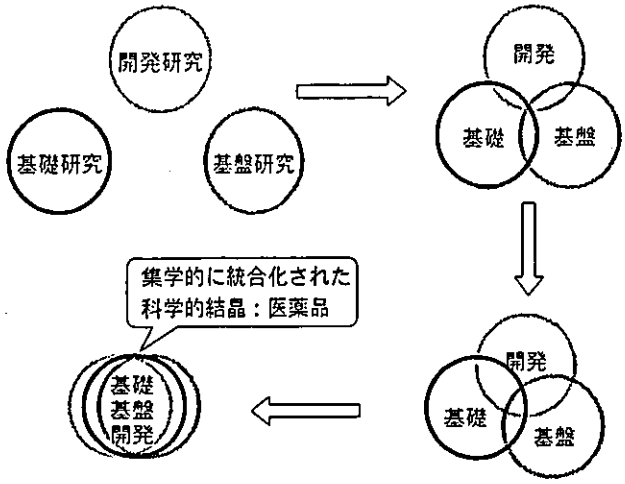


図27　基礎研究、基盤研究、開発研究の効率的連携と集学的な統合化による医薬品創製

しかし、こうした結晶を生み出すためには、基礎研究、基盤研究、開発研究がそれぞれ自律的に進歩、発展を遂げることももちろんですが、創薬を目指す場合には、これらが不統合では都合が悪く、それらの効率的、有機的連携と集学的な統合化が不可欠です。これまでのバイオ医薬品の例を振り返ると、結局、スムーズな開発に成功したバイオ医薬品は、生命科学の基礎および応用研究の成果を合理的にかつ効率良く医療への応用に結びつけたものです。そして、これからはさらに倫理的妥当性、社会的理解・認知などの問題をクリアしたものでなければなりません。

3.4　科学的妥当性、倫理的妥当性、社会的理解・認知、経済的許容性の確保

すなわち、人から得た材料をベースに個人の遺伝子情報も含め研究を進めたり、人から得た細胞組織などを医療に応用する、あるいは遺伝子治療を行なう、ファーマコゲノミクスに基づいてテーラーメード医療を行なうなどの先端的医療分野において特に明らかなことですが、科学的妥当性、倫理的妥当性、社会的理解・認知がこれからますます重要な要素、避けては通れない極めて重要でエッセンシャルな要素となります（図 28 参照）。

またこれらの諸要素をいかに調整、調和させていくかを考える必要があります。さらに、経済的妥当性の確保といった問題も解決していかなければならないことです。

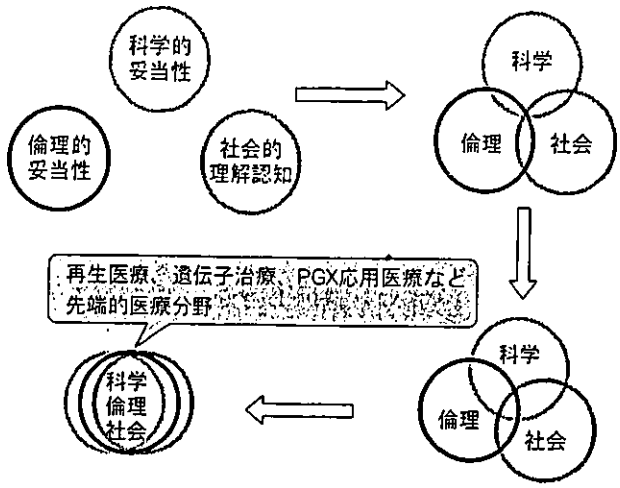


図28　先端的医療分野に必要な科学、倫理、社会的理解の調和

3.5　産・学・官の連携

次に、別の次元から見た重要な要素に、産・学・官の連携が挙げられます（図 29 参照）。産・学・官の連携の場合に、技術的な面での連携とコミュニケーションという面での連携があります。現在、技術面では、ミレニアムプロジェクト、疾患プロテオーム、トキシコゲノムプロジェクト、創薬総合研究事業などが、直接に創薬を意識したものとしてありますし、コミュニケーション面では、バイオロジクスフォーラムや医療機器フォーラム、品質フォーラムなどがあります。さらに積極的に優良な医薬品の開発のために産・学・官が技術的連携をする、また、コミュニケーションの場を作り、情報交流の面での連携を深めることが、国際化時代にあって全日本として対応していくために非常に重要であると思います。

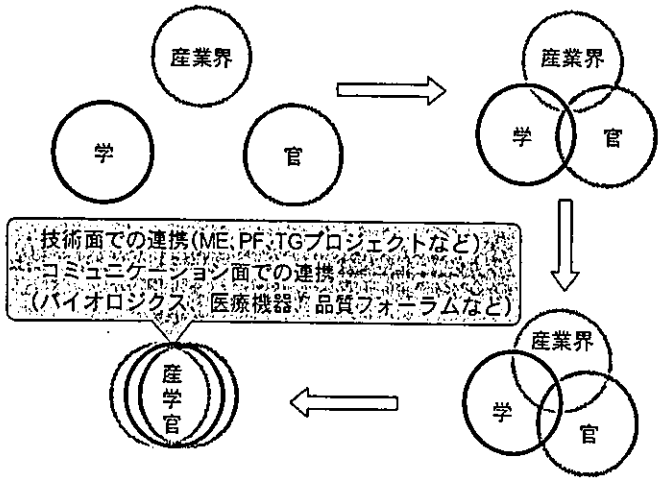


図29　産・学・官の連携

3.6　国際共同活動と規制基準の国際調和

さらに、別の視点からの医薬品開発の効果的推進に必要な要素として、国際共同活動と、規制基準の国際調和が挙げられます。医薬品分野の国際調和の最も代表的な例は、ICH と薬局方の国際調和活動です。いずれも新医薬品開発のほとんどを占める日・米・欧の三極が行なっている活動です（図 30 参照）。

ICH の目的は、「より優れた医薬品を国や地域を越えて少しでも早く患者のもとに届ける」ことです。「より優れた医薬品」とは、高い品質、有効性、安全性が確保された画期的な新薬に代表されるものです。それらを「国や地域を越えて少しでも早く患者のもとに届ける」ために、医薬品先進地域である日・米・欧三極における Q（品質）/S（安全性）/E（有効性）に関する承認審査基準や必要とされるデータの違いを極力解消しようとする国際共同活動が ICH です[33]。

ICH 活動の具体的内容は、医薬品開発および評価にあたって、どのように試験項目や試験方法を選択し評価すれば合理的であり、適正であるかについて、三極の製薬メーカおよび規制当局が同じテーブルにつき論議し、共通に活用できる国際ガイドラインを作製し、それらを各極でのルールの基本とすることです。ICH 活動の期待される成果は、医薬品開発で求められる品質評価試験、非臨床安全性試験、臨床試験などにおける各極間の不必要な重複がなくなり、治験に参加するヒトや非臨床試験での動物資源などが最小限になり、いずれの極で開発された製品でもそのデータが他極でも受け入れられ、適正に評価され、速やかな医薬品開発が進むことにあります。つまり ICH には規制という側面もありますが、創薬の推進という側面も大きいということを強調しておきます。

次に、今後バイオ医薬品分野で国際調和が期待される課題について述べます。

1)　先発バイオ医薬品のコンパラビリティ（同等性・同質性）：品質版、非臨床版、臨

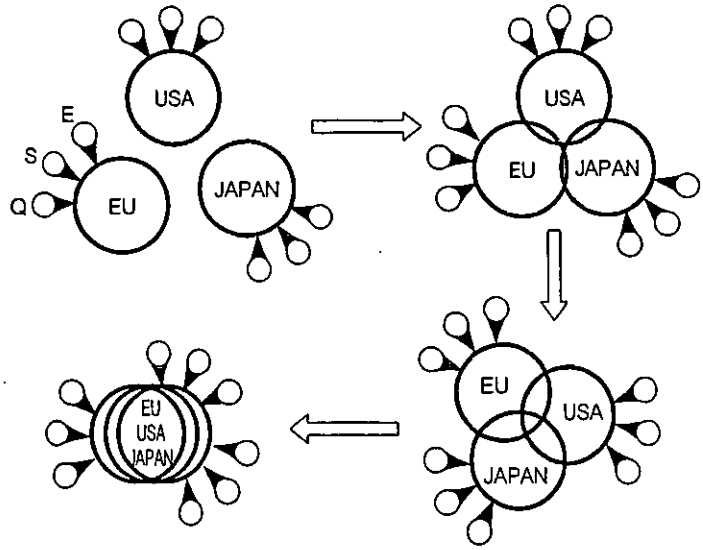


図30　日・米・欧三極による国際調和

床版の完成

2) バイオジェネリックスのコンパラビリティ

3) プロセスバリデーション

4) 製造工程

5) 既存の GL（Q5A、Q5B、Q5C、Q5D、Q6B）の改訂

6) 遺伝子治療薬

7) 細胞治療薬

などです。

ちなみにコンパラビリティとは、たとえば図 31 に示すように、開発段階で製法 X で開発していた製品を、製法 Y と一部変更した場合、新製品の旧製品との同等性・同質性をどのように立証していけばよいかという問題です[12,34,35]。

