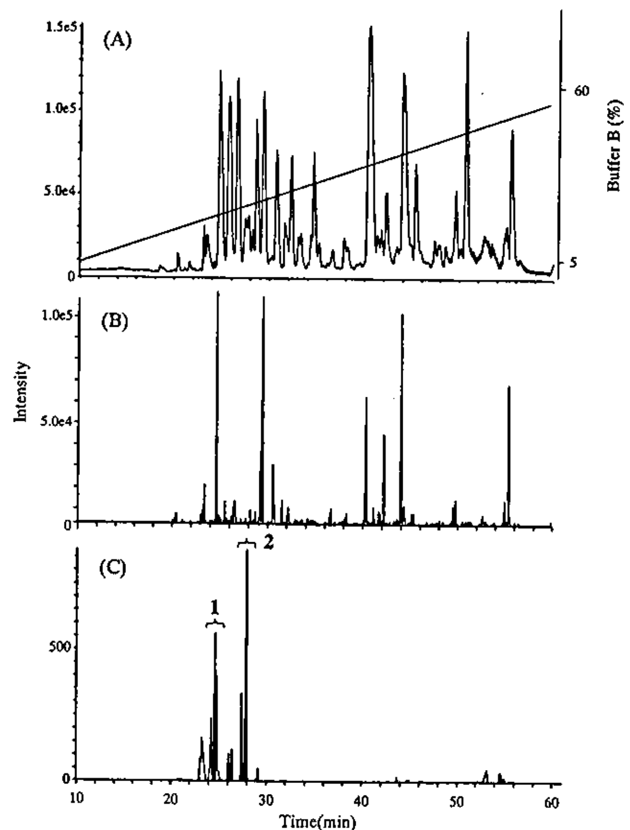


Fig. 1. LC/MS/MS of AFP, (A) TOF-MS full scan at *m/z* 700–2000, (B) product ion scan at *m/z* 100-2000, (C) product ion scan at *m/z* 204.

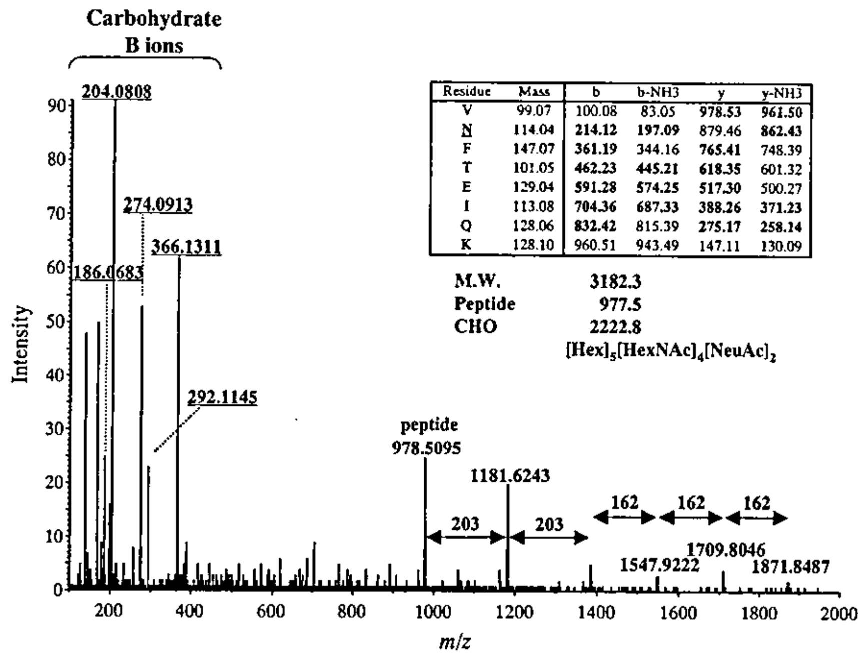


Fig. 2. Product ion spectrum of a glycopeptide (*m/z* 1061.83^{3+}) at 24 min.

Inset table shows theoretical *m/z* values of b and y ions, and ions detected by MS/MS are indicated by bold face.

Table 1. Glycosylation analysis of AFP

Peak No.	Peptide sequence (theoretical peptide mass[a])	Retention time (min)	Observed *m/z* (charge)	Carbohydrate composition	Calculated Carbohydrate mass	Theoretical carbohydrate mass[a]
1	KVNFTEIQKL (977.5)	23	1013.43(3)	$[dHex]_1[Hex]_5[HexNAc]_4[NeuAc]_1$	2077.8	2077.7
		23	1519.67(2)	$[dHex]_1[Hex]_5[HexNAc]_4[NeuAc]_1$	2077.8	2077.7
		23	1081.29(3)	$[dHex]_1[Hex]_5[HexNAc]_5[NeuAc]_1$	2281.4	2280.8
		23	1621.44(2)	$[dHex]_1[Hex]_5[HexNAc]_5[NeuAc]_1$	2281.4	2280.8
		23	1446.62(2)	$[Hex]_5[HexNAc]_4[NeuAc]_1$	1931.8	1931.7
		23	1118.67(2)	$[Hex]_4[HexNAc]_3$	1275.9	1275.5
		23	1264.05(2)	$[Hex]_4[HexNAc]_3[NeuAc]_1$	1566.6	1566.6
		23	1110.48(3)	$[Hex]_4[HexNAc]_3[NeuAc]_1$	1566.6	1566.6
		24	1665.44(2)	$[dHex]_1[Hex]_5[HexNAc]_4[NeuAc]_2$	2369.4	2368.8
		24	1178.33(3)	$[dHex]_1[Hex]_5[HexNAc]_5[NeuAc]_2$	2572.5	2571.9
		24	1061.78(3)	$[Hex]_5[HexNAc]_4[NeuAc]_2$	2222.9	2222.8
		25	1592.17(2)	$[Hex]_5[HexNAc]_4[NeuAc]_2$	2222.9	2222.8
2	KFTKVNFTEIQKL (1353.7)	27	1236.04(3)	$[dHex]_1[Hex]_5[HexNAc]_4[NeuAc]_2$	2369.4	2368.8
		27	1853.29(2)	$[dHex]_1[Hex]_5[HexNAc]_4[NeuAc]_2$	2369.4	2368.8
		27	1187.17(3)	$[Hex]_5[HexNAc]_4[NeuAc]_2$	2222.8	2222.8
		28	1780.23(2)	$[Hex]_5[HexNAc]_4[NeuAc]_2$	2222.8	2222.8
		28	1303.56(3)	$[dHex]_1[Hex]_5[HexNAc]_5[NeuAc]_2$	2572.0	2571.9
		28	1138.84(3)	$[dHex]_1[Hex]_5[HexNAc]_4[NeuAc]_1$	2077.8	2077.7
		28	1206.52(3)	$[dHex]_1[Hex]_5[HexNAc]_5[NeuAc]_1$	2280.9	2280.8
		28	1090.14(3)	$[Hex]_5[HexNAc]_4[NeuAc]_1$	1931.7	1931.7
		28	1322.75(3)	$[Hex]_5[HexNAc]_6[NeuAc]_2$	2629.6	2628.9

[a] Monoisotopic mass value.

機能に深く関わっていると考えられている[15]．しかし，微量膜タンパク質であることから，精製が困難で，解析に必要な量が得られず，多くの糖タンパク質の糖鎖構造は明らかにされていない．タンパク質混合物の分離に適した電気泳動と LC/MS/MS を活用することによって，これらの糖鎖構造を解析できると期待される．以下は，GPI を介して膜に結合する GPI アンカー型タンパク質の糖鎖解析を行った例である．

ラット脳の膜画分から，Phosphatidylinositol-specific phospholipase C (PIPLC) 消化によって得られた可溶性 GPI アンカー型タンパク質群を，還元カルボキシアミドメチル化後，SDS-PAGE で分離した（Fig. 3）．通常，ゲル内のタ

ンパク質同定は，ゲル内プロテアーゼ消化後，抽出されたペプチドのみを用いて行われているが，糖鎖構造解析を行う場合，すべての糖ペプチド断片を同程度の収率で回収する必要がある．そこで，ゲルより糖タンパク質を抽出後，トリプシン消化を行う方法を選んだ．

Fig. 4 (A) は，バンド1を切り出し，1%SDS を用いてタンパク質を抽出後，トリプシン消化を行い，LC/MS/MS 分析を行ったものである．まず，データベース検索の結果，バンド1は，Thy-1 と同定された．Thy-1 は，免疫グロブリンスーパーファミリーに属する GPI アンカー型タンパク質であり，3 箇所の N- 結合型糖鎖付加部位 (Asn23, 74, 98) を有するアミノ酸残基数 111 の糖タンパク質である (Fig. 5)．

