A simple micro-method for determining precise oligosaccharidic specificity of mannose-binding lectins

Henri Debray¹,², Bernadette Coddeville², Liezelotte R Bomfim³, and Márcio V Ramos⁴

¹Unité de Glycobiologie Structurale et Fonctionnelle UMR CNRS/U STL n° 8576 Université des Sciences et Technologies de Lille, 59655 Villeneuve d’Ascq Cedex, France; ²Centro de Ciências, Faculdade de Educação de Ceará, Universidade Estadual do Ceará, Crateús Ceará 63700-000, Brasil; and ³Departamento de Bioquímica e Biologia Molecular Universidade Federal do Ceará Campus do Pici, Caixa Postal 6033 Fortaleza, Ceará 60451-970, Brasil

Received on May 10, 2008; revised on June 15, 2009; accepted on June 17, 2009

A simple and inexpensive method was developed to rapidly define the specificity of mannose-specific lectins toward oligomannoside-type structures. The method involved the interaction of a mixture of N-[¹⁴C]-acetylated glycoasparagines, prepared by exhaustive pronase digestion of bovine pancreatic ribonuclease B and N-[¹⁴C]-acylation with [¹⁴C]-acetic anhydride and containing all the possible oligomannoside-type N-glycans, with the lectin immobilized on Sepharose-4B. After exhaustive desalting, the obtained fractions were separated by high-performance thin-layer chromatography on silica gel plates and visualized by autoradiography with intensifying screen. As an example of the usefulness of this method, the fine specificity of artocarpin, the mannose-specificity lectin isolated from seeds of jackfruit (Artocarpus integrifolia) toward oligomannoside-type structures is presented. On the basis of such a determination, the best oligomannosidic ligand recognized by a mannose-specific lectin can be selected for studies of crystal structures of the lectin in complex with the defined ligand. Furthermore, some of these immobilized lectins, after definition of their precise specificities with the method, could represent valuable tools for the fractionation and characterization of oligomannoside-type structures, present in complex mixtures.

Keywords: artocarpin/autoradiography with intensifying screen/HPTLC/immobilized mannose-specific lectins/ribonuclease B glycoasparagines

Introduction

Very often, plant lectins are still classified according to the monosaccharide which inhibits the interaction between a lectin and a cell or which allows the specific elution of a bound glycoconjugate from an immobilized lectin column (Sharon and Lis 2003). However, it is well known that in most cases, complex oligosaccharides are several 1000-fold more potent inhibitors of lectins than monosaccharides. It is very important to know this complex specificity before using a lectin either as a probe in the exploration of cell surface glycoconjugates or as a tool to fractionate various glycoconjugates by affinity chromatography.

Fine specificity and kinetic parameters of lectins can be studied now with sophisticated methods including isothermal titration microcalorimetry (Christensen and Toone 2003), surface plasmon resonance (Duverger et al. 2003), frontal affinity chromatography (Ohyama et al. 1985) associated either with fluorescence detection (Hirabayashi et al. 2003) or coupled with electrospray mass spectrometry (Zhang et al. 2001), capillary affinity electrophoresis (Nakajima et al. 2004), nuclear magnetic resonance (Siebert et al. 2003), and X-ray diffraction techniques (Williams et al. 2005).

However, all these methods require expensive devices and need also large quantities of oligosaccharides often available in limited amounts. For instance, determination of carbohydrate binding specificity of a fucose-specific lectin from Aspergillus orizae by surface plasmon resonance with a BiaCore apparatus needs micromole amounts of glycans (Matsumura et al. 2007). To define the fine specificity of various mannose/glucose or of true mannose-specific lectins, we chose to study the interaction of radiolabeled oligomannoside-type glycoasparagines with the lectin immobilized on Sepharose-4B. Even if interactions between immobilized lectins and radiolabeled glycans of known structures are now a rather old method, it has allowed to define the precise specificity of numerous lectins (for a review, see Debray and Monfreuil 1992). In particular, we used this method to study the specificities of several plant lectins (Debray and Rougé 1984; Debray et al. 1986, 1994; Debray and Monfreuil 1989; Ayoub et al. 1992; Ramos et al. 2000, 2001). However, in the case of mannose-binding lectins, as the complete collection of each of the high-mannose structures is very difficult to isolate or too expensive to buy, the novelty of our approach was to apply on an immobilized lectin column less than a nanomole of the whole mixture of ¹⁴C-labeled oligomannoside-type glycoasparagines prepared from bovine pancreatic ribonuclease B and to analyze by autoradiography the content of the obtained fractions by high-performance thin-layer chromatography on silica gel plates. As an example of the usefulness of this simple and inexpensive method, the specificity of artocarpin, a mannose-specific lectin isolated from seeds of Artocarpus integrifolia, toward high-mannose structures is presented.

