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INHIBITION OF ALKALINE PHOSPHATASE BY SIALIC ACID

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Summary

The interaction of human organ alkaline phosphatases (orthophosphoric-monoester phosphohydrolases (alkaline optimum), EC 3.1.3.1) with sugars was studied. Hexosamines, *N*-acetylneuraminic acid (NANA or sialic acid), *N*-acetylmuramic acid and *N*-acetylglycolylneuraminic acid inhibited human organ alkaline phosphatase activities. Of these, sialic acid was the most effective inhibitor.

The pH profiles for the enzymes in the absence and presence of sialic acid were similar. The sialic acid · enzyme complex was more heat stable than the free enzyme between 20 and 45°C.

Lineweaver-Burk plots of $1/v$ versus $1/S$ at various concentrations of sialic acid showed intersecting straight lines indicating that the mechanism of inhibition was a mixed type. The K_i value obtained from the plots of $1/v$ versus the square of sialic acid concentration was 0.07 mM for the hepatic, sialidase-treated hepatic, and intestinal alkaline phosphatases. The respective Hill coefficients varied somewhat with the alkaline phosphatase isoenzyme. Hyperbolic curves were obtained when the percentage of remaining activity was plotted against the substrate concentration at different concentrations of sialic acid. The Hill coefficient was lowered in the presence of sialic acid. The sialidase-treated hepatic enzymes used gave the most effective conversion.

Partial denaturation of the enzyme with urea, or pronase digestion had a little if any effect on the sialic acid inhibition with constant time.

Introduction

Many workers have reported that hepatic, placental, bone and kidney alkaline phosphatases (orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) are glycoproteins containing sialic acid [1—4]. We have also reported that human hepatic and human placental alkaline phosphatases are sialoglycoproteins, while calf and human intestinal alkaline phosphatases have little if any sialic acid [5,6]. These glycoprotein enzymes contain glucosamine,

galactosamine, galactose, fucose, mannose and glucose, the saccharide contents being different for each enzyme [7]. We have suggested that the interaction of human organ alkaline phosphatases with concanavalin A, a specific carbohydrate-binding protein, differs according to the source organ. Previous reports have discussed some roles of the carbohydrate moiety in the alkaline phosphatase molecule: protection from the effects of heat denaturation and proteinase susceptibility, and the determination of enzyme-substrate affinity [7,8].

Many attempts have been made to differentiate the nature of the active site and the essential groups of alkaline phosphatase isoenzymes by means of organ-specific inhibitors [9—11], as well as by non-organ-specific inhibitors [12—14]. We have also reported the organ-specific inhibition of intestinal alkaline phosphatase using spermine as the polycation [15].

In this study, we investigated the interactions of the human organ alkaline phosphatases with several saccharides using sialic acid, the most effective inhibitor of the enzyme.

Some preliminary results of this work have already been reported [16].

Materials and Methods

Materials

Lithium 3,5-diiodosalicylate, L-fucose, and D-mannosamine were purchased from Wako Pure Chemicals Co. Ltd. (Osaka); Triton X-100, D-galactosamine, and D-glucosamine from Nakarai Chemicals Co. Ltd. (Kyoto); sialidase (neuraminate glycohydrolase from *Vibrio Cholerae*) from BDH Laboratory Chemicals Division; concanavalin A (three times crystallized) from Miles Laboratories Inc.; α-methyl-D-mannoside, *N*-acetyl-D-glucosamine, *N*-acetylmuramic acid, *N*-acetylneuraminic acid (sialic acid: synthetic type IV) and *N*-acetylglycolylneuraminic acid were from Sigma Chemicals Co.; *N*-acetyl-D-manosamine from Nutritional Biochemicals Co.; *N*-acetyl-D-galactosamine from Seikagaku Indust. Co. (Tokyo), and pronase-P from Kaken Chemicals Co. Ltd. (Tokyo). All other chemicals were analytical grade from Wako Pure Chemicals Co. Ltd.

Tissue extracts

Alkaline phosphatases of human liver was purified according to the method of Sussman et al. [17], with slight modifications which included a further purification with concanavalin A/Sepharose 4B affinity chromatography [6].

