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Relationship Between Protease Activity and a Sialoglycopeptide Inhibitor Isolated From Bovine Brain

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We have recently described the isolation and purification to homogeneity of a new sialoglycopeptide from bovine brain cell surfaces that reversibly inhibits protein synthesis and DNA synthesis of normal but not transformed cells. Active inhibitory preparations, however, were shown to contain a protease activity that was not lost upon purification. Several experiments were performed to establish the relationship between the proteolytic activity of the sialoglycopeptide and the biological inhibitory activity. Both the protease activity and inhibitory activity were stable at pH 6–8 but were reduced or completely destroyed below pH 4 and above pH 9. Acid inactivation was reversible and upon dialysis, both the biological inhibitory and protease activities were regained. Deglycosylation and CNBr cleavage indicated that the polypeptide backbone, rather than carbohydrate moiety, played an important role in the protease and biological inhibitory activities. Furthermore, chemical modification of amino and tyrosine groups indicated that both residues are essential for both activities. Thus, the biological inhibitory activity and protease activity are very closely related and most likely reside with the same polypeptide sequence.

Key words: growth regulation, sialoglycopeptide inhibitor, protease protein synthesis inhibition

In recent years there has been a remarkable advance in our understanding of the nature and mechanism of cell growth regulation. A number of studies have clearly demonstrated that proliferation of quiescent normal cells can be initiated by proteases such as trypsin, pronase, or thrombin [1]. It has been reported that protease treatment stimulates cell division and DNA synthesis in mouse 3T3, chick embryo fibroblasts, human diploid foreskin, and bovine embryonic brachial cells [1]. However, several lines of evidence have indicated that cell proliferation is under both positive and negative control. Inhibition of cell growth can occur either with soluble growth inhibitors derived from the conditioned media of monkey kidney cells [2], mouse embryo fibroblasts [3] and mouse 3T3 cells [4], or from membrane-associated inhibitors [5–8].

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We have purified a growth inhibitor from bovine cerebral cortex cells that reversibly inhibits protein synthesis and DNA synthesis of normal but not transformed cells [9]. The inhibitor has been purified to apparent homogeneity and migrated at 18 KD by SDS-PAGE analysis and a single band by isoelectric focusing at a pI of 3.0 [9]. During the course of purification, however, we observed a protease activity associated with the growth inhibitory preparation that was not eliminated during purification. This prompted us to carry out the present studies in which we attempted to separate the two activities by several methods including pH inactivation, temperature inactivation, and chemical modifications of the inhibitor.

EXPERIMENTAL PROCEDURES

Material

All chemicals used were of reagent grade or higher quality. All general chemicals were purchased from Sigma Chemical Company (St. Louis, MO) and citraconic anhydride was purchased from Pierce Chemical (Rockford, IL). *Limulus polyhemus* lectin (LPA) was from E-Y Laboratories (San Mateo, CA). [^{35}S]-Methionine was obtained from Amersham Co. (Arlington Heights, IL).

Cell Culture

BALB/C 3T3 cells were propagated at 37°C as monolayers in humidified incubators with 5% CO_2:95% air atmosphere as described by Kinders and Johnson [8]. Cells were harvested from subconfluent monolayers for inhibitor assays by scraping the cells from the culture vessel surface with a sterile rubber policeman.

Protein Synthesis Assay

Protein synthesis assays were carried out essentially as described by Kinders and Johnson [8]. Briefly, 40 μl of the growth inhibitor in HKM buffer (10 mM Hepes, 120 mM KCl, 5 mM $MgCl_2$, pH 7.1) were added to 5×10^5 3T3 cells in 100 μl of DMEM-HEPES (pH 7.1). The cells were incubated at 37°C for 45 min and at the end of this incubation period 2.0 μCi of [^{35}S]-methionine, in 10 μl of DMEM-HEPES, pH 7.1, were added and the cells were reincubated at 37°C for 1 hr. Cells were then pelleted by centrifugation, lysed with deionized water, and the proteins were precipitated by the addition of an equal volume of 20% ice-cold trichloroacetic acid. The precipitates were collected by centrifugation, the supernatant fluid was discarded, and the pellet resolubilized in 0.1 N NaOH, reprecipitated with 20% trichloroacetic acid, and the macromolecules collected by centrifugation. The precipitated material was again resuspended in distilled water, resolubilized in 0.1 N NaOH, and an aliquot was taken for liquid scintillation counting while another aliquot was used for protein determination [10] using bovine serum albumin as a protein standard.