Results

Preparation of the RNase B glycoasparagine mixture

Bovine pancreatic ribonuclease B is a 14.9–15.5 KDa glycoprotein which possesses a single N-glycosylation site at Asn-34,
occupied by heterogeneous oligomannose-type glycans containing 5–9 mannose residues. A detailed structural characterization of these glycans, released by peptide/N-glycosidase F (PNGase F) action, by $^1$H NMR spectroscopy and mass spectrometry has shown that RNase B contains nine distinct oligomannose-type oligosaccharides presented in Table I and their relative proportions were estimated (Fu et al. 1994).

The whole mixture of glycoasparagines, prepared here by exhaustive pronase digestion of RNase B, contains all the possible oligomannose-type glycans, potential ligands for different mannose-binding lectins. A MALDI-TOF spectrum of this glycoasparagine mixture after N-[$^{14}$C]-acetylation is shown in Figure 1. The peaks observed at $m/z$ 1414.9, 1576.8, 1738.8, 1900.8, and 2062.7 correspond to the (M + Na)$^+$ of N-[$^{14}$C]-acetylated Man5 GlcNAc2 Asn, Man6 GlcNAc2 Asn, Man7 GlcNAc2 Asn, Man8 GlcNAc2 Asn, and Man9 GlcNAc2 Asn, respectively. These mass values are in good accordance with the calculated masses.

It can be seen that the mixture of glycoasparagines contains also ions that correspond respectively to (M + Na)$^+$ of N-[$^{12}$C]-acetylated Man5 to Man8-GlcNAc2 Asn, which were not acetylated by the addition of [$^{14}$C] acetic anhydride, but were N-acetylated either by the unlabeled acetic anhydride present in the used reagent or when an excess of cold acetic anhydride was added in the reaction vial after 1 h. The N-[$^{12}$C]-acetylated glycoasparagines correspond to the peaks found at $m/z$ 1412.9 (Man5), 1574.8 (Man6), 1736.8 (Man7), and 1898.8 (Man8). Finally, peaks at $m/z$ 1434.8 and 1436.8, 1596.8 and 1598.8, 1758.8 and 1760.8, 1920.8 and 1922.8, and 2082.8 and 2084.8 were assigned to (M + Na)$^+$ of Man5-GlcNAc2 Asn, Man6-GlcNAc2 Asn, Man7-GlcNAc2 Asn, Man8-GlcNAc2 Asn, and Man9-GlcNAc2 Asn, respectively.

### Table I. Structures and molar ratio of glycoasparagines from bovine pancreatic ribonuclease B according to Fu et al. (1994)

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure and Mol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man5</td>
<td>$\alpha$-D-Man-(1→6)$\alpha$-D-Man-(1→3)$\beta$-D-Man-(1→4)$\beta$-D-GlcNAc-(1→4)-GlcNAc-Asn 57</td>
</tr>
<tr>
<td>Man6</td>
<td>$\alpha$-D-Man-(1→6)$\alpha$-D-Man-(1→3)$\beta$-D-Man-(1→4)$\beta$-D-GlcNAc-(1→4)-GlcNAc-Asn 31</td>
</tr>
<tr>
<td>Man7 (1)</td>
<td>$\alpha$-D-Man-(1→6)$\alpha$-D-Man-(1→3)$\beta$-D-Man-(1→4)$\beta$-D-GlcNAc-(1→4)-GlcNAc-Asn 1.5</td>
</tr>
<tr>
<td>Man7 (2)</td>
<td>$\alpha$-D-Man-(1→2)$\beta$-D-Man-(1→3)$\beta$-D-Man-(1→4)$\beta$-D-GlcNAc-(1→4)-GlcNAc-Asn 1.5</td>
</tr>
<tr>
<td>Man7 (3)</td>
<td>$\alpha$-D-Man-(1→2)$\beta$-D-Man-(1→3)$\beta$-D-Man-(1→4)$\beta$-D-GlcNAc-(1→4)-GlcNAc-Asn 1.0</td>
</tr>
<tr>
<td>Man8 (1)</td>
<td>$\alpha$-D-Man-(1→2)$\alpha$-D-Man-(1→6)$\alpha$-D-Man-(1→3)$\beta$-D-Man-(1→4)$\beta$-D-GlcNAc-(1→4)-GlcNAc-Asn 5.9</td>
</tr>
<tr>
<td>Man8 (2)</td>
<td>$\alpha$-D-Man-(1→2)$\beta$-D-Man-(1→3)$\beta$-D-Man-(1→4)$\beta$-D-GlcNAc-(1→4)-GlcNAc-Asn 0.4</td>
</tr>
<tr>
<td>Man8 (3)</td>
<td>$\alpha$-D-Man-(1→2)$\alpha$-D-Man-(1→6)$\beta$-D-Man-(1→4)$\beta$-D-GlcNAc-(1→4)-GlcNAc-Asn 0.7</td>
</tr>
<tr>
<td>Man9</td>
<td>$\alpha$-D-Man-(1→2)$\alpha$-D-Man-(1→3)$\alpha$-D-Man-(1→4)$\alpha$-D-Man-(1→5)$\alpha$-D-Man-(1→6)$\beta$-D-Man-(1→4)$\beta$-D-GlcNAc-(1→4)-GlcNAc-Asn 1.0</td>
</tr>
</tbody>
</table>
Establishing the fine specificity of mannose-binding lectins