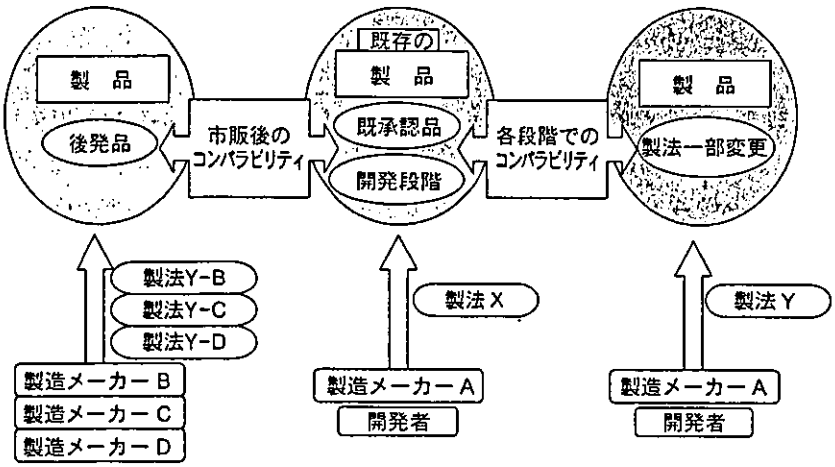


図31 コンパラビリティ

3.7 品質・有効性・安全性確保

次に、バイオ創薬の適正かつ効果的な進展に必要な要素として品質・有効性・安全性の確保が挙げられることは言うまでもありません。

品質・有効性・安全性、いずれの要素も確保できたものが医薬品として認知されるわけですが、いかにこれらの要素を合理的に効果的にバランスをとり、統合化していくかが、医薬品開発推進の鍵になるのです（図 32 参照）。より優良な医薬品を目指して、より高いレベルでの品質、有効性、安全性の確保を図ることも、当然望まれています。

まさに、各関係者のそれぞれの立場での腕の見せ所でもあり、認識の共有化も必要なところです。

技術的な視点からいえば、たとえばバイオテクノロジーという画期的な医薬品製造技術の応用が、医薬品に結実していくためには、新たな技術により得られた新たな製品の

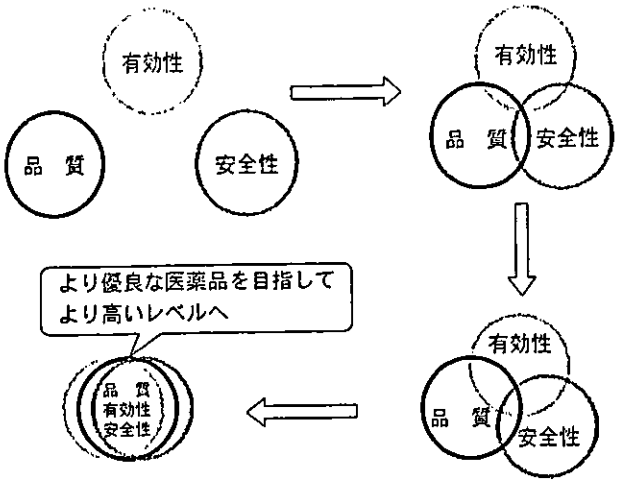


図32　優良な医薬品を目指した品質・有効性・安全性の確保

特徴に応じた品質、安全性および有効性に関する適切な試験方法や評価方法の開発というもう一つの要素が不可欠であるということです。

もともと、どのような時代、製品にあっても、新たな医薬品の創製（創薬）は、技術的には新たな医薬品シーズの探索・発見や新たな医薬品製造技術の進歩と、これに対応するよりレベルの高い、新たな製品の品質、安全性、有効性確保のための適切な試験方法や評価方法の開発といういわば車の両輪によって推進、達成されます。製造技術の開発と並んで試験・評価法を開発し、あるいは評価方法に関する適切な指針を提示することは、当面の問題を解いていくためばかりではなく、将来、新たな医薬品開発を適正にかつ効率よく促進させるための先導的基盤的要素にもなるということです。

そうした認識のもと、国内外の関係者はそれぞれの立場で、この目標を達成すべく注力してきましたが、さらに強力に推進していくことが改めて期待されています。

特性解析、評価、管理、使用に関して、特に課題を抱える新規医薬品の例としては、

1)　巨大糖タンパク質
2)　機能性人工タンパク質
3)　各種細胞・組織製品
4)　増殖性ウイルスベクター
5)　自己複製性 RNA を用いた新規組換えワクチンなどの遺伝子ベースの製品
6)　分子標的薬
7)　テーラーメード型医薬品
8)　ナノテクノロジーなどを応用した新規 DDS 製剤

などがあります。

幹細胞・前駆細胞由来製品については、

1) 通常の分化誘導や、分化系列を越えての分化誘導（trans-differentiation）の徹底したコントロールとモニタ、および製品の特性解析と品質管理が必要です。そして
2) この目的を達成するための関連技術の開発が必要ですが、
3) いかに critical バイオマーカを見い出すか、いかに最終細胞製品の力価や機能を測定するか
4) 貴重な試料の非破壊的測定もしくは微量化など

も重要な課題になってくると思います。

複合的バイオロジクスについては

1) いかに規制基準を作成し、適用するか
2) いかに各構成要素および最終製品について品質・有効性・安全性に関連して特性解析し、評価するか
3) いかに製造工程を評価/バリデートするか
4) いかに製造工程や最終製品の恒常性を維持・保証するか

などが重要な課題であると思われます。

次に、ウイルス安全性確保の技術面での今後の課題を挙げます。たとえば、

1) 新興・再興感染症関連ウイルスの高感度・高精度検出法の開発
2) 各種ウイルスに対する感染性 PCR 法の開発
3) マイクロアレイ技術などを応用した多種類のウイルス同時検出法・同定法の開発と標準化
4) プロテオミクスの応用による既知および想定される感染性ウイルス検出法の開発
5) 非破壊的方法の開発による試料量の節約
6) ナノテクノロジー：フロースルーアッセイの応用
7) ウイルスに関する新規不活化・除去法の開発

などが挙げられます。

度々述べていることですが、これからは、先端技術をどの局面でいかに活用するかが非常に重要になってくると考えられます。

1) 「ゲノミクス」「プロテオミクス」「バイオインフォマティクス」などの新技術を特性解析、評価あるいは管理方法としていかに適切に活用するか
2) 研究開発、承認審査、承認後の品質管理などのステージ、局面でいかにこれらの技術を活用するか
3) 実験動物による安全性、有効性評価試験から生化学的解析、ゲノミクス、プロテオミクスによる評価試験への移行をいかに合理的に図るか
4) 先端技術を駆使して開発された製品を適正に迅速に科学的評価するための、規制当

局の先端技術に対する理解度をいかに深めるか
などが、重要なポイントと考えられます[36]。

3.8 トランスレーショナルリサーチの推進

ポストゲノム時代において、有望な医薬品候補をより迅速・的確にピックアップし、効率的な開発の推進を図るためには、トランスレーショナルリサーチ（TR：探索的臨床研究）を適正に実施することが、極めて重要な要素になると言われています[9,19,37]。

図33に、TRにおける基本的要素を示します。

前述したように、バイオ創薬は、生命科学分野での学問的解明や技術開発の進歩の延長線上にあり、基礎研究、基盤技術研究から臨床応用にいかにスムーズに、合理的に至るかというポイントは、ここに示した要素の連携をいかに効率よく行なうか、いかに必要な各要素を最終目標に向かって統合化していくかが重要になります。システム的には、そうした実施環境・体制をいかに構築するかが重要なのです。

規制環境の整備も科学的妥当性や倫理的妥当性をいかに考えるかというガイダンスを示すかも含めて、ポジティブに捉え、活用できれば、TR を適正に推進する上で非常に重要な要素になると思います。

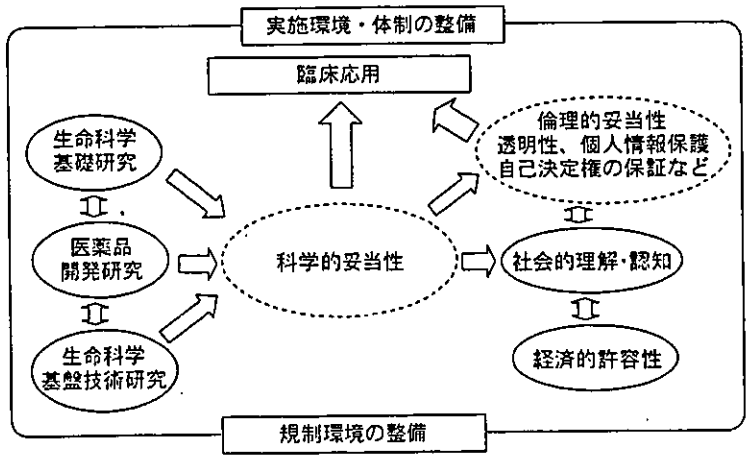


図33　トランスレーショナルリサーチの基本的要素

4 まとめ

これからは医療技術的にも新しく、また、社会的理解・認知、倫理的妥当性の面でもあらかじめ用意された答えはない、いわば新たな挑戦となるものが次々と出てくること

が予測されます。これらについては、産・学・官そして全ての医療関係者がより優良な医薬品や適正な医療技術を患者に1日でも早く提供するという観点に立ち、蓄積された知識や経験と新たな英知を結集して、医療の進歩と課題の克服にあたることが重要であると思われます。