Purification of the Sialoglycopeptide

Purification of the growth inhibitor was essentially as described by Sharifi et al [9]. Briefly, bovine cerebral cortex cells were treated with a dilute solution of pronase at 37°C for 15 min. The macromolecules were precipitated with ethanol, extracted with chloroform/methanol, and lyophilized. The lyophilized sample was resuspended in 0.02 M acetate buffer (pH 5.0) and applied to a DEAE ion exchange column and eluted with 0.4 M NaCl in 0.02 M acetate buffer, pH 5.0. The DEAE purified growth

inhibitor was then applied to a wheat germ agglutinin column and the unbound fraction, which contained the inhibitory activity, was collected and concentrated by lyophilization. The wheat germ purified sample was then applied to a TSK 3000G gel permeation HPLC column that previously was equilibrated with 0.2 M phosphate buffer (pH 6.5), and the peak corresponding to a molecular weight of 18,000 daltons contained the sialoglycopeptide inhibitor. The gel permeation HPLC purified sample was resuspended in 0.1 M phosphate buffer, 10% glycerol, and 55% ammonium sulfate. In some cases the sample was further purified by hydrophobic interaction HPLC with a phenyl hydrophobic column with a linear gradient of a decreasing concentration of ammonium sulfate [9]. The fraction that contained inhibitory activity was dialyzed and concentrated and stored at −70°C until further study.

Radioiodination

The sialoglycopeptide was radioiodinated as described previously [9]. Briefly, 1 μg of the sialoglycopeptide was incubated with carrier-free ^{125}I and chloramine-T. The reaction was quenched by the addition of 100 μl of a saturated tyrosine solution. The bulk of the free iodine was separated from the sialoglycopeptide by gel filtration and the radioiodinated samples were dialyzed against 0.1 M KI overnight at 4°C.

Proteolytic Activity Assay

The sialoglycopeptide inhibitor was suspended in HKM buffer and proteolytic activity was measured using Bio-Rad protease substrate gel tablets as described by the manufacturer. A 5-mm hole was cut in the agar and inhibitory solution was pipetted into the hole. After incubation overnight at room temperature, the gel was developed by flooding with 3% acetic acid for 15 min. The width of the transparent ring around the sample wells was measured and the activities are reported in millimeters.

Protease Inhibitor Assay

The protease inhibitors were examined according to Barrett [11]. Briefly, various protease inhibitors were added to the inhibitor solution in HKM or PBS buffers, incubated for 1 hr at room temperature, and the proteolytic activity was measured as described by Barrett [11]. The sample was then dialyzed against water at 4°C, concentrated by lyophylization, and protein synthesis inhibition was measured as described by Kinders and Johnson [8].

pH Stability

Various pH solutions were prepared using trifluoroacetic acid and ammonium hydroxide. The gel permeation HPLC purified inhibitor was dissolved in the various pH solutions and incubated at room temperature for 1 hr. After the incubation period, the sample was dried under a stream of nitrogen gas, resuspended in HKM buffer and assayed for proteolytic and inhibitory activity as described. In some cases the inhibitor was dialyzed against HKM buffer overnight at 4°C and then assayed for proteolytic and inhibitory activities.

Deglycosylation of Inhibitor

Deglycosylation of the sialoglycopeptide inhibitor was performed as described previously [9]. Briefly, the inhibitor was resuspended in trifluoroacetic acid and a

stream of HBr gas was passed through the solution for 2 hr. After the reaction period the solution was dried under a stream of nitrogen gas. The samples were then dialyzed overnight against water at 4°C and the proteolytic and inhibitory activities measured as described. As a control, an inhibitor sample was resuspended in trifluoroacetic acid, incubated at room temperature for 2 hr, dialyzed against water, and assayed.

We dissolved 10 mg CNBr in 0.05 M phosphate buffer (pH 5.5) and the sialoglycopeptide inhibitor was dissolved in this solution and incubated at room temperature for 15 hr in the dark. After the incubation period the inhibitor and the controls (phosphate buffer containing CNBr and the inhibitor dissolved in phosphate buffer without CNBr) were lyophylized, resuspended in 100 μl of water and both activities were measured as described.

Chemical Modifications of the Sialoglycopeptide

The procedure used to modify the sialoglycopeptide inhibitor with citraconic anhydride was described by Singhal and Atassi [12]. Briefly, aliquots of inhibitor were resuspended in either 400 μl of PBS (pH 7.0) or 0.05 M borate saline buffer (BSB) (pH 8.5). We then added 100 μl of citraconic anhydride to the above solutions and incubated for 30 min at 4°C. After the incubation period, the sample was dialyzed against HKM buffer (pH 7.2) overnight at 4°C. The samples were then assayed for proteolytic and inhibitory activity as described. As a control, inhibitor samples were resuspended in PBS or BSB without citraconic anhydride. Unblocking of the modified inhibitor was performed by dialyzing the modified inhibitor overnight against acetate buffer (0.1 M, pH 5.5) at 4°C. The sample was then dialyzed against HKM buffer, pH 7.2, for 24 hr at 4°C and assayed for activities as described.