Fig. 1. Matrix-assisted laser desorption time of flight mass spectra of the N-[14C]-acetylated glycoasparagines mixture from ribonuclease B.

and 1758.8 and 1922.8 correspond to the (M + 2Na)⁺ of N-[12C]- and N-[14C]-acetylated glycoasparagines with 5, 6, 7, and 8 mannose residues, respectively.

A recent study involving isotopic labeling of oligosaccharides with 13C methyl iodide indicates that the presence of unlabeled 12C methyl iodide in the labeling reagent affects the isotopic distribution of the 13C-labeled oligosaccharides and that the height of the monoisotopic peak in the 13C-labeled glycans is affected by the presence of 12C in the labeling reagent. Moreover, the effect is dependent on the mass of the glycan (Alvarez-Manilla et al. (2007)). Here, in the case of the labeling of glycoasparagines with [1–14C] acetic anhydride containing 12C acetic anhydride in an unknown proportion, distribution of 14C-labeled glycoasparagines as well as the height of the monoisotopic peak in the 14C-labeled glycoasparagines may also be affected by the presence of glycoasparagines containing 12C acetyl group.

As estimated by the peak height ratio of unlabeled Man 5 (1412.9) and labeled Man 5 glycoasparagines (1414.9) of Figure 1, labeled glycoasparagines represents here approximately 75% of the total glycoasparagines.

Although not quantitative, MALDI analysis of a mixture of similar compounds, such as glycoasparagines, gives also a rather good estimation of the relative proportions of oligomannosidic glycans associated with RNase B disregarding the three Man 7 and Man 8 glycoforms. As estimated by peak height ratios of N-[14C] glycoasparagines observed in the MALDI spectra, Man5 to Man9 GlcNAc2 Asn exist in a relative molar ratio of 53: 30.4: 6.1: 8.1: 2.4%, respectively. As shown in Table II, these values are close to those reported by Fu et al. (1994), but are significantly different from the values given by Rudd et al. (1992) and determined also by mass spectrometry of the glycans released by hydrazinolysis. They are also slightly different from the values obtained by Gutiérrez Gallego et al. (2004) after HPLC analysis of 2-aminobenzamide-labeled glycans enzymatically released by PNGase F. These discrepancies may be due to the ribonuclease B batches used in the different studies.

Analysis of N-[14C]-acetylated glycoasparagines by HPTLC

Figure 2 presents the resulting image when 10³ disintegrations min⁻¹ corresponding to 20 ng of N-[14C]-acetylated glycoasparagines are submitted to an ascending chromatography with n-propanol/methanol/0.25% KCl (60: 10: 35, v/v/v) on Silica Gel 60 HPTLC plates and autoradiography with the Kodak BioMax transcreen LE-intensifying screen system. Five populations of oligomannoside-type glycoasparagines are separated according to their molecular weight, i.e., to the number of mannose residues they contain. The lightest man 5-containing structures present the fastest migration rate while the heaviest Man 9-containing glycoasparagines have the slowest.

The relative proportions of the five glycoasparagine populations can be roughly estimated visually to 50, 30, 7, 10, and Table II. Relative proportions (Mol%) of oligomannoside-type N-glycans of ribonuclease B determined by different studies

<table>
<thead>
<tr>
<th>Glycoforms</th>
<th>Methods previously used</th>
<th>Methods used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>¹H NMR/MS</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>Man6</td>
<td>31</td>
<td>21</td>
</tr>
<tr>
<td>Man7</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Man8</td>
<td>7</td>
<td>16</td>
</tr>
<tr>
<td>Man9</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>
Affinity chromatography of N-[^14]C-acetylated glycoasparagines on immobilized artocarpin-Sepharose 4B column

The elution profile obtained when N-[14C]-acetylated glycoasparagines from ribonuclease B were applied to an immobilized artocarpin-Sepharose column is shown in Figure 3. Two fractions, weakly interacting with the lectin, are eluted from the column by the starting buffer as retarded fractions FR1 (between fractions 6 and 10) and FR2 (between fractions 11 and 26). A fraction FE (between fractions 44 and 46) with a strongest specific interaction with the immobilized lectin is eluted with 0.01 M methyl α-D-mannopyranoside in the starting buffer. Fractions FR1, FR2, and FE represent 10, 54, and 36% of the [14C]-glycoasparagines applied to the artocarpin column, respectively.

