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# THE INTERACTION OF *RICINUS COMMUNIS* AGGLUTININ WITH NORMAL AND TUMOR CELL SURFACES

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SUMMARY

Two plant agglutinins were isolated and purified from *Ricinus communis* beans by affinity chromatography. Both of the agglutinins were inhibited by sugars containing terminal β-D-galactose-like residues, but the smaller agglutinin (mol. wt approximately 60 000) was additionally inhibited by *N*-acetyl-D-galactosamine-like sugars. The larger agglutinin (mol. wt approximately 120 000) was used to specifically agglutinate several normal and transformed cell lines. The agglutinin consistently agglutinated transformed cells at much lower concentrations than those required to agglutinate normal cell lines unless the normal cells were first treated with low concentrations of trypsin. The significance of the change in agglutinability of transformed or trypsinized cells is discussed in relation to the topology of the cell surface.

Several lectins or carbohydrate specific plant agglutinins have been shown to specifically agglutinate certain viral transformed cell lines at concentrations which do not cause agglutination of the normal untransformed cell lines from which they were derived[1–6]. Plant agglutinins that show these properties are: wheat germ agglutinin[1,2] (specific for *N*-acetyl-D-glucosamine-like residues[2]), concanavalin A[3,4] (specific for α-D-mannose-like residues[7]), and soy bean agglutinin[5,6] (specific for *N*-acetyl-D-galactosamine-like residues[5]). Here we report on the purification of two plant agglutinins by affinity chromatography from *Ricinus communis* beans, and the differential agglutination by one of these agglutinins of tumor cells through their surface oligosaccharides.

The agglutinins were isolated from local wild *R. communis* beans** by blending 100 g of beans in 1 l of 0.2 M NaCl–0.005 M sodium phosphate buffer, pH 7.2. After 3 h the NaCl–sodium phosphate buffer extract was filtered through cheesecloth and centrifuged at 10 000 × *g* for 30 min. The clear supernatant was removed without disturbing the floating lipid layer and was centrifuged again, as above. The solution

Abbreviations: $RCA_{60}$ and $RCA_{120}$, *Ricinus communis* agglutinins of mol. wt 60 000 and 120 000, respectively.

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** *R. communis* extracts are extremely toxic and great care must be exercised in isolating these proteins.

containing the *R. communis* agglutinins was adjusted to 0.6 saturation with ammonium sulfate[8] and was left overnight at 5 °C. After centrifugation for 30 min at 10000 × *g*, the pellet containing hemagglutinating activity was dissolved in NaCl–sodium phosphate buffer and exhaustively dialyzed against NaCl–sodium phosphate buffer at 5 °C to remove ammonium sulfate. The dialyzed agglutinin solution was centrifuged as before and the supernatant applied to a 4 cm × 40 cm column of (Biorad) Agarose A-0.5 m. The column was washed with NaCl–sodium phosphate buffer until the absorbance at 280 nm was below 0.05; usually about 2–3 l of NaCl–sodium phosphate buffer was required. The agglutinins were eluted by either a solution of 0.2 M D-galactose in NaCl–sodium phosphate buffer, or a linear gradient of 0.01–0.5 M lactose in NaCl–sodium phosphate buffer. When the linear lactose gradient was used, one peak (containing approximately 200 mg protein per initial 100 g of beans) was obtained on the affinity column (Fig. 1). This peak was found to contain two agglutinins which could be separated by chromatography on a 2 cm × 40 cm column of Sephadex G-100, eluted with NaCl–sodium phosphate buffer (Fig. 2). The first Sephadex peak contained one protein of approximately 120000 molecular weight as determined by electrophoresis in sodium dodecylsulfate-equilibrated (5%) polyacrylamide gels[9]. The second peak contained a protein of 60000 molecular weight. When the amino acid analyses of these proteins were compared, the agglutinin of 120000 mol. wt ($RCA_{120}$) had considerably less (on a molar basis) of the amino acids phenylalanine, methionine and valine compared to the agglutinin of 60000 mol. wt ($RCA_{60}$).

The specificities of $RCA_{60}$ and $RCA_{120}$ determined by agglutination inhibition were similar with one major exception (Table I). Both agglutinins were inhibited by lactose, D-galactose, melibiose, raffinose, D-fucose, methyl-β-D-galactoside and D-galactosyl-1,4-β-D-mannoside, but not by L-fucose, L-arabinose, methyl-α-D-mannoside, methyl-α-D-glucoside, D-xylose, D-ribose, D-mannose, maltose or *N*-