次に，AFP と同様に，*m/z* 204 を指標として，全ペプチドのプロダクトイオンスペクトルの中から，糖ペプチドのプロダクトイオンスペクトルを選択的に取り出した (Fig. 4 (C))．それらのプロダクトイオンスペクトル及びプリカーサーイオンの TOF-MS から，T1 及び T2 に溶出された糖ペプチドは，それぞれ高マンノース型糖鎖が結合した Asn23 を含む糖ペプチド，His21-Phe33，及び His21-Arg37 であり，T3 及び T4 は，複合型及び混成型糖鎖が結合した Asn74 を含む糖ペプチド，Val69-Lys78 であることが判った (Table 2)．

Fig. 6 は，T3 に溶出された糖ペプチドのうち，*m/z* 1532.2^{+2} を示す糖ペプチドのプロダクトイオンスペクトルである．低分子量側に，AFP で検出されたイオン，*m/z* 168, 186, 204, 366 に加え，*m/z* 528 ($[Hex]_2[HexNAc]^+$)，*m/z* 569 ($[Hex][HexNAc]_2^+$) や，*m/z* 512 ($[Hex][HexNAc][dHex]^+$) 等の B イオンが検出されており，Gal1-3(4)(Fuc1-4(3))-GlcNAc 構造を含むことが推定される．高分子量側には，ペプチドに由来するイオン (*m/z* 1107.6) と b 及び y イオンが検出され，VLTLANFTTK と同定された (Fig. 6 中の表)．また，ペプチドに HexNAc, HexNAc-HexNAc, HexNAc-HexNAc-Hex が結合したイオンが検出されている他，さらに，これらに dHex が 1 分子結合したイオンが検出されていることから，結合糖鎖のコア部分がフコシル化されていることが判る．結合糖鎖は，TOF-MS で得られた糖ペプチドの分子量 (3062.4 Da) から，ペプチドの理論分子量 (1106.6 Da) を差し引くことにより得られた糖鎖分子量 (1973.7 Da) から，Fig. 6 中に示すような構造であると推定される．Table 2 は，同様に解析した結果，Thy-1 に結合すると推定された糖鎖構造である．

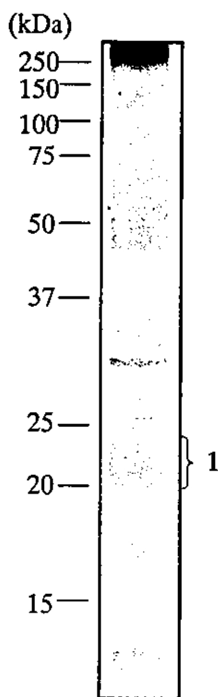


Fig. 3. SDS-PAGE of lipid-free GPI-anchored protein prepared from rat brain.

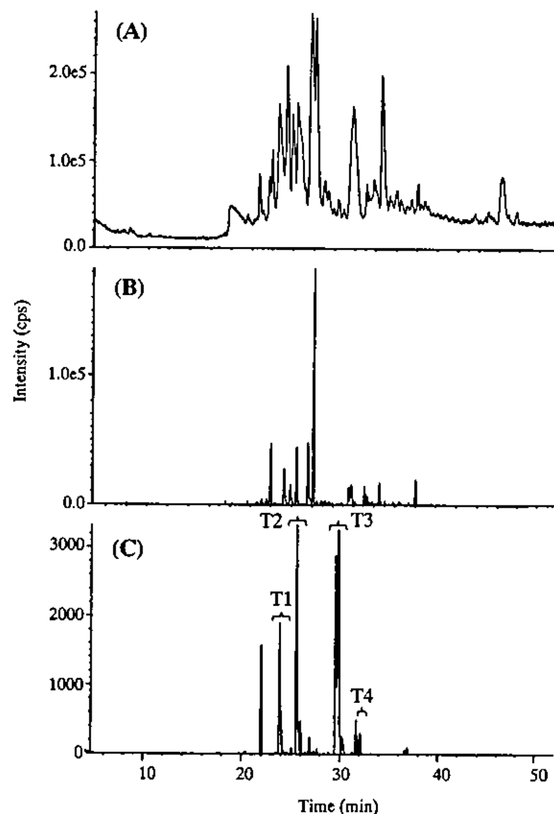


Fig. 4. LC/MS/MS of rat brain Thy-1, (A) TOF-MS full scan at *m/z* 700–2000, (B) product ion scan at *m/z* 100–2000, (C) Product ion scan at *m/z* 204.

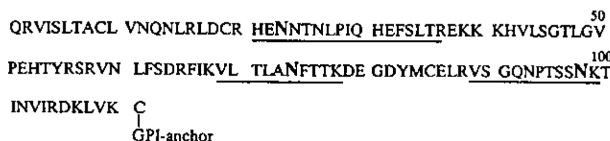
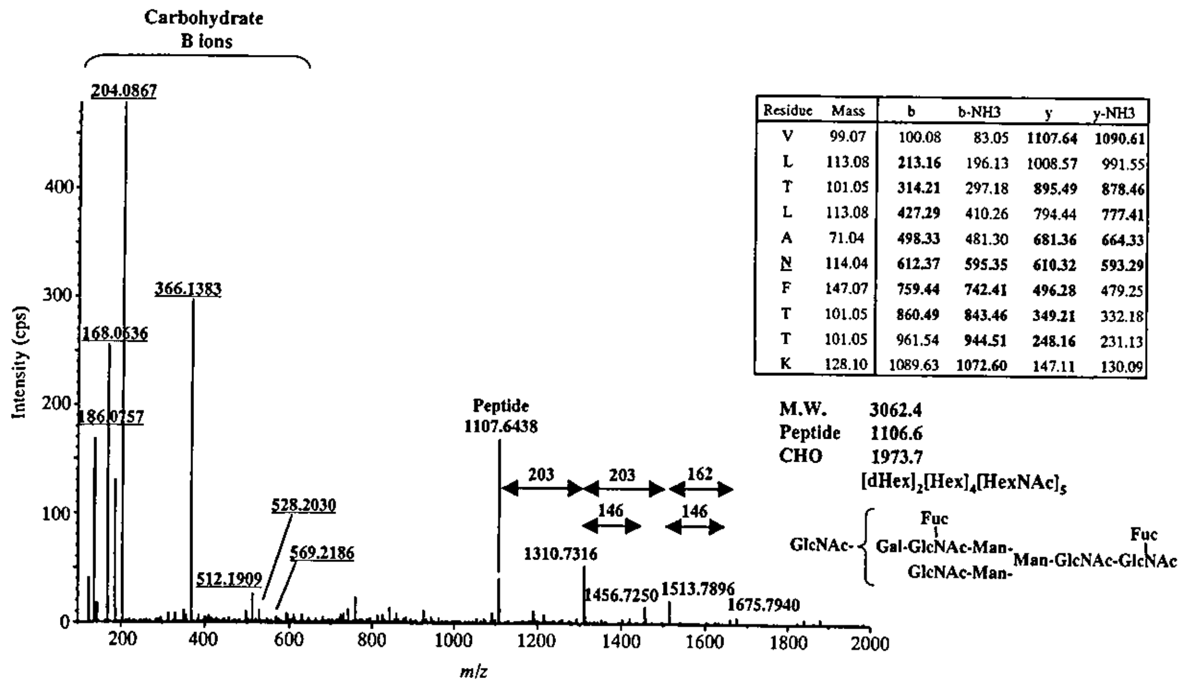


Fig. 5. Amino acid sequence of rat Thy-1.

Glycopeptides produced by trypsin are underlined. N-glycosylation sites are indicated by bold face.



Residue	Mass	b	b-NH3	y	y-NH3
V	99.07	100.08	83.05	**1107.64**	**1090.61**
L	113.08	**213.16**	196.13	1008.57	991.55
T	101.05	**314.21**	297.18	**895.49**	**878.46**
L	113.08	**427.29**	410.26	794.44	**777.41**
A	71.04	**498.33**	481.30	**681.36**	**664.33**
N	114.04	**612.37**	**595.35**	**610.32**	**593.29**
F	147.07	**759.44**	**742.41**	**496.28**	479.25
T	101.05	**860.49**	**843.46**	**349.21**	332.18
T	101.05	961.54	**944.51**	**248.16**	231.13
K	128.10	1089.63	**1072.60**	147.11	130.09

Fig. 6. Product ion spectrum of a glycopeptide (*m/z* 1532.2^{2+}) at peak 29.5 min. Inset table shows theoretical *m/z* values of b and y ions, and ions detected by MS/MS are indicated by bold face.