【引用・参考文献】

1) 早川堯夫, 石井（渡部）明子：先端的バイオロジクス開発の現状と新たなバイオ創薬に向けての課題, 医薬品研究, 33, pp. 693-728 (2002).
2) 早川堯夫：バイオテクノロジーを応用した医薬品の特性解析, 品質及び安全性確保の評価科学-組換え医薬品, 細胞培養医薬品, 遺伝子治療用医薬品, 細胞治療用医薬品, トランスジェニック動物由来タンパク質性医薬品, トランスジェニック動物由来細胞治療医薬品-, 衛研報告, 117, pp. 1-38 (1999).
3) 厚生省医薬安全局審査管理課長通知, 生物薬品（バイオテクノロジー応用医薬品/生物起源由来医薬品）製造用細胞基材の由来, 調製および特性解析, 医薬審第 873 号（平成 12 年 7 月 14 日).
4) 厚生省医薬安全局審査管理課長通知, 組換え DNA 技術を応用したタンパク質生産に用いる細胞中の遺伝子発現構成体の分析, 医薬審第 3 号（平成 10 年 1 月 6 日).
5) 厚生省医薬安全局審査管理課長通知, ヒト又は動物細胞株を用いて製造されるバイオテクノロジー応用医薬品のウイルス安全性評価, 医薬審第 329 号（平成 12 年 2 月 22 日).
6) 厚生省医薬安全局審査管理課長通知, 生物薬品（バイオテクノロジー応用医薬品/生物起源由来医薬品）の安定性試験, 医薬審第 6 号（平成 10 年 1 月 6 日).
7) 厚生労働省医薬局審査管理課長通知, 生物薬品（バイオテクノロジー応用医薬品/生物起源由来医薬品）の規格および試験方法の設定, 医薬審第 571 号（平成 13 年 5 月 1 日).
8) 厚生省医薬安全局審査管理課長通知, バイオテクノロジー応用医薬品の非臨床における安全性評価, 医薬審第 326 号（平成 12 年 2 月 22 日).
9) 早川堯夫：バイオテクノロジー応用医薬品, 臨床試験, 内藤周幸編：薬事日報社, pp. 155-179 (2003).
10) 早川堯夫：製品の特性解析・品質規格、安定性および Comparability：バイオ医薬品の品質・安全性評価, 早川堯夫, 山崎修道, 延原正弘編：エル・アイ・シー, pp. 205-230 (2001).
11) N. Kawasaki, M. Ohta, S. Hyuga, M. Hyuga and T. Hayakawa : Application of liquid chromatography / mass spectrometry and liquid chromatography with tandem mass spectrometry to the analy sisofthesite - specific carbohydrate

heterogeneity in erythropoietin, *Anal. Biochem.*, 285, pp. 82−91 (2000).
12) 早川堯夫：製品の特性解析・品質規格，安定性および Comparability：バイオ医薬品の品質・安全性評価，早川堯夫，山崎修道，延原正弘編：エル・アイ・シー，pp. 205−230 (2001).
13) 厚生労働省医薬局長通知，遺伝子治療用医薬品の品質および安全性の確保に関する指針，医薬発第 0329004 号，（平成 14 年 3 月 29 日).
14) 早川堯夫：遺伝子治療用医薬品の品質，安全性等の確保，早川堯夫，山崎修道，延原正弘編：バイオ医薬品の品質・安全性評価，エル・アイ・シー，pp. 341−350 (2001).
15) 厚生労働省医薬局長通知，細胞・組織利用医薬品等の取扱いおよび使用に関する基本的考え方，医薬発第 266 号（平成 13 年 3 月 28 日).
16) 早川堯夫，永田龍二：再生医療分野における指針・ガイドライン：再生医療の適切かつ効果的な推進を目指して，再生医療，8, pp. 11−19 (2004).
17) 厚生省医薬安全局長通知，ヒト由来細胞・組織加工医薬品等の品質および安全性の確保に関する指針，医薬発第 1314 号（平成 12 年 12 月 26 日).
18) 早川堯夫，山口照英，押沢正：日局生物薬品の品質・安全性確保に関する研究―ウイルス安全性確保の基本要件―，医薬品研究，33, pp. 210−230 (2002).
19) 早川堯夫：バイオ創薬におけるレギュラトリーサイエンスの新展開，衛研報告，121, pp. 128−143 (2003).
20) H. Mizuguchi, M. A. Kay, and T. Hayakawa : A pproaches for generating recombinant adenovirus vectors, *Adv. DrugDeliv. Rev.*, 52, pp. 165−176 (2001).
21) 水口裕之，早川堯夫:遺伝子機能解析のための遺伝子導入ベクター−ウイルスベクターを中心として−，蛋白質核酸酵素，48, pp. 1653−1662 (2003).
22) H. Mizuguchi, N. Koizumi, T. Hosono, N. Utoguchi, Y. Watanabe, M. A. Kay and T. Hayakawa : Asimplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob, *Gene Ther.*, 8, pp. 730−735 (2001).
23) N. Koizumi, H. Mizuguchi, T. Hosono, A. Watabe−Ishii, E. Uchida, N. Utoguchi, Y. Watanabe and T. Hayakawa : Efficient gene transfer by fiber−mutant adenoviral vectors containing RGD peptide, *Biochim Biophys Acta*, 1568, pp. 13−20 (2001).
24) N. Koizumi, H. Mizuguchi, N. Utoguchi, Y. Watanabe and T. Hayakawa : Generation of fiber−modified adenovirus vectors containing heterologous peptides in both the HI loop and C−terminus of the fiber knob, *J.Gene Med*, 5, pp. 267−276 (2003).
25) F. Sakurai, H. Mizuguchi and T. Hayakawa : Efficient gene transfer into human $CD34^+$ Cells by an adenovirus type 35 vector, *Gene Ther*, 10, pp. 1041−1048 (2003).
26) F. Sakurai, H. Mizuguchi, T. Yamaguchi and T. Hayakawa : Characterization of in vitro and in vivo gene transfer properties of adenovirus serotype 35 vector, *Mol.*

Ther, 8, pp. 813−821 (2003).

27) H. Mizuguchi and T. Hayakawa : Characteristics of adenovirus－mediated tetracycline controllable expression system, *Biochim Biophys Acta*, 1568, pp. 21−29 (2001).

28) H. Mizuguchi, M. A. Kay, T. Hayakawa : In vitro ligation−based cloning of foreign DNAs into the E3 as well as E1 deletion region for generation of recombinant adenovirus vector, *Bio Techniques*, 30, pp. 1112−1116 (2001).

29) H. Mizuguchi and T. Hayakawa : The tet−off system is more effective than the tet−on system for regulating transgene expression in a single adenovirus vector . *J. Gene Med.*, 4, pp. 240−247 (2002).

30) H. Mizuguchi, Z. Xu, F. Sakurai, T. Mayumi and T. Hayakawa : Tight positive regulation of transgene expression by asingle adenovirus vector containing the rt TA and tTS expression cassettes in separate genome regions. *Hum. Gene Ther.*, 14, pp. 1265−1277 (2003).

31) T. Hosono, H. Mizuguchi, K. Katayama, Z. Xu, F. Sakurai, A. Ishii−Watabe, K. Kawabata, T. Yamaguchi, S. Nakagawa, T. Mayumi and T. Hayakawa : Adenovirus vector−mediated doxycycline−inducible RNA interference, *Human Gene Ther.*, 15, pp. 813−819 (2004).

32) T. Hosono, H. Mizuguchi, K. Katayama, N. Koizumi, K. Kawabata, T. Yamaguchi, S. Nakagawa, Y. Watanabe, T. Mayumi, and T. Hayakawa : RNA interference of PPARg using fiber−modified adenovirus vector efficiently suppresses preadipocyte−to−adipocyte differentiation in 3T3−L1 cells (Submitted).

33) 早川堯夫：バイオテクノロジー医薬品分野における ICH の進展，ファルマシア，34：pp. 992−994 (1998).

34) T. Hayakawa : Perspective on assessing comparability of biotechnology products−aview from Japan−Biologics 2000 Brown F, Lubiniecki A, Murano G (eds) , *Dev Biol Stand.*, Basel, Karger, 109, pp. 27−40 (2002).

35) 早川堯夫：品質 (Quality) 分野［バイオ］，ICH6 最前線−国際調和の新潮流−，日刊薬業別冊，特別企画，じほう，pp. 137−144 (2003).

36) 早川堯夫：バイオ医薬品の新展開と課題，ICH6 最前線−国際調和の新潮流−，日刊薬業別冊，特別企画，じほう，pp. 109−111 (2003).

37) 早川堯夫，石井（渡部）明子：生物薬品の開発の現状とトランスレーショナルリサーチへの条件，医学のあゆみ，200, pp. 539−543 (2002).