Purification of human intestinal alkaline phosphatase was modified according to the methods of Moss et al. [18] and of Brenna et al. [19] which including the solubilization of the tissue homogenates with 0.2% Triton X-100 and 0.2 M lithium 3,5-diiodosalicylate in 0.25 M sucrose followed by acetone fractionation (33—50% saturation), ammonium sulfate fractionation (45—70% saturation), and affinity chromatographic purification with concanavalin A/Sepharose and tyraminyl derivative/Sepharose [7]. The purified enzyme preparations had specific activities of 38.4 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (human liver) and 513.2 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein (human intestine).

Enzyme assays

The reaction mixture contained 2 mM of disodium *p*-nitrophenyl phosphate

in 50 mM carbonate/bicarbonate, pH 10.0 at 37° C. The mixture was incubated in the cuvette of a spectrophotometer (UV-200, from Shimazu Instrument Co. Ltd., Kyoto) equipped with a thermospacer connected to a constant temperature water bath. Enzyme activity was determined by reading the increase in absorption at 405 nm. The μmol per ml of *p*-nitrophenolate liberated during a particular interval were plotted against corresponding time.

A mixture of the enzyme and saccharide was incubated for about 20 min at 25° C and an aliquot was assayed for phosphatase activity.

The effects of pH on the phosphatase activities were measured using 0.1 M Tris · HCl buffer in the 8.5—9.0 pH range and 0.1 M carbonate/bicarbonate buffer in the 8.8—10.9 pH range.

All measurements were made at least in triplicate.

Protein determination

Protein concentration was estimated according to the method of Hartree [20] using bovine serum albumin as a standard.

Hydrolysis of alkaline phosphatase by sialidase and pronase-P

For the hydrolysis of alkaline phosphatase by sialidase, a solution containing 0.5 unit sialidase per ml was added to an equal volume of the enzyme in 5 mM $CaCl_2$/24 mM acetate buffer, pH 5.5. A mixture of the enzyme and sialidase was incubated for 24 h at 37° C.

For the hydrolysis of alkaline phosphatase by pronase-P, a solution containing 100 mg pronase per ml was added to 1/50 (w/w) volume of the enzyme in the presence of 0.01 M Tris · borate buffer, pH 8.6 at 37° C. Aliquots of these treatment mixtures taken at various time intervals were diluted with 3 volumes of cold water to stop the digestion and then assayed for phosphatase activity.

Results

Effects of some saccharides on hepatic and intestinal alkaline phosphatase activities

As shown in Table I, the alkaline phosphatase activities were strongly inhibited by sialic acid, *N*-acetylmuramic acid, and glucosamine, while *N*-acetylglycolylneuraminic acid, mannosamine and galactosamine were less affected. Of these saccharides, sialic acid was the most effective inhibitor of the alkaline phosphatase activities.

Effect of sialic acid concentrations on alkaline phosphatase activity

The inhibition of the alkaline phosphatase activity by sialic acid was dependent on inhibitor concentration (0, 5, 10, 15 mM). At 10 mM sialic acid, which is the concentration used for the present study (the final inhibitor concentration), 58% of the human liver and 27% of the human intestinal alkaline phosphatase activities were inhibited, as shown in Fig. 1. As can be seen from the results, the hepatic enzyme activity was more effectively inhibited by sialic acid than was the intestinal enzyme. To examine whether such inhibition could be due to metal ions in the sialic acid molecules, 0.01 mM ethylenediamine tetraacetic acid was added to the sialic acid, this amount being too low to

TABLE I

INHIBITION OF HUMAN LIVER AND HUMAN INTESTINAL ALKALINE PHOSPHATASE ACTIVITIES BY VARIOUS SACCHARIDES

Enzyme concentrations were used with 1.2 μg human liver and 0.21 μg human intestine. The concentrations of saccharides were 10 mM for this study. Enzyme assays are the same as Fig. 1.

Saccharides	Human liver	Human intestine
No addition	100	100
Mannose	100	97
Glucose	100	99
Galactose	104	97
N-Acetylglycolylneuraminate	85	89
N-Acetylneuraminate	52	72
N-Acetylmuraminate	73	88
Mannosamine	89	81
Galactosamine	90	80
Glucosamine	85	76
N-Acetylmannosamine	98	99
N-Acetylgalactosamine	101	99
N-Acetylglucosamine	99	102

affect the activity of the two alkaline phosphatases. The enzyme activities of the hepatic and intestinal alkaline phosphatase · sialic acid complex with and without the chelating reagent were compared, and found to be almost identical.

