LECTIN-INDUCED INHIBITION OF PLASMA MEMBRANE 5'-NUCLEOTIDASE: SENSITIVITY OF PURIFIED ENZYME

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SUMMARY

To determine whether the lectin-induced inhibition of plasma membrane 5'-nucleotidase resulted from direct interaction of the lectin with the enzyme or indirectly from a membranous change due to lectin binding to other membrane glycoproteins, the enzyme was purified and its sensitivity tested in the absence of other membrane components. A 5000 fold purification was achieved by solubilization in Lubrol PX followed by gel filtration (Sephadex G-100), anion exchange (DEAE-Biogel A) and selective adsorption (hydroxylapatite) chromatography. The purified enzyme was even more sensitive to inhibition by high concentrations of concanavalin A, wheat germ agglutinin or *Ricinus communis* agglutinin than was the membrane-bound enzyme indicating that inhibition is due to direct binding of the lectins to the glycoprotein enzyme itself. Divalent succinyl Con A inhibited neither form of the enzyme suggesting the need for crosslinking for inhibition by the native lectin. The purified enzyme could not be activated by low concentrations of lectins which stimulated the membrane bound enzyme.

Among a wide variety of different structural and functional features of cell surface plasma membranes (1-8), the activities of a number of plasma membrane enzymes (9-17) are modified as a result of binding of plant lectins to carbohydrate on the membrane surface. We have shown that the ecto-enzyme, 5'-nucleotidase of liver plasma membranes is modified in a biphasic manner by the binding of increasing concentrations of concanavalin A[1] (11). Activity was enhanced in a cooperative manner by the binding of low concentrations of the lectin (<0.05 μM) and then progressively inhibited at higher concentrations. Carraway and coworkers (15-17) have since observed the inhibitory phase of the modification in membranes of rat mammary ascites carcinoma and Stefanovic et al (18) have shown that it occurs at the surface of intact cultured glioma cells.

[1]The abbreviations used are: Con A (concanavalin A), WGA (wheat germ agglutinin) and RCA-I (first agglutinin from *Ricinus communis*).

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We now show that when the enzyme is purified to apparent homogeneity, sensitivity to inhibition by Con A and a number of other lectins is retained. These findings are consistent with other evidence that the enzyme is itself a glycoprotein (32) and indicate that inhibition results from its direct interaction with lectin. An abstract describing part of this work has already appeared (19).

METHODS

Plasma membranes from the livers of male Wistar rats (130-175 g) were isolated according to Ray (20). 5'-nucleotidase activity at 37° in the presence of 0.05 M Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 10 mM AMP was assayed radiochemically using tracer amounts of [8-^{14}C] AMP (21). Protein was determined by the fluorometric procedure of Böhlen et al (22).

Concanavalin A from Sigma was purified by affinity chromatography on Sephadex G-100 (23). Wheat germ agglutinin was purified from crude wheat germ lipase (Sigma) according to Marchesi (24). Fucose binding protein from *Lotus tetragonolobus* and soybean agglutinin were from Miles Laboratories, Inc. The leuco-agglutinin from *Phaseolus vulgaris* was from Pharmacia. The first of the two agglutinins from *Ricinus communis* was purified according to Nicolson and Blaustein (25). Succinyl Con A was prepared by reaction of native Con A with succinic anhydride according to Gunther et al (26). The product eluted from a Biogel P-100 (Biorad) column in approximately the same volume as ovalbumin (molecular weight 45,000). A sedimentation coefficient of 3.86 ± 0.06 was determined by sedimentation velocity analysis (27), in good agreement with values obtained by other workers for the dimeric succinyl Con A (26,28).