Chromatography of N-[14C]-acetylated RNAse B glycoasparagines on undervoid Sepharose 4B and on Arum maculatum-Sepharose 4B columns

In order to check if the elution profile obtained with the artocarpin-Sepharose column reflects specific interactions of the immobilized lectin with glycoasparagines, the N-[14C]-acetylated glycoasparagines mixture was also applied to a column of undervoid Sepharose 4B (20 × 0.6 cm) equilibrated in PBS. In this case, it can be seen (Figure 4A) that all the glycoasparagines are eluted at the void volume of the column (between fractions 5 and 7), reflecting a complete lack of interaction between undervoid Sepharose 4B and glycoasparagines.

The whole glycoasparagine mixture was also applied to a column of Arum maculatum agglutinin-Sepharose 4B (20 × 0.6 cm) equilibrated in PBS. As shown in Figure 4B, two fractions, weakly interacting with this immobilized lectin, are eluted from the column as retarded fractions FR1 (between fractions 8 and 14) and FR2 (between fractions 17 and 31). No fraction is eluted with methyl α-D-mannopyranoside. Fractions FR1 and FR2 represent 17 and 83% of the glycoasparagines applied to the Arum maculatum agglutinin-Sepharose column, respectively. Moreover, HPTLC analysis of these two fractions (results not shown) shows that fraction FR1 contains all the Man 9 glycoasparagines, half of Man 8 and most of the Man 7 glycoasparagines. These results obtained with immobilized Arum maculatum agglutinin as well as results obtained with other manno-binding lectins (unpublished results) clearly show that each elution profile is characteristic of the used immobilized lectin.

Affinity chromatography of N-[14C]-acetylated individual glycopeptides on artocarpin-Sepharose column

In order to determine if the presence in the starting mixture of the nine glycoasparagines, in different amounts and with different affinities for the immobilized lectin, may affect the elution profile, the chromatographic behavior of three individual glycoasparagines on the artocarpin column was assessed. Figure 5A shows the elution profile obtained when pure N-[14C] Man 9 glycoasparagine, isolated from soybean agglutinin, is applied to the artocarpin column. One fraction, weakly

3% for the Man 5 to Man 9 glycoforms, respectively. However, clearer evaluation can be obtained by a quantitative analysis of the film performed by densitometry.

An identical image is also obtained when the same HPTLC plate is exposed for 1 h to 14C Phosphor Storage screen, further read by a Storm 860 PhosphorImager (not shown). Analysis with the ImageQuant software gives the relative proportions of 42, 26, 11, 17, and 4% for the Man 5 to Man 9 structures, respectively (Table II). The observed discrepancy between values obtained by mass spectrometry and PhosphorImager analysis (Table II) may be explained by the fact that intensity of signal given by Man 5 glycoasparagines is over the range of 5 orders of magnitude for which response remains linear. Shorter exposure times would solve this problem. However, as the PhosphorImager system is 10 times more sensitive than standard X-ray film and has a linear response over a range of 5 orders of magnitude, it is the solution of choice for those who have access to such an equipment.

Affinity chromatography of N-[14C]-acetylated glycoasparagines on Silica Gel 60 HPTLC plate.

Man 5 to Man 9 containing glycoasparagines were separated with one ascent of n-propanol/methanol/0.25% KCl (60: 10: 35, v/v/v) at 20 °C and visualized by autoradiography with the Kodak BioMax transcreen LE-intensifying screen system. Arrow indicates direction of migration. Values in brackets correspond to the molecular mass (Da) of the five groups of oligomannoside glycoasparagines.
Establishing the fine specificity of mannose-binding lectins

Fig. 4. Elution profiles of N-[14C]-acetylated glycoasparagines from ribonuclease B (A) on an underived Sepharose 4B column and (B) on an Arum maculatum agglutinin-Sepharose 4B column. Labeled glycoasparagines (35 × 10³ disintegrations min⁻¹) were applied either on an underived Sepharose 4B column or to an Arum maculatum agglutinin-Sepharose 4B column (20 × 0.6 cm) equilibrated at room temperature in the 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride (PBS). FNR: nonretained fraction; FR1 and FR2: retarded fractions. Interacting with the lectin, is eluted from the column with PBS between fractions 7 and 10 as was eluted fraction FR1 containing all the Man 9, one third of the Man 8 and half of the Man 7 glycoasparagines, when the whole glycoasparagine mixture was applied to the column.

In the same way, Figure 5B shows that pure N-[14C] Man 6 glycopeptides isolated from Viscum album lectin I are also eluted as a weakly retarded fraction FR2 with PBS between fractions 11 and 18 as was eluted fraction FR2 containing all the Man 6, the other half of Man 7 and two-thirds of the Man 8 glycoasparagines, when the whole glycoasparagine mixture was applied to the column. Finally, Figure 5C shows the elution profile of pure N-[14C] Man 5 glycoasparagines, first isolated from the whole glycoasparagine mixture with the artocarpin column, desalted and reapplied to the same column. Here again, Man 5 glycoasparagines are strongly bound and must be eluted from the artocarpin column with 0.01 M methyl α-D-mannopyranoside.