Table 2. Glycosylation analysis of rat brainThy-1

Peak No.	Peptide sequence (theoretical peptide mass[a])	Glycosylation site	Retention time (min)	Observed *m/z* (charge)	Carbohydrate composition	Calculated carbohydrate mass	Theoretical carbohydrate mass[a]
T1	H21-F33 (1591.73)	N23	23.9	991.1(3)	$[Hex]_6[HexNAc]_2$	1396.6	1396.5
			23.9	1486.2(2)	$[Hex]_6[HexNAc]_2$	1396.6	1396.5
			23.9	1045.1(3)	$[Hex]_7[HexNAc]_2$	1558.7	1558.5
			24.0	1567.2(2)	$[Hex]_7[HexNAc]_2$	1558.6	1558.5
			24.0	937.1(3)	$[Hex]_5[HexNAc]_2$	1234.5	1234.4
			24.0	1405.1(2)	$[Hex]_5[HexNAc]_2$	1234.5	1234.4
T2	H21-R37 (2048.99)	N23	25.5	1197.6(3)	$[Hex]_7[HexNAc]_2$	1558.6	1558.5
			25.6	1143.5(3)	$[Hex]_6[HexNAc]_2$	1396.6	1396.5
			25.6	857.9(4)	$[Hex]_6[HexNAc]_2$	1396.6	1396.5
			25.7	1089.5(3)	$[Hex]_5[HexNAc]_2$	1234.5	1234.4
			25.7	817.4(4)	$[Hex]_5[HexNAc]_2$	1234.6	1234.4
			25.9	1633.8(2)	$[Hex]_5[HexNAc]_2$	1234.5	1234.4
T3	V69-K78 (1106.62)	N74	29.5	1686.3(2)	$[dHex]_3[Hex]_5[HexNAc]_5$	2282.0	2281.9
			29.6	1124.5(3)	$[dHex]_3[Hex]_5[HexNAc]_5$	2282.0	2281.9
			29.6	1532.2(2)	$[dHex]_2[Hex]_4[HexNAc]_5$	1973.9	1973.7
			29.6	1021.8(3)	$[dHex]_2[Hex]_4[HexNAc]_5$	1973.9	1973.7
			29.7	1162.6(2)	$[Hex]_5[HexNAc]_2$	1234.5	1234.4
			29.7	1491.2(2)	$[dHex]_2[Hex]_6[HexNAc]_3$	1891.8	1891.7
			29.7	1592.8(2)	$[dHex]_2[Hex]_6[HexNAc]_4$	2094.9	2094.8
			29.8	1378.2(2)	$[dHex]_1[Hex]_3[HexNAc]_5$	1665.7	1665.6
			29.9	919.1(3)	$[dHex]_1[Hex]_3[HexNAc]_5$	1665.7	1665.6
			29.8	1438.7(2)	$[dHex]_1[Hex]_5[HexNAc]_4$	1786.8	1786.7
			30.3	959.5(3)	$[dHex]_1[Hex]_5[HexNAc]_4$	1786.8	1786.7
			29.9	1511.7(2)	$[dHex]_2[Hex]_5[HexNAc]_4$	1932.8	1932.7
			30.0	1008.1(3)	$[dHex]_2[Hex]_5[HexNAc]_4$	1932.8	1932.7
			30.0	1519.7(2)	$[dHex]_1[Hex]_6[HexNAc]_4$	1948.8	1948.7
			30.0	1572.2(2)	$[dHex]_2[Hex]_7[HexNAc]_3$	2053.9	2053.7
			30.1	1499.2(2)	$[dHex]_1[Hex]_7[HexNAc]_3$	1907.8	1907.7
			30.2	1276.6(2)	$[dHex]_1[Hex]_3[HexNAc]_4$	1462.6	1462.5
			30.2	1337.2(2)	$[dHex]_1[Hex]_5[HexNAc]_3$	1583.7	1583.6
			31.7	1860.4(2)	$[dHex]_2[Hex]_5[HexNAc]_6[NeuAc]_1$	2630.1	2630.0
			31.7	1240.6(3)	$[dHex]_2[Hex]_5[HexNAc]_6[NeuAc]_1$	2630.1	2630.0
			31.9	1706.3(2)	$[dHex]_1[Hex]_4[HexNAc]_6[NeuAc]_1$	2321.9	2321.9
T4	V69-K78 (1106.62)	N74	32.1	1563.7(2)	$[dHex]_1[Hex]_6[HexNAc]_3[NeuAc]_1$	2036.8	2036.7
			32.1	1482.7(2)	$[dHex]_1[Hex]_5[HexNAc]_3[NeuAc]_1$	1874.7	1874.7
			32.1	1584.2(2)	$[dHex]_1[Hex]_5[HexNAc]_4[NeuAc]_1$	2077.8	2077.8

[a] Monoisotopic mass value.

ラット脳のThy-1のN-結合型糖鎖については，これまでに抗体カラム等を用いて精製されたThy-1をトリプシン消化後，糖ペプチドごとに糖鎖を切り出し，ゲルろ過や，エキソグリコシダーゼ消化等を用いて分析した例が報告されている．Asn23には，高マンノース型（M5，6）が，Asn74には，コアがフコシル化された複合型糖鎖と，マイナー糖鎖としてハイブリッド型糖鎖及び高マンノース型糖鎖が結合していることが明らかにされているが[16,17]，電気泳動とLC/MS/MSを組み合わせることによって，微量の膜タンパク質を，抗体を使用せずに簡便且つより詳細に解析できることがわかる．尚，Asn98に結合する糖鎖及びGPIアンカーについては，トリプシン消化によって得られた糖ペプチドの親水性が高く，LCカラムに保持されなかったため，他の酵素消化によって得られた糖ペプチドを解析することによって明らかにしている（データ省略）．

以上のように，LC/MS/MSによるペプチド・糖ペプチドマッピングにおいて，糖鎖に特徴的なオキソニウムイオンを用いて糖ペプチドのプロダクトイオンスペクトルを取り出す方法は，タンパク質を同定すると同時に，糖鎖結合部位を決定し，結合部位ごとの糖鎖構造を推定することを可能にする．2次元HPLCとの組み合わせや，2次元電気泳動で分離された糖タンパク質の解析も可能であることから，今後，グライコプロテオミクスの分野で役立つことが期待される．

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要　約

LC/MS/MSは，アミノ酸配列情報に加え，糖鎖構造に関する情報についても得ることができ，糖ペプチドの解析にも有用である．QqTOF-MSを用いたLC/MS/MSは，糖ペプチドのピークを特定し，ペプチドを同定し，結合糖鎖構造に関する情報も得ることができる．本稿では，糖ペプチドの解析例として，APF及び電気泳動で分離されたGPIアンカー型タンパク質の解析例を示す．

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Kinetic Analysis of Pepsin Digestion of Chicken Egg White Ovomucoid and Allergenic Potential of Pepsin Fragments

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Key Words

Ovomucoid · Allergen · Digestion · Simulated gastric fluid · Fragment, pepsin-digested · Human serum IgE

Abstract

Background: The allergenic potential of chicken egg white ovomucoid (OVM) is thought to depend on its stability to heat treatment and digestion. Pepsin-digested fragments have been speculated to continue to exert an allergenic potential. OVM was digested in simulated gastric fluid (SGF) to examine the reactivity of the resulting fragments to IgE in sera from allergic patients. ***Methods:*** OVM was digested in SGF and subjected to SDS-PAGE. The detected fragments were then subjected to N-terminal sequencing and liquid chromatography/mass spectrometry/mass spectrometry analysis to confirm the cleavage sites and partial amino acid sequences. The reactivity of the fragments to IgE antibodies in serum samples from patients allergic to egg white was then determined using Western blotting (n = 24). ***Results:*** The rate of OVM digestion depended on the pepsin/OVM ratio in the SGF. OVM was first cleaved near the end of the first domain, and the resulting fragments were then further digested into smaller fragments. In the Western blot analysis, 93% of the OVM-reactive sera also bound to the 23.5- to 28.5-kDa fragments, and 21% reacted with the smaller 7- and 4.5-kDa fragments. ***Conclusion:*** When the digestion of OVM in SGF was kinetically analyzed, 21% of the examined patients retained their IgE-binding capacity to the small 4.5-kDa fragment. Patients with a positive reaction to this small peptide fragment were thought to be unlikely to outgrow their egg white allergy. The combination of SGF-digestibility studies and human IgE-binding experiments seems to be useful for the elucidation and diagnosis of the allergenic potential of OVM.

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Introduction

Chicken egg white is one of the strongest and most frequent causes of food allergies among young children [1–5]. Egg white contains several allergens, including ovalbumin, ovotransferrin, lysozyme and ovomucoid (Gal d 1, OVM). OVM accounts for about 11% of all egg white proteins [6] and has a molecular weight of 28 kDa, containing a carbohydrate content of 20–25% [7]. OVM is known to be stable to digestion and heat, and cooked eggs can cause allergic reactions in OVM-specific allergic patients [8–11]. One possible reason for this is that OVM contains linear epitopes that are only slightly affected by conformational changes induced by heat denaturation.

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OVM consists of 186 amino acids divided into three domains of about 60 amino acids each; the third domain has been reported to be the most important domain with regard to allergenicity [12]. In a previous report, N-glycans in the third domain were suggested to be essential for allergenicity [13]; however, a recent report found that the deletion of the N-glycans did not affect the allergic reactivity.

We previously reported the digestibility of 10 kinds of food proteins in simulated gastric fluid (SGF) [8, 14]. OVM was digested relatively rapidly, but several fragments were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue (CBB) staining. The reactivity of these fragments with IgE antibodies from the sera of patients with egg white allergy is very important to understanding the mechanism of OVM allergy.