Rate of substrate hydrolysis

Time/activity curves with and without sialic acid were linear for the first 30—40 min with *p*-nitrophenyl phosphate, the reaction apparently observing zero-order kinetics. The initial velocity rates for the substrate were measured at the 15-min point for the data present in Figs. 2—6.

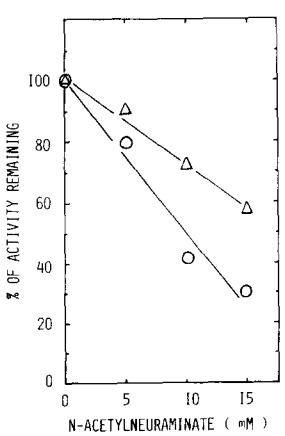

Fig. 1. Inhibitory effects of sialic acid on alkaline phosphatase isoenzyme activities. ○, liver enzyme; △, intestinal enzyme. Enzyme assays were carried out at 37°C with 1 mM *p*-nitrophenyl phosphate as substrate in 50 mM carbonate/bicarbonate buffer, pH 10.0. Enzyme preparations contained 71.6 ng(liver) and 6.3 ng(intestine) proteins, respectively. The percentages of inhibition by sialic acid were calculated against the control tube without sialic acid.

Effect of optimum pH on the inhibited-enzyme activity

The enzyme activities following interaction of human liver and human intestinal isoenzymes respectively with 10 mM sialic acid were pH dependent. As in the present study, pH 10.0—10.5 was the optimum for the hepatic enzyme with or without sialic acid, and the optimum pH of the intestinal alkaline phosphatase activity was pH 9.5—9.8 with or without sialic acid.

The influence of pH on inhibition by sialic acid indicated that the inhibition of the alkaline phosphatase activities was pH independent.

Studies on heat stability of the enzyme · sialic acid complex

The effect of temperature variation on the two alkaline phosphatase activities was studied, the temperature ranging from 20 to 55° C. The enzyme activity was more stable in the alkaline phosphatases complexed with sialic acid than in the non-complexed enzyme. In particular, the activities at 45° C were significantly protected from heat denaturation. The enzyme complexed with sialic acid underwent a shift in optimum temperature from near 37° C to 45° C. At 50° C significant heat-stable conversion did not take place.

Lineweaver-Burk (1/v versus 1/S) and 1/v versus square of sialic acid concentrations plots for the alkaline phosphatase isoenzyme

The mechanism of inhibition by sialic acid was investigated by studying the effect of substrate concentration on the enzyme activity at different concentrations of the inhibitor. The sialic acid concentrations used were 5 and 10 mM, and enzyme activity was assayed at 37° C with substrate concentrations ranging from 0.05 to 4 mM. When the results were plotted as $1/v$ versus $1/S$ for the various concentrations of the inhibitor, the series of intersecting straight lines obtained for the alkaline phosphatases activities indicated 'mixed type' inhibition, as shown in Fig. 2.

The inhibition constant (K_i) was obtained by plotting $1/V$ versus various sialic acid concentrations (0, 2, 5, 10 and 15 mM), where the value of $1/V$ was obtained from Fig. 2 by extrapolating to the vertical axis. The $1/V$ versus the square of various concentrations of sialic acid for the alkaline phosphatase activities in a reaction mixture containing 1 mM or 3 mM *p*-nitrophenyl phosphate as substrate are replotted. A series of straight lines was obtained for the alkaline phosphatase activities and the K_i value was identical for both human liver and human intestinal alkaline phosphatases. It is interesting that the K_i value of sialidase-treated human hepatic enzyme for sialic acid is calculated to be the same (K_i = 0.07 mM) as that of the non-treated hepatic enzyme.

Mechanism of the alkaline phosphatase interaction with sialic acid

The inhibition of the enzyme activities by sialic acid was studied by examining the effect of sialic acid concentration on the inhibition. Plots of the resulting inhibition against sialic acid concentration gave apparently sigmoidal curves, as shown in Fig. 3.