<早川　堯夫>

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1067 (2005) 145–152
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Isotope tag method for quantitative analysis of carbohydrates by liquid chromatography–mass spectrometry

Jin Yuan[1], Noritaka Hashii, Nana Kawasaki*, Satsuki Itoh, Toru Kawanishi, Takao Hayakawa

Division of Biological Chemistry and Biologicals, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Available online 15 December 2004

Abstract

We have previously demonstrated that liquid chromatography/mass spectrometry equipped with a graphitized carbon column (GCC-LC/MS) is useful for the structural analysis of carbohydrates in a glycoprotein. Here, we studied the monosaccharide composition analysis and quantitative oligosaccharide profiling by GCC-LC/MS. Monosaccharides were labeled with 2-aminopyridine and then separated and monitored by GCC-LC/MS in the selective ion mode. The use of tetradeuterium-labeled pyridylamino (d_4-PA) monosaccharides as internal standards, which were prepared by the tagging of standard monosaccharides with hexadeuterium-labeled 2-aminopyridine (d_6-AP), afforded a good linearity and reproducibility in ESIMS analysis. This method was successfully applied to the monosaccharide composition analysis of model glycoproteins, fetuin, and erythropoietin. For quantitative oligosaccharide profiling, oligosaccharides released from an analyte and a standard glycoprotein were tagged with d_0- and d_6-AP, respectively, and an equal amount of d_0- and d_4-PA oligosaccharides were coinjected into GCC-LC/MS. In this procedure, the oligosaccharides that existed in either analyte or a standard glycoprotein appeared as single ions, and the oligosaccharides that existed in both analyte and a standard glycoprotein were detected as paired ions. The relative amount of analyte oligosaccharides could be determined on the basis of the analyte/internal standard ion-pair intensity ratio. The quantitative oligosaccharide profiling enabled us to make a quantitative and qualitative comparison of glycosylation between the analyte and standard glycoproteins. The isotope tag method can be applicable for quality control and comparability assessment of glycoprotein products as well as the analysis of glycan alteration in some diseases.

Keywords: Monosaccharides; Oligosaccharides; Pyridylamination; Isotope tag

1. Introduction

A variety of recombinant glycoproteins and modified glycoproteins are developed as medical agents, and most of them exist in heterogeneous forms because of the various combinations of oligosaccharides. Alteration of glycosylation is known to affect the biological activity, mobilization, and biophysical properties of glycoproteins [1], so assessments of their carbohydrate structure and heterogeneity are essential in many stages of development and quality control of glycoprotein products. Since glycosylation varies in response to changes in the manufacturing condition, monosaccharide composition analysis and/or oligosaccharide profiling are needed for the characterization and as a test for constancy and comparability assessments of glycosylation [2]. Several analytical procedures using HPLC have been reported for oligosaccharide profiling and structural analysis of carbohydrates [3–5]. The oligosaccharide profiling using liquid chromatography/mass spectrometry (LC/MS) is especially known to provide structural information from their chromatographic behavior and molecular mass [6–8]. We have developed mass spectrometric oligosaccharide profiling using a graphitized carbon column (GCC), which can separate

Abbreviations: AP, 2-aminopyridine; d_0, non-deuterium-labeled; d_4, tetradeuterium-labeled; d_6, hexadeuterium-labeled; Fuc, fucose; Gal, galactose; GalN, galactosamine; GalNAc, *N*-acetylgalactosamine; GCC, graphitized carbon column; Glc, glucose; GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; PA, pyridylamino; R.S.D., relative standard deviation; SIM, selected ion mode; TFA, trifluoroacetic acid; TIC, total ion chromatogram

* Corresponding author. Tel.: +81 3 3700 1141; fax: +81 3 3707 6950.
E-mail address: nana@nihs.go.jp (N. Kawasaki).
[1] Present address: Chendu Institute of Biological Products, 610063, Chengdu Wai Dong Bao Jiang Qiao, Sichuan Province, China.

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.11.070

oligosaccharides based on subtle differences in branch, position, and linkage with volatile solution [9,10]. This method enables us to distinguish the glycosylation among some glycoprotein products produced in different cells [11].

A use of internal standards is known to improve the precision and linearity in quantitative analyses. Isotopic analogs of the analytes are currently the preferred internal standards for quantification by mass spectrometry (MS) procedures. For instance, Gygi et al. [12] demonstrated the approach for the accurate quantification of the proteins within complex mixture using isotope-coded affinity tags (ICATs). The use of the isotope-labeled carbohydrates as internal standards can make it possible to quantify the carbohydrates by LC/MS. Reductive pyridylamination is frequently used for the tagging of carbohydrates in HPLC analysis [13,14]. This derivatization is known to afford higher sensitivity in MS analysis [15], and PA oligosaccharides were reported to be separated by GCC [16]. Here, we study quantitative analysis of carbohydrates using tetradeuterium-labeled pyridylamino (d_4-PA) carbohydrates as internal standards. First, we study the monosaccharide composition analysis by using d_4-PA monosaccharides as internal standards. Next, the isotope tag method is used for the quantitative oligosaccharide profiling using recombinant human chorionic gonadotropin (rhCG) and human chorionic gonadotropin (hCG) as an analyte and standard glycoproteins, respectively.

2. Materials and methods

2.1. Materials

All monosaccharide standards were purchased from Seikagaku-kogyo (Tokyo, Japan). The pyridylamination apparatus (PALSTATION), reagents for the pyridylamination reaction, and PA monosaccharide standards were available from TaKaRa Biomedicals (Otsu, Japan). The hexadeuterium-labeled 2-aminopyridine (d_6-AP) was purchased from Wako (Osaka, Japan). Human chorionic gonadotropin (hCG) and recombinant hCG (rhCG) were bought from Sigma (St. Louis, MO, USA). *N*-glycosidase F was purchased from Roche Diagnostics. All other chemicals and reagents were of analytical grade and were commercially available.

2.2. Pyridylamination of monosaccharides

For the pyridylamination of amino sugars, free amino groups of monosaccharides (GlcN, GalN, 1–1000 pmol) were acetylated by incubation in 50 μl of methanol/pyridine/distilled water (30/15/10, v/v/v) with 2 μl of acetic anhydride for 30 min at room temperature. The mixture was dried using a vacuum centrifuge evaporator without heating. Acetic acid (50 μl), methanol (60 μl), and 10 μl of coupling reagent prepared by mixing 100 mg of AP was added to monosaccharides (Fuc, Gal, Glc, Man, GlcNAc, GalNAc, 1–1000 pmol). The mixture was heated at 90 °C for 20 min by PALSTATION, and the excess reagents were removed by evaporation under a stream of nitrogen gas at 60 °C for 20 min. Then 10 μl of a reducing reagent, prepared just before use by mixing 6 mg of borane–dimethylamine complex and 100 μl of acetic acid, was added, and the mixture was heated at 90 °C for 35 min. The reaction mixture was dried three times under a stream of nitrogen gas at 50 °C for 10 min. The residue was dissolved in water for LC/MS analysis. For the preparation of isotope analogs, the tetradeuterium-labeled PA (d_4-PA) monosaccharide, d_0-AP was just replaced by d_6-AP (Fig. 1).

2.3. Monosaccharide composition analysis of a glycoprotein

A glycoprotein (25 pmol) was placed in a hydrolysis tube fitted with a Teflon-lined screw cap. Fifty microliters of 2 M HCl-2M trifluoroacetic acid (TFA) was added to the sample, which was then heated at 100 °C for 6 h. Simultaneously, a set of monosaccharide standards, 100 pmol of Gal, Man, Glc, Fuc, GlcN, and GalN, was treated identically as the analytes. The solution obtained was freeze-dried. The monosaccharides obtained from the analyte glycoproteins and standard monosaccharides were tagged with non-deuterium-labeled 2-aminopyridine (d_0-AP) and d_6-AP, respectively. Each tagged oligosaccharide mixture was dissolved into purified water, and a mixture of d_0- and d_4-PA monosaccharides was injected into the GCC-LC/MS.

2.4. Preparation of N-linked oligosaccharides

N-linked oligosaccharides were released from hCG as described previously [17]. Briefly, hCG and rhCG (100 μg)

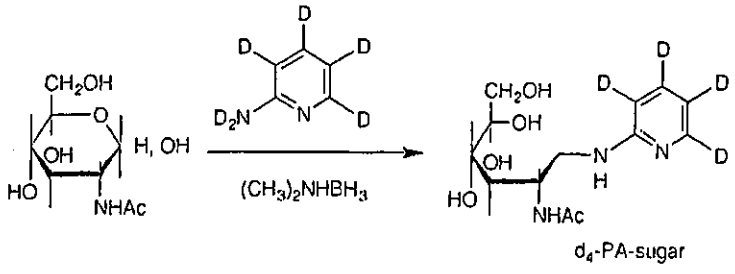


Fig. 1. Synthesis of d_4-PA monosaccharide internal standard.

were dissolved in 360 μl of 0.5 M Tris–HCl buffer (pH 8.6), containing 8 M guanidine hydrochloride and 5 mM ethylenediaminetetra-acetic acid (EDTA). After an addition of 2.6 μl of 2-mercaptethanol, the mixture was allowed to stand at room temperature for 2 h. To this solution, 7.56 mg of monoiodoacetic acid was added, and the resulting mixture was incubated at room temperature for 2 h in the dark. The reaction mixture was applied to a PD-10 column (Amershambioscience, Uppsala, Sweden) to remove the reagents, and the eluate was lyophilized.