A few previous reports have described the reactivity of IgE in sera from patients with egg white allergies with OVM-derived fragments. Kovacs-Nolan et al. [15] separated pepsin-digested fragments of OVM using high-performance liquid chromatography (HPLC) and examined the IgE-binding activities of each fragment using an enzyme-linked immunosorbent assay (ELISA). Besler et al. [16] investigated the reactivity of pepsin-digested fragments with patient IgE using Western blotting and showed that the fragments retain their binding capacity to human IgE in some serum samples from OVM-allergic patients. However, little attention has been paid to the digestive conditions, and the number of serum samples has been somewhat small in these studies. Urisu et al. [17] reported that the sera of subjects that tested positive or negative during an oral egg white challenge exhibited a significant difference in their reactivity with pepsin fragments.

In the present report, kinetic data for different generations of SGF-stable OVM fragments were obtained, and the reactivity of the fragments with serum IgE from patients with egg white allergies was investigated using Western blotting.

Materials and Methods

Pepsin (catalog number P6887) and chicken egg white OVM (T2011, Trypsin Inhibitor, Type III-O) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). The concentration of the OVM test solution was 5 mg/ml of water. The gels and reagents used for the SDS-PAGE analysis were purchased from Invitrogen (Carlsbad, Calif., USA).

Serum Specimens

Sera from 24 patients with egg white allergies and a healthy volunteer were used after obtaining informed consent from the patients and ethical approval by the Institutional Review Board of the National Institute of Health Sciences. Twenty-two of the patients had been diagnosed as having an egg white allergy at hospitals in Japan, based on their clinical histories and positive IgE responses to egg white proteins by radioallergosorbent test (RAST), while the remaining 2 allergen-specific sera were purchased from Plasma Lab International (Everett, Wash., USA); the commercial sera originated from adult Caucasians who had been diagnosed as having several food allergies, including egg white, based on their clinical history and skin tests. The commercial sera also showed positive IgE responses to egg white proteins when examined using RAST.

Preparation of SGF

Pepsin (3.8 mg; approximately 13,148 units of activity) was dissolved in 5 ml of gastric control solution (G-con; 2 mg/ml NaCl, pH adjusted to 2.0 with distilled HCl), and the activity of each newly prepared SGF solution was defined as the production of a ΔA280 of 0.001/min at pH 2.0 and 37 °C, measured as the production of trichloroacetic acid-soluble products using hemoglobin as a substrate. The original SGF was prepared at a pepsin/OVM concentration of 10 unit/μg, and this solution was diluted with G-con for the experiments performed at pepsin/OVM concentrations of 1 and 0.1 unit/μg. The SGF solutions were used within the same day.

Digestion in SGF

SGF (1,520 μl) was incubated at 37 °C for 2 min before the addition of 80 μl of OVM solution (5 mg/ml). The digestion was started by the addition of OVM. At each scheduled time point (0.5, 2, 5, 10, 20, 30, and 60 min), 200 μl of the reaction mixture was transferred to a sampling tube containing 70 μl of 5 × Laemmli buffer (40% glycerol, 5% 2-mercaptoethanol, 10% SDS, 0.33 *M* Tris, 0.05% bromophenol blue, pH 6.8) and 70 μl of 200 m*M* Na_2CO_3. For the zero-point samples, the OVM solution (10 μl) was added to neutralized SGF (190 μl of SGF, 70 μl of 5 × Laemmli buffer, and 70 μl of 200 m*M* Na_2CO_3). All neutralized samples were then boiled at 100 °C for 3 min and subjected to SDS-PAGE.

SDS-PAGE Analysis and Staining Procedure

Samples (15 μl/lane) were loaded onto a 10–20% polyacrylamide Tris/Tricine gel (Invitrogen, Carlsbad, Calif., USA) and separated electrophoretically. The gels were fixed for 5 min in 5% trichloroacetic acid, washed for 2 h with SDS Wash (45.5% methanol, 9% acetic acid), stained for 10 min with CBB solution (0.1% Coomassie Brilliant blue R, 15% methanol, 10% acetic acid), and destained with 25% methanol and 7.5% acetic acid. The stained gel images were then analyzed using Image Gauge V3.1 (Fuji Film, Tokyo, Japan), and the density of each band was quantified. Periodic acid-Schiff (PAS) staining [18] was used to detect the glycosylated fragments.

N-Terminal Sequence Analysis

OVM (1.5 mg) was digested in SGF containing 1 unit/ml pepsin, concentrated by centrifugation using Centriprep YM-3 (Millipore Corporation, Bedford, Mass., USA) and subjected to SDS-PAGE followed by electrical transblotting to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad, Richmond, Calif., USA) and CBB staining. The detected fragment bands were then cut out and sequenced using a Procise 494HT Protein Sequencing System (Applied Biosys-

tems, Foster City, Calif., USA) or an HP G1005A Protein Sequencing System (Hewlett-Packard, Palo Alto, Calif., USA); each fragment was analyzed for 5 cycles.

Carboxymethylation and Peptide Mapping Using Liquid Chromatography/Mass Spectrometry/Mass Spectrometry (LC/MS/MS)

The digested OVM sample was separated electrophoretically as described above, stained with CBB, and the stained bands were cut out. The gel pieces were homogenized in 20 m*M* Tris-HCl (pH 8.0) containing 0.1% SDS and the proteins were extracted. The extracts were concentrated and purified by acetone precipitation. The acetone precipitates were incubated with 2-mercaptethanol (92.5 m*M*) in 72 µl of 0.5 *M* Tris-HCl buffer (pH 8.6) containing 8 *M* guanidine hydrochloride and 5 m*M* EDTA at room temperature for 2 h. To this solution, 1.5 mg of monoiodoacetic acid was added, and the mixture was incubated at room temperature for 2 h in the dark. The reaction mixture was desalted using a MicroSpin G-25 column (Amersham Bioscience, Uppsala, Sweden) and lyophilized. Reduced and carboxymethylated proteins were digested with trypsin (50 ng/µl in 50 m*M* NH_4HCO_3).

Tandem electrospray mass spectra were recorded using a hybrid quadruple/time-of-flight spectrometer (Qstar Pulsar i; Applied Biosystems, Foster City, Calif., USA) interfaced to a CapLC (Magic 2002; Michrom BioResources, Auburn, Calif., USA). Samples were dissolved in water and injected into a C18 column (0.2 × 50 mm, 3 µm, Magic C18, Michrom BioResources). Peptides were eluted with a 5-36% acetonitrile gradient in 0.1% aqueous formic acid over 60 min at a flow rate of 1 µl/min after elution with 5% acetonitrile for 10 min. The capillary voltage was set to 2,600 V, and data-dependent MS/MS acquisitions were performed using precursors with charge states of 2 and 3 over a mass range of 400-2,000.

Western Blotting of Digested Fragments with Human Serum IgE

The digested OVM samples were applied to a 10-20% polyacrylamide Tris/Tricine 2D gel, followed by electrical transfer to a nitrocellulose membrane. The membrane was then blocked with 0.5% casein-PBS (pH 7.0) and cut into 4-mm strips. The strips were incubated with diluted human serum (1/4 to 1/5) in 0.2% casein-PBS (pH 7.0) at room temperature for 1 h and then at 4°C for 18 h. After washing with 0.05% Tween 20-PBS, the strips were incubated with rabbit anti-human IgE (Fc) antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands) at room temperature for 1 h, and then with horseradish peroxidase-conjugated donkey anti-rabbit Ig antibodies (Amersham Biosciences, Little Chalfont, UK) at room temperature for 1 h. Finally, the strips were reacted with Konica ImmunoStain HRP-1000 (Konica, Tokyo, Japan), according to the manufacturer's protocol.

Results

Kinetics of OVM Digestion by Pepsin

OVM was digested in SGF containing various concentrations of pepsin, and the fragments were separated by SDS-PAGE and stained with CBB (fig. 1). The molecular weight of OVM, based on its amino acid sequence, is about 20 kDa, but a broad band representing intact OVM appeared at about 34-49 kDa in the SDS-PAGE gel because of the presence of five N-linked sugar chains. The pepsin band was detected at 39 kDa, overlapping with the intact OVM band, and lysozyme (14 kDa) contamination was detected in the OVM sample that was used. Intact OVM rapidly disappeared within 0.5 min in SGF (pepsin/OVM = 10 unit/µg), and a fragment band was detected at 7 kDa. When the pepsin content in SGF was reduced to 1 and 0.1 unit/µg, the digestion rate markedly decreased. Intact OVM was still detected after 30 min when the pepsin/OVM ratio was 0.1 unit/µg. The fragment bands were clearer (fig. 2) when a concentrated SGF-digested OVM solution (pepsin/OVM = 1 unit/µg, digestion times 5 and 30 min) was used, followed by SDS-PAGE. As shown in figure 2, a strong 23.5- to 28.5-kDa band (FR 1) was detected at 5 min, while 10- (FR 2), 7- (FR 3) and 4.5- to 6-kDa (FR 4) bands were detected after 30 min. FR 1 and FR 2 were both positively stained by PAS, suggesting that the FR 1 and FR 2 fragments have high carbohydrate contents. The time courses for the amounts of intact OVM and the four fractions are plotted in figure 3, where the pepsin/OVM ratio is 1 unit/µg. FR 1 rapidly increased but slowly disappeared after 2 min. FR 2 and FR 3 also rapidly reached maximum values at 5 min and then slowly disappeared. On the other hand, FR 4 gradually increased throughout the entire period of the experiment.