The inset in Fig. 3 shows the apparent straight-line relationship between the $\log[(V-v)/v]$ and the log(sialic acid concentration), where v is the enzyme activity in the presence of various concentrations of sialic acid with 1 mM disodium *p*-nitrophenyl phosphate, and V the activity without the inhibitor under

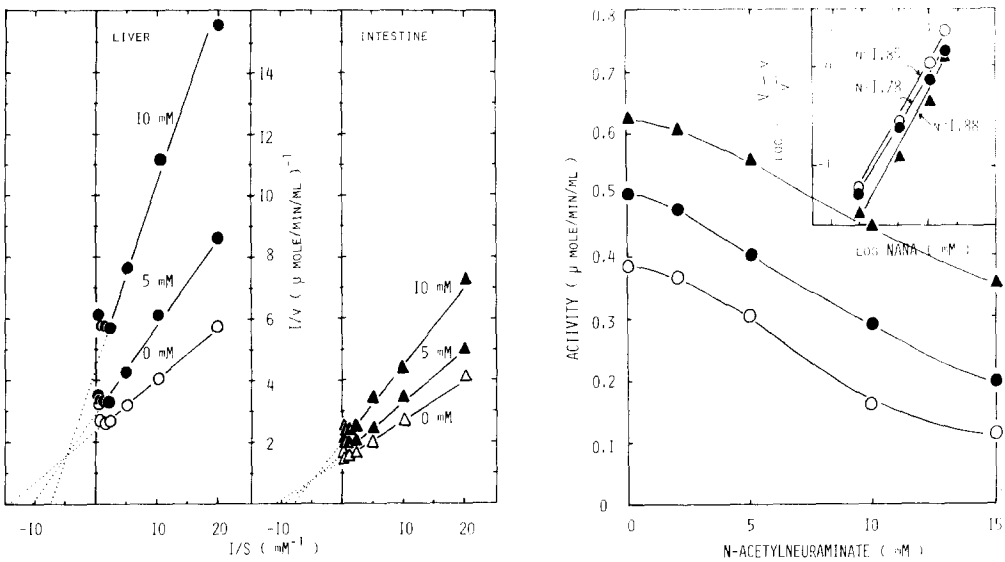

Fig. 2. Double-reciprocal plots of velocity against substrate concentration at different concentrations of sialic acid for both alkaline phosphatases. Enzyme assays are the same as in Fig. 1.

Fig. 3. Effect of sialic acid concentrations on the inhibition of alkaline phosphatase activity by sialic acid, and linear plots of $\log[(V-v)/v]$ versus log(sialic acid concentrations). ○, with non-treated hepatic enzyme; ●, with sialidase-treated hepatic enzyme; ▲, with non-treated or sialidase-treated intestinal enzyme. Enzyme assays are the same as in Fig. 1. V is the control activity of the enzyme without sialic aicid and v is the activity with sialic acid. NANA, *N*-acetylneuraminic acid.

the same conditions. From the slope, the values of n obtained were 1.85 with human liver, 1.78 with sialidase-treated human liver and 1.88 with non-treated human intestinal alkaline phosphatase.

Effect of urea, and pronase digestion on the inhibition

To examine the effects of urea on the enzyme activities and their inhibition by sialic acid, the enzyme preparations were incubated in the presence of 3.5 M urea at 37°C. At various time intervals during the incubation, portions of the enzyme/urea mixture were withdrawn, diluted with cold water, and immersed in an ice bath to stop the denaturation. Activities of the urea-treated enzyme samples, with and without 10 mM sialic acid, showed that both liver and intestinal alkaline phosphatases were progressively inactivated when incubated with urea, while the percentage of inhibition by sialic acid remained fairly constant, as shown in Fig. 4.

The effects of digestion with pronase-P on alkaline phosphatase activities and inhibition by sialic acid are shown in Fig. 5. An aliquot of the enzyme/pronase-P mixture was withdrawn at various times, the digestion stopped (the conditions were as described in Materials and Methods), and the samples assayed for phosphatase activity with and without 10 mM sialic acid. The percentage of activity remaining after digestion was calculated from the control tube in which the enzyme was incubated with buffer only. It was found that 40% of the liver enzyme and 30% of the intestinal enzyme were inactivated after 40 min of

