Studies on Hemagglutinins from *Maackia amurensis* Seeds*

(Received for publication, October 20, 1973)

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**SUMMARY**

Two phytohemagglutinins (strongly hemagglutinating *Maackia amurensis* hemagglutinin and strongly mitogenic *Maackia amurensis* hemagglutinin (MAH and MAM)), which have mitogenic activity against human peripheral lymphocytes, have been purified from the seeds of *Maackia amurensis* by affinity chromatography on porcine thyroglobulin-Sepharose columns followed by SE-Sephadex chromatography and gel filtration on Sepharose 6B. The preparations were homogeneous by ultracentrifugal analysis and disc electrophoresis, and had $s_{20,w}$ values of 5.7 S (MAH) and 7.4 S (MAM), respectively. The approximate molecular weight of 130,000 was estimated by gel filtration for both hemagglutinins. From chemical analyses these hemagglutinins were found to be glycoproteins and only small differences in chemical composition were found between them. However, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed that MAH is a tetramer of subunits having an approximate molecular weight of 33,000 and MAM is a dimer of disulfide-containing subunits of an approximate molecular weight of 75,000. In hemagglutinating activity, MAH is much stronger than MAM, whereas MAM is a more potent mitogen than MAH. Binding experiments with $^{125}$I-labeled hemagglutinins indicate that normal human erythrocytes bind approximately $5.2 \times 10^7$ molecules of MAH and $7.8 \times 10^7$ molecules of MAM with apparent association constants of $2.9 \times 10^7$ M$^{-1}$ and $1.2 \times 10^7$ M$^{-1}$, respectively, and, furthermore, normal human lymphocytes bind approximately $1.1 \times 10^6$ molecules of MAH and $2.4 \times 10^7$ molecules of MAM with apparent association constants of $2.1 \times 10^7$ M$^{-1}$ and $1.1 \times 10^7$ M$^{-1}$, respectively. Inhibition assays using various sugars and glycoproteins as hapten inhibitors revealed that these hemagglutinins from *M. amurensis* seeds differ from each other in their specificities for sugars. Porcine submaxillary mucin (PSM) having blood group H activity was a potent hapten inhibitor against MAH and even against the mitogenic activity of MAH.

In this paper we report the purification and characterization of two hemagglutinins from *M. amurensis* seeds which have mitogenic activity against human peripheral lymphocytes. The detailed specificities of the purified hemagglutinins are also reported in this paper and, based on these results, the nature of their receptor sites on the cell surface are discussed in relation to their biological activities.

**EXPERIMENTAL PROCEDURES**

*Materials*-The seeds of *M. amurensis* were purchased from F. W. Schumacher, Sandwich, Mass. *Phaseolus vulgaris* hemagglutinin was Wellcome purified PHA (MR 69, lot K 1275; Wellcome Research Laboratories, Beckenham, U. K.). Concanavalin A was purified from jack bean (Sigma) according to the method of Agra-wal and Goldstein (8). The glycopeptide A and B from porcine thyroglobulin was prepared according to the procedure described by Fukuda and Egami (3). The structures of these glycopeptides have been proposed as shown in Fig. 1 (4-9). Sequential degradation of glycopeptide B with purified glycosidases and the characterization of the resulting residual glycopeptides were carried out according to the methods previously described (5). The liberation of monosaccharides during the sequential enzymic degradation of glycopeptide B and the carbohydrate composition of the residual glycopeptides were exactly as expected from the structure shown in Fig. 1. The details of these experimental data were already reported in a previous paper (5). Porcine submaxillary mucin was purified from porcine submaxillary glands showing blood group H activity according to the method of Katzman and Eylar (7). Desialization of the glycopeptides and glycoproteins was performed by a mild acid hydrolysis at pH 1 for 1 hour at 80°C and the released sialic acid was removed by gel filtration on a column of Sephadex G-25. By this method nearly quantitative liberation...
FIG. 1. Proposed structures of glycopeptide A (GPA) and glycopeptide B (GPB) from porcine thyroglobulin.