Preheating (at 100°C for 5 or 30 min) of the OVM solution (5 mg/ml in water) did not influence the digestion pattern (fig. 1).

Table 1. N-Terminal sequences of pepsin fragments

Digestion period	Fraction	Fragment	Residues	Sequence	Ratio %[a]
5 min	FR 1	1-1	50-54	FGTNI	73.1
		1-2	51-55	GTNIS	11.6
		1-3	1-5	AEVDC	6.9
5 min	FR 2	2-1	1-5	AEVDC	68.8
		2-2	134-138	VSVDC	28.2
5 min	FR 3	3-1	1-5	AEVDC	48.4
		3-2	134-138	VSVDC	24.3
		3-3	104-108	NECLL	9.6
		3-4	85-89	VLCNR	6.5
30 min	FR 4	4-1	134-138	VSVDC	30.6
		4-2	104-108	NECLL	24.0
		4-3	19-23	VLVCN	20.6

[a] Molar ratios of the fragments to the total amount in each fraction.

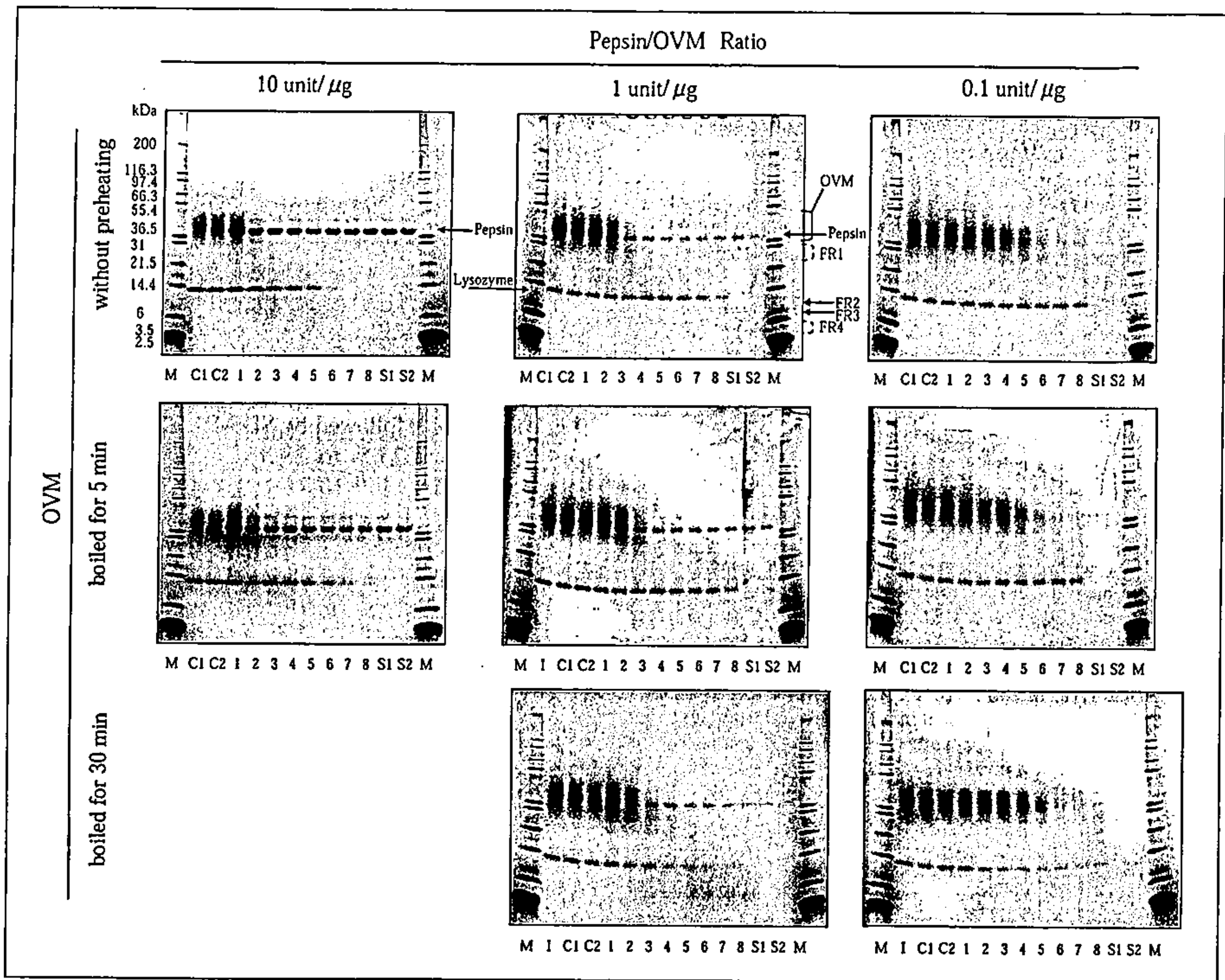

Fig. 1. Kinetic patterns of OVM digestion in SGF-containing pepsin. Digested samples were analyzed by SDS-PAGE followed by CBB staining. The digestion patterns of OVM without preheating (upper panels), preheated at 100°C for 5 min (middle panels), and preheated at 100°C for 30 min (lower panels) are shown. The ratio of pepsin to OVM was 10 unit/1 μg (left), 1 unit/1 μg (middle), and 0.1 unit/1 μg (right). Lane M = Molecular weight markers; lanes C1 and C2 = OVM without pepsin at 0 (C1) and 60 (C2) min; lanes 1–8 = SGF-digested OVM at 0, 0.5, 2, 5, 10, 20, 30 and 60 min, respectively; lanes S1 and S2 = SGF alone at 0 (S1) and 60 (S2) min; lanes I = OVM without preheating; FR 1 = fraction 1 containing a fragment at 23.5–28.5 kDa; FR 2 = fraction 2 containing a 10-kDa fragment; FR 3 = fraction 3 containing a 7-kDa fragment; FR 4 = fraction 4 containing 4.5- to 6-kDa fragments.

Sequence Analysis of OVM Fragments

The sequences of the five N-terminal residues in each fragment were analyzed, and the data are summarized in table 1. Figure 4 schematically depicts the identified fragments; the arrows in the upper panel indicate the sites of pepsin cleavage.

The internal sequences of the FR 1, FR 3, and FR 4 fragments were also identified by LC/MS/MS and are shown in table 2 and in the upper panel of figure 4.

Reactivity of the Fragments with Serum IgE from Patients with Egg White Allergy

Western blot analysis using patient sera as the source of the primary antibodies was performed to identify sera that reacted with intact OVM and the SGF fragments. Representative blotting data are shown in figure 5, and all the results are listed in table 3. Ninety-two percent of the serum samples from allergic patients reacted with OVM, and 93% of the OVM-positive sera reacted with FR 1

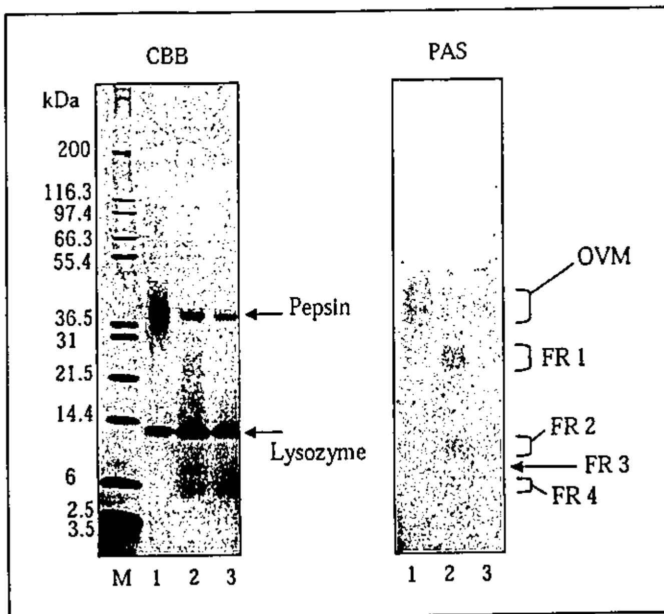

Fig. 2. CBB and PAS staining of OVM fragments following digestion in SGF (pepsin/OVM = 1 unit/μg) for 5 and 30 min. Lane M = Molecular weight markers; lane 1 = original OVM (2.5 μg/lane); lanes 2 and 3 = OVM digested for 5 and 30 min, respectively, and concentrated (12 μg, equivalent to the original OVM/lane). Samples were applied to two SDS-PAGE gels and electrophoresed. One plate (left panel) was stained with CBB reagent, and the other (right panel) was stained with PAS reagent.

