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Association of Placental Alkaline Phosphatase Activity with Preparations of the Human Placental Inhibitor to Hemagglutination by H-1 Virus* (33735)

M. USATEGUI-GOMEZ, NIMAI K. GHOSH,[1] AND H. W. TOOLAN

Putnam Memorial Hospital Institute for Medical Research, Bennington, Vermont, 05201; and Institut du Cancer de Montreal, Laboratories de Recherche, Hopital Notre Dame, Montreal, Canada

Recent studies (1, 2) described the purification of a glycoprotein observed by Toolan (3) in all human placental fluids, that inhibits hemagglutination by H-1 and HB viruses, but not hemagglutination by the closely related RV or H-3 viruses. The inhibitor contains 73–78% protein, 9–11% hexose, 7–8% hexosamine, and 4–6% sialic acid (1). Purified preparations, when subjected to acrylamide or paper electrophoresis, show one band near the origin in the gel and one band which coincides with the slow alpha or fast beta globulin region on paper (1, 2). After purification by the use of sulfosalicylic acid, the inhibitor exhibits heterogeneity when examined by the analytical centrifuge. Such heterogeneity could be explained on the basis of polymerization of a single species (2). Preparations obtained with more gentle procedures and centrifuged in a sucrose gradient with $alpha_2M$ macroglobulin, a 19S serum protein used as a marker, show inhibitor activity in the same area as the $alpha_2M$ (2). The glycoprotein is very stable to heat and a wide pH range.

In a recent investigation, Ghosh and Fishman (4, 5) purified and studied the properties of molecular weight variants of human placental alkaline phosphatase. The molecular weights for the two major variants, designated A and B, were 69,000 and over 200,000 respectively, on the basis of sucrose density gradient ultracentrifugation. Placental alkaline phosphatase was reported to be a sialoprotein (6, 7) containing a large carbohydrate moiety (5). It was observed (8) that highly purified preparations of placental alkaline phosphatase contain glucosamine, galactosamine, mannose, galactose, fucose, and glucose in addition to terminal neuraminic acid residues. These placental phosphatases appear in starch gel patterns near the origin (5) and also in the $alpha_2$ and beta globulin zones. The thermal stability of the placental enzyme (9) is a property that has been exploited to inactivate contaminating phosphatase and other enzymes (5). Heat stability of the enzyme is also used to detect, measure, and characterize placental isoenzyme of alkaline phosphatase in pregnancy sera (9–11).

The similarities in physical and chemical properties between the human placental alkaline phosphatases and the hemagglutination inhibitor raises the question of whether they might be the same glycoprotein. The present report describes the association of placental alkaline phosphatase activity with purified preparations of human placental in-

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[1] Present address: Department of Medicine Research Laboratories, Roger Williams General Hospital, Brown University, 825 Chalkstone Avenue, Providence, Rhode Island.

TABLE I. HA-I and Alkaline Phosphatase Activity of Various Fractions Obtained during Purification of Placental Inhibitor.

Sample	HA-I titer[a]	Total units ($\times 10^4$) of HA-I activity[b]	Enzyme activity[c]	Total enzyme activity	Total protein (mg)
Original placental fluid	1280	11.1	1.61	160.4	6250
Kaolin-absorbed fluid	160	11.0	0.34	228.6	3271
Centrifugation pellet	5120	13.2	3.04	128.6	1430
Ammonium sulfate supernatant fluid	5120	13.2	2.89	57.8	214
Active Sephadex column eluate	10240	15.6	0.04	3.42	12
Active sucrose gradient fractions	10240	14.3	0.03	1.04	8

[a] HA-I titers are read as the reciprocal of the highest dilution of glycoprotein that completely prevented agglutination of H-1 virus with guinea pig red blood cells.

[b] Titer times number of ml of material.

[c] The alkaline phosphatase activity is expressed in micromoles of phenol/ml/min released at 37° from 70 m*M* phenylphosphate in 0.05 *M* carbonate–bicarbonate buffer (pH 10.7) containing 10 m*M* $MgCl_2$.

hibitor for the hemagglutination of red cells by H-1 virus.

Materials and Methods. Placental fluid was obtained from unwashed placentas as described previously (2). Only fluids showing the 1-1 hemehaptoglobin patterns were used since the larger 2-2 and 2-1 haptoglobins contaminate the final fractions in the purification procedure. A volume of 100 ml of fluid was employed for each run. The hemagglutination-inhibition (HA-I) tests and the chemical techniques employed were described in detail elsewhere (1, 2).