FIG. 2. Affinity chromatography of crude hemagglutinin on porcine thyroglobulin glycopeptides-Sepharose 4B column. Crude hemagglutinin, 100 mg, was dissolved in 1 ml of 0.15 M NaCl-1 mM phosphate buffer (pH 7) and applied to a column (2 X 16 cm) equilibrated against the same buffer. Elution was carried out with the same buffer. Fractions of 4 ml were collected at 8 ml per hour at 4°C. Mitogenic activity was tested on each 10 μl of the fractions.

of sialic acid from PSM1 and glycopeptide B was achieved. Highly purified samples of β-galactosidase, β-N-acetylglucosaminidase, and α-mannosidase from Turbo cornutus were kindly provided by Seikagaku Kogyo Co. Ltd. Each purified enzyme used in this study was found to be virtually devoid of other glycosidase activity.

Preparation of Porcine Thyroglobulin Glycopeptides-Sepharose 4B Adsorbent-A mixture of glycopeptides prepared from porcine thyroglobulin by pronase digestion followed by gel filtration according to the method of Fukuda and Egami (3) was coupled to activated Sepharose 4B according to the method previously described (8).

Purification of M. amurensis Hemagglutinins—Finely powdered M. amurensis seeds (100 g) were suspended in 1 liter of 0.15 M NaCl and allowed to stand overnight at 4°C with stirring. To the yellow clear supernatant obtained by centrifugation, solid (NH4)2SO4 was added to give 50% and later 80 and 100% saturation. The bulk of both hemagglutinating and mitogenic activities was detected in the fraction precipitating between 50 to 80% saturation.

Further purification of this fraction was achieved by chromatography successively on affinity adsorbent prepared by coupling porcine thyroglobulin glycopeptides to activated Sepharose 4B, SE-Sephadex C-50 and Sepharose 6B as described in the legends of Figs. 2, 3, and 4, respectively.

Ultracentrifugation—Measurement of the sedimentation velocity of hemagglutinins was performed according to the band sedimentation method (9) in a Spinco model E ultracentrifuge equipped with an ultraviolet optical system at a speed of 50,100 rpm at 20°C in 1 M NaCl.

Disc Electrophoresis—Disc electrophoresis in polyacrylamide gels was carried out in 7.5% gels in Tris-HCl buffer at pH 8.9 according to Ornstein (10) and Davis (11). Staining was performed with Amido black in 7% acetic acid, and destaining in an electric field with 7% acetic acid.

The sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out according to Weber and Osborn (12). Twenty micrograms of protein were placed onto a 7.5% gel in 0.01 M phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate. Experiments were performed both in the presence and absence of 1% β-mercaptoethanol.

Molecular Weight Estimation by Gel Filtration—A column of Bio-Gel P-200 (1 X 100 cm) was equilibrated with 0.15 M NaCl-5 mM phosphate buffer (pH 7). The relationship between elution volume and the logarithm of the molecular weights of various proteins was established on this column according to the pro-
The cells were then washed with 0.25% bovine serum albumin in 0.15 M NaCl and buffy coat, was washed three times by centrifugation with 0.3 ml of 0.15 M NaCl-5 mM phosphate buffer (pH 7) each time carefully removing the top layer of cells. The centrifugation was performed at 400 g for 20 min. The whiteuffy interface layer between the serum phase and the Urografin-Ficoll phase was removed by aspiration. The approximate proportion of lymphocytes in this layer was higher than 98%, and the viability of the cells was found to be 98 to 100% by the Trypan blue exclusion test. The viability of lymphocytes in this layer was higher than 98%, and the viability of the cells was found to be 98 to 100% by the Trypan blue exclusion test.

### RESULTS

**Ammonium Sulfate Fractionation**—Table I summarizes data pertaining to the purification of hemagglutinins from *M. amurenensis* seeds. From Table I, it can be seen that most of the hemagglutinating and mitogenic activities were precipitated by (NH₄)₂SO₄ fractionation at 50 to 80% saturation (crude hemagglutinin).