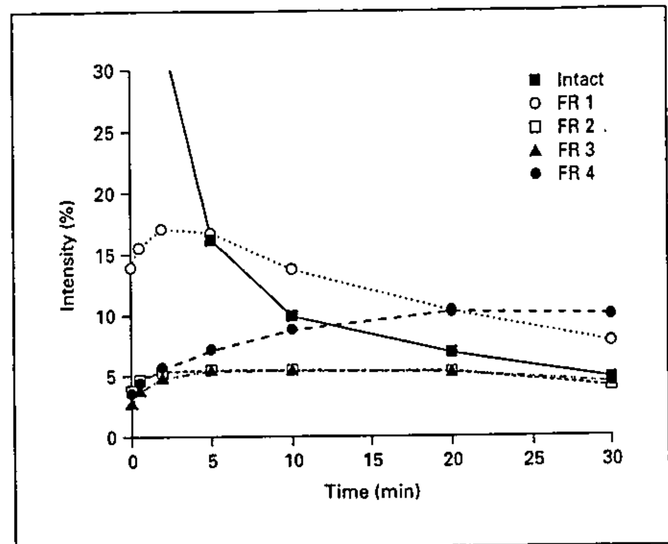

Fig. 3. Quantification of the SGF-digestion pattern of intact OVM and the digestion fragments at a pepsin/OVM ratio of 1 unit/μg. The intensity of each band was calculated using the ratio of the band's density to the total density of the originally detected band at t = 0. Values are the mean of duplicate analyses. Similar results were observed in another set of experiments.

