Inhibition of Human Acrosin by Monosaccharides and Related Compounds: Structure-Activity Relationships

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The inhibition of human acrosin by monosaccharides and related compounds was examined in an attempt to identify structural determinants which render such compounds acrosin inhibitors. The data suggest that the spacial configuration of the last four asymmetric carbons (the anomeric carbon being designated as the first carbon) is important in determining the efficacy of monosaccharides as acrosin inhibitors. Acrosin inhibition was optimal for monosaccharides which contained six carbons, although chain length did not appear to be an important structural determinant. Polyols, such as glycerol, D-sorbitol, and inositol, inhibited acrosin, suggesting that neither the presence of ketone nor aldehyde moieties are absolute requirements. Carbohydrates were inhibitory when in either cyclic or open-chain forms. Preliminary kinetic analysis of acrosin inhibition by fructose revealed no identifiable type of inhibition (i.e., competitive, noncompetitive, or uncompetitive) but showed the inhibition to be of "mixed" type.

The penetration of ova by mammalian spermatozoa is thought to be mediated by hydrolytic enzymes localized within the spermatozoal acrosome, a lysosomal-like structure which surrounds the anterior portion of the sperm head. Among these enzymes is the serine proteinase, acrosin (EC 3.4.21.10). Acrosin is believed to facilitate the migration of the spermatozoon through the innermost layer (the zona pellucida) which surrounds the ovum.[3-5] The availability of specific acrosin inhibitors would permit the investigation of the role of the enzyme in fertilization. Early studies had indicated acrosin to be a trypsin-like enzyme, with substrate specificity and inhibitor sensitivity similar to that of bovine pancreatic trypsin.[6,7] More recent studies have shown several differences between human acrosin and trypsin, including differences in molecular weight[8,9] and inhibition constants for several known trypsin inhibitors.[10] One notable difference between acrosin and trypsin is the carbohydrate content of acrosin. The glycoprotein nature of acrosin represents a unique property of this enzyme as compared to trypsin and has been found in acrosin from several mammalian species, including boar,[11] ram,[12] rabbit,[13] and man.[14] These observations prompted our laboratory to examine the interaction of acrosin with monosaccharides and related compounds, with respect to their effect on enzymatic activity. An earlier communication has described the reversible inhibition of human acrosin by selected monosaccharides.[15] It was the purpose of the present report to more extensively examine the structure-activity relationship of several monosaccharides and related compounds as inhibitors of acrosin and to attempt to establish minimal structural requirements for inhibitory

Table I. Inhibition of Human Acrosin by Ketohexoses[a]

ketohexose	anomer	% inhibn (n)[b]
D-fructose	β	66 ± 3 (6)[c]
D-fructose 6-phosphate	β	32 ± 5 (3)
D-tagatose	mixed	58 ± 6 (3)[c]
L-sorbose	mixed	18 ± 2 (4)[c]

[a] Ketohexoses (200 mM) were evaluated as acrosin inhibitors by preincubating the sugars with acrosin for 5 min prior to initiating the enzymatic reaction by addition of 5×10^{-5} M BAEE or 3×10^{-4} M BAPA (see Experimental Section). [b] Values are expressed as percent inhibition of control rates obtained in the absence of inhibitor and represent the average plus or minus standard error of three to six separate determinations. Values from this and subsequent tables were derived from a total of four separate acrosin preparations. [c] Data taken from Anderson et al.[15]

Table II. Inhibition of Human Acrosin by Aldohexoses[a]

aldohexose	anomer	confign	% inhibn (n)[b]
mannose	mixed	D	60 ± 4 (4)
α-methyl mannoside (100 mM)		D	96 ± 4 (5)
glucose	α	D	28 ± 2 (4)
		L	0 ± 6 (3)
gulose	mixed	D	0 ± 5 (3)[c]
galactose	α	D	34 ± 5 (5)
		L	12 ± 5 (3)
fucose	mixed	D	62 ± 4 (4)
		L	20 ± 4 (3)

[a] Aldohexoses were evaluated as acrosin inhibitors, as described in the legend to Table I. [b] Values represent the average plus or minus standard error of the percent inhibition of control rates obtained in the absence of aldohexose.