The modification of tyrosyl groups in the sialoglycopeptide inhibitor was performed using tetranitromethane, to nitrate the tyrosyl residues, and iodination using the chloramine T method. Nitration of tyrosyl residues was performed essentially as described by Dorner [13] at two different pH values. Aliquots of the sialoglycopeptide inhibitor were dissolved in 400 μl of PBS (pH 7.0) or BSB (pH 8.0). We added 25 μl of tetranitromethane and incubated the reaction mixture at room temperature for 30 min. After the incubation period the sample was dialyzed against HKM buffer and assayed for activities as described. A control sample did not receive tetranitromethane.

Iodination of tyrosyl residues was performed basically as described by Greenwood et al [14] as modified by Binion and Rodkey [15]. After incubation at room temperature for 1 min, 200 μl of a saturated solution of tyrosine was used to quench the reaction. Excess iodine was then removed by passing the sample through a G-25 column equilibrated with PBS. Fractions corresponding to the void volume were dialyzed overnight against HKM buffer and assayed for activities. Control samples received various concentrations of chloramine-T without NaI.

RESULTS

Characterization of the Sialoglycopeptide

The hydrophobic interaction HPLC profile of the sialoglycopeptide is shown in Figure 1. A shallow ammonium sulfate gradient was used to increase the resolution of the peaks. Each peak was assayed for protein synthesis inhibitory and protease activities. Only one peak, with a retention time of 22.80 min, contained the protein synthesis inhibitory and protease activities. Because the highly purified inhibitor has

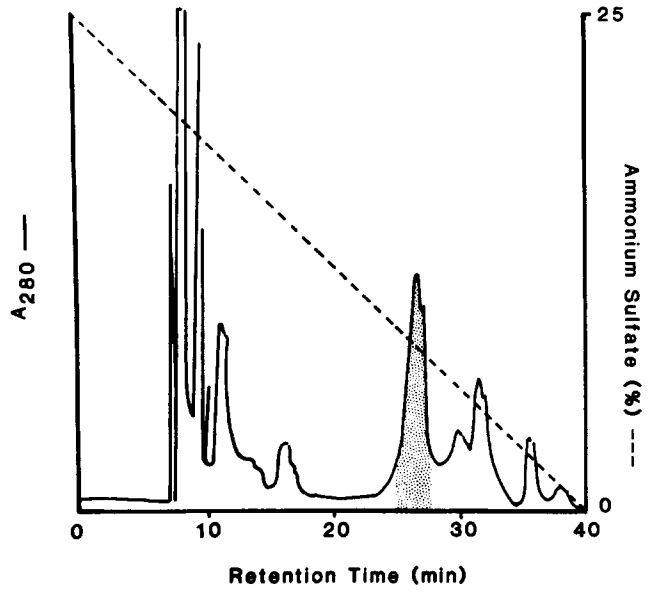

Fig. 1. Hydrophobic interaction HPLC of the sialoglycopeptide. The gel permeation purified sample (50 μg) was loaded on a 4.6 × 25 cm phenyl column [9]. The material was eluted from the column with a decreasing ammonium sulfate gradient. The flow rate was 0.5 ml/min at room temperature. All peaks were assayed for inhibition of protein synthesis and protease activity. The shaded peak was the only peak that had protein synthesis inhibitory and protease activities. All others were negative for both.

protease activity, it is possible that some of the peaks observed in the gel permeation and hydrophobic interaction HPLC profile could have arisen from self digestion of the sialoglycopeptide. Since we could not detect any biological activity or protease activity in any other peaks of the hydrophobic interaction purified inhibitor, we carried out the following experiments with the gel permeation HPLC purified sialoglycopeptide.

The SDS-PAGE analysis of HPLC purified growth inhibitor is shown in Figure 2. The results indicated that the HPLC purified growth inhibitor appears homogeneous, does not have subunit structure, and migrates with a molecular weight of 18,000.

Protein Synthesis Inhibition

Gel permeation HPLC purified sialoglycopeptide was tested in protein synthesis inhibition with suspensions of BALB/c 3T3 cells. A linear response in the inhibition of [^{35}S]-methionine incorporation into cellular proteins was measured with 30–130 ng of the bovine sialoglycopeptide (Fig. 3). Under these experimental conditions the presence of approximately 50 ng of the sialoglycopeptide inhibited 3T3 cell protein synthesis by 25% (defined as one unit of biological activity). In contrast to the inhibition of protein synthesis, the sialoglycopeptide had little influence on the uptake of [^{35}S]-methionine into the intracellular acid-soluble pools (Fig. 3). This observation is similar to an earlier study that showed the sialoglycopeptide to inhibit cellular DNA synthesis in BALB/c cells without altering [^{3}H]-thymidine uptake [9].