Carboxymethylated hCG and rhCG were dissolved in 100 μl of 0.1 M sodium phosphate buffer, pH 7.2, and incubated with 5 units of PNGase F at 37 °C for 2 days. Protein was precipitated with 340 μl of cold ethanol, and the supernatant was dried.

2.5. Pyridylamination of oligosaccharides from hCG

To the lyophilized oligosaccharides released from rhCG we added 10 μl of coupling reagent prepared by mixing 300 mg of d_0-AP, and 100 μl of acetic acid, and the reaction mixture was heated at 90 °C for 60 min. Then, 10 μl of a reducing reagent, prepared just before use by mixing 20 mg of borane–dimethylamine complex and 100 μl of acetic acid, was added, and the mixture was heated at 80 °C for 60 min. The reaction mixture was dried three times under a stream of nitrogen gas at 60 °C for 10 min. The residue was dissolved in water for LC/MS analysis. For the preparation of the tetradeuterium-labeled (d_4)-PA oligosaccharide isotope analogs, d_0-AP was just replaced by d_6-2-aminopyridine.

2.6. LC/MS analysis

LC was carried out using a Magic 2002 HPLC system (Michrom BioResources Inc., Auburn, CA, USA) using a Hypercarb column (0.2 mm × 150 mm, Thermoelectron, San Jose, CA, USA). The flow rate was set at 2–3 μl/min through a splitter system. The mobile phases were 5 mM ammonium acetate (pH 8.5) with 2% of acetonitrile (pump A) and 80% of acetonitrile (pump B). A gradient of 10–35% of B in 60 min was used for the monosaccharide analysis. For oligosaccharide profiling, we used a gradient of 5–20% of B in 20 min, 20–70% of B in 15 min, and 70–95% of B in 5 min. The mass spectrometer used was a TSQ 7000 (Thermoelectron) equipped with a nanoelectrospray ion source (AMR Inc., Tokyo, Japan). The ESI voltage was set to 2000 V (positive ion mode) or 1500 V (negative ion mode), and the capillary temperature was 175 °C.

3. Results

3.1. Monosaccharide composition analysis using the isotope tag method

First, we examined the possibility of the isotope-tag method for the monosaccharide composition analysis of gly-

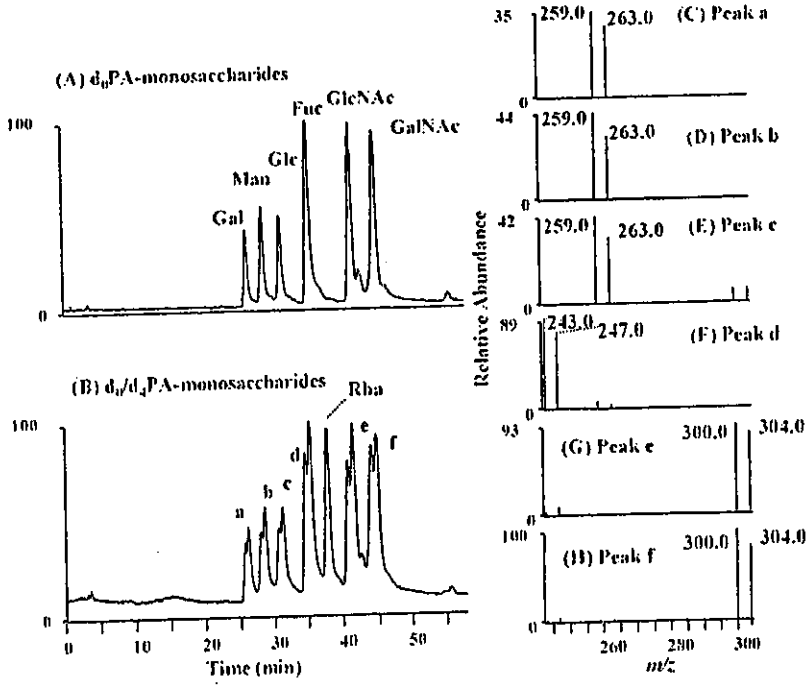


Fig. 2. (A) Extracted ion chromatogram (set *m/z* values, 243, 259, and 300) of d_0-PA monosaccharides (1 pmol Gal, Man, Glc, Fuc, GlcNAc, and GalNAc). (B) Extracted ion chromatogram (set *m/z* values, 243, 247, 259, 263, 300, and 304) of a mixture of d_0- and d_4-PA monosaccharides (1 pmol Gal, Man, Glc, Fuc, Rham, GlcNAc and GalNAc). (C) Mass spectra of peaks a (C), b (D), c (E), d (F), e (G), and f (H).

coproteins. An equal molar of each d_0-PA monosaccharide (Gal, Man, Glc, Fuc, GlcNAc, and GalNAc, 1 pmol each) was analyzed by GCC-LC/MS in the positive ion mode. The ions monitored were *m/z* 259 (for d_0-PA-Gal, d_0-PA-Man, and d_0-PA-Glc), *m/z* 243 (d_0-PA-Fuc), and *m/z* 300 (d_0-PA-GlcNAc and d_0-PA-GalNAc). Fig. 2A shows the mass chromatogram of the d_0-PA monosaccharides. All six d_0-PA monosaccharides were retained and separated by GCC. The detection limit at a signal-to-noise ratio of 3 was 45 fmol.

The d_4-PA monosaccharides were prepared as internal standards by tagging of standard monosaccharides with d_6-AP and combined with d_0-PA monosaccharides. Fig. 2B shows the chromatogram of a mixture of d_0-, d_4-PA monosaccharides and PA-labeled Rhamnose, which is frequently used as an internal standard in the monosaccharide composition analysis. Paired ions with a difference of *m/z* 4 were detected in the mass spectra of peaks a–f (Fig. 2C–H). When 0.5 pmol d_0-PA monosaccharides were determined in the presence of d_4-PA monosaccharides or Rhamnose by GCC-LC/MS, the relative standard deviation ($n = 5$) was 1.8–4.8% or 5.6–8.3%, respectively.

To assess the linearity and reproducibility of the whole procedure, including reacetylation, pyridylamination, the removal of excess derivatization reagents, and GCC-LC/MS, we tagged different amounts of monosaccharides (Gal, Man, Glc, Fuc, GlcN, and GalN, 1–1000 pmol) with d_0-AP, and d_4-PA monosaccharides (4 or 20 pmol) were added to the d_0-PA monosaccharides (1–10 pmol or 10–1000 pmol, respectively). The whole process of the isotope tag method was found to be linear for all six monosaccharides over the tested range of 1–1000 pmol (Fig. 3). The accuracy of this method was approximately 80–100% (Fig. 3), and the relative standard deviations (%R.S.D.) were less than 7.2% for all monosaccharides (based on the peak area ratio of monosaccharides from five samples).

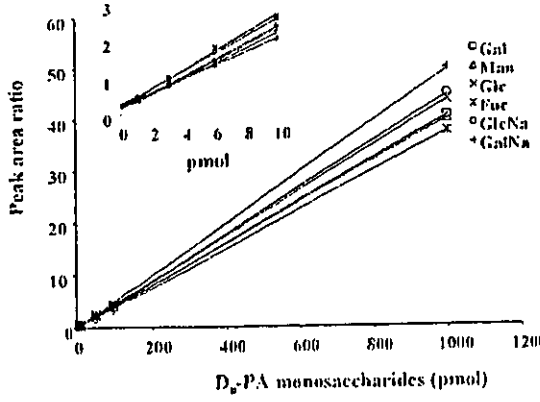


Fig. 3. Linearity on the isotope-tag method for monosaccharide quantification. For the internal standards, 1000 pmol monosaccharides were derivatized to d_4-PA monosaccharides. Different amounts of monosaccharides were derivatized to d_0-PA monosaccharides and co-injected with 4 pmol (A) or 20 pmol (B) internal standards into GCC-LC/MS.

We used this method for the monosaccharide composition analysis of fetuin and erythropoietin. Accuracy in the monosaccharide composition analysis of a glycoprotein relies on the condition of hydrolysis. Fan et al. [18] studied the hydrolysis of N-linked oligosaccharides and recommended 4 h with 2 M TFA at 100 °C for neutral sugars, and 6 h with 4 M HCl at 100 °C for amino sugars. While these hydrolysis conditions result in the complete release of neutral and amino sugars with no degradation, it takes two hydrolyses for a single sample. To quantify both neutral and amino sugars in glycoproteins in the same run, fetuin and erythropoietin (25 pmol) were heated in 2 M HCl-2M TFA at 100 °C for 6 h [19], and a set of monosaccharide standards, 100 pmol of Gal, Man, Glc, Fuc, GlcN, and GalN, was treated identically as the analyte glycoproteins. After hydrolysis, the analyte and standard monosaccharides were tagged with d_0- and d_6-AP, respectively. Fig. 4A and E show the mass chromatogram of monosaccharides prepared from fetuin and erythropoietin in the presence of d_4-PA monosaccharides, respectively.