Table 2. Identified inside sequences in pepsin- and trypsin-digested OVM

Pepsin digestion	Fraction	Residues	Sequence
5 min	FR 1	83–89	VMVLCNR
		90–103	AFNPVCGTDGVTYD
		90–112	AFNPVCGTDGVTYDNECLLCAHK
		90–122	AFNPVCGTDGVTYDNECLLCAHKVEQGASVDKR
		113–122	VEQGASVDKR
5 min	FR 3	90–112	AFNPVCGTDGVTYDNECLLCAHK
		90–122	AFNPVCGTDGVTYDNECLLCAHKVEQGASVDKR
		104–111	NECLLCAH
		104–112	NECLLCAHK
		104–121	NECLLCAHKVEQGASVDK
		104–122	NECLLCAHKVEQGASVDKR
		113–122	VEQGASVDKR
		134–159	VSVDCSEYPKPDCTAEDRPLCGSDNK
		165–185	CNFCNAVVESNGTLTLSHFGK
30 min	FR 4	90–112	AFNPVCGTDGVTYDNECLLCAHK
		104–111	NECLLCAH
		104–112	NECLLCAHK
		104–122	NECLLCAHKVEQGASVDKR
		112–122	KVEQGASVDKR
		113–121	VEQGASVDK
		113–122	VEQGASVDKR
		165–185	CNFCNAVVESNGTLTLSHFGK

```
    1         11        21        31        41        51
    |         |         |         |         |         |
1   AEVDCSRFPN ATDKEGKDVL VCNKDLRPIC GTDGVTYTND CLLCAYSIEF GTNISKEHDG   60
61  ECKETVPMNC SSYANTTSED GKVMVLCNRA FNPVCGTDGV TYDNECLLCA HKVEQGASVD  120
121 KRHDGGCRKE LAAVSVDCSE YPKPDCTAED RPLCGSDNKT YGNKCNFCNA VVESNGTLTL  180
181 SHFGKC
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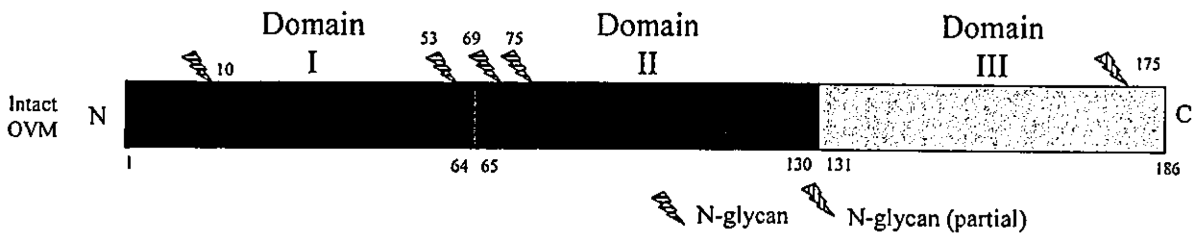

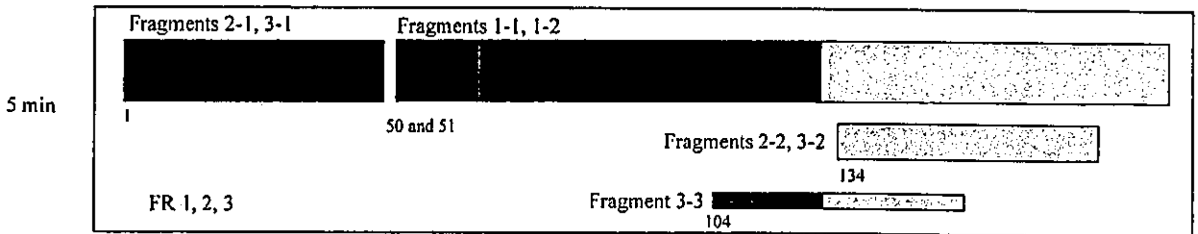

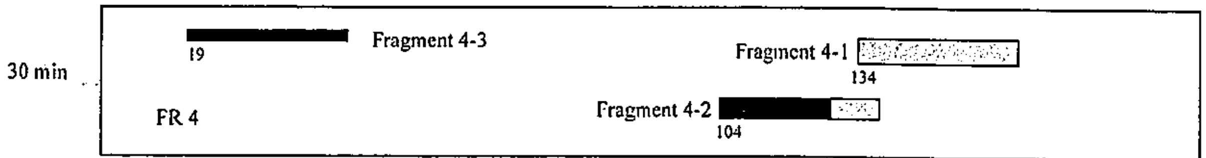

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after 5 min of digestion. Three of the serum samples also reacted with FR 2, FR 3, and FR 4 after 30 min of digestion.

The three samples that react with FR 2, FR 3, and FR 4 were obtained from patients who exhibited persistent allergies to egg white. One of these serum samples, No.4, was obtained from a 3-year-old girl who is presently 6 years old; her total IgE level has decreased slightly to 4,450 IU/ml, but the specific IgE level for egg white remains at more than 100 IU/ml, and the patient has not outgrown her hypersensitivity to eggs. Another patient, No. 13, was a 1-year-old boy; 7 years later, his total and egg white-specific IgE levels had been reduced to 947 and 6.85 IU/ml, respectively, but eating raw eggs still caused allergic symptoms. The third FR 4-positive patient, No. 19, was an 11-year-old boy whose total IgE level decreased to 3,940 IU/ml and whose egg white-specific IgE decreased to 13.5 IU/ml after a period of about 2 years; however, this patient has also not outgrown his allergies. These cases and our previously reported data [17] indicate that the induction of egg white tolerance may be difficult in patients whose serum IgE exhibits binding activity to digested small fragments of OVM.

Discussion

In the SGF-digestion system, preheating the OVM (100°C for 5 or 30 min) did not affect the OVM digestion pattern (fig. 1), consistent with the results of previous reports [9] in which heat treatment did not markedly decrease the allergenicity of OVM. On the other hand, a decrease in the pepsin/OVM ratio dramatically reduced the digestion rate, suggesting that digestibility may vary depending on the amount of OVM intake and the conditions of the individual's digestion system. In its native state, OVM possesses serine protease inhibitor activity. Fu et al. [11] and our group [10] previously reported that intact OVM was stable for 60 min in simulated intestinal fluid. Kovacs-Nolan et al. [15] also reported that pepsin-

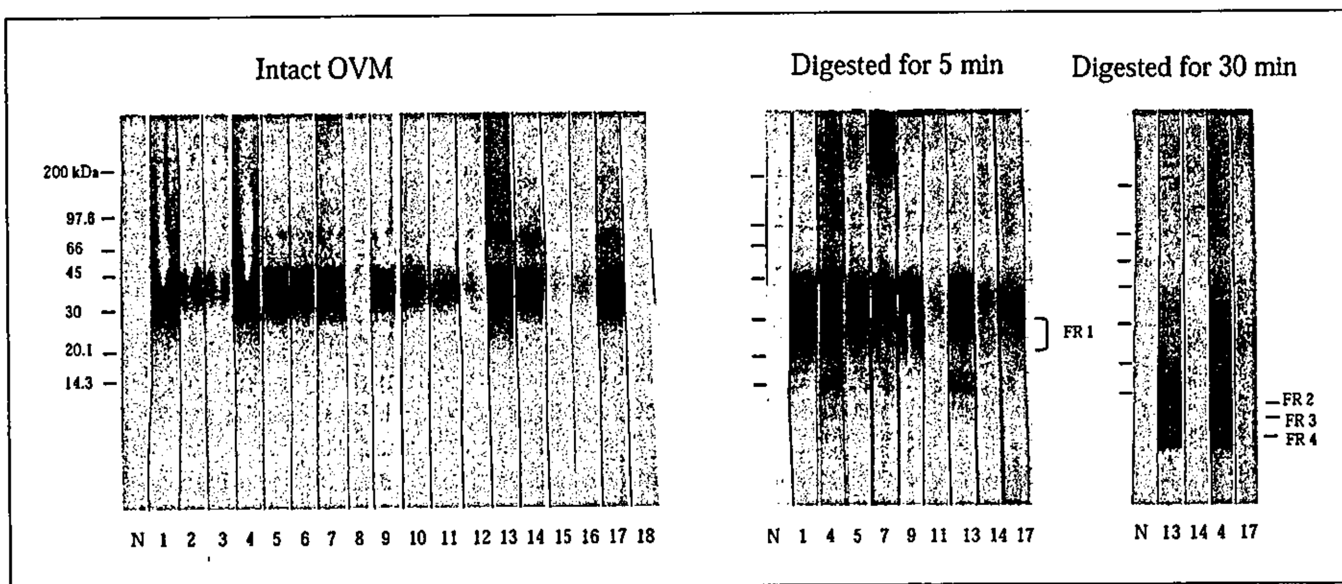

Fig. 4. Amino acid sequence and schematic representation of the SGF-digestion pattern of OVM. The amino acid sequence of OVM is shown in the upper panel. The arrows indicate the SGF-digested points according to the results of an N-terminal analysis of the OVM fragments (table 1); and the underlined regions indicate sequences identified by LC/MS/MS. Solid line = FR 1; dotted line = FR 3; dashed line = FR 4. Shaded areas represent reported human IgE epitopes [16]. The lower panel is a summary of the OVM digestion pattern according to N-terminal analysis.

Fig. 5. Western blot analysis of intact OVM and the fragments with serum IgE from egg white allergic patients and a normal volunteer. The fragments were prepared as described in the legend of figure 2. The number of each strip corresponds to the sample numbers in table 2.

Table 3. Reactivity of OVM and pepsin fragments with patient serum IgE

No.	IgE content, IU/ml		Reactivity with patient IgE[1]				
	total	egg white-specific	intact OVM	FR 1	FR 2	FR 3	FR 4
1	3,700	>100	+++	++	–	–	–
2	402	3.74	+	n.d.	n.d.	n.d.	n.d.
3	251	6.85	+	n.d.	n.d.	n.d.	n.d.
4	6,510	>100	+++	+++	+	+	++
5	2,060	>100	++	++	–	–	–
6	1,240	12.4	++	n.d.	n.d.	n.d.	n.d.
7	4,180	31.3	++	++	–	–	–
8	56	20.1	±	n.d.	n.d.	n.d.	n.d.
9	1,355	50.7	++	++	–	–	–
10	22,810	2.11	+	n.d.	n.d.	n.d.	n.d.
11	1,463	4.65	+	–	–	–	–
12	14,230	0.70–3.49	±	n.d.	n.d.	n.d.	n.d.
13	8,000	>100	+++	+++	+	+	++
14	22,490	1.05	+++	±	–	–	–
15	934	66.3	+	n.d.	n.d.	n.d.	n.d.
16	345	20.1	+	n.d.	n.d.	n.d.	n.d.
17	1,500	80	++	+	–	–	–
18	3,300	>10	–	n.d.	n.d.	n.d.	n.d.
19	20,500	26.8	+++	++	±	±	±
20	138	45.4	++	+	–	–	–
21	940	2.44	+	+	–	–	–
22	91	0.70–3.49	+	±	–	–	–
23	828	0.9	++	+	–	–	–
24	21	3.50–17.4	–	n.d.	n.d.	n.d.	n.d.
	positive/tested		22/24 (92%)[2]	13/14 (93%)[3]	3/14 (21%)[3]	3/14 (21%)[3]	3/14 (21%)[3]

n.d. = Not done.

[1] Intensity of the reactivity of each band was evaluated by the ratio to normal serum: – = <1; ± = 1–2; + = 2–5; ++ = 5–10; +++ = >10.

[2] Percent of egg white-positive samples.

[3] Percent of intact OVM-positive samples.

digested OVM retains its trypsin inhibitor activity. Therefore, OVM and its pepsin-digested fragments were thought to be stable in the small intestine.

At a pepsin/OVM ratio of 1 unit/µg, FR 1 reached a maximum level after 2 min of digestion, while both FR 2 and FR 3 reached maximum levels after 5 min of digestion; thereafter, FR 1, FR 2, and FR 3 gradually decreased. However, FR 4 increased continuously throughout the 30-min period of digestion and the major fragments were seen after 30 min of digestion (fig. 3). FR 4 was mainly composed of three fragments whose N-terminals were 134V, 104N and 19V (table 1). A C-terminal sequence, 165N–185C, was also identified in FR 4 (table 2). These fragments contain known IgE epitopes [19] and therefore may cause allergic responses. Three of the OVM-positive sera from patients with egg white allergy reacted positively with the FR 4 fragments (table 3).

The present results are consistent with the previous finding that pediatric subjects with a higher IgE-binding activity to pepsin-treated OVM were unlikely to outgrow their egg allergy [17]. For peanut allergies, differences in IgE-binding epitopes have been reported between the patients with clinically active peanut allergies and those who developed a tolerance, regardless of the presence of high or low peanut-specific IgE levels [20].

The N-terminal residue of the major fragment (4-1) of FR 4 was Val-134 (30%; table 1). This fragment retains most of domain III, which has been reported to have significantly higher human IgG- and IgE-binding activities than those of domains I and II [12]. A domain-III OVM

variant has also been reported to cause a reduction in immunogenicity and allergenicity [21].

Domains I, II, and III contain one, three, and one N-glycosylation sites, respectively [7]. The possible relation between the carbohydrate chain in domain III and allergenicity is interesting. One report suggested that this carbohydrate chain may play an important role in allergenic determinants against human IgE antibody [13], and another report suggested that the carbohydrate chains of OVM may protect against peptic hydrolysis [22]. However, the carbohydrate moieties have been shown to have only a minor effect on allergenicity [23]. As shown in figure 2, intact OVM, FR 1, and FR 2 fragments were detected using PAS staining, suggesting the presence of carbohydrate chains, but FR 4 was not stained with the PAS reagent, despite being clearly detected with CBB. Therefore, FR 4 might contain little or no carbohydrate chains. Since FR 4 seems to maintain its allergenic potential, as described above, the absence of the carbohydrate chains in FR 4 suggests that they are not necessary for OVM allergenicity. Since the minimum peptide size capable of eliciting significant clinical symptoms of allergic reactions is thought to be 3.1 kDa [24], FR 4 may be able to trigger mast cell activation and elicit clinical symptoms.

In this report, the SGF-digestion kinetic pattern of OVM was investigated in detail, and the partial sequences of the fragments in the 4 fractions separated by SDS-PAGE were determined. Furthermore, the reactivity of the fragments with a number of serum samples from patients with egg white allergies was detected using Western blotting. The four fractions were separated according to their molecular weight and consisted of more than one fragment, as determined by N-terminal analysis. The identified sequences that started at Asn-104 and Val-134 in FR 3, as determined using LC/MS/MS (table 2), coincided with the 3-2 and 3-3 fragments in the N-terminal analysis (table 1), and the sequence that started at Asn-104 in FR 4 coincided with fragment 4-2. Moreover, the LC/MS/MS analysis indicated that FR 3 and FR 4 contained other parts of domain II and the C-terminal sequence N165–C185, which are thought to be minor components of these fractions. The combination of SGF digestion and patient IgE may provide useful information for the diagnosis and prediction of potential OVM allergenicity.

Acknowledgement

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Neutralizing Antibody Evasion Ability of Adenovirus Vector Induced by the Bioconjugation of Methoxypolyethylene Glycol Succinimidyl Propionate (MPEG-SPA)