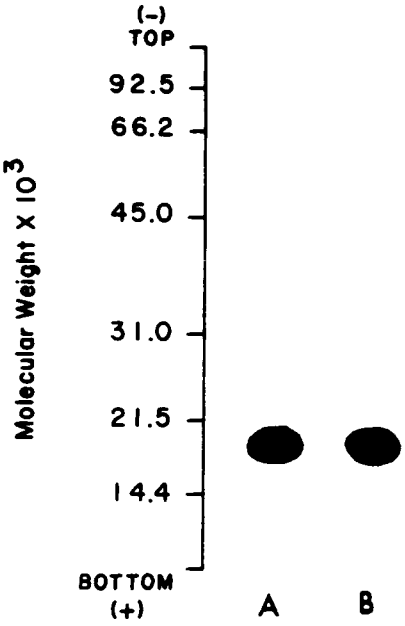

Fig. 2. SDS-PAGE analysis of the hydrophobic interaction HPLC purified [^{125}I]-radiolabeled sialoglycopeptide. The gel was run under reducing (lane A) and nonreducing (lane B) conditions with a linear gradient of 7.5–25% acrylamide. After electrophoresis the gel was exposed to x-ray film overnight using an intensifying screen. The protein standards included phosphorylase B (92,500), bovine serum albumin (66,000), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).

Assay of Protease Activity

The results of protease activity are shown in Figure 4. The assay is very sensitive in that above 40 ng of sialoglycopeptide a linear proteolytic response was observed while below 40 ng a break occurred.

Effect of Various Protease Inhibitors on the Protease and Inhibitory Activities of the Sialoglycopeptide

We have examined the effect of various protease inhibitors on the protease and biological inhibitory activities of the bovine sialoglycopeptide. The concentration of protease inhibitors that have been used in this study is five times higher than have been reported in the literature [11,16,17]. The data presented in Table I demonstrate that phenylmethylsulfonyl fluoride (PMSF) treatment did not inactivate the protease activity of the inhibitor, suggesting that either the protease activity is not a serine type protease or that the active site serine may not be available to react with PMSF. N-α-P-tosyl-L-lysine chloromethyl ketone (TLCK). TLCK and benzamidine treatment gave similar results. Leupeptin, a competitive inhibitor of trypsin that inhibits various proteases, such as plasmin, trypsin, kallikrein, elastase, thrombokinase, papain and cathepsin B [17], was not effective in inhibiting protease activity. This indicated that the inhibitor does not have trypsin-like activity and further supports the previous data which showed that the growth inhibitor is not a serine protease. To determine whether

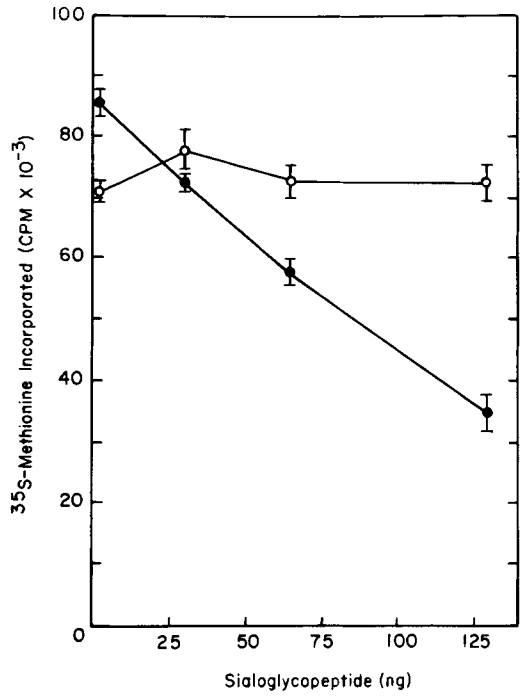

Fig. 3. Protease synthesis inhibition. Various amounts of gel permeation HPLC purified sialoglycopeptide, in 40 μl of HKM buffer, were incubated with 1 × 10^5 BALB/c 3T3 cells and 2.0 μCi of [^{35}S]-methionine in 100 μl of DMEM/HEPES (pH 7.1) at 37°C for 45 min. After incubation the cells were pelleted by centrifugation, resuspended and repelleted in DMEM/HEPES two times, and the radioactivity in trichloroacetic acid-insoluble proteins (●) and the acid-soluble intracellular pools (○) determined [9]. The data represent the average and range of duplicate determinations.