Fig. 5. Elution profiles of individual N-[14C]-acetylated glycopeptides on an immobilized artocarpin-Sepharose 4B column. (A) Man 9 glycoasparagines from soybean agglutinin. (B) Man 6 glycopeptides from Viscum album lectin I. (C) Man 5 glycoasparagines isolated from ribonuclease B glycoasparagines. Labeled glycopeptides (34 × 10³, 6.5 × 10³, and 5.5 × 10³ disintegrations min⁻¹, respectively) were applied to the artocarpin-Sepharose column (20 × 0.6 cm) equilibrated at room temperature in the 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride (PBS). FR1 and FR2: retarded fractions; FE: fraction exhibiting high affinity for the immobilized lectin. Arrow indicates the start (fraction 32) of elution with 0.01 M methyl α-D-mannopyranoside.
H Debray et al.

Fig. 6. HPTLC analysis of the three glycoasparagine fractions obtained after affinity chromatography on artocarpin-Sepharose. A: unfractionated glycoasparagine mixture; FR1: glycoasparagines with low affinity for artocarpin; FR2: glycoasparagines with medium affinity for artocarpin; FE: glycoasparagines strongly recognized by artocarpin and eluted from the artocarpin column with 0.01 M methyl α-D-mannopyranoside. Arrow indicates direction of migration. Molecular mass of glycoasparagines are shown in Figure 2.

Table III. Oligomannose-type glycoasparagines recovered in the three fractions obtained after affinity chromatography on artocarpin-Sepharose

<table>
<thead>
<tr>
<th>Glycoforms</th>
<th>FR1</th>
<th>FR2</th>
<th>FE 10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man5</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man6</td>
<td></td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Man7 (1)</td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Man7 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man7 (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man8 (1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man8 (2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man8 (3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Man9</td>
<td></td>
<td></td>
<td>+</td>
</tr>
</tbody>
</table>

These results highlight the fact that the presence of an excess of a glycoasparagine or glycoasparagines in the mixture (Man 5 representing 50% and Man 6 30.4% of the whole glycoasparagine mixture, respectively) does not interfere with the relative affinity of the immobilized artocarpin for each of the nine glycoasparagines. This is particularly obvious here in the behavior of Man 9 glycoasparagine, representing only 2.4% of the glycoforms and for which the affinity of the lectin is the lower.

HPTLC analysis of the fractions recovered from the artocarpin-Sepharose column

After extensive desalting by gel filtration on Bio-Gel P2 and on microcolumns of graphitized carbon, 10⁶ disintegrations min⁻¹ of each fraction recovered after affinity chromatography on the artocarpin-Sepharose column, together with 10⁵ disintegrations min⁻¹ of the starting N-[14C]-glycoasparagines mixture as a standard, were submitted to HPTLC on Silica Gel plate. Figure 6 and Table III present the results of this analysis. A classical edge effect, with a more rapid migration of the solvent on each side of the plate, giving the observed concave migration front, explains why Man 5 glycoasparagines present in the standard seem to migrate faster than Man 5 glycoasparagines of fraction FE or why Man 9 glycoasparagines present in FR1 seems to migrate slower than Man 9 glycoasparagines present in standard. In spite of this edge effect, it can be easily seen from the spot intensities that the weakly retarded fraction FR1 contains all the Man 9 glycoasparagines, one third of Man 8 glycoasparagines and half of Man 7 glycoasparagines. The more retarded fraction FR2 contains all the Man 6 glycoforms, the other half of Man 7 glycoforms and two-thirds of the Man 8 glycoforms. The immobilized lectin presents the strongest affinity for all Man 5 glycoasparagines which were recovered in the fraction eluted with 0.01 M methyl α-D-mannopyranoside (fraction FE). From this simple survey of the film and keeping in mind the structures and relative proportions given by Fu et al. (1994) and Table I, for the three Man 7 and Man 8 glycoisomers, it can be deduced that artocarpin presents the strongest affinity for Man 5-containing glycoasparagines presenting the trimannosidic Man α-1,3-[Man α-1,6]-Man sequence shown in Table IV, recovered in the fraction eluted with 0.01 M methyl α-D-mannopyranoside (fraction FE).

Artocarpin presents a weaker affinity for Man 6 and Man 7(1) glycoasparagines which also contain the Man α-1,3-[Man α-1,6]-Man sequence, here less accessible due to the steric hindrance of Man α-1,2-linked residue. Man 7(3) and Man 8(1) glycoasparagines, containing a terminal nonreducing α-1,3-linked mannose residue shown in Table IV, are recognized to the same extent by artocarpin. This indicates that the α-1,3-linked mannosine may occupy the primary binding site of the lectin.