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Although adenovirus vectors (Ad) which possesses high transduction efficiency are widely used for gene therapy in animal models, clinical use is very limited. One of the main reason is that nearly 80% of human beings possess anti-Ad antibodies. In this study, we tried to modify Ad with methoxypolyethylene glycol (MPEG) activated by succinimidyl propionate, and, the neutralizing antibody evasion ability of PEGylated Ad was evaluated. The results demonstrated that PEG-Ad showed stronger protection ability against anti-Ad neutralizing antibody compared to that with unmodified-Ad. Considering there are many people carrying neutralizing antibody against Ad and readministration of Ad was necessary for treating chronic diseases, this strategy, which was also applicable to other vectors, can be used for developing improved vectors.

Key words adenovirus vector; polyethylene glycol; antibody; gene therapy

Although, the gene therapy for cancer or an incurable disease has attracted considerable attention, clinical use is very limited by the problem of vectors for transgenics. Vectors based on human adenoviruses have been developed as efficient vehicles for therapeutic transgenes in a wide variety of animal models.[1—3] Gene manipulated adenovirus vectors (Ad) have commanded considerable attention as gene delivery carriers since high-titer replication-deficient recombinant viral preparations can be easily generated, the viruses can be engineered to accommodate large DNA inserts, and they can show high gene expression in a wide variety of dividing and nondividing cells.[4,5] However, first-generation adenoviruses possess a fundamental problem that both cellular and humoral immune responses prohibit gene transfer on readministration of Ad due to neutralization of viral particles by antibodies produced against capsid proteins.[6,7] Furthermore, many people carry immunity to Ad and a large amount of Ad administration causes side effects. Therefore, clinical application of Ad was very limited.[8—10] PEGylation, the covalent attachment of activated polyethylene glycol (PEG) to free lysine groups on Ad surface, is a promising strategy for overcoming these limitations. PEG-modification is a well-established technique for the modification of therapeutic peptides and proteins, and the validity of PEGylation has been reported by us and some other groups.[11,12] This method enabled transgenics in the presence of neutralizing antibodies of Ad without the necessity of recombining a gene in a vector like a gutless vector.[13,14] In this study, we used monomethoxypolyethylene glycol (MPEG) activated by succinimidyl propionate, which reacts preferentially with the ε-amino terminal of lysine residues, to covalently attached to the surface of Ad. And, we assessed the effects of biochemical modification of viral capsids with functionalized PEG on the neutralizing antibody against first-generation Ad.

MATERIALS AND METHODS

Cells and Animals HEK 293 cells, A549 human lung carcinoma cells were cultured with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Female ICR mice were purchased from Nippon SLC (Kyoto, Japan) and used at 6 weeks-old stage.

Adenovirus ad expressing firefly Luciferase under the control of cytomegalovirus (CMV) promoters were amplified in 293 cells, using a modification of established methods and purified from cell lysates by banding twice on CsCl gradients, dialyzed and stored at −80 °C. The Ad used in this study were constructed by an improved *in vitro* ligation method as described previously.[15] Viral particle titer was spectrophotometrically determined by the established method.[16]

PEGylation of Ad Activated methoxypolyethylene glycol succinimidyl propionate (MPEG-SPA, MW 5000, Shearwater Corporation) was used in this study (Fig. 1). Ad was reacted with 100 molar excess of mPEG-SPA for viral lysine residue at 37 °C for 45 min with gentle stirring (300 rpm). The particle size of PEG-Ad was measured by ZETASIZER 3000HS (Malvern, U.K.).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis SDS-PAGE analysis was conducted for detecting the PEGylated viral proteins. In short, the unmodified-Ad or PEG-Ad suspensions were mixed with the same volume of 2XSDS protein gel loading solution (Quality Biological, Inc. U.S.A.) and 5% (v/v) 2-

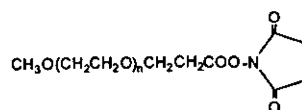

Fig. 1. mPEG-SPA Methoxypolyethylene Glycol Succinimidyl Propionate MW 5000.

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mercaptothanol was added to the final solution. After heating at 95 °C for 5 min, the mixture was added to 4—20% of polyacrylamide gel (PAG Mini 4/20) and electrophoresis was carried out using SDS-PAGE buffer. Full range Rainbow™ molecular weight marker (Amersham Life Science, U.S.A.) was used as the marker in this study. The gel was stained for viral hexon using Coomassie blue. The bioconjugation ratio of PEGylated Ad was calculated by the colority of the hexon band and the PEGylated hexon using NIH Image software.

Preparation of Ad Antiserum Ad antiserum was obtained from ICR mouse according to the approach described previously.[17,18] In brief, female ICR mouse (6 weeks old) was administered hypodermically with a dose of 10^{10} viral particles of conventional Ad with Freund's complete adjuvant in 100 μl of PBS. Another 10^{10} viral particles of conventional Ad were hypodermically administered with Freund's incomplete adjuvant after 2 and 4 weeks. Then serum of the mouse was collected after 1 week and filtered, and stored at −20 °C.

Transduction Efficiency of PEG-Ad and Unmodified-Ad into A549 Cells in the Presence or Absence of Ad Antiserum A549 cells (1×10^4 cells) were seeded into a 48-well plate with 500 μl medium. On the following day, the cells were transduced with 1000 particles/cell of unmodified-Ad or PEG-Ad respectively in a final volume of 500 μl in the presence of 0, 42, 125 ng protein/well of Ad antiserum. After 24 h cultivation, luciferase activity was measured using Luciferase Assay System (Promega, U.S.A.) and Microlumat Plus LB 96 (Perkin Elmer, U.S.A.) after cells were lysed with Luciferase Cell Culture Lysis Reagent (Promega, U.S.A.) according to the manufacturer's instruction.

Table 1. Viral Particle Sizes of PEGylated Ad and Unmodified-Ad

Ratio (Ad : PEG)[a]	Vector size (nm)
1 : 0 (unmodified)	113.3±0.76
1 : 100 (PEG-Ad)	123.8±0.98

a) Amount of PEG to lysine residue of adenovirus vector capsid protein (mol : mol).

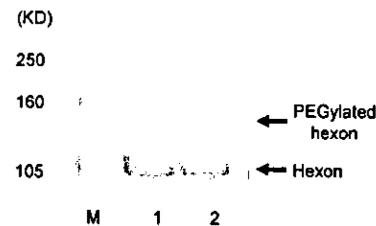

Fig. 2. SDS-PAGE Analysis of PEGylated Hexon

Lane M, protein marker; lane 1, unmodified-Ad; lane 2, PEG-Ad. Further details are described in Materials and Methods.

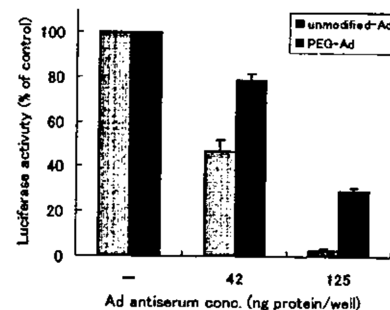

Fig. 3. Transduction Efficiency of Unmodified-Ad and PEG-Ad in the Presence or Absence of Ad Antiserum

A549 cells (1×10^4 cells) were transduced with 1000 particles/cell of unmodified-Ad and PEG-Ad in the presence or absence of Ad antiserum respectively. Luciferase expression was measured after 24 h. Each point was represented as mean±S.D. (n=3).

RESULTS AND DISCUSSION

In this study, we tried to modify Ad with MPEG activated by succinimidyl propionate which was safety and widely used.[19] A simple and practicable method for constructing of PEG-Ad was employed. After reaction, the sizes of PEG-Ad and unmodified-Ad were measured. The results showed that the particle size of Ad was increased with PEGylation. The average viral particle size of PEG-Ad was about 10 nm bigger than that observed in the unmodified-Ad (Table 1). And SDS-PAGE analysis showed the presence of a new band of PEGylated viral capsid protein, hexon (Fig. 2). We also demonstrated that all Ad were conjugated by activated PEG, and there was no unmodified Ad mixed with the PEGylated ones (data not shown).

For evaluating the protection ability of PEG-Ad against the neutralizing antibodies, PEG-Ad and unmodified-Ad were added to A549 cells in the presence or absence of neutralizing antibodies against adenovirus capsid proteins, and the transduction levels were compared. Transduction efficiency of the unmodified-Ad was significantly reduced by the neutralizing antibodies (Fig. 3). In the presence of neutralizing antiserum of 42 ng, the luciferase gene expression of unmodified-Ad was less than half of that without antiserum, whereas PEG-Ad remained about 80% of its gene expression. In the presence of antibodies of 125 ng, the gene expression of unmodified-Ad was only 2.5% compared to that in the absence of antibodies, however, PEG-Ad showed more than 10-fold of antibody evasion ability compared to unmodified-Ad. As anticipated, the absolute level of gene expression of PEG-Ad was decreased to about 1/300 compared to that of unmodified-Ad in the absence of antibodies due to the effect that PEG chains prevented the interaction between Ad and coxsackie-adenovirus receptor (CAR). For developing novel Ad which possess a targeting ability, it is indispensable to block the route of Ad infection through CAR because of its broad expression in many tissues. So as the next step, we are trying to construct Ad which was conjugated by PEG with targeting molecule on the tip of the PEG. And we expect that this novel Ad will enhance the transduction efficiency of PEG-Ad at the same time keep the evading ability from the neutralizing antibodies.

One of the goals of Ad PEGylation is to provide a means to overcome the major limitation of Ad gene therapy, namely, antibody neutralization. The results presented here demonstrate that PEGylation can notably improve the ability of Ad to escape antibody neutralization *in vitro* by the steric hindrance of PEG chains. This antibody evasion ability is essential for clinical applications, because nearly 80% of human beings possess anti-Ad antibodies and readministration is indispensable in some cases like treating chronic diseases, such as cystic fibrosis and hemophilia. Furthermore, antibody evasion ability enabled decrease of the amount of medical re-