the protease activity has thiol-protease-type activity, the growth inhibitor was treated with N-ethylmaleimide and iodoacetamide, which irreversibly alkyates thiol groups [1]. As Table I demonstrates, N-ethylmaleimide and iodoacetamide were not effective in inhibiting the protease activity of the growth inhibitor preparation. These data suggest that the growth inhibitor does not have cathepsin-B1-type activity, further supporting the results obtained with leupeptin. Cathepsin-D-like activity, which is found in lysosomes of many cell types and is the major protease produced by activated macrophages, was not associated with the growth inhibitor because pepstatin, which blocks the activity of cathepsin D, was not effective in inhibiting the protease activity of the bovine sialoglycopeptide. The results observed with 6-aminohexanoic acid treatment also support the absence of a cathepsin-D-type protease. Neither EDTA nor phosphoramidone were effective in inhibiting the protease activity, suggesting the absence of metalloprotease type activity. Finally, 2-mercaptoethanol treatment was also not effective in blocking the protease and biological inhibitory activities.

Control experiments were carried out to be certain that protease inhibitors would block proteolysis under the experimental conditions shown in Figure 4 and Table I. Trypsin (0.02 units, 10-mm zone) was completely inactivated by TLCK, 6-aminohexanoic acid, PMSF, and iodoacetamide; papain (0.02 units, 7-mm zone)

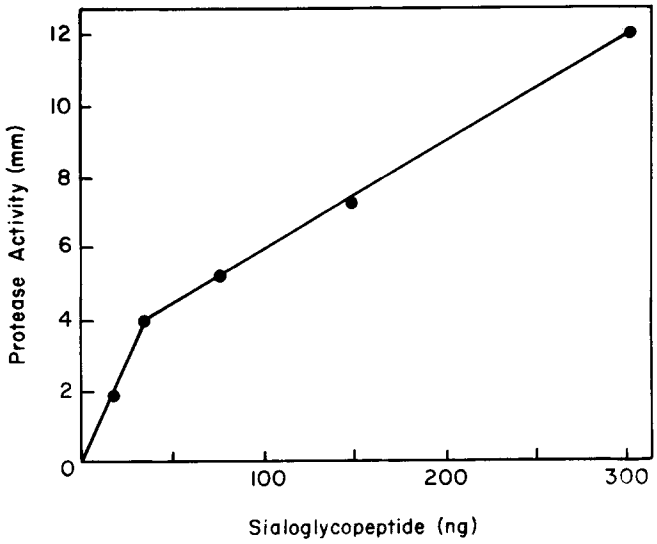

Fig. 4. Protease activity assay. Aliquots (40-μl) of inhibitor solutions in HKM buffer containing 18–300 ng of the sialoglycopeptide were incubated at room temperature for 24 hr and then overlayed with 3% acetic acid. The width of transparent ring (mm) around the sample wells was plotted versus nanograms of inhibitor.

TABLE I. Effect of Various Protease Inhibitors on Proteolytic and Inhibitory Activity of Bovine Sialoglycopeptide

Protease inhibitor	Concentration per 1 μg of sialoglycopeptide (mM)	Class of protease inhibited	Protease activity (mm)	Protein synthesis inhibition (units)[a]
None (inhibitor control)	—	—	7.0	3.3
PMSF	10	Serine	7.0	3.0
N-ethylmaleimide	500	Thiol	6.5	2.8
β-Mercaptoethanol	500	Nonspecific	7.0	3.0
TLCK	10	Serine	7.0	3.0
Iodoacetamide	20	Nonspecific	6.0	2.5
6-Aminohexanoic acid	100	Cathepsin D	7.0	ND[b]
EDTA	10	Metallo	7.0	ND
Benzamidine	5	Trypsin-like	6.0	ND
Leupeptin	10	Estrase	7.0	ND
Pepstatin	10	Carboxy	7.5	ND
Phosphoramidone	5	Metallo	6.0	ND

We suspended 1.5 μg (30 inhibitory units) of the sialoglycopeptide in 180 μl of PBS containing protease inhibitor of interest and removed 30 μl (250 ng) to measure protease activity. PBS was then added to the remainder to a final volume of 1.0 ml, the samples were dialyzed overnight against distilled water at 4°C, lyophilized to dryness, the samples resuspended in 0.3 ml of HKM buffer, and protein synthesis inhibition was measured with 40-μl aliquots (three inhibitory units) as described in the text.

[a]One unit of biological activity is defined as 25% inhibition of protein synthesis (Fig. 3).