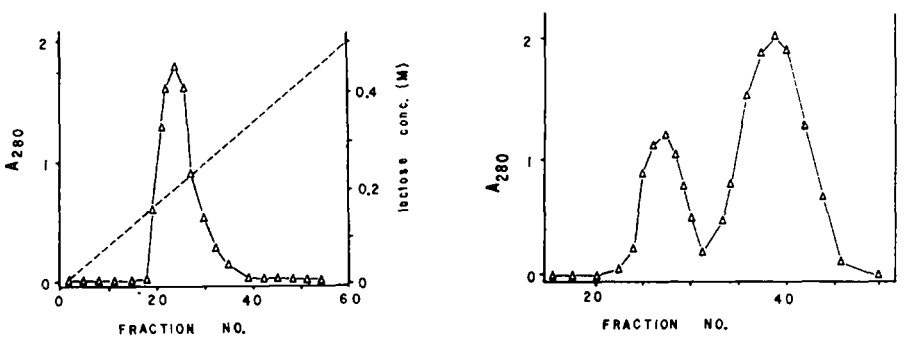


Fig. 1. Agarose affinity chromatography of ammonium sulfate-fractionated *R. communis* agglutinins. The dialyzed salt fractionated agglutinins from approximately 25 g of beans were applied to a 4 cm × 40 cm Agarose A-0.5 m column at 5 °C. After washing the column with 2–3 l of buffer, the agglutinins were eluted with a linear lactose (1 l of a 0.01 M lactose solution in the mixing chamber; 1 l of a 0.5 M lactose solution in the reservoir chamber) gradient. Absorbance at 280 nm was recorded for each 10-ml fraction.

Fig. 2. Sephadex chromatography of the *R. communis* agglutinins purified by affinity chromatography. Approximately 25 mg of the *R. communis* agglutinins in 8 ml were applied to a 2 cm × 40 cm Sephadex G-100 column and the agglutinins were eluted with buffer. Absorbance at 280 nm was recorded for each 2-ml fraction. Both peaks contained considerable hemagglutinating activity.

TABLE I

SPECIFICITY OF *R. communis* AGGLUTININS AS MEASURED BY HEMAGGLUTINATION INHIBITION

The two *R. communis* agglutinins were adjusted in concentration to have equal titers against rabbit erythrocytes ($2 \cdot 10^8$ cells/ml) in saline. Hemagglutination inhibition was determined by titering the agglutinins both with and without a standard molarity of sugar present. The inhibition values were normalized by setting inhibition by the standard concentration of lactose ($3 \cdot 10^{-5}$ M) equal to 100%.

| *Sugar* | *Relative % inhibition* * | |
|---|---|---|
| | $RCA_{120}$ (*2 μg/ml*) | $RCA_{60}$ (*18 μg/ml*) |
| Lactose (D-Gal-1,4-β-D-Glc) | 100 | 100 |
| D-Galactose | 26 | 21 |
| Melibiose (D-Gal-1,6-α-D-Glc) | 50 | 34 |
| Raffinose (D-Gal-1,6-α-D-Glc-1,2-β-D-Fru) | 43 | 9 |
| D-Gal-1,4-β-D-Man | 125 | 100 |
| Methyl-β-D-Gal | 34 | 28 |
| D-Fucose (6-deoxy-D-Gal) | 24 | 26 |
| L-Fucose | <5 | <5 |
| L-Arabinose | <5 | <5 |
| Methyl-α-D-Man | <5 | <5 |
| Methyl-α-D-Glc | <5 | <5 |
| D-Xylose | <5 | <5 |
| D-Ribose | <5 | <5 |
| Maltose (D-Glc-1,4-α-D-Glc) | <5 | <5 |
| *N*-Acetyl-D-glucosamine | <5 | <5 |
| *N*-Acetyl-D-galactosamine | <5 | 116 |

* Average of four determinations.

acetyl-D-glucosamine. The exception was the strong inhibition of $RCA_{60}$ by *N*-acetyl-D-galactosamine. These results are similar to the inhibition data of Drysdale *et al.*[10] using a salt fractionated *R. communis* preparation with the exception that L-arabinose and L-fucose were not inhibitory to the purified agglutinins.

Normal and tumor cells were compared by their agglutinability using $RCA_{120}$. Mouse fibroblasts (3T3) and their SV40 transformed (SV 3T3) or SV40-polyoma doubly transformed (SVPy 3T3) lines and hamster fibroblasts (BHK) and their polyoma transformed (Py BHK) lines were grown to confluency in Dulbecco's modified Eagle's medium supplemented with unheated 10% calf serum[11]. After removal from Petri dishes with isotonic 0.02% disodium versenate[4], the cells were washed three times with 0.6% sodium chloride–0.05 M sodium phosphate buffer, pH 7.4, and suspended at a concentration of $2 \cdot 10^6$ cells/ml. The cell viability was usually $\geqslant 95\%$ as determined by dye exclusion. For the agglutination assays, 0.25-ml aliquots of the cell suspensions were added to equal volumes of various concentrations of $RCA_{120}$ in 0.6% sodium chloride–0.05 M sodium phosphate buffer, pH 7.4. The incubation was performed on plastic trays (FB 54, Linbro Chemical) in 2-ml wells, and the agglutination was scored after 30-min incubation with gentle mixing at room temperature[5]. The cell agglutination was specific, since 0.2 M lactose (final) in the buffer prevented cell agglutination, and the agglutination could be reversed if the aggregated cells were incubated with 0.2 M lactose (final) a short time after agglutination. The transformed cells (SV 3T3,