All these glycoasparagines are recovered in the retarded fraction FR2 and the found relative proportions of the Man 7(1)+3 isomers (about 50%) and of the Man 8(1) glycoisomers (about 66%) are closely related to their abundance in ribonuclease B (Table I) given by Fu et al. (1994).

Man 7(2), Man 8(2 and 3), and Man 9 glycoasparagines, found in the less retarded fraction FR1, do not possess either the trimannosidic Man α-1,3-[Man α-1,6]-Man sequence or the nonreducing α-1,3-linked mannose residue and are only weakly recognized through their terminal nonreducing α-mannose residues by a multivalency effect. In this fraction also, the found relative proportions of the Man 7(2) isomers (about 50%) and Man 8(2) + (3) isomers (about 33%) correlates well with the data of Fu et al. (1994) and Table I.

Discussion

A simple and inexpensive method was developed to rapidly define the specificity of mannose-specific lectins toward oligomannoside-type structures. The method involved the interaction of a mixture of N-[14C]-acylated glycoasparagines, prepared by exhaustive pronase digestion of bovine pancreatic ribonuclease B and N-[14C]-acylation with [14C]-acetic anhydride and containing all the possible oligomannoside-type N-glycans, with the lectin immobilized on Sepharose-4B. After exhaustive desalting, the obtained fractions were separated by high-performance thin-layer chromatography on silica gel plates and visualized by autoradiography with intensifying screen. As an example of the usefulness of this method, the fine specificity of artocarpin, the mannose-specificity lectin isolated from seeds of jackfruit (Artocarpus integrifolia) toward oligomannoside-type structures is presented.

Our results obtained with this simple method on the specificity of artocarpin for oligomannose-type structures are in complete accordance with those previously reported by Misquith et al. (1994), Rani et al. (1999), and Jeyaprakash et al. (2004) and obtained either by an enzyme-linked lectin absorbent assay (Misquith et al. 1994), by isothermal titration calorimetry measurements (Rani et al. 1999), or by studies...
Establishing the fine specificity of mannose-binding lectins

Table IV. Structural determinants involved in the interaction of oligomannose-type glycoasparagines with artocarpin

<table>
<thead>
<tr>
<th>Glycoforms</th>
<th>Structural determinants are in bold face</th>
</tr>
</thead>
<tbody>
<tr>
<td>Man7</td>
<td>α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>(2)</td>
<td>FR1&lt;br&gt;α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>Man8</td>
<td>α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>(2)</td>
<td>FR1&lt;br&gt;α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>Man8</td>
<td>α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>(3)</td>
<td>FR1&lt;br&gt;α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>Man9</td>
<td>α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>Man6</td>
<td>α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>FR2</td>
<td>α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>Man6</td>
<td>α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>(1)</td>
<td>FR2&lt;br&gt;α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>Man7</td>
<td>α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>(3)</td>
<td>FR2&lt;br&gt;α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>Man8</td>
<td>α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>(1)</td>
<td>FR2&lt;br&gt;α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>Man5</td>
<td>α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
<tr>
<td>FE</td>
<td>α-D-Man-(1→6)&lt;br&gt;α-D-Man-(1→3)&lt;br&gt;β-D-Man-(1→4)-β-D-GlcNAc-(1→4)-GlcNAc-Asn&lt;br&gt;α-D-Man-(1→2)-α-D-Man-(1→3)</td>
</tr>
</tbody>
</table>

Structures weakly recognized (FR1); recognized (FR2) and strongly recognized (FE).

of the crystal structures of artocarpin in complex with oligosaccharides (Jeyaprakash et al. 2004). However, these previous studies have needed expensive oligosaccharides and a delicate enzyme-linked lectin absorbance assay (Misquith et al. 1994) or both expensive oligosaccharides and equipments: titration calorimeter (Rani et al. 1999) or X-ray generator (Jeyaprakash et al. 2004).

In contrast, the method described here needs only nanogram amounts of N-[14C]-labeled glycoasparagines mixtures containing all the possible oligomannose glycans found on glycoproteins and allows a rapid determination of the precise specificities of all mannose-specific algal, plant, and animal lectins. The validity of the proposed method was shown here by the determination of the specificity of artocarpin. The fine
specifications of several other true mannose-specific or glucose/mannose-specific algal and plant lectins were also determined (articles in preparation). On the basis of such determinations, the best oligomannosidic ligand(s) recognized by a mannose-specific lectin can be selected for the determination of the structural basis of the carbohydrate specificity through studies of crystal structures of the lectin in complex with the defined ligand. Furthermore, on the basis of their precise specificities defined with the method, different immobilized mannose-specific lectins can represent valuable tools for the fractionation and characterization of oligomannosidic structures of various origins. Particularly, glycan moieties of glycopeptides isolated with such immobilized mannose-specific lectins could be structurally characterized by MALDI-TOF MS and MALDI-QIT-TOF MS/MS methods as recently proposed by Kubota et al. (2008).