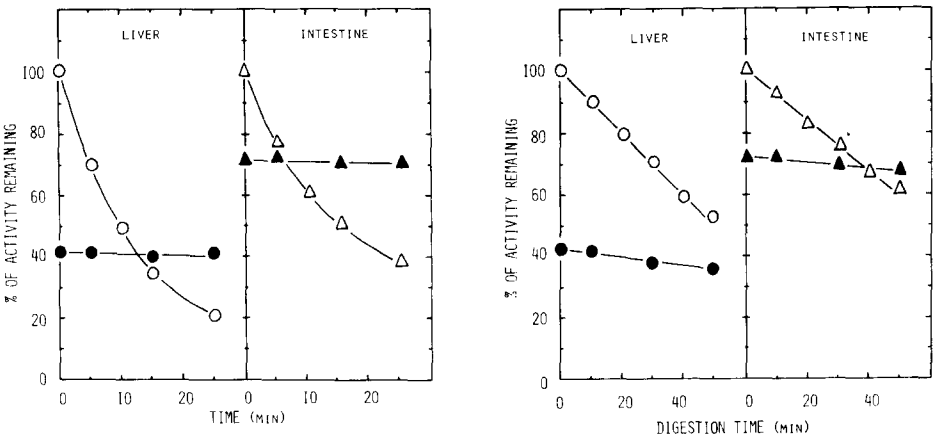

Fig. 4. Effects of urea on enzyme activity and inhibition by sialic acid of hepatic and intestinal alkaline phosphatases. ○, ●, liver enzyme; △, ▲, intestinal enzyme; ○, △, without sialic acid; ●, ▲, with 10 mM sialic acid. Urea, 3.5 M. Enzyme preparations used were the same as in Fig. 1.

Fig. 5. Effects of pronase-P on enzyme activity and inhibition by sialic acid. Symbols used were the same as in Fig. 4, and enzyme preparations used were the same as described in legend for Fig. 1.

incubation with pronase-P. The effects of pronase digestion on enzyme activity and inhibition by sialic acid were similar to those obtained in the urea denaturation study, in that both isoenzymes were progressively inactivated on incubation while the percentage of inhibition by sialic acid remained at 57—59% of the hepatic enzyme and 26—28% of the intestinal enzyme activity throughout the 50 min of incubation.

The affinity of the substrate and the alkaline phosphatases for sialic acid

In the Hill plot derived from Fig. 2, the Hill coefficient for the hepatic alkaline phosphatase activity was 0.98 without sialic acid, 0.93 with 5 mM sialic acid and 0.89 with 10 mM sialic acid; that for the intestinal alkaline phosphatase activity was 1.03 without sialic acid, 0.96 with 5 mM sialic acid and 0.93 with 10 mM sialic acid. The results obtained by varying the sialic acid concentration revealed that one molecule of substrate couples with one active site of alkaline phosphatase during the inhibition reaction.

The sialic acid-induced change in substrate affinity for sialidase-treated hepatic alkaline phosphatase

To investigate the role of sialic acid in the hepatic enzyme molecule, the interaction of sialic acid with the sialidase-treated hepatic enzyme was studied by the effect of substrate concentration on the sialidase-treated hepatic enzyme activity at different concentrations of the inhibitor. The concentrations of sialic acid used were 0, 5 and 10 mM, and enzyme activity was assayed at 37°C with substrate concentrations ranging from 0.02 to 4 mM. The curves of v against S exhibit an apparent hyperbolic nature.

The Hill plots of $\log[v/(V-v)]$ versus log(substrate concentration) for the sialidase-treated enzyme activity are shown in Fig. 6. The Hill coefficients for the alkaline phosphatase activity were: 1.01 without sialic acid; 0.83 with 5

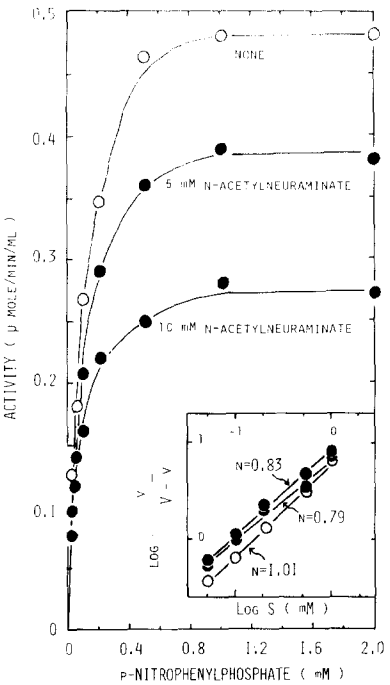

Fig. 6. Effects of sialic acid concentration at different concentrations of substrate on the inhibition of sialidase-treated hepatic alkaline phosphatase activity and linear plots of $\log[v/(V-v)]$ versus log(substrate concentration). The value of V was obtained from the velocity of sialidase-treated enzyme at 2 mM *p*-nitrophenyl phosphate without sialic acid.