For solubilization membranes were incubated with gentle mixing at a concentration of about 1 mg per ml in 0.05 M NaH_2PO_4, pH 7.5 containing 0.7% Lubrol PX for 1 h at 37°. After centrifugation at 40,000 g for 1 h the supernatant was concentrated 7 fold by ultrafiltration (Amicon PM 30) to a volume of about 5 ml containing about 40 mg of protein and applied to a column (2.5 x 90 cm) of Sephadex G-100 (40-120 μ particle diameter) with elution in 0.05 M NaH_2PO_4, pH 7.5 containing 0.1% Lubrol-PX at 4°. Five milliliter fractions were collected and monitored for absorbance at 280 nm and enzyme activity. The fractions comprising the enzyme peak were pooled, dialysed, concentrated 11 fold by ultrafiltration to a volume of 6 ml containing 10 mg of protein and applied to a DEAE Biogel A column (1.5 x 25 cm). Elution was performed in 0.001 M NaH_2PO_4, pH 7.5 with a linear salt gradient to 0.3 M NaCl. Fractions containing the enzyme were pooled, dialysed, concentrated 7 fold by ultrafiltration to a volume of 7 ml containing 0.8 mg protein and applied to a hydroxylapatite (Biorad) column (0.9 x 30 cm) and eluted with a linear gradient from 0.001 M to 0.1 M NaH_2PO_4, pH 7.5.

RESULTS

The results of the chromatographic steps used to purify the enzyme are shown in fig. 1. The profile of elution of the solubilized material from Sephadex G-100 appears as fig. 1a. The first very small peak of A_{280} is

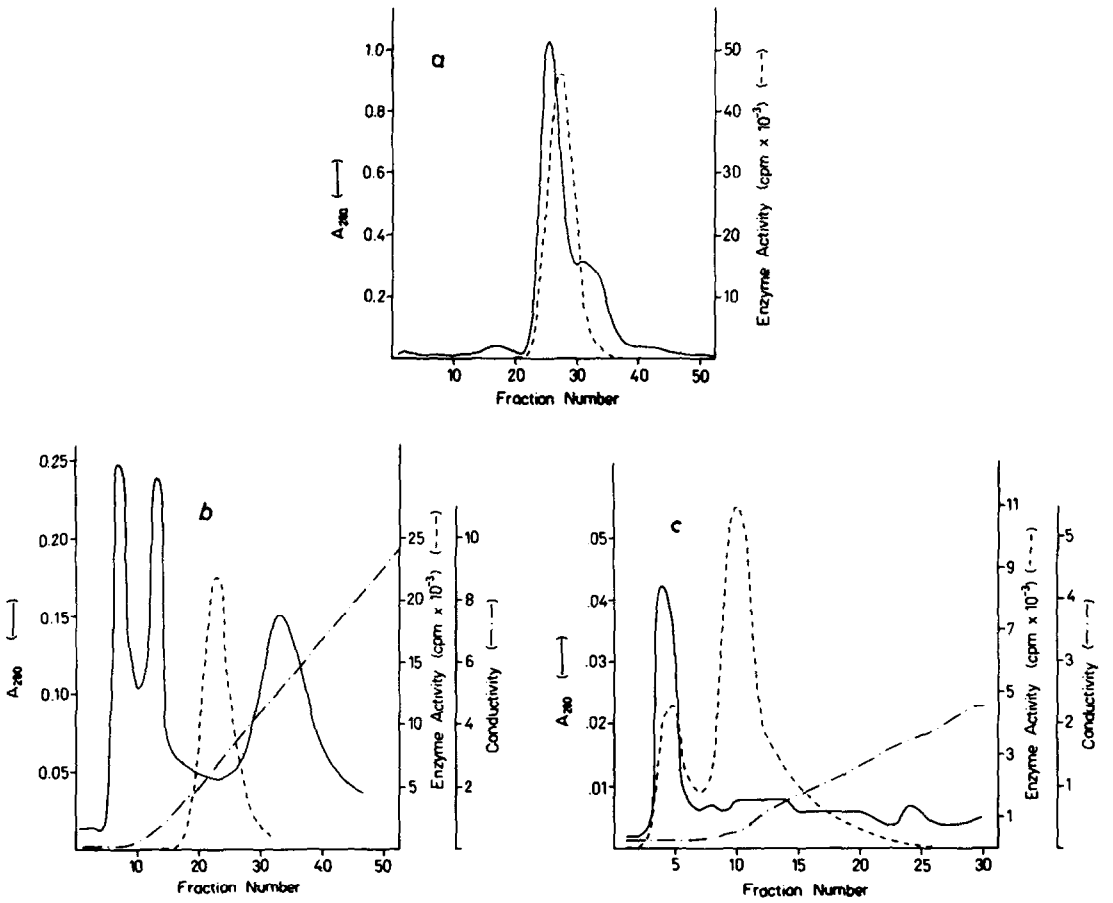

Fig. 1a Gel filtration of ultrafiltrate (Amicon PM 30) of Lubrol-PX soluble fraction of plasma membrane on a Sephadex G-100 column (2.5 x 90 cm) in 50 mM NaH_2PO_4, pH 7.5 containing 0.1% Lubrol-PX. 5 ml fractions were collected and monitored for absorbance at 280 nm and 5'-nucleotidase activity.