**Affinity Chromatography on Porcine Thyroglobulin Glycopeptides-Sepharose 4B**—The crude hemagglutinin was applied to a column of porcine thyroglobulin glycopeptides-Sepharose 4B affinity column in 0.15 M NaCl-1 mM phosphate buffer (pH 7.0) and elution was performed by the same buffer as shown in Fig. 2. After the bulk of protein was eluted in the void volume of the column, strong hemagglutinating activity appeared first, and strong mitogenic activity was recovered in the more retarded fractions. Thus, most of the inert proteins were effectively removed and, furthermore, the presence of two hemagglutinins differing in biological activity was ascertained by this chromatography. These overlapping active fractions were combined (Fraction A) and subjected to SE-Sephadex column chromatography to clearly separate these two hemagglutinins. In another experiment, the crude hemagglutinin was applied to a column of Sepharose 4B having the same size as the column in the case of the glycopeptides-Sepharose 4B adsorbent. The hemagglutinating and mitogenic activities were, however, not separated from each other and recovered together as a single peak in the void volume of the column.

**Chromatography on SE-Sephadex C-50**—Fraction A obtained by affinity chromatography was concentrated by ultrafiltration, dialyzed against 50 mM phosphate buffer (pH 5) and applied to a column of SE-Sephadex C-50 in the same buffer. After a large peak (Fraction B1) was eluted, gradient elution was performed as shown in Fig. 3, and another protein peak (Fraction B2) was obtained. The bulk of mitogenic activity was recovered in Fraction B1, which had relatively weak hemagglutinating activity.

### Table I

**Details of purification of crude hemagglutinins**

| Fraction | Yield from 100 g seeds | Hemagglutinating activity* | Mitogenic activity* |
|----------|-----------------------|----------------------------|---------------------|
|          | *mg*                  | *O* A B                     |                     |
| Crude extract |                       | 5000                        | 13                  |
| (NH₄)₂SO₄ fractions |              |                              |                     |
| 0–50% saturation |                   | 9400                          | 12                  |
| 50–80% saturation |             | 1300                          | 8.0                 |
| 80–100% saturation |           | 1730                          | 7.5                 |
| Affinity adsorbent Fraction A |             | 280                           | 6.5                 |
| SE-Sephadex fractions |              |                              |                     |
| Fraction B1 |                   | 220                           | 1.5                 |
| Fraction B2 |             | 280                           | 0.18                |
| Sepharose 4B fractions |           | 200                           | 1.5                 |
| Fraction C1 |           | 240                           | 0.18                |
| Fraction C2 |           | 240                           | 0.18                |

*Minimum hemagglutinating dose against human O, A, or B erythrocytes.

*Mitogenic dose to give 10,000 cpdm of [3H]thymidine incorporation against 3 X 10⁴ lymphocytes.
ity, and very strong hemagglutinating activity was observed in Fraction B2, which had relatively weak mitogenic activity. These two fraction pools were further purified by passage over Sepharose 6B to remove trace contaminants.

Chromatography on Sepharose 6B—Both Fractions B1 and B2 obtained by SE-Sephadex chromatography were further purified by gel filtration on Sepharose 6B. As shown in Fig. 4, strong mitogenic and strong hemagglutinating activities were recovered in Fractions C1 and C2, respectively, each as a single peak. These fractions were designated MAM (strongly mitogenic) and MAH (strongly hemagglutinating).

The minimum hemagglutinating dose of MAH was 0.18 µg per ml against human erythrocytes regardless of their blood group types, whereas the mitogenic activity of this fraction against human peripheral lymphocytes was 5.3 µg per ml. On the other hand, the mitogenic activity of MAH against human peripheral lymphocytes was 1.2 µg per ml, and its minimum hemagglutinating dose against human erythrocytes was found to be 1.5 µg per ml. These data are also listed in Table I.

Homogeneity and Molecular Weight Estimation—Ultracentrifugation of both MAH and MAM yielded a single peak in each case during the whole of the run in the densitometer chart obtained from the experiment. The sedimentation coefficients (s20,w) of MAH and MAM calculated from the sedimentation velocity data were 7.1 and 7.4 S, respectively.

The electrophoretic homogeneity of MAH or MAM was confirmed by disc electrophoresis on polyacrylamide gel. A single band was observed in both instances at pH 8.9, as shown in Fig. 4.