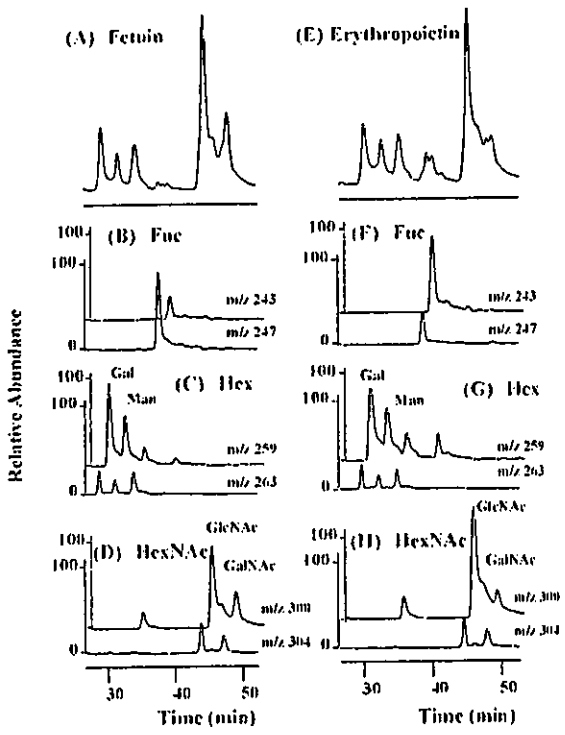


Fig. 4. Monosaccharide composition analysis of glycoproteins. Extracted ion chromatograms of d_0-PA monosaccharides from fetuin and d_4-PA standard monosaccharides (set *m/z* values, 243, 247, 259, 263, 300, and 304) (A), d_0-PA Fuc from fetuin and d_4-PA standard Fuc (set *m/z* values, 243 and 247) (B), d_0-PA Hex from fetuin and d_4-PA standard Hex (set *m/z* values, 259 and 263) (C), and d_0-PA HexNAc from fetuin and d_4-PA standard HexNAc and (set *m/z* values, 300 and 304) (D). Extracted ion chromatograms of d_0-PA monosaccharides from erythropoietin and d_4-PA standard monosaccharides and (set *m/z* values, 243, 247, 259, 263, 300, and 304) (E), d_0-PA Fuc from erythropoietin and d_4-PA standard Fuc (set *m/z* values, 243 and 247) (F), d_0-PA Hex from erythropoietin and d_4-PA standard Hex (set *m/z* values, 259 and 263) (G), and d_0-PA HexNAc from erythropoietin and d_4-PA standard HexNAc (set *m/z* values, 300 and 304) (H).

Table 1
Monosaccharide composition analysis by isotope-tag method

Glycoprotein	Monosaccharide	mol/mol[a]	mol/mol
Fetuin	Fuc	0.3	0 [20]
	Gal	10.4	12
	Man	7.6	9
	GlcNAc	14.7	15
	GalNAc	3.4	3
Erythropoietin	Fuc	3.4	4.1 [21]
	Gal	12.8	13.8
	Man	8.1	8.7
	GlcNAc	15.6	17.2
	GalNAc	1.5	0.9

[a] Values were expressed as mol detected in 1 mol glycoprotein.

Fig. 4B, and F show the mass chromatograms of d_0-, and d_4-PA fucose, Fig. 4C and G indicate those of d_0-, d_4-PA hexose, and Fig. 4D and H show those of d_0-, d_4-PA HexNAc. The monosaccharide compositions of fetuin and erythropoietin calculated from the peak area ratios (d_0-PA/d_4-PA monosaccharides) were in good agreement with the reported values (Table 1) [20,21]. By heating the standard monosaccharides simultaneously the decomposition of monosacchrides during hydrolysis can be corrected, and a use of isotope analogs as the internal standards can reduce deviation in ESIMS analysis.

3.2. Quantitative oligosaccharide profiling using the isotope tag method

Next, we explored the capability of the isotope-tag method for the quantitative oligosaccharide profiling. When d_0-PA oligosaccharides prepared from an analyte glycoprotein are analyzed with an equal part of d_4-PA oligosaccharides prepared from a standard glycoprotein, oligosaccharides which link to both the analyte and the standard glycoproteins are expected to appear as paired ions with a difference of 4 Da, and the individual oligosaccharides in the analyte glycoprotein can be quantified based on the analyte/internal standard ion-pair intensity ratio. On the other hand, any oligosaccharides that link to either the analyte or the standard glycoprotein ought to be detected as single ions. Oligosaccharides released from rhCG and hCG were tagged with d_0- and d_6-AP, respectively, and the tagged oligosaccharides were analyzed by GCC-LC/MS in both positive and negative ion modes.

Fig. 5A and B show the mass spectra of the peak which was detected at 21.5 min in the positive and the negative ion mode, respectively. In the positive ion mode, ions at *m/z* 863.0, 1359.4 and 1197.2 were detected (Fig. 5A), and they can be assigned to d_4-PA $[Hex]_5[HexNAc]_4^{2+}$ (an asialobiantennary oligosaccharide), d_4-PA$[Hex]_3[HexNAc]_4^{+}$ (a fragment of the asialobiantennary form) and d_4-PA$[Hex]_4[HexNAc]_4^{+}$ (a fragment of the asialobiantennary form), respectively. In contrast, only an ion at *m/z* 860.9 (d_4-PA$[Hex]_5[HexNAc]_4^{2-}$, asialobiantennary oligosaccharide) was detected in the negative ion mode (Fig. 5B). This result suggests that mass spectra

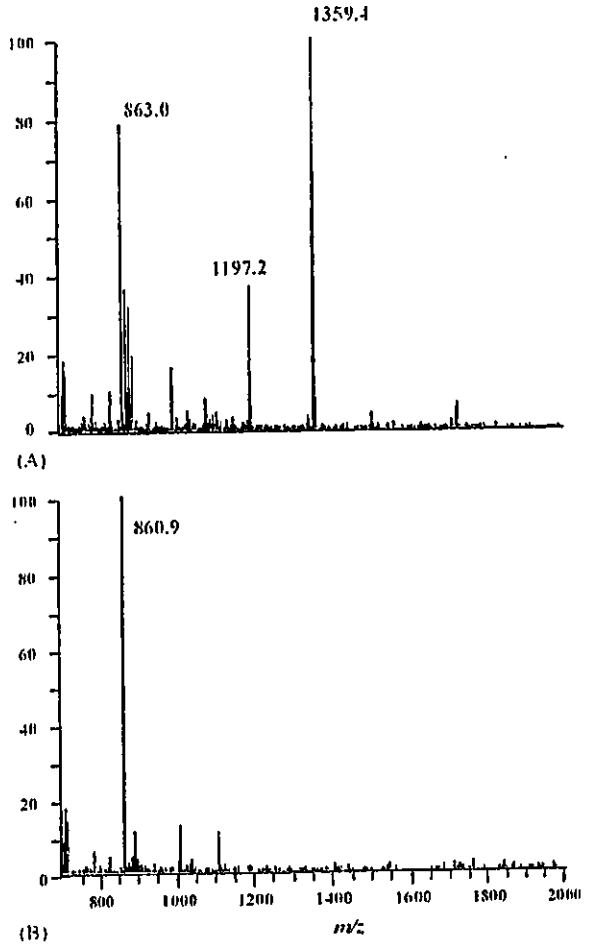


Fig. 5. Mass spectra of d_4-PA oligosaccharide. D_4-PA oligosaccharide eluted at 21.5 min from GCC was analyzed by ESIMS in the positive ion mode (A) and negative ion mode (B).

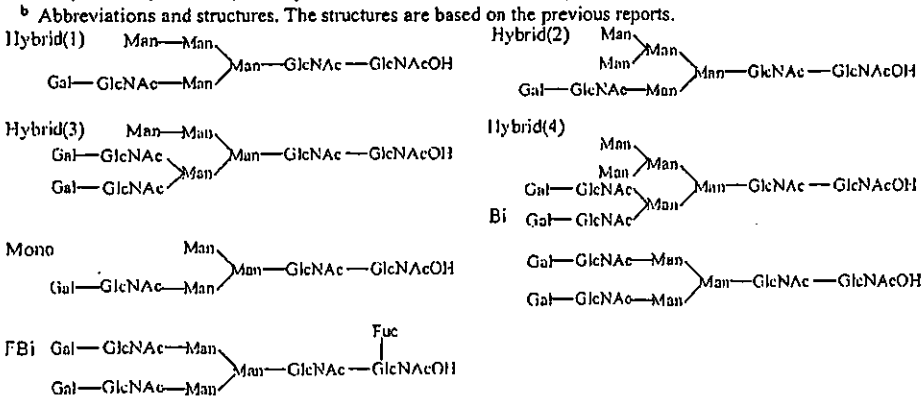
of PA oligosaccharides become complicated due to fragmentation in the positive ion mode, while only molecular ions can be detected in the negative ion mode. Therefore, ESI analysis in the negative ion mode was chosen for the PA oligosaccharide profiling.