Fig. 1b Anion exchange chromatography of pooled gel filtration fractions (23 to 32 of Fig. 1a) on a DEAE Biogel A column (2.5 x 18 cm). Elution was with a salt gradient from 0 to 0.3 M NaCl which resulted in the conductivity (mmho) rise shown.

Fig. 1c Hydroxylapatite chromatography of pooled DEAE fractions (18 to 28 of Fig. 1b) on a 0.9 x 25 cm. column. Elution was by a linear gradient from 0.001 M to 0.1 M NaH_2PO_4, pH 7.5 which resulted in the conductivity (mmho) changes indicated.

coincident with column void volume. Since material smaller than the equivalent of 30,000 daltons was removed by the ultrafiltration step, the remainder of the profile is simple, exhibiting one major peak of A_{280} with a leading shoulder. The enzyme activity eluted as a well defined peak slightly behind

Table I

Purification of 5'-nucleotidase

Fraction	Specific Activity (μmoles/mg prot/h)	Purification factor		Total Activity	Recovery (%)
Liver homogenate	2.7	-	(1)	-	-
Plasma membrane	48.9	1	(18.1)	2910	100
Solubilized enzyme	81.1	1.7	(30.0)	2728	93.7
G-100 peak	197.3	4.0	(73.1)	2407	82.7
DEAE peak	2,263	46.3	(838.2)	1262	43.3
Hydroxylapatite Peak I	3,883	79.4	(1438.1)	163	5.6
Hydroxylapatite Peak II	13,279	271.6	(4918.0)	285	13.2

Numbers in parentheses in the purification column are purification factors relative to the specific activity of the homogenate defined as 1. The actual specific activity given for the homogenate is the mean of the values of the 6 homogenates from which the plasma membranes were purified. The total volumes of some of these homogenates were not accurately recorded so that the total activity of the homogenate could not be calculated. Recoveries of enzyme were calculated relative to the pooled plasma membranes used.

the major protein peak but was not coincident with it. Fig 1b shows the elution diagram from DEAE Biogel A chromatography of the pooled enzyme-containing fractions obtained by the gel filtration step. Three large protein peaks are resolved with enzyme eluting as a sharp peak between the last two. The fractions comprising this peak were chromatographed on hydroxyapatite (fig 1c). A peak containing nearly all of the protein appeared very early, whereas, the majority of the enzyme eluted later in association with a very small amount of protein. A smaller proportion of the enzyme eluted nearly coincident with the major protein peak.

The degree of purification and recovery of the enzyme attained in each of the steps are contained in table I. The specific activity of the enzyme in the plasma membranes was nearly 20 times greater than that of the liver homogenate. After solubilization in Lubrol-PX the specific activity is nearly doubled with little loss of enzyme. Despite the fact that not a great deal of resolution appeared to be obtained on gel filtration, the specific activity

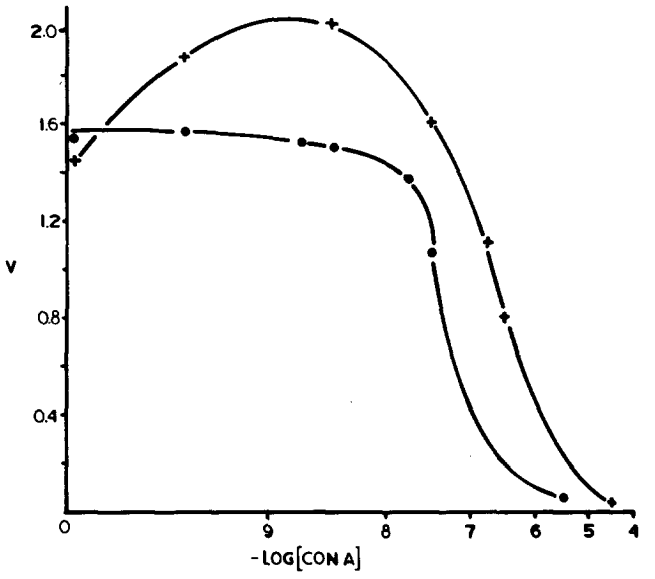

Fig. 2 Influence of increasing Con A concentrations on the velocity (v) (c.p.m./2 min. x 10^{-4}) of the reaction catalysed by purified 5'-nucleotidase (6.2 ng protein) (●) or by plasma membrane (25 μg protein) (+) at 37^{0}.