[b]These samples were not tested for protein synthesis inhibition because the interaction of the protease inhibitors with their respective substrates is reversible (competitive).

was completely inactivated by N-ethylmaleimide and iodoacetamide; and carboxypeptidase A (0.02 units, 5-mm zone) was completely inactivated by β-mercaptoethanol, EDTA, and phosphoramidone (data not shown). The samples that have been treated with the competitive inhibitors were not assayed for protein synthesis inhibitory activity due to the reversible interaction of these protease inhibitors with substrates. All other protease inhibitors were shown to have no effect on the protein synthesis inhibitory activity. These results demonstrated that the protease activity of the bovine sialoglycopeptide is unique and cannot be grouped with the four major classes of proteases that have been proposed by Barrett [1]; ie, serine, thiol, carboxy, and metalloproteases.

pH Stability of Protease and Biological Inhibitory Activity

To investigate pH stability the sialoglycopeptide inhibitor was dissolved in HKM buffer and the pH was adjusted with either trifluoroacetic acid or ammonium hydroxide. The samples were incubated for 1 hr at room temperature, dried under a stream of nitrogen gas, and then assayed for protease and protein synthesis inhibitory activities. Figure 5 shows that below pH 4 and above pH 9, both activities were markedly reduced or completely destroyed. The protease and biological inhibitory activities were stable between pH 6–8, which is typical of most proteins.

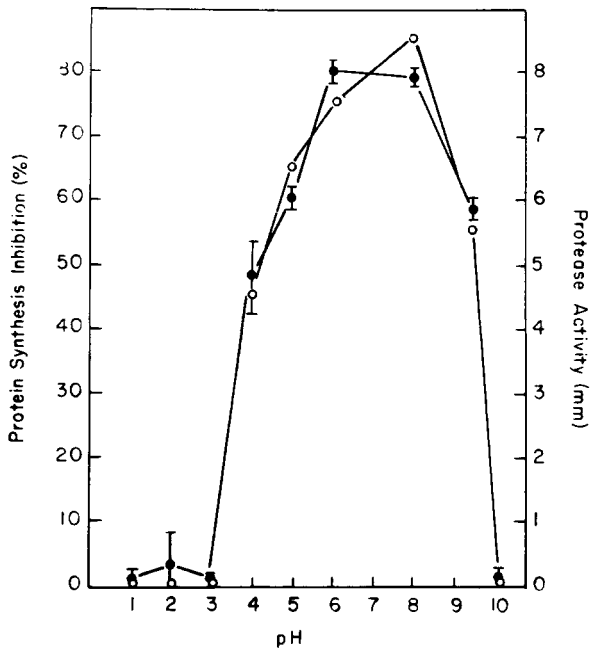

Fig. 5. The effect of pH on the stability of the protease and the protein synthesis inhibitory activities. We incubated 2.0 μg of the sialoglycopeptide, in 500 μl of the various pH buffers described in the experimental section, for 1 hr at room temperature. After incubation the samples were dried under a stream of nitrogen gas and resuspended in 500 μl of HKM buffer. We used 40 μl (160 ng) to measure protein synthesis inhibition (●) and proteolytic activity (○). The data represent the average ± range of duplicate determinations.

Previously we have used an isoproponal-urea solvent system (pH 3.0) to purify the growth inhibitor by reversed phase HPLC [18]. After elution of the growth inhibitor with an isoproponal-urea solvent system, the proteins were dialyzed exhaustively against water at 4°C and assayed for protein synthesis inhibitory activity. In contrast to the results that suggested that the inhibitor is sensitive to an acidic pH (Fig. 5), the reversed phase HPLC data demonstrated that biological inhibitory activity was acid stable [18]. Thus it was important to determine whether the apparent resistance of inhibitory activity to acid inactivation was due to the presence of urea, which may have stabilized the inhibitory activity, or whether the inhibitory activity was regained during dialysis at 4°C. We incubated the sialoglycopeptide in pH 3.0 buffer at room temperature for 1 hr and the acid-inactivated inhibitor was then dialyzed at 4° C overnight and assayed both for protein synthesis inhibition and protease activity. When the acid-inactivated sialoglycopeptide was dialyzed overnight the biological inhibitory and protease activities were regained (Table II) suggesting that acid inactivation of the inhibitor was a reversible phenomenon and that the protease and the biological inhibitory activities were inseparable.

Kinetics of Heat Inactivation

We have also examined the kinetics of heat inactivation of the biological inhibitory and protease activities. The bovine sialoglycopeptide was dissolved in HKM buffer and heated at 90°C for various time periods as shown in Figure 6. After 5 min at 90°C, there was no reduction in either the growth inhibitory or protease activities. However, when the sample was heated for 20 min, 50% of the protease and biological inhibitory activities were lost. When the heat-inactivated sample was dialyzed overnight, the activities were not regained (Table II). This suggested that heat inactivation of the growth inhibitor, unlike acid-inactivation, was an irreversible process.