Fig. 6A and B show the TIC of a mixture of equal parts of d_0-PA oligosaccharides prepared from rhCG and d_4-PA oligosaccharides from hCG, and its two-dimensional display (retention time versus *m/z*), respectively. The carbohydrate structures, which can be deduced from *m/z* values, are indicated in Table 2. Paired ions at *m/z* 757.5, 759.5 were observed in the mass spectrum of peak a1. Based on carbohydrate composition $[Hex]_5[HexNAc]_3$, it can be assigned to a hybrid type oligosaccharide. Likewise, peak 11, 12, 14, 15, p1, p2, and p4 consisted of paired ions and can be assigned to monosialylated (11, 12, 14, 15) and disialylated (p1, p2) biantennary oligosaccharide without Fuc. Fig. 7 shows TIC of d_0-, d_4-PA oligosaccharides (A), extracted ion chromatograms of d_0-PA (B), d_4-PA (C), and d_0-, d_4-PA monosialylated biantennary form (D). The mass spectra of peaks 11–15 are shown in Fig. 7E–I. Peak 13 was not observed in Fig. 7D and only

Table 2
Structural assignment of peaks in Fig. 6B

Peak nos.	Carbohydrate composition[a]	Deduced structure[b]	Theoretical mass (d_0-PA-sugar)	Observed *m/z*			Ion-pair intensity ratio d_0/d_4
				d_0-PA-rhCG	d_4-PA-hCG		
				M^{2-}	M^{3-}	M^{2-}	
a1	$[Hex]_5[HexNAc]_3$	Hybrid (1)	1517.5	757.5		759.5	0.27
b1	$[Hex]_5[HexNAc]_4[NeuNAc]_2$	$Bi+NA_2$	2303.1		768.2		
c1	$[Fuc]_1[Hex]_5[HexNAc]_4[NeuNAc]_2$	$FBi+NA_2$	2449.3		816.7		
d1	$[Hex]_4[HexNAc]_3[NeuNAc]_1$	Mono+NA	1646.6			824.3	
d2	$[Hex]_4[HexNAc]_3[NeuNAc]_1$	Mono+NA	1646.6			824.0	
e1	$[Hex]_6[HexNAc]_3$	Hybrid (2)	1679.6	838.6			
e2	$[Hex]_6[HexNAc]_3$	Hybrid (2)	1679.6			840.6	
f1	$[Hex]_5[HexNAc]_4$	Bi	1720.7	858.9			
f2	$[Hex]_5[HexNAc]_4$	Bi	1720.7			861.2	
g1	$[Hex]_5[HexNAc]_3[NeuNAc]_1$	Hybrid (1)+NA	1807.7	902.9			
g2	$[Hex]_5[HexNAc]_3[NeuNAc]_1$	Hybrid (1)+NA	1808.7			905.0	
h1	$[Fuc]_1[Hex]_5[HexNAc]_4$	FBi	1866.8			934.0	
i1	$[Hex]_6[HexNAc]_4$	Hybrid (3)	1882.8	940.2			
j1	$[Hex]_5[HexNAc]_5$	Bi+GN	1924.9			962.7	
k1	$[Hex]_6[HexNAc]_3[NeuNAc]_1$	Hybrid (2)+NA	1970.8			986.8	
k2	$[Hex]_6[HexNAc]_3[NeuNAc]_1$	Hybrid (2)+NA	1970.8			986.2	
l1	$[Hex]_5[HexNAc]_4[NeuNAc]_1$	Bi+NA	2011.9	1004.7		1006.7	0.77
l2	$[Hex]_5[HexNAc]_4[NeuNAc]_1$	Bi+NA	2011.9	1004.6		1007.3	0.56
l3	$[Hex]_5[HexNAc]_4[NeuNAc]_1$	Bi+NA	2011.9	1004.6			
l4	$[Hex]_5[HexNAc]_4[NeuNAc]_1$	Bi+NA	2011.9	1004.6		1006.5	0.67
l5	$[Hex]_5[HexNAc]_4[NeuNAc]_1$	Bi+NA	2011.9	1004.6		1006.4	0.49
m1	$[Hex]_7[HexNAc]_4$	Hybrid (4)	2044.9	1021.4			
n1	$[Fuc]_1[Hex]_5[HexNAc]_4[NeuNAc]_1$	FBi+NA	2158.0			1079.8	
n2	$[Fuc]_1[Hex]_5[HexNAc]_4[NeuNAc]_1$	FBi+NA	2158.0			1079.8	
n3	$[Fuc]_1[Hex]_5[HexNAc]_4[NeuNAc]_1$	FBi+NA	2158.0			1079.8	
o1	$[Hex]_6[HexNAc]_4[NeuNAc]_1$	Hybrid (3)+NA	2174.0	1085.6			
o2	$[Hex]_6[HexNAc]_4[NeuNAc]_1$	Hybrid (3)+NA	2174.0	1085.7			
p1	$[Hex]_5[HexNAc]_4[NeuNAc]_2$	$Bi+NA_2$	2303.1	1150.3		1152.1	5.76
p2	$[Hex]_5[HexNAc]_4[NeuNAc]_2$	$Bi+NA_2$	2303.1	1150.2		1152.2	5.92
p3	$[Hex]_5[HexNAc]_4[NeuNAc]_2$	$Bi+NA_2$	2303.1	1150.1			
p4	$[Hex]_5[HexNAc]_4[NeuNAc]_2$	$Bi+NA_2$	2303.1	1150.3		1152.4	0.45

[a] Hex, hexsose; HexNAc, *N*-acetyl hexsosamine; NeuNAc, *N*-acetyl neuraminic acid; Fuc, fucose.
[b] Abbreviations and structures. The structures are based on the previous reports.



single ion was detected in Fig. 7G. These results suggest that one of monosialylated binantenary oligosaccharides isomers links to only rhCG.

We determined relative amounts of some oligosaccharides in rhCG on the basis of ion-pair intensity ratios (Table 2). The amount of monosialylated biantennary forms (l1, l2, l4, and l5) linked to rhCG were 50–70% of those to hCG. The amount of disialylated biantennary forms (p1 and p2) linked to rhCG was five-fold of those to hCG, and the linkage of p4 to rhCG was one-half of that of hCG. The isotope tag method clearly shows the difference in distribution of isomers between rhCG and hCG.

In this procedure, oligosaccharides linked to either rhCG or hCG were detected as single ions. As shown in Table 2, nine oligosaccharides were detected as single ions in rhCG, and they are reduced to hybrid type and complex type.

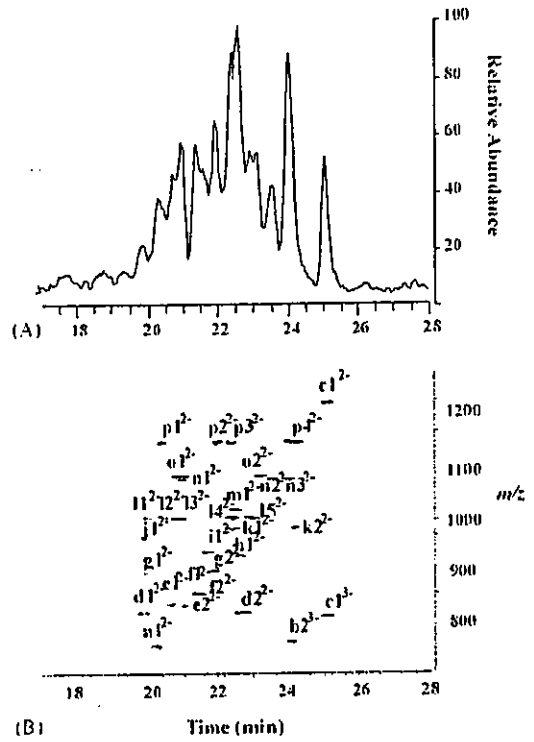


Fig. 6. TIC of a mixture of equal amount of d_0-PA N-linked oligosaccharides from rhCG and d_4-PA N-linked oligosaccharides from hCG (A), and its 2D display (B). Oligosaccharides (from 2 μg rhCG and hCG) were analyzed by GCC-LC/MS in the negative ion mode.

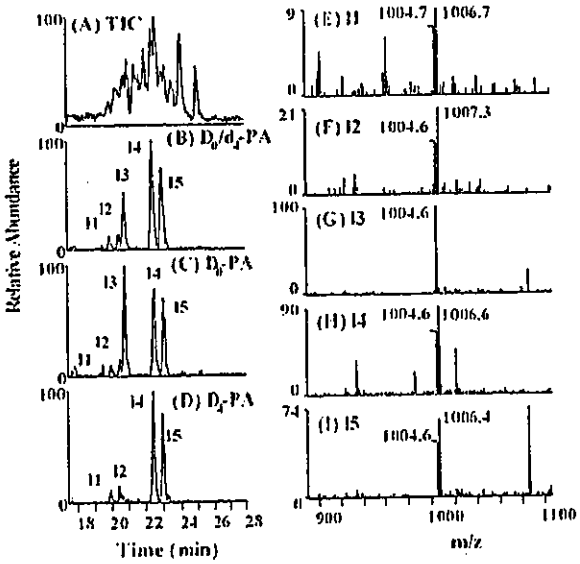


Fig. 7. TIC of a mixture of equal amount of d_0-PA N-linked oligosaccharides from rhCG and d_4-PA N-linked oligosaccharides from hCG (A). Extracted ion chromatograms of d_0-, and d_4-PA monosialylated biantennary (set *m/z* values, 1004–1007) (B), d_0-PA monosialylated biantennary (set *m/z* values, 1004–1005) (C), and d_4-PA monosialylated biantennary oligosaccharides (set *m/z* values, 1006–1007) (D). Mass spectra of peak I1–I5 (E–I).