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Table III. Inhibition of Human Acrosin by Aldopentoses [a]

pentose	anomer	confign	% inhibn (n) [b]
xylose	α	D	37 ± 3 (4)
		L	40 ± 6 (3)
lyxose	α	D	35 ± 2 (3)
		L	50 ± 3 (3)
ribose	β	D	65 ± 2 (5)
arabinose	β	D	50 ± 3 (5)
		L	65 ± 8 (3)

[a] The indicated aldopentoses were evaluated as acrosin inhibitors, as described in the legend to Table I. [b] Values represent the average plus or minus standard error of the percent inhibition of control rates obtained in the absence of added sugar.

Table IV. Inhibition of Human Acrosin by Aldoses and Ketoses: Effect of Chain Length [a]

monosaccharide	carbon chain length	% inhibn (n) [b]
Aldoses		
D-erythrose	4	40 ± 5 (3)
D-arabinose	5	36 ± 2 (4)
D-mannose	6	48 ± 2 (7)
Ketoses		
dihydroxyacetone	3	15 ± 2 (3)
D-ribulose	5	38 ± 3 (3)
D-fructose	6	47 ± 2 (5)
D-mannoheptulose	7	41 ± 3 (3)

[a] Homologous aldoses or ketoses were evaluated as acrosin inhibitors. All monosaccharides were evaluated at a concentration of 100 mM, rather than 200 mM, due to the high absorbtivity of D-erythrose and D-ribulose. BAPA (3×10^{-4} M) was used as substrate. [b] Values represent the average plus or minus standard error of the percent inhibition of control activity.

activity. Additionally, preliminary kinetic data are presented which describe the nature of inhibition of human acrosin by fructose.

Results and Discussion

Several monosaccharides and polyols were evaluated as acrosin inhibitors in an attempt to construct a structure–activity relationship concerning acrosin inhibition. Inhibition of acrosin by monosaccharides was in excellent agreement with data previously published.[15] There does not appear to be an absolute structural requirement for aldehyde as opposed to ketone moieties, since no consistent difference in inhibition was noted between aldohexoses and ketohexoses (Tables I and II).

When the configurations of either carbon-2 or carbon-4 (the second or the fourth from the last asymmetric carbons) differed from that of fructose, as in D-glucose, D-gulose, or D-galactose, acrosin inhibition was reduced. On the other hand, 6-deoxygalactose (fucose) was nearly as inhibitory as fructose. The removal of certain hydroxyl groups may therefore provide for a better "fit" between the monosaccharide and acrosin. L-Hexoses were less inhibitory than their D derivatives (Tables I and II).

Similar to the hexoses, when the configuration of either carbon-1 or carbon-3 (the second or the fourth from the last asymmetric carbon) of aldopentoses differed from that of fructose (D-xylose and D-lyxose), less than 50% inhibition of acrosin resulted. When the configurations of these carbons were the same as fructose (D-ribose and D-arabinose), inhibition was 50% or greater (Table III). Unlike the hexoses, L-aldopentoses were at least as effective as their D derivatives.

Table V. Acrosin Inhibition by Phosphorylated Monosaccharides [a]

compd	anomer	% inhibn (n) [b]
fructose	β	46 ± 6 (5)
fructose 1-phosphate	β	9 ± 9 (3)
fructose 6-phosphate	β	7 ± 4 (4)
fructose 1,6-diphosphate	β	18 ± 6 (6)
ribose	β	45 ± 9 (4)
ribose 5-phosphate	β	15 ± 5 (3)
galactose	α	0 ± 5 (4)
galactose 1-phosphate	α	10 ± 3 (3)
galactose 6-phosphate	α	3 ± 1 (4)

[a] All compounds were evaluated at 100 mM, due to limited solubility at pH 8.0 (the barium salts of the phosphorylated monosaccharides were used). All compounds were in the D configuration. The parent sugar of each phosphorylated compound was evaluated at 100 mM, for purposes of comparison. Reactions were conducted as described in the legend to Table I. Barium chloride (200 mM) had no effect on enzyme activity. [b] Values represent the average plus or minus standard error inhibition of rates obtained in the absence of monosaccharide or phosphorylated monosaccharide.