was increased by more than two fold with the total loss of enzyme still less than 20%. As had been indicated by the DEAE Biogel A elution profile, a major purification (approximately 12 fold) was achieved in this step while more than 40% of the original membrane enzyme was retained. The hydroxylapatite step increased the specific activity a further 6 fold (major enzyme peak) and this material contained 13% of the original enzyme. A further 6% was contained in the minor enzyme peak from the hydroxylapatite step. The most highly purified major peak has a specific activity 270 times greater than the plasma membranes or about 5000 times greater than the liver homogenate.

Fig 2 shows the effects of increasing concentrations of Con A on the membrane-bound and purified forms of 5'-nucleotidase. The response of the former (upper curve) is biphasic in agreement with earlier observations (11,29) while the latter shows only one phase, an abrupt inhibition at Con A concentrations above approximately 10^{-8}M. This inhibitory phase is shifted to the left by about one order of magnitude from that of the membrane-bound enzyme. These

Table II

Inhibition of 5'-nucleotidase by lectins.

Lectin	% Inhibition	
	Membrane enzyme	Purified enzyme
Succ. Con A	6	8
Con A	85	94
WGA	16	31
RCA-I	53	66

Enzymes (25 μg of membrane protein or 6.2 ng of purified enzyme protein) were preincubated with lectins (0.5 mg.ml) for 30 min at 37° prior to assay. The effects of Con A, WGA, and RCA were reversed by 0.3M α-methyl-D glucoside, N-acetyl-D-glucosamine, or D-galactose respectively.

observations confirm the fact that activation of the enzyme at low Con A concentrations requires interactions involving components of the membrane other than the enzyme itself which can be disrupted by detergent solubilization (11). On the other hand, they indicate that the inhibition requires only the enzyme and, therefore, must be a direct result of the binding of the lectin to carbohydrate of this glycoprotein enzyme. In addition the increased sensitivity of the purified enzyme to this inhibition suggests the membrane normally restricts either the binding of lectin to the enzyme or the subsequent rearrangement within the membrane which may be responsible for the inhibition.

The inability of the divalent succinyl Con A to inhibit either form of the enzyme (table II) tends to support the idea that extensive cross linking of enzyme molecules by native lectin is responsible for the inactivation. This is the only evidence that the lectin does not act simply by a stoichiometric binding to enzyme which could result in blockage of the substrate site or induction of an alteration in the conformation of the enzyme molecule itself. Although these changes may occur, the greater valency of the native lectin does facilitate the inhibition. Hence, its capacity to link neighbouring enzyme molecules probably is an important component of the inhibitory mechanism.

Table II also shows that WGA and RCA-I, two other lectins which are inhibitory, inactivate the purified enzyme even more readily than the membrane-associated enzyme. Among other lectins tested, soybean agglutinin, the leucoagglutinin from *Phaseolus vulgaris*, and the fucose-binding protein from *Lotus tetragonolobus* were without influence on either form of the enzyme.

DISCUSSION

As one means of assessing the mode of inhibition of plasma membrane 5'-nucleotidase by some plant lectins, the enzyme was purified and the relative sensitivities of the two forms of the enzyme tested. Solubilization of all of the enzyme with a single non-ionic detergent followed by conventional methods of protein purification yielded an enzyme showing a single protein staining band with a molecular weight of about 70,000 on polyacrylamide gel electrophoresis in sodium dodecyl sulfate (30). This and the purification factor of 5000 compares well with the results of the two other methods of purification reported (31,32).

The purified enzyme retained susceptibility to inactivation by Con A, wheat germ agglutinin and the first component of the *Ricinus communis* lectin while divalent succinyl Con A inhibited neither it nor membrane-bound enzyme. Therefore, it seems likely that inhibition is a result of direct binding and cross-linking by native lectins of the glycoprotein enzyme. Inhibition of this enzyme by antibodies to liver plasma membranes may occur by an analogous mechanism (33).

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