Material and methods

Preparation of glycoasparagines from bovine pancreatic ribonuclease B

Hundred milligrams of bovine pancreatic ribonuclease B (Sigma-Aldrich) was dissolved in a 15 mL screwcap tube into 6 mL of distilled water and 1 mL of 1 M Tris-HCl pH 8.4, 0.1 M CaCl₂. To prevent bacterial contaminations, a few drops of toluene were added and the glycoprotein was digested with 5 mg of pronase from Streptomyces griseus (B grade, Calbiochem) for 72 h at 37°C, a further 5 mg being added at 24 h and 48 h.

The pronase digest was chromatographed on a column (90 × 2 cm) of Bio-Gel P2 (200–400 mesh) equilibrated in distilled water at a flow rate of 9 mL h⁻¹. Aliquots (2 μL) of each collected fraction (1.5 mL) were applied on a Silica Gel aluminum plate (Kieselgel 60, Merck, Germany). After drying up, the plate was sprayed with a 0.2% (w/v) orcinol solution in 20% (v/v) sulfuric acid to detect glycopeptides. Fractions containing hexoses were pooled, concentrated under vacuum and submitted to a second identical pronase digestion. After a second gel filtration chromatography on the Bio-Gel P2 column, the recovered glycopeptide fraction was submitted to a 10 h preparative descending paper chromatography on Whatman 3 MM paper using the pyridine/ethyacetate/acetic acid/water, 5:5:1:3 (by vol.) solvent system, to eliminate contaminating peptides. The starting area containing the nonmoving glycopeptides was eluted with distilled water, concentrated under vacuum and submitted to a new gel filtration chromatography on the Bio-Gel P2 column. Fractions containing glycopeptides were pooled and lyophilized.

Labeling of glycopeptides

Glycopeptides (1 mg) were labeled with 3.7 MBq of [1-¹⁴C] acetic anhydride (0.37–1.1 GBq mmol⁻¹, The Radiochemical Centre, Amersham, UK) as described (Koide and Muramatsu 1974) and purified by gel filtration on a Bio-Gel P2 column (90 × 2 cm) equilibrated in distilled water at a flow rate of 9 mL h⁻¹. Fractions (1.5 mL) were collected and aliquots counted in a Beckman LS-6000TA scintillation counter. Fractions containing radiolabeled glycopeptides were pooled and after lyophilization, re-dissolved into 3 mL of distilled water, and stored at −20°C.

Mass spectrometric analysis of N-[¹⁴C]-acylated glycopeptides derived from ribonuclease B

Mass spectra were recorded on an Applied Biosystems (Framingham, MA) Voyager DE-STR time-of-flight mass spectrometer equipped with a N₂ laser (337 nm, 3 ns pulse width, 20 Hz repetition rate). All mass spectra were acquired in the reflectron positive mode with a delayed extraction. External mass calibration was performed with calibration mixture 2 from Applied Biosystems containing angiotensin (m/z 1297.51), bovine insulin (m/z 5730.60) and three fragments of ACTH (m/z 2093.08; 2465.19 and 3657.92, respectively) 2,5-dihydroxy-benzoic acid (10 mg mL⁻¹) dissolved in methanol/water, 50: 50 (v/v) was used as a matrix. Samples were prepared by mixing 1 μL of a one-fortieth dilution of the glycopeptide solution with 1 μL of matrix.

Purification and immobilization of artocarpin

Artocarpin was isolated from an extract of jackfruit (Artocarpus integrifolia) seeds using mannose-Sepharose affinity chromatography as described (Misquith et al. 1994). The lectin was coupled with Sepharose 4B (Pharmacia) that had been CNBr-activated according to the procedure of March et al. (1974). The amount of artocarpin bound to Sepharose was estimated to be 3.7 mg per mL of gel by subtracting the quantity of unbound protein found in the supernatant and washing solutions after coupling. Arum maculatum agglutinin (AMA), isolated from tubers of Arum maculatum according to the procedure of Allen (1995), was also coupled with CNBr-activated Sepharose 4B at a concentration of 3.5 mg per mL of gel.

Affinity chromatography of N-[¹⁴C]-acylated RNAse B glycoasparagines on artocarpin-Sepharose column

N-[¹⁴C]-Acetylated RNAse B glycoasparagines (35 × 10³ disintegrations min⁻¹, 0.8 nmol) were applied to the column of artocarpin-Sepharose 4B (20 × 0.6 cm) equilibrated at room temperature in the 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl (PBS). Elution was performed first with PBS at a flow rate of 9 mL h⁻¹ and then with PBS containing 0.01 M methyl α-D-mannopyranoside (Sigma-Aldrich). Fractions (0.85 mL) were collected and aliquots counted in a Beckman LS-6000TA scintillation counter. Recovery of radioactivity from the artocarpin column was always higher than 95%.