Table VI. Inhibition of Human Acrosin by Polyols [a]

polyol	% inhibn (n) [b]
D-glycerol	28 ± 4 (5)
D-sorbitol	38 ± 5 (3)
inositol	30 ± 6 (3)
D-mannitol	29 ± 3 (5)

[a] Human acrosin was assayed after a 5-min preincubation of the enzyme in the presence or absence of 200 mM polyol. [b] Inhibited rates are expressed as percent inhibition of control rate (average plus or minus standard error). Control rates for the four acrosin preparations used in these studies ranged from 38 to 62 μmol of BAEE hydrolyzed min^{-1} (mg of protein)$^{-1}$ (5×10^{-5} M BAEE).

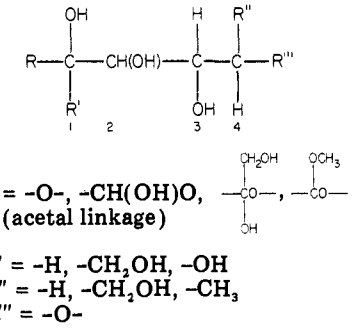
There was no absolute requirement for the anomeric form of the monosaccharide. Although the β-sugars tended to be slightly more inhibitory (Tables I–III), the most potent inhibitor was α-methyl mannoside, in which the anomeric hydroxyl group of mannose is masked (and committed to the α position).

Phosphorylation of monosaccharides reduced their activity (Table V). When the phosphorylated derivatives were examined at 100 mM, there was no apparent effect of the positioning of the phosphate group on inhibitory activity. Whether decreased inhibition by phosphorylated monosaccharides is due to the negative charge of the phosphate group or another property inherent to the phosphate moiety is presently unknown.

Acrosin inhibition was reduced by 78% when the concentration of fructose 6-phosphate was reduced from 200 to 100 mM (Tables I and V). In contrast, acrosin inhibition was reduced by approximately 30% by decreasing the concentration of either fructose or ribose from 200 to 100 mM (Tables I, III and V). On the other hand, 200 mM D-galactose inhibited acrosin by 32% (Table II), while 100 mM D-galactose had no effect (Table V). These data indicate that the sensitivity of acrosin to inhibition by increasing concentrations of monosaccharide is dependent upon the specific monosaccharide under investigation.

Acrosin inhibition by homologous series of aldoses and ketoses varied as a function of chain length, with hexoses displaying the highest inhibitory activity (Table IV). Acrosin inhibition by polyols was not greatly influenced by chain length, since glycerol (three carbons), sorbitol, and inositol (six carbons each) inhibited acrosin to approximately the same extent (Table VI).

Chart I. Inhibition of Acrosin by Aldoses and Ketoses: General Structure Requirements[a]



[a] The configuration presented above is common to all monosaccharides which inhibited acrosin by 50% or greater. Carbon-4 represents the asymmetric carbon most remote from the anomeric carbon.

Cyclicity was not a major determinant of acrosin inhibition, since similar activity was observed in the presence of sorbitol (a six-carbon open-chain structure) and inositol (a six-carbon cyclic polyol). D-Erythrose and D-ribulose, both open-chain structures, were as effective acrosin inhibitors as several of the higher chain-length sugars, which exist as cyclic structures (Table I–IV).

Analysis of the reaction mechanism of trypsin has led to the identification of a tetrahedral reaction intermediate adduct of the enzyme and substrate, whose structure resembles an aldehyde.[21] Potent, naturally occurring serine proteinase inhibitors, such as antipain and leupeptin, may act by mimicking structural properties of the reaction intermediate, since these inhibitors contain aldehyde moieties. Because acrosin is a trypsin-like enzyme and is strongly inhibited by antipain,[22] the reaction mechanism for acrosin catalysis probably also contains an aldehyde-like reaction intermediate. Acrosin inhibition by monosaccharides could be attributed to their aldehyde (or ketone) moieties. Acrosin inhibition by polyols (Table VI), however, demonstrated the lack of an absolute requirement for either aldehyde or ketone moieties, although the extent of polyol-induced inhibition indicated that the presence of either of these functional groups enhances the inhibitory properties of the carbohydrate.

In general, the most monosaccharide inhibitors were those in which the penultimate and terminal asymmetric carbons (starting from the anomeric carbon) had the same configuration. The configuration of the next asymmetric carbon was not critical, although the hydroxyl group was usually in the α position (below the plane of the D-sugar) in the most effective acrosin inhibitors. Finally, the configuration of the fourth from the last asymmetric carbon was important in determining the potency of the inhibition. In fructose and other sugars with similar inhibitory potency, the hydroxyl group of this carbon was in the β position (above the plane of the D-sugar). The determinant for inhibitory potency, therefore, centers around the spacial configuration of the last four asymmetric carbons of the molecule. If we take the above observations into consideration, Chart I presents the structural features of monosaccharides which appear to be optimal for acrosin inhibition.