The procedure followed for purifying the placental inhibitor was the same as reported by Usategui-Gomez and Morgan (2) except that the active Sephadex G-200 column eluate layered on a sucrose gradient was spun for 19 hr at 35,000 rpm instead of 22 hr.

The method for determining alkaline phosphatase activity which involves diazocoupling of phenol released from disodium phenylphosphate was described in earlier reports (5, 12). The measurements of *l*-phenylalanine inhibition of the enzyme, the heat stability, and the ratio of alkaline phosphatase activity at pH 10.7 versus 9.8 were done as reported previously (12–16).

Results. In all steps in the purification procedure, the fractions containing HA-I activity also showed alkaline phosphatase activity (Table I). That the alkaline phosphatase present in the preparations of placental inhibitor exhibited characteristic parameters of purified placental alkaline phosphatase is shown by the results presented in Table II. The extent of L-phenylalanine inhibition, the ratio of alkaline phosphatase activity at pH 10.7 versus pH 9.8, and the stability of enzyme at 55° for 16 min were similar to those found for human placental alkaline phosphatase. The D-phenylalanine had no inhibitory effect on the alkaline phosphatase activity of the inhibitor preparations. This characteristic feature of the enzyme present in the virus inhibitor is in harmony with the properties of placental and intestinal alkaline phosphatases which undergo uncompetitive homosteric inhibition by L-phenylalanine but not by its D-enantiomorph (5, 12–16).

Viral hemagglutination inhibition appeared as expected in the first fractions eluted from the Sephadex G-200 column, *i.e.*, the macroglobulin area (Fig. 1). Three peaks, corresponding to alkaline phosphatase activity were observed: the first in fractions 4–11, the second in fractions 19–28 and the third in fractions 29–44. The presence of several peaks of enzyme activity could be expected due to the molecular weight variants of the enzyme (4, 5, 12). The first enzyme peak characterized as variant B (5, 12) came off in the void volume of the column and coincided with the HA-I activity. The third peak

TABLE II. Comparison of the Properties of Alkaline Phosphatase in Placental Fluid Inhibitor with those of Purified Placental Alkaline Phosphatase.

Sample	L-Phenylalanine (5 m*M*) inhibition[a]	10.7/9.8*R*[b]	Heat stability (%) at 55°, 16 min
Original placental fluid[c]	75%	1.10	100
Second centrifugation pellet[c]	73%	1.00	100
Ammonium sulfate supernatant[c]	74%	1.05	100
Alkaline phosphatase purified from human placental tissue[d]	75%	1.10	100

[a] The L-phenylalanine inhibition was measured as described by Ghosh and Fishman (5, 11) using 5 m*M* D-phenylalanine in the control digests with phenylphosphate as substrate in 0.05 *M* carbonate–bicarbonate buffer, pH 9.6.

[b] The ratio of alkaline phosphatase activity at pH 10.7 versus 9.8 using 2 m*M* phenylphosphate as substrate; the ratio was measured as described previously (11, 16).

[c] Inhibitors to hemagglutination by H-1 virus were prepared as described previously by Usategui-Gomez (2).

[d] Alkaline phosphatase was purified by the procedure of Ghosh and Fishman (4, 5).

of enzyme activity which corresponded to the lower molecular weight variant A of Ghosh and Fishman (5, 12) did not show viral inhibition under the experimental conditions emloyed.

When the viral inhibitor Sephadex G-200 fractions were centrifuged on a sucrose gradient for 19 hr, the viral placental inhibitor appeared in fractions 1–20 (Fig. 2). The enzyme activity was also obtained in the same fractions. It should be noted that there seems to be more than just one molecular form of the enzyme as indicated by the different peaks of activity. Results shown in Fig. 2, indicate that the same finding is true for the viral HA-I activity. The serum protein, alpha$_2$M, used as a marker was detected in fractions 6-17. These were the same fractions where the peak alkaline phosphatase and viral HA-I were found.

Discussion. The similarities in physical and chemical properties (1, 2, 4–7, 12) between the human placental inhibitor to hemagglutination by H-1 virus and the large molecular-weight variants of placental alkaline phosphatases, raises the question of whether the enzyme and the inhibitor are the same glycoprotein with the enzyme active site in the polypeptide part of the molecule and the hemagglutination inhibitory site active in the carbohydrate region or, whether, alternatively, the two glycoproteins are different. Neuraminidase and sodium periodate treatments which are carbohydrate specific reagents, destroyed the HA-I titer indicating that sugar groups are involved in the HA-I reaction. It is apparently also necessary that the protein part of the molecule be intact in order that HA-I occur, since treatment with proteolytic enzymes such as trypsin, chymotrypsin, papain, and pepsin either destroyed or drastically reduced the HA-I activity (2). Neuraminidase treatment of placental alkaline phosphatase releases sialic acid and lowers the electrophoretic mobility (6, 7, 12) but does not alter the enzyme activity (6) since the enzyme active site is in the polypeptide region.