mM sialic acid and 0.79 with 10 mM sialic acid. The results obtained after sialidase treatment of the enzyme with no addition of sialic acid revealed that one molecule of the substrate couples with one substrate-binding site of the enzyme molecule. Sialic acid addition following the sialidase treatment of the enzyme significantly reduced the affinity of the substrate for the active sites of the sialidase-treated enzyme.

Discussion

In the course of studies on the structure-function relationship of alkaline phosphatase, it became especially necessary to understand physiological roles of protein and carbohydrate moieties in the glycoprotein enzyme.

We have reported that some functions of the carbohydrate moiety in the alkaline phosphatase molecule are (1) providing protection from heat denaturation and protease action [7,8], (2) determining the affinity of the enzyme for the substrate [6], and (3) serving as a specific carbohydrate-binding protein in the interaction of the enzyme with concanavalin A [7].

The present paper investigated the interactions of several sugars with alkaline phosphatase isoenzymes.

The alkaline phosphatases from human organs were strongly inhibited by sialic acid, *N*-acetylmuramic acid, *N*-acetylglycolylneuraminic acid and a few hexosamines. Among these, sialic acid was the strongest inhibitor. The hexo-

samines with only one amino group inhibited the intestinal enzyme activity more strongly than the hepatic enzyme activity. However, *N*-acetylhexosamines in which the above-mentioned amino group is acetylated hardly affected either of the alkaline phosphatase activities. It has been reported that the inhibitor amino group and binding affinity may determine organ specificity [14,15]. Sialic acid, *N*-acetylmuramic acid and *N*-acetylglycolylneuraminic acid which have an acetylated amino group and a carboxyl group were more effective inhibitors than the hexosamines with only one amino group. Moreover, the inhibition of the enzyme activities by 10 mM pyruvic acid (sialic acid consists of *N*-acetylmannosamine and pyruvic acid) was slight, only 8.9% of the hepatic enzyme and 5.5% of the intestinal enzyme activity. As can be seen, the inhibition of alkaline phosphatase activity by these sugars are indicative of specific inhibitory mechanisms.

From these results, it was presumed that the sensitive site and essential groups of the inhibitor for the alkaline phosphatase activity must demand a specific structure in the inhibitor molecules. We suggest that an inhibitor such as an amino acid, having both an amino group and a carboxyl group, is more strongly inhibitory than one having only a single amino or carboxyl group [15]. This is of considerable interest because of its similarity to the inhibition of enzyme activity by the addition of sialic acid to alkaline phosphatase preparations such as sialoglycoprotein, and the inhibition of enzyme activity by Zn^{2+}, e.g. as in zinc-containing metalloenzymes [21,22].

The inhibition of the alkaline phosphatase activities by sialic acid was independent of pH variation and the sialic acid · enzyme complex was resistant to heat denaturation. Sialic acid complexing also caused a shift in the optimum temperature from 37 to 45°C. This shift towards a higher optimum temperature for phosphatase activity in the presence of inhibitor indicates that the binding of the inhibitor to the enzyme · substrate complex may also cause the enzyme molecule to go into a more thermostable conformation or that the inhibitor may stabilize the enzyme in its active form [11]. But, at higher temperature, or with the enzymes of longer digestion as shown Fig. 5, the sialic acid · enzyme complex becomes very labile. The reason why the sialic acid · enzyme complex shows such marked lability under the respective conditions has not yet been determined.

Partial inactivation of the enzyme with urea did not prevent it from being inhibited by sialic acid. The results of these experiments show that the sialic acid-binding site on the enzyme molecule is not affected by urea.

According to the Lineweaver-Burk plots, the inhibition of the alkaline phosphatase activity by sialic acid occurs by a mixed type mechanism.

The Hill plot shows that approximately two sialic acid molecules couple with one molecule of the enzyme during the inhibition reaction, the enzyme-substrate affinities being slightly lower in the presence of sialic acid.

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