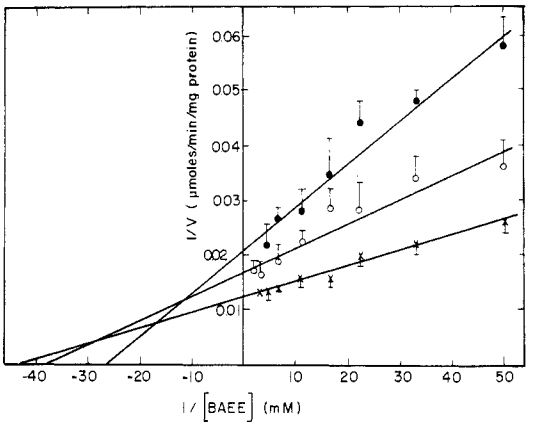


Figure 1. Kinetic analysis of acrosin inhibition by fructose. Plots of reciprocal reaction velocity vs. reciprocal concentration of BAEE were constructed at 0 (×), 50 (○), and 100 mM (●) fructose. Assays were carried out as described under Experimental Section. Vertical bars represent the standard deviation of each value, averaged from three to four determinations. Lines of regression were drawn through the data by using least-squares analyses.

Kinetic analysis with fructose showed the inhibition to be reversible and of a mixed type (Figure 1). The precise mechanism of inhibition by monosaccharides and polyols is presently unknown. Human, boar, and ram acrosins are glycoproteins. This is based upon the ability of acrosin to bind to conconavalin A linked resin[11] and upon sugar analysis of purified acrosin preparations.[12] Our laboratory has shown that wheat germ agglutinin and concanavalin A inhibit human acrosin (unpublished observations). These findings suggest that the carbohydrate moiety of acrosin may be located near, or at least affects, the active site of the protein. Detailed sugar analysis has been performed only on acrosin's zymogen (proacrosin) from rabbit.[13] Whether the carbohydrate content remains the same after zymogen activation or is similar to that of human acrosin remains to be established.

Reversible enzyme inhibitors often exert a stabilizing effect upon enzyme activity. Proacrosin extraction, for example, is most efficiently effected when benzamidine (an acrosin inhibitor) is included in the extraction medium.[23] Glycerol inhibited acrosin when present in incubation mixtures at a concentration of 200 mM (Table VI). Increased extractable acrosin activity in the presence of 10% glycerol[24] may be due to glycerol's stabilizing effect as an acrosin inhibitor.

Acrosin inhibitors have been tested against acrosin mainly on the basis of their inhibitory action toward other serine proteinases, especially trypsin. Preliminary results from our laboratory (unpublished) indicate that neither human nor bovine pancreatic trypsin is affected by carbohydrates. The present findings should lead to new directions in regards to the development of a specific acrosin inhibitor, so that the putative function(s) of acrosin during fertilization can be confirmed. Kinetic studies are currently being directed toward the detailed description of acrosin inhibition by monosaccharides.

Conclusions

Human acrosin, a sperm-specific serine proteinase, is inhibited by a variety of monosaccharides and polyols. Kinetic analysis of the inhibition of acrosin by fructose revealed the inhibition to be of a mixed type. In general,

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monosaccharides were more effective than polyols as acrosin inhibitors. These data suggest that the aldehyde and ketone moieties are not absolute requirements for inhibitory activity, although the presence of these groups renders the carbohydrates more effective acrosin inhibitors. The most important determinant of inhibitory activity was the spacial configuration of the hydroxy groups attached to the last four asymmetric carbons of the monosaccharide. Preliminary studies have indicated that the interaction between monosaccharides and acrosin is not common to other serine proteinases such as trypsin. Structure–activity studies of acrosin inhibition by monosaccharides and related compounds could be useful in the design of a specific acrosin inhibitor. A specific acrosin inhibitor would be particularly useful in elucidation of the role of acrosin in essential enzymatic processes related to fertilization.