An approximate molecular weight of 130,000 was calculated for both MAH and MAM from the curve relating the elution volume to logarithm of molecular weights of various standard proteins on a Bio-Gel P-200 column.

Polyacrylamide gel electrophoreses of MAH and MAM in the presence of sodium dodecyl sulfate without β-mercaptoethanol treatment gave a single discrete band in each case (Fig. 5). The molecular weights of these subunits were estimated to be 33,000 and 75,000, respectively. When MAH and MAM were subjected to electrophoresis in the presence of sodium dodecyl sulfate, the same was true for MAH but not for MAM. In the case of MAM with β-mercaptoethanol treatment, two discrete bands were observed at molecular weights of 33,000 and 35,000, respectively. These facts strongly suggest that MAH consists of four subunits of the approximate molecular weight of 33,000 and MAM consists of two disulfide-containing subunits of the approximate molecular weight of 75,000.

Chemical Composition—Results of amino acid and carbohydrate analyses of MAH and MAM are shown in Table II. The most notable feature of the amino acid composition of these proteins is the absence of cysteine in MAH and the high proportion of acidic and hydroxy amino acids in both hemagglutinins. Major carbohydrate constituents of both MAH and MAM were mannose and glucosamine. The remainder of the carbohydrate was made up of smaller amounts of fucose, xylose, and arabinose in MAH, and fucose, xylose, arabinose, and galactose in MAM. Total carbohydrate contents of MAH and MAM were 9.5% and 8.7%, respectively.

Time and Dose Response for Maximum Incorporation of [6-3H]Thymidine—Maximum incorporation of [6-3H]thymidine by lymphocytes stimulated with MAH or MAM took place in approximately 3 days.

In order to demonstrate [6-3H]thymidine incorporation as a function of MAH or MAM added in comparison with P. vulgaris hemagglutinin and concanavalin A, lymphocytes were treated with various quantities of the hemagglutinins. The results are shown in Fig. 6. Maximum incorporation doses per 3×10⁶ cells were: 2 µg per ml for MAM, 20 µg per ml for MAH, 3 µg per ml for P. vulgaris hemagglutinin and 7 µg per ml for concanavalin A.

Binding to Erythrocytes and Lymphocytes—The binding of 125I-labeled MAH and MAM to human group O erythrocytes and h.
FIG. 6. Dose response curves of various mitogens. Each value represents the average of four cultures. MAM (▲—▲); MAH (●—●); P. vulgaris hemagglutinin (○—○); concanavalin A (△—△).

FIG. 7. Bindings of MAM and MAH to human erythrocytes and lymphocytes (see “Experimental Procedures”). The data have been plotted by the method of Steck and Wallach (24) according to the equation

\[
[\text{PHA bound}] = \frac{1}{K_n} \times \frac{1}{[\text{PHA free}]} + \frac{1}{n}
\]

where [PHA] = concentration of phytohemagglutinin (M), \(n\) = number of phytohemagglutinin molecule bound per cell, and \(K\) = the association constant of phytohemagglutinin. \(^{131}\text{I-MAM (▲—▲)}; ^{131}\text{I-MAH (●—●)}.

human lymphocytes was carried out and the data were plotted according to the method of Steck and Wallach (24) (Fig. 7). From the data, the apparent association constants and the average number of molecules bound per cell were calculated assuming molecular weights of 130,000 for both MAH and MAM, as shown in Table III. These data demonstrate that MAH or MAM binds to either erythrocytes or lymphocytes with almost the same association constant, but the apparent association constants of MAH for these cells are twice those of MAM. Of interest are the findings that the number of binding sites for strongly mitogenic MAM on lymphocytes is much larger than

that on erythrocytes, and strongly hemagglutinating MAH binds more on erythrocytes than on lymphocytes.

**Inhibition of Hemagglutination with Various Sugars and Glycopeptides**—The results of inhibition tests of hemagglutination with sugars and glycopeptides on MAH and MAM are given in Table IV. Although most of the monosaccharides and oligosaccharides tested were not inhibitory, lactose and \(N\)-acyethylneuraminic acid had weak inhibitory activity against both MAH and MAM.