Fourteen oligosaccharides were detected only in hCG, and most of them were fucosylated complex type. These results show the differences in glycosylation between rhCG and hCG and suggest that many hybrid type oligosaccharides linked to rhCG, while fucosylated oligosaccharides attach to hCG.

4. Discussion

Alteration of glycosylation is known to cause many changes in the biological activity as well as the physical properties of proteins. Several procedures of oligosaccharide profiling have been reported for the assessment of alteration of glycosylation, however, most of them can be used for only either qualitative or quantitative analysis. Although mass spectrometric oligosaccharide profiling is useful for the qualitative analysis, it has a problem on precision, and some isomers are still indistinguishable if their retention times are closed to others. In this study, we demonstrated that the use of isotope-tagged internal standards and GCC-LC/MS made it possible to do both quantitative and qualitative carbohydrate analysis.

First, we demonstrated the monosaccharide composition analysis using the isotope tag method. The use of internal standards that were heated under the same hydrolysis condition as an analyte glycoprotein resulted in good precision and accuracy in the monosaccharide composition analysis. Several HPLC methods for determination of monosaccharides have been reported. High-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) has been widely used for monosaccharide composition analysis [20,22–25]. Although HPAEC-PAD gives high resolution of all common monosaccharides and has the advantage of not requiring derivatization, this method is also known to have a disadvantage of limited selectivity [26]. The isotope tag method with SIM mode is equal to the HPAEC-PAD in sensitivity and is better than it in selectivity.

Next, we demonstrated the potentiality of the isotope tag method for quantitative oligosaccharide profiling using rhCG and hCG as model glycoproteins. hCG consists of an α subunit (MW 14.7 kDa) and a β subunit (MW 23.0 kDa), and oligosaccharides link to Asn52, and 78 in the α subunit and Asn13 and 30 in the β subunit. It has been reported that the majority of N-linked oligosaccharides in rhCG and hCG are fucosylated or non-fucosylated di-, tri-, and tetra-antennary forms with a various level of sialylation [27–30]. We prepared d_0-PA oligosaccharides and d_4-PA oligosaccharides from rhCG and hCG, respectively, and an equal part of d_0-PA and d_4-PA oligosaccharides was injected into LC/MS. We demonstrated that the oligosaccharides existing in one side protein were detected as single ions, whereas common oligosaccharides were detected as paired ions. We could easily realize that monosialo-, and disialobiantennary oligosaccharides linked to both hCG and rhCG, while fucosylated oligosaccharides and some hybrid type oligosaccharides linked to only hCG and rhCG, respectively. In addition, we demonstrated the pos-

sibility of the quantitative comparison the oligosaccharides between two quite similar glycoproteins. This quantitative oligosaccharide profiling is expected to be a powerful tool in various stages, including quality control and comparability assessment of glycoprotein products, and elucidation of glycan alteration in some diseases.

Acknowledgements

This study was supported in part by the Japan–China Sasakawa Medical Fellowship (J.Y.) and by a grant-in-aid for Research on Health Sciences focusing on Drug Innovation from The Japan Health Sciences Foundation (N.K.).

References

[1] A. Varki, Glycobiology 3 (1993) 97.
[2] T. Hayakawa, in: Y.-y.H. Chiu, J.L. Gueriguian (Eds.), Drug Biotechnology Regulation. Scientific Basis and Practices, Marcel Dekker Inc., New York, 1991, p. 468.
[3] N. Takahashi, H. Nakagawa, K. Fujikawa, Y. Kawamura, N. Tomiya, Anal. Biochem. 226 (1995) 139.
[4] R.R. Townsend, M.R. Hardy, O. Hindsgaul, Y.C. Lee, Anal. Biochem. 174 (1988) 459.
[5] G.R. Guile, P.M. Rudd, D.R. Wing, S.B. Prime, R.A. Dwek, Anal. Biochem. 240 (1996) 210.
[6] L. Royle, T.S. Mattu, E. Hart, J.I. Langridge, A.H. Merry, N. Murphy, D.J. Harvey, R.A. Dwek, P.M. Rudd, Anal. Biochem. 304 (2002) 70.
[7] J. Delaney, P. Vouros, Rapid Commun. Mass Spectrom. 15 (2001) 325.
[8] K.A. Thomsson, H. Karlsson, G.C. Hansson, Anal. Chem. 72 (2000) 4543.
[9] N. Kawasaki, M. Ohta, S. Hyuga, M. Hyuga, T. Hayakawa, Anal. Biochem. 285 (2000) 82.
[10] N. Kawasaki, S. Itoh, M. Ohta, T. Hayakawa, Anal. Biochem. 316 (2003) 15.
[11] N. Kawasaki, M. Ohta, S. Itoh, M. Hyuga, S. Hyuga, T. Hayakawa, Biologicals 30 (2002) 113.
[12] S.P. Gygi, B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb, R. Aebersold, Nat. Biotechnol. 17 (1999) 994.
[13] S. Hase, T. Ikenaka, Y. Matsushima, Biochem. Biophys. Res. Commun. 85 (1978) 257.
[14] S. Hase, S. Hara, Y. Matsushima, J. Biochem. (Tokyo) 85 (1979) 217.
[15] S. Suzuki, K. Kakehi, S. Honda, Anal. Chem. 68 (1996) 2073.
[16] M. Wuhrer, H. Geyer, M. von der Ohe, R. Gerardy-Schahn, M. Schachner, R. Geyer, Biochimie 85 (2003) 207.
[17] S. Itoh, N. Kawasaki, M. Ohta, T. Hayakawa, J. Chromatogr. A 978 (2002) 141.
[18] J.Q. Fan, Y. Namiki, K. Matsuoka, Y.C. Lee, Anal. Biochem. 219 (1994) 375.
[19] H. Takemoto, S. Hase, T. Ikenaka, Anal. Biochem. 145 (1985) 245.
[20] M.R. Hardy, R.R. Townsend, Y.C. Lee, Anal. Biochem. 170 (1988) 54.
[21] H. Sasaki, B. Bothner, A. Dell, M. Fukuda, J. Biol. Chem. 262 (1987) 12059.
[22] M.R. Hardy, Methods Enzymol. 179 (1989) 76.
[23] C.C. Ip, V. Manam, R. Hepler, J.P. Hennessey Jr., Anal. Biochem. 201 (1992) 343.
[24] A. Lampio, J. Finne, Anal. Biochem. 197 (1991) 132.
[25] Y.C. Lee, Anal. Biochem. 189 (1990) 151.
[26] M. Weitzhandler, C. Pohl, J. Rohrer, L. Narayanan, R. Slingsby, N. Avdalovic, Anal. Biochem. 241 (1996) 128.
[27] A. Kobata, J. Cell Biochem. 37 (1988) 79.
[28] Y. Endo, K. Yamashita, Y. Tachibana, S. Tojo, A. Kobata, J. Biochem. (Tokyo) 85 (1979) 669.
[29] A. Amoresano, R. Siciliano, S. Orru, R. Napoleoni, V. Altarocca, E. De Luca, A. Sirna, P. Pucci, Eur. J. Biochem. 242 (1996) 608.
[30] A. Gervais, Y.A. Hammel, S. Pelloux, P. Lepage, G. Baer, N. Carte, O. Sorokine, J.M. Strub, R. Koerner, E. Leize, A. Van Dorsselaer, Glycobiology 13 (2003) 179.

Original Paper

International Archives of Allergy and Immunology

Int Arch Allergy Immunol 2005;136:23–32
DOI: 10.1159/000082581

Received: April 27, 2004
Accepted after revision: September 1, 2004
Published online: December 8, 2004

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

Kayoko Takagi[a] Reiko Teshima[a] Haruyo Okunuki[a] Satsuki Itoh[a]
Nana Kawasaki[a] Toru Kawanishi[a] Takao Hayakawa[a] Yoichi Kohno[b]
Atsuo Urisu[c] Jun-ichi Sawada[a]

[a]National Institute of Health Sciences, Tokyo; [b]Department of Pediatrics, Graduate School of Medicine, Chiba University, Chiba, and [c]Department of Pediatrics, Fujita Health University School of Medicine, Aichi, Japan

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.

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.

KARGER
Fax + 41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1018–2438/05/1361–0023$22.00/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Dr. Reiko Teshima
National Institute of Health Sciences, Division of Biochemistry and Immunochemistry
1-18-1 Kamiyoga, Setagaya-ku
Tokyo 158-8501 (Japan)
Tel. +81 3 3700 1141, ext. 243, Fax +81 3 3707 6950, E-Mail rteshima@nihs.go.jp

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 $\Delta A280$ 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-