Experimental Section

D-Seduheptulose was a product of P.L. Biochemicals (Milwaukee, WI). Other carbohydrates, as well as N^{α}-benzoyl-L-arginine ethyl ester (BAEE) and N^{α}-benzoyl-DL-arginine-*p*-nitroanilide (BAPA), were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the finest quality commercially available.

Measurement of Enzyme Activity. Esterolytic activity of acrosin was determined spectrophotometrically with either BAEE or BAPA as substrates. Assay mixtures consisted of 50 mM sodium phosphate, pH 7.5, the K_m concentration of substrate (empirically determined as 3×10^{-5} and 3×10^{-4} M for BAEE and BAPA, respectively), and enzyme protein (0.04–0.2 μg), in a total volume of 1.0 mL. A change in absorbance at 253 nm of 1.15 and 9.9 corresponded to the hydrolysis of 1.0 μmol of BAEE and BAPA, respectively.[14]

Isolation and Purification of Human Acrosin. α-Acrosin was isolated from frozen human spermatozoa by acetic acid extraction and purified by sequential steps of Sephadex G-150 and CM-cellulose chromatography, as previously described.[9,15] The purification procedure resulted in α-acrosin preparations with specific activities which ranged from 80 to 140 μmol of BAEE hydrolyzed min^{-1} (mg of protein)$^{-1}$, when assayed in the presence of 0.5 mM BAEE. The protein content of the purified acrosin preparations was estimated by reaction with fluorescamine.[16] Bovine serum albumin was used as a protein standard. Polyacrylamide gel electrophoresis of the preparation at pH 4.3[17] yielded a single band of protein after staining with Coomassie brilliant blue.[18]

Inhibition Studies. Monosaccharides and related compounds were evaluated as acrosin inhibitors by preincubation with acrosin at pH 7.5 for 5 min at ambient temperature prior to initiating the reaction by the addition of either BAEE or BAPA. All inhibitors were present in reaction mixtures at a concentration of 200 mM, unless otherwise indicated.

Kinetic Studies. Michaelis constants were determined by use of plots of reciprocal velocity vs. reciprocal substrate concentration.[19] Kinetic analysis of acrosin inhibition by fructose was performed by varying the substrate concentration (BAEE) in the presence of fixed concentrations of fructose. Enzyme was preincubated in the presence of inhibitor for 5 min prior to the addition of substrate to initiate the reaction.

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2-L-Rhamnopyranosyl[1,2,4]triazolo[1,5-*a*]pyridine. 4′ and 3′ Oxidation Products. Synthesis and Structure–Activity Relationships[1a,b]

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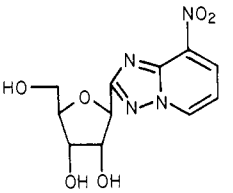
A series of 2-α-L-rhamnopyranosylnitro[1,2,4]triazolo[1,5-*a*]pyridine C-nucleosides was synthesized from the condensation of a thioiminoether with nitro-2-pyridylhydrazines. Catalytic reduction afforded the corresponding amino derivative. A 1′,2′ unsaturated C-nucleoside was also obtained by two different routes. Selective oxidation gave the 3′- and 4′-ketonucleosides. The cytotoxic properties of the nucleosides, as well as their effect on viral transformation and replication, were described. The nitro derivatives inhibit viral replication, but at toxic doses; the introduction of a keto function leads to a product which inhibits the replication of murine leukemia virus (MuLV) at noncytotoxic concentrations. The amino derivatives have no significant antiviral effect.

In the last decade, after the discovery of the pharmacological properties of C-nucleosides,[2] a number of studies have been devoted to the synthesis of such products.[3] We recently described the obtention of some structural analogues of the formycines, including 2-β-D-ribosyl[1,2,4]-triazolo[1,5-*a*]pyridines and 3-β-D-ribosyl[1,2,4]triazolo-[4,3-*a*]pyrazines.[4] One of these products, the 2-β-D-ribofuranosyl-8-nitro[1,2,4]triazolo[1,5-*a*]pyridine (**1**), presented interesting cytotoxic and antiviral properties.



1 (CT 5394)

In order to establish some structure-activity relationships, we decided to prepare a new series of compounds containing the same base, [1,2,4]triazolo[1,5-*a*]pyridine, and the L-rhamnopyranose as the glycon. Our purpose in choosing this sugar was to determine if modifications of

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