SVPy 3T3, Py BHK) were always agglutinated at much lower agglutinin concentrations than their normal lines (3T3 and BHK), but brief trypsinization of the normal cells (10 $\mu$g/ml trypsin for 2 min at 37 °C) rendered them more agglutinable (Fig. 3).

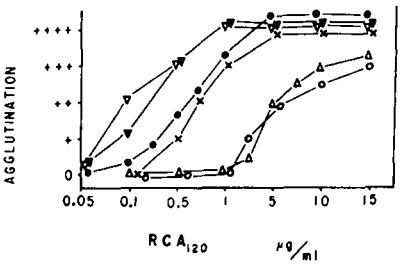


Fig. 3. The agglutination of normal and transformed cells by $RCA_{120}$. The agglutination procedures generally follow the method of Sela *et al.*[5]. Cells at a concentration of $2 \cdot 10^6$–$4 \cdot 10^6$ per ml were mixed with an equal volume of agglutinin solution and mixed for 30 min at room temperature. Agglutination was scored on a qualitative scale from 0 (no agglutination) to ++++ (complete agglutination) with the appropriate concentration of $RCA_{120}$. Controls with 0.2 M lactose present showed no agglutination. Cell lines are indicated as follows: △--△, normal 3T3; ▼—▼, SV 3T3; ▽—▽, SVPy 3T3; X—X, trypsinized-normal 3T3; ○—○, normal BHK; ●—●, Py BHK.

Several theories have been presented to explain the difference in agglutinability of normal cells, transformed cells, and cells treated with proteolytic enzymes. Burger[2] proposed that the differential agglutination of normal and transformed cell lines by wheat germ agglutinin could be due to a complete *de novo* synthesis of agglutinin sites, a net increase of sites, or an unmasking of buried or cryptic agglutinin sites when a cell is transformed. Since the agglutinin sites on normal cells could be "unmasked" by proteolytic enzymes the transformation process was thought to uncover "cryptic" agglutinin sites.

Recently, several laboratories have found that there is little or no difference in the number of wheat germ agglutinin[12], concanavalin A[12–14] or soy bean agglutinin[15] sites on normal, trypsinized-normal or transformed cells. These findings have led to the suggestion that the topological distributions of these agglutinin sites are different on normal *versus* transformed and trypsinized-normal cell surfaces[12,14–17]. The experimental demonstration of differences in the topological distributions of agglutinin-binding sites by one of us[17] using ferritin-conjugated concanavalin A on normal and transformed cell surfaces indicates that the surface distributions of these agglutinin sites may be responsible for the differences in cell agglutinability. When normal 3T3 cell surfaces were examined, the ferritin–concanavalin A molecules were found to be in a dispersed distribution. But when trypsinized-normal 3T3 cells or SV40 transformed 3T3 cells were examined, the ferritin–concanavalin A molecules were in a more clustered distribution[17]. It is tempting to suggest that the $RCA_{120}$ binding sites may also be more dispersed on normal cells compared to the transformed or trypsinized cells. We are currently attempting to quantitate the $RCA_{120}$ sites and determine the surface distributions on several types of cells using $^{125}I$-labeled $RCA_{120}$ and ferritin-conjugated plant agglutinins[18,19].

We have also carried out experiments designed to specifically kill tumor cells *in vivo* and *in vitro*, using $RCA_{60}$ and $RCA_{120}$. Preliminary results indicate that

$RCA_{60}$ or $RCA_{120}$ can be used to suppress growing mammary tumors in rats. Lin *et al.*[20] have isolated a phytotoxin from *R. communis*, Ricin D, that also has tumor-suppressive and hemagglutinating properties. The molecular weight of Ricin D (65 000)[21,22] is very similar to $RCA_{60}$, but the toxicity ($LD_{50}$ = 0.2 $\mu$g per 20 g mouse)[21] is much higher than $RCA_{60}$ ($LD_{50}$ = 10–15 $\mu$g per 20 g mouse) (G. L. Nicolson and W. T. Shier, unpublished data). Other investigators[8,23] have found that extensively purified Ricin D may have no hemagglutinating activity. The possibility that $RCA_{60}$ is, in fact, Ricin D must await further characterization in progress in our laboratory.

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