TABLE II. Inactivation and Reactivation of Protease and Inhibitory Activities

Experiment	Sialoglycopeptide (ng)	Treatment	[^{35}S]-Methionine incorporation (CPM)	Protein synthesis inhibition (%)	Protease activity (mm)
1	0	—	64,000 ± 2,500	0	0
	150	—	25,600 ± 2,400	60	6
	150	pH 3.0	66,680 ± 3,200	0	0
2	0	—	68,840 ± 2,200	0	0
	150	Dialyzed	22,720 ± 2,100	67	6
	150	pH 3.0, dialyzed	19,760 ± 3,100	77	7

In experiment 1, 2.0 μg of the sialoglycopeptide were incubated in 500 μl of HKM buffer or a 500 μl aqueous solution of trifluoroacetic acid (TFA) (pH 3.0) for 1 hr at room temperature. After incubation the samples were dried under a stream of nitrogen gas and resuspended in 500 μl of distilled water (HKM sample) or HKM buffer (TFA sample). We then used 40 μl (150 ng) of the sample to assay for protein synthesis inhibition and protease activity. The data represent the average ± range of duplicate measurements.In experiment 2, 2.0 μg of the sialoglycopeptide were incubated with HKM buffer or TFA as described above. The samples were resuspended in HKM buffer and dialyzed overnight against HKM buffer at 4°C. After dialysis 40 μl (150 ng) of the samples were assayed for protein synthesis inhibition and proteolytic activity. The data represent the average ± range of duplicate measurements.

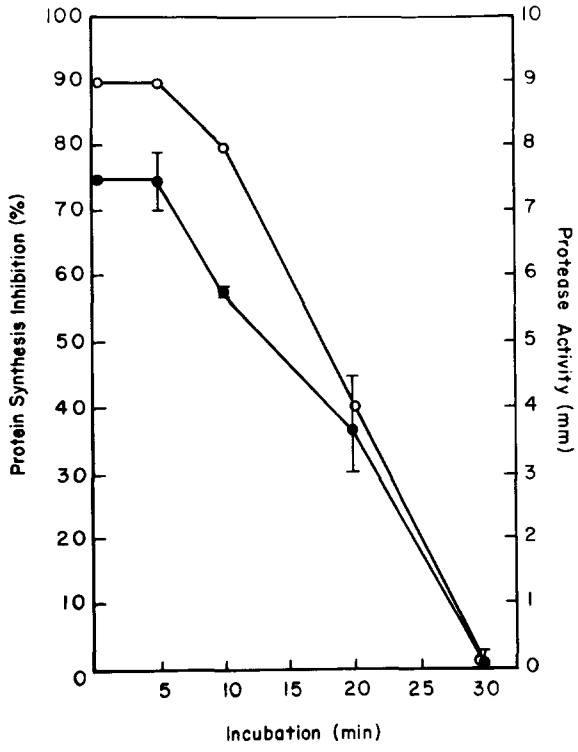

Fig. 6. The kinetics of heat inactivation. We dissolved 5.0 μg of the sialoglycopeptide in 1 ml HKM buffer and heated at 90°C for various time periods. After cooling the sample to room temperature the protease and the biological inhibitory activities were measured. The data represent the average ± range of triplicate determinations. Protein synthesis inhibition (●), protease activity (○).

TABLE III. Effect of Deglycosylation on Protease and Inhibitory Activities

Sialoglycopeptide preparation	*Limulus polyhemus* lectin affinity column	Protein synthesis inhibition (%)	Protease activity (mm)
Glycosylated	Bound	86 ± 2	8
Glycosylated	Unbound	4 ± 1	0
Deglycosylated	Bound	11 ± 4	0
Deglycosylated	Unbound	83 ± 1	8

1.5-μg aliquot of the sialoglycopeptide to be deglycosylated was dissolved in 1.0 ml of TFA containing 1.0% (v/v) ethylmethylsulfide. A stream of HBr gas was passed through the solution for 2 hr at room temperature. The deglycosylated sample and another 1.5-μg sample of the sialoglycopeptide in PBS (not deglycosylated with HBr) were then dried under a stream of nitrogen gas, resuspended in 500 μl of PBS, and applied to the lectin column. The unbound fractions, and the bound material released from the lectin column with 100 mM sialic acid in PBS, were then dialyzed against distilled water overnight at 4°C. After dialysis the samples were concentrated by lyophilization, resuspended in 280 μl of HKM buffer and 40-μl aliquots were used to measure protein synthesis inhibition and proteolytic activity. The data represent the average ± range of duplicate determinations.