Size of a glycoprotein seems to be a determinant factor in reactions with viruses. Fazekas de St. Groth and Gottschalk (17), demonstrated that the number of negatively charged terminal sialic acid residues attached simultaneously to complementary groupings at the virus surface, is a primary factor in determining the inhibitory potency of glycoproteins. In short, the higher the number of sialic acid residues available per glycoprotein molecule, the stronger the inhibitory action of the molecule will be. This concept would explain the finding that ovine submaxillary glycoprotein (OSM) with a molecular weight of 1×10^6 has an inhibitory titer against inactive influenza viruses of 2.25×10^6/mg

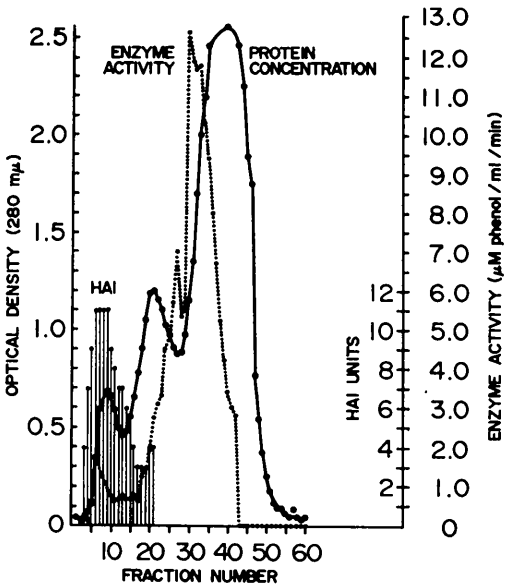

FIG. 1. Elution diagram of sephadex G-200 fractions: protein concentration (●); enzyme activity (•); and HA-I activity (vertical lines) were measured in each fraction. Protein was determined by measuring the absorance at 280 mμ; HA-I titer is expressed as that dilution of glycoprotein which completely prevented agglutination of H-1 virus with guinea pig red blood cells; titers are expressed as 1 = 10; 2 = 20, etc. The alkaline phosphatase activity is expressed in arbitrary units directly proportional to the optical density at 490 mμ of the pink color of the diazotized solutions of phenol (12) liberated in 15 min at 37° from 70 m*M* disodium phenylphosphate in 0.05 *M* carbonate–bicarbonate buffer (pH 10.7) containing 10 m*M* $MgCl_2$.

substance, whereas OSM treated with a very small dose of trypsin under controlled conditions to produce glycopeptides with a molecular weight of approximately 50,000, has a titer of only 100 (18). Intact OSM (mol. wt. $= 1 \times 10^6$) and the urinary glycoprotein (mol. wt. $= 4 \times 10^6$) of Tamm and Horsfall (19) are very potent inhibitors of influenza virus hemagglutination. In both glycoproteins several hundred heterosaccharides are attached to the polypeptide chain and each heterosaccharide is terminated by a sialic acid residue. The serum components fetuin (mol. wt. 48,400) and orosomucoid (mol. wt. 44,100), on the other hand, are poor inhibitors. They contain only a few heterosaccharides per molecule (17). Orosomucoid can become a potent inhibitor of hemagglutination by a number of influenza virus strains when it is polymerized (20, 21). Springer (22) testing *in vitro* blood group activity of human MM and MN antigens as measured with human sera and the capacity of these antigens to inhibit hemagglutination by some myxoviruses, found a marked relationship between the molecular size of the isolated glycoproteins and their activity. He observed that the MN glycoproteins possess molecular weights which are multiples of 30,000 and that these large molecules tend to disaggregate upon manipulation. Springer also found that blood-group as well as antiviral activities were highest for the largest molecules. The difference in inhibitory activities expressed on a molar basis between molecules of mol. wt. 12×10^6 and 3.1×10^4 was 10^4- to 10^6-fold. It is not surprising, therefore, that only placental fluid preparations containing the large molecular-weight variants of placental alkaline phosphatase which is a sialoprotein (6) are capable of drastically inhibiting the hemagglutination of guinea pig red blood cells by the H-1 virus. One may postulate that in order to inhibit hemagglutination of the virus a critical number of negatively charged sialic acid residues of the glycopro-