### Table III

**MAH and MAM binding to human erythrocytes and lymphocytes**

| Cells       | Association constant | Number of molecules bound per cell |
|-------------|----------------------|-----------------------------------|
|             | \(\times 10^6\) M\(^{-1}\) | \(\times 10^9\)                    |
| Erythrocytes| 2.9                  | 0.32                              |
| Lymphocytes | 2.1                  | 1.1                               |

### Table IV

**Inhibitory activity of various sugars and glycopeptides toward MAH and MAM**

The following sugars are not inhibitory at a concentration of 400 pmol per ml: \(n\)-galactose, \(n\)-mannose, \(l\)-fucose, methyl \(a\)-\(n\)-galactopyranoside, methyl \(\beta\)-\(n\)-galactopyranoside, melibiose, di-\(N\)-acyethylchitobiose (100 pmol per ml).

| Compounds tested | Minimum amount completely inhibiting four hemagglutinating doses |
|------------------|---------------------------------------------------------------|
|                  | \% control pmol/ml                                            |
| None             | 100                                                           |
| Glycopeptide A\(^d\) | >5.0 0.46                                                 |
| Glycopeptide B\(^d\) | >5.0 0.012                                                  |
| Treated with sialidase \(\beta\)-galactosidase | >1.3 0.014                                                           |
| Treated with sialidase \(\beta\)-galactosidase, \(\beta\)-\(N\)-acyethylglucosaminidase | >1.3 0.13                                                        |
| Treated with sialidase \(\beta\)-galactosidase, \(\beta\)-\(N\)-acyethylglucosaminidase, \(\alpha\)-mannosidase | >1.3 0.25                                                        |
| Porcine submaxillary mucin (PSM) | >825*                                                     |
| Desialized PSM | >825* >100(260)*                                             |
| \(N\)-Acetyl-\(\alpha\)-galactosamine | >400 4(50)                                                |
| \(N\)-Acetylneuraminic acid | 40 80                                                   |
| Maltose          | >400 11(25)                                                  |
| Lactose          | 69 >100(25)                                                   |

\(^a\) Average value of four experiments.

\(^b\) 5 \(\mu\)g of MAH per ml.

\(^d\) Porcine thyroglobulin glycopeptides were tested at concentrations no higher than 1.3 or 5 pmol per ml because of their limited availability.

\(*\) Micrograms per ml.
Among various glycopeptides and glycoproteins tested, porcine submaxillary mucin (PSM) was found to demonstrate marked inhibitory activity only against MAH, but desialylation of the glycoprotein gave rise to a remarkable loss of inhibitory activity. On the other hand, glycopeptide B (Fig. 1) from porcine thyroglobulin exerted potent inhibitory activity only against MAH. In this case, removal of sialic acid did not affect the inhibitory activity, but the sequential enzymatic degradation with purified \( \beta \)-galactosidase, \( \beta \)-N-acetylgalactosaminidase, and \( \alpha \)-mannosidase gradually diminished the inhibitory activity against MAH, leaving appreciable inhibitory activity even after \( \alpha \)-mannosidase treatment. Glycopeptide A from porcine thyroglobulin, whose structure has been shown to consist of a mannose oligomer and a core di-\( \alpha \)-N-acetylhexosamine (4), was less inhibitory than glycopeptide B against MAH.

Inhibition on [\( ^{3} \text{H} \)]Thymidine Incorporation in Human Peripheral Lymphocyte Stimulation—Table IV summarizes the effect of various sugars and glycoproteins on [\( ^{3} \text{H} \)]thymidine incorporation of human peripheral lymphocytes exposed to MAH and MAM. Among the simple sugars and oligosaccharides tested, only \( \text{N-acetyl-d-galactosamine} \) and \( \text{malto} \) have moderate inhibitory activity against both MAH and MAM. In spite of the fact that glycopeptide B from porcine thyroglobulin is not inhibitory against MAH in the hemagglutination-inhibition assays, this glycopeptide and its sequential enzymatic degradation products are potent inhibitors against the mitogenic activity of MAH. The fact that the glycopeptide after \( \alpha \)-mannosidase treatment still retains significant inhibitory activity against both hemagglutinins indicates that the core region of the glycopeptide can interact with the hemagglutinins.