Chromatography of N-[¹⁴C]-acylated RNAse B glycoasparagines on underived Sepharose 4B and on Arum maculatum-Sepharose 4B columns

N-[¹⁴C]-Acetylated RNAse B glycoasparagines (35 × 10³ disintegrations min⁻¹, 0.8 nmol) were applied to columns of either underived Sepharose 4B or of Arum maculatum agglutinin immobilized on Sepharose 4B (20 × 0.6 cm) equilibrated at room temperature in PBS. Elution and counting were performed as described previously.

Affinity chromatography of N-[¹⁴C]-acylated individual glycopeptides on the artocarpin-Sepharose column

Man 9 glycoasparagines (Dorland et al. 1981), isolated from a pronase digest of soybean agglutinin purified by affinity chromatography (Gordon et al. 1972), were labeled with [1-¹⁴C] acetic anhydride according to the method described previously. N-[¹⁴C]-Acetylated Man 9 glycoasparagines (34 × 10³
disintegrations min⁻¹) were applied to the same artocarpin-Sepharose 4B column (20 × 0.6 cm) equilibrated in PBS. Elution and counting were performed as described previously.

Man 6 glycopeptides, isolated from a pronase digest of mistletoe (*Viscum album*) lectin I by affinity chromatography on Concanavalin A-Sepharose (Debray et al. 1992), were also N-[¹⁴C]-acetylated according to Koide and Muramatsu (1974).

N-[¹⁴C]-Man 6 glycopeptides (6.5 × 10³ disintegrations min⁻¹) were then applied to the artocarpin-Sepharose column and elution and counting performed as described previously.

Man 5 glycosparagines (5.5 × 10³ disintegrations min⁻¹), obtained by affinity chromatography of N-[¹⁴C]-acetylated RNase B glycoasparagines on the artocarpin-Sepharose column, were reapplied on the same artocarpin-Sepharose column. Elution and counting were performed as described previously.

Analysis of the fractions obtained by affinity chromatography on artocarpin-Sepharose by high-performance thin-layer chromatography

The fractions obtained after affinity chromatography on the artocarpin column were first desalted by gel filtration on a column of Bio-Gel P2 (90 × 2 cm) equilibrated in distilled water, concentrated to 2 mL and lyophilized. Each fraction was then recovered in 200 µL of 0.1% trifluoroacetic acid (TFA) solution in distilled water and desalted on microcolumns of 5 mg of nonporous graphitized carbon (Alltech, Deerfield, IL) packed in 200 µL filter pipet tips. The microcolumns were first sequentially washed with 2 mL methanol and 2 mL 0.1% TFA. Glycosparagines of a recovered fraction dissolved in 200 µL of 0.1% TFA were applied to a microcolumn which was washed with 1 mL of 0.1% TFA. Elution of glycosparagines was obtained by the application of 2 mL of 50% acetonitrile in 0.1% TFA. The desalted fractions were lyophilized and dissolved in 200 µL of distilled water. Aliquots of 2 µL were counted in a Beckman LS-6000TA scintillation counter and the fractions stored at −20°C until HPTLC analysis.

Silica Gel 60 (20 × 20 cm or 10 × 20 cm) HPTLC glass plates (Merck, Germany) were spotted with a sufficient volume of the desalted fractions obtained by affinity chromatography and of the starting glycosparagine mixture in order to have 10³ disintegrations min⁻¹ per spot. The HPTLC plate was irrigated with n-propanol/methanol/0.25% KCl (60:10:35, v/v/v) at 20°C. The solvent was allowed to ascend to the top of the plate, which was dried in a ventilated hood.

Glycosparagines present in each fraction were then visualized using the Kodak Biomax transcreen LE intensifying screen system with Kodak Biomax MS films (Eastman Kodak company, Rochester, NY), placed for 1 week into a freezer (−80°C) to maximize the sensitivity of the system. Films were then processed according to Kodak recommendations.

Detection and quantitation of [¹⁴C]-labeled glycoasparagines after HPTLC with Storm 860 PhosphorImager

After migration, HPTLC plates were exposed to a reusable ¹⁴C Phosphor Storage screen which was analyzed by a Storm 860 PhosphorImager (Amersham Pharmacia Biotech). Obtained data were quantified by using ImageQuant software (Amersham Pharmacia Biotech).

Funding

Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/Comitê (CAPES)/Comité Français d’Evaluation de la Coopération Universitaire et Scientifique avec le Brésil (COFECUB) (N.269/99); International Foundation for Science-IFS (N.F/3070-3) (M.R.V); Centre National de la Recherche Scientifique-CNRS; European Community (FEDER); the Région Nord-Pas de Calais (France); the Université des Sciences et Technologies de Lille.

Acknowledgements

We are indebted to the service commun d’imagerie cellulaire de l’Université de Lille II for providing access to the Storm 860 PhosphorImager.

Conflict of interest statement

None declared.

References


1425