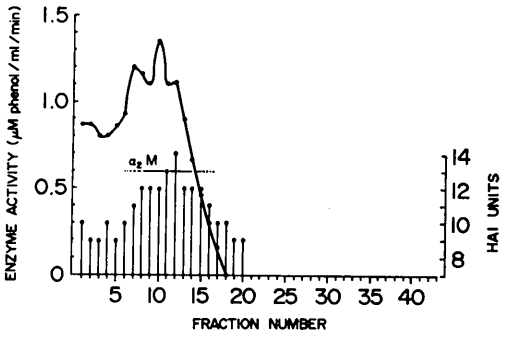

FIG. 2. Comparison of the alkaline phosphatase (●) and HA-I(verticle lines) activities of sucrose density gradient fractions. See Fig. 1 for expression of HA-I titer units and for the conditions of alkaline phosphatase activity assay. Incubation time for assays in Fig. 2 was 150 min and the substrate concentration 72 m*M*. The solid horizontal line indicates the $alpha_2M$ distribution as determined by diffusion in agar gel; trace amounts are shown by broken lines.

tein molecule must be attached simultaneously to the virus surface. It is probable that the smaller molecular weight phosphatase variants can attach themselves reversibly to the virus particles in the absence of competing cellular receptors. Fazekas de St. Groth and Gottschalk (17) have shown that several sialic acid containing glycoproteins independent of the degree of their capacity to inhibit influenza virus hemagglutination, can attach themselves reversibly to enzymically inactive virus particles (9).

It should be noted that there was a loss in specific enzyme activity in the sucrose gradient fractions. This may have been due to the fact that during Sephadex G-200 gel filtration the enzyme preparations are diluted and hence the active Sephadex column eluates are pooled and concentrated 30-fold before the sample is put on the sucrose gradient. It is conceivable that in the concentrated sample the protein is exposed to tremendously high salt and sucrose concentrations which may be deleterious to the active center of the enzyme but may not have any detrimental effect on the site responsible for hemagglutination inhibition. In general, the observed specific activity of alkaline phosphatase in the purified preparation of the inhibitor was not comparable to that of a highly purified preparation of placental alkaline phosphatase. This may be due to the fact that the isolation methods employed in this study were geared to purify the placental inhibitor without taking necessary precautions to protect the catalytic site of the enzyme in the polypeptide chain. It has been found that the inhibitor preparations made by the treatment with sulfosalicylic acid (1) which would destroy the active site of the enzyme, do not show appreciable enzyme activity (23). However, viral hemagglutination inhibitory activity was demonstrated in purified placental alkaline phosphatase (5) which exhibits over 1000-fold higher specific enzyme activity than that of the original placental homogenate (24).

The results of this investigation indicate: (i) that all fractions containing HA-I activity in the different steps of the purification procedure, also contain alkaline phosphatase activity; (ii) that in the macroglobulin fractions eluting first from the Sephadez G-200 column (which have been shown to contain only $alpha_2M$ glycoprotein of serum as a major impurity) the alkaline phosphatase activity peak coincides with the viral inhibitor activity peak; (iii) that in the sucrose gradient fractions, the enzyme and viral inhibitor activities also coincide; and finally (iv) that the L-phenylalanine inhibition, thermal stability and the ratio of enzyme activity at pH 10.7 versus 9.8, indicate that the alkaline phosphatase activity present has all the properties of a placental alkaline phosphatase. Since the viral inhibitor has only been found in placental fluid and is considered to be a product of the placenta, it appears likely that the H-1 viral inhibitor and the large molecular-weight variants of placental alkaline phosphatase are the same glycoprotein.

It may be noted that L-phenylalanine sensitive "placental type" isoenzyme of alkaline phosphatase has recently been found in pregnancy sera (9–12), blood specimens of a few cancer patients, occasional malignant tumor tissues and neoplastic pleural effusions (25–28). The H-1 virus was isolated from a transplantable human tumor and has been found in human tumors and embryos (3, 29). It was studied extensively during the last decade (30). The possible identity of the placental alkaline phosphatase isoenzyme and the placental inhibitor to hemagglutination by H-1 virus may, therefore, have some relevance in oncology (31, 32).

Summary. Purified preparations of the glycoprotein found in human placental fluid that inhibits the hemagglutination of H-1 and HB viruses, have also been shown to contain alkaline phosphatase activity. This phosphatase activity has been identified as a placental phosphatase by comparing its characteristic behavior with that of purified human placental alkaline phosphatase. It is probable that the viral inhibitor and the large molecular-weight variant of placental alkaline phosphatase are the same glycoprotein.

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