DISCUSSION

A recent survey in our laboratory on the hemagglutinating and mitogenic activities of the extracts of many plant seeds revealed that \( \text{M. amurenensis} \) seeds contained strong hemagglutinating and mitogenic activities. These activities were effectively separated from inactive proteins by affinity chromatography using a column prepared by coupling the glycopeptides obtained by pronase digestion of porcine thyroglobulin to activated Sepharose. It was found that the affinity column prepared by coupling porcine thyroglobulin itself to the activated Sepharose had a smaller capacity than the glycopeptide-Sepharose column. Thus, two hemagglutinins (MAH and MAM) were purified by further chromatography on SE-Sephadex followed by gel filtration on Sepharose 6B.

In biological activities, a notable difference exists between MAH and MAM. As shown in Table I, the hemagglutinating activity of MAH is stronger than that of MAM, but the mitogenic activity of MAH is much weaker than that of MAM. Interestingly, a good correlation was found between these biological characteristics of these hemagglutinins and the results of binding studies of the \( ^{3} \text{H} \)-labeled hemagglutinins to erythrocytes and lymphocytes. The apparent association constant of MAH to human erythrocytes was about twice that of MAM, and the number of receptor sites on erythrocytes for MAH was four times greater than for MAM. In contrast, the number of receptor sites on lymphocytes for MAH was approximately half of that for MAM, which was in turn 30 times more abundant than on erythrocytes. More striking differences between MAH and MAM can be seen in their specificities for sugars. In hemagglutination inhibition assays, PSM was found to be a good inhibitor for MAH, whereas it was almost devoid of inhibitory activity against MAM. Since the desialylation of PSM results in a remarkable diminution of the inhibitory activity, and since lactose and \( \text{N-acetylenouraminic} \) acid have weak, but definite inhibitory activity against MAH, it seems likely that MAH binds primarily to the galactose \( \rightarrow \) (sialic acid \( \rightarrow \)) \( \text{N-acetylgalactosamine} \) portion of the sugar chain of the PSM molecule and possibly to a similar structure in the \( O \)-glycosidically linked sugar chain of human erythrocyte cell surface. On the other hand, \( N \)-glycosidically-linked glycopeptide B from porcine thyroglobulin and its enzymic degradation products exerted strong inhibitory activity against MAM, but they were not inhibitors against MAH in the hemagglutination. It is of interest to note that the residual core glycopeptide after \( \alpha \)-mannosidase treatment still retains appreciable inhibitory activity, suggesting that MAM has a stronger affinity to the inner core of the glycopeptide. From these facts, it can be assumed that MAM binds primarily to \( N \)-glycosidically-linked oligosaccharide chain on the erythrocyte cell surface as the one reported by Kornfeld and Kornfeld (25). Good correlations were generally observed between the results of hemagglutination inhibition and those of the inhibition of thymidine incorporation in the case of MAM. However, the correlation is not quite as good as in the case of MAH. A major discrepancy is that glycopeptide B and its enzymic degradation products, especially the inner part of the glycopeptide, have definite inhibitory activity against the mitogenic activity of MAH in spite of the fact that these glycopeptide are virtually devoid of inhibitory activity against the hemagglutinating activity of MAH. Since the contamination of MAH with a small amount of strongly mitogenic MAM can be excluded on the basis of their chromatographic behavior and the physiological assays, a possible explanation for this discrepancy may lie in the assumptions that the transformation of lymphocytes can be triggered by the binding of mitogen to the \( N \)-glycosidically-linked sugar chain on the lymphocyte cell surface (5, 26), and that the affinity of MAH to glycopeptide B is so small compared to its affinity to \( O \)-glycosidically linked sugar chain on the erythrocyte cell surface that the glycopeptide cannot cause detectable effects on the hemagglutination inhibition assay.

Acknowledgments—We acknowledge the excellent technical assistance of Mrs. S. Shimizu. We thank Dr. K. Suzuki for carrying out ultracentrifugal analyses.

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*J. Biol. Chem.* 1974, 249:2786-2792.

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