Role of Carbohydrate and Polypeptide Moieties on the Protease and Inhibitory Activities

In a previous study, we demonstrated that the bovine sialoglycopeptide binds to *Limulus polyhemus* lectin and, therefore, is a glycopeptide containing sialic acid [9]. To determine whether the protease and biological inhibitory activities were associated with the carbohydrate or the polypeptide backbone, we either deglycosylated the glycoprotein or cleaved the polypeptide with CNBr and then measured both protein synthesis inhibition and protease activity.

To evaluate the role of carbohydrates in the protease and biological inhibitory activities, the sialoglycopeptide was deglycosylated as previously described [9]. The inhibitor was dissolved in trifluoroacetic acid and a stream of hydrogen bromide gas was passed through the solution at room temperature for 2 hr. The deglycosylated growth inhibitor did not bind to the *Limulus polyhemus* lectin column indicating that sialic acid was removed; however, the deglycosylated inhibitor retained both biological inhibitory and protease activities. This suggested that sialic acid is not necessary for either activity. These results further substantiated our earlier data that show the close relationship between protease and protein synthesis inhibitory activities [9]. These results also were consistent with the suggestion of Warren et al [19] that, as a rule, removal of sialic acid does not affect the biological activities of glycoproteins.

CNBr cleavage of the sialoglycopeptide inhibitor was performed to investigate the role of the polypeptide in the protease and inhibitory activities. CNBr cleavage has usually been performed under acidic conditions (formic or trifluoroacetic acid) but, we did not use these acidic solvents due to the sensitivity of the growth inhibitor to acidic solutions (Fig. 5). Furthermore, we wanted to avoid the possible loss of an active polypeptide fragment during the dialysis step that follows such treatment. Therefore, 10 mg CNBr was dissolved in 0.05 M phosphate buffer (pH 5.5), the inhibitor was added, and the mixture was incubated in the dark overnight at room temperature. Data presented in Table IV demonstrate that CNBr completely inactivated the protease and biological inhibitory activities of the sialoglycopeptide. The CNBr treated samples were assayed directly for protease and growth inhibitory activities without dialyzing the samples. Therefore, the inactivation was not due to the loss of a small fragment that might have been lost during dialysis. Furthermore, CNBr alone did not interfere with the protein synthesis assay (Table IV). Thus,

TABLE IV. CNBr Inactivation of the Protease and Inhibitory Activity

CNBr (mg)	Sialoglycopeptide (ng)	[^{35}S]-Methionine incorporation (CPM)	Protein synthesis inhibition (%)	Protease activity (mm)
0	0	102,100 ± 1,500	0	0
0	190	15,320 ± 1,277	85	8
0.5	0	92,760 ± 1,720	9	0
0.5	190	100,570 ± 2,400	1	0

We dissolved 1.0 μg of the sialoglycopeptide in 50 μl of phosphate buffer (0.05 M, pH 5.5), added 50 μl of CNBr (10 mg/ml) in phosphate buffer, and incubated the samples overnight at room temperature. After incubation the samples were lyophilized, dissolved in 100 μl of distilled water, and 100 μl of PBS added. Control sialoglycopeptide samples were treated in the same manner with the exception that CNBr was not added. We used 40 μl (190 ng) of the sialoglycopeptide to measure protein synthesis inhibition and proteolytic activity. The data represent the average ± range of duplicate determinations.

deglycosylation and CNBr treatment of the sialoglycopeptide have also strongly suggested that the carbohydrate moiety was not important for the protease or inhibitory activities although the polypeptide was essential for both.

Chemical Modification of the Sialoglycopeptide

Another approach to separate the protease activity from the biological inhibitory activity was to modify the sialoglycopeptide chemically and then investigate the effect of the modification on both activities. First, amino groups of the sialoglycopeptide inhibitor were modified using citraconic anhydride. The use of citraconic anhydride over maleic anhydride (a common amino group modifier) was based on the fact that unblocking of the amino group with citraconic anhydride requires less acidic conditions than maleic anhydride. Amino group modification has been shown to be pH dependent because the unprotonated amino group is a reactive moiety [20]. Data presented in Table V demonstrate that modification of the inhibitor at pH 7.0 resulted in a 15% decrease in biological inhibitory activity and a 30% decrease in protease activity. Modification at pH 8.5 completely destroyed both activities. When the modified protein was unblocked, both activities were essentially restored (Table V). Although the primary target of the citraconic anhydride is an amino group, we can not rule out the possible modification of histidyl and tyrosyl residues. Nevertheless, it was clear that the biological and the protease activities both were lost by citraconic anhydride modification.

Because we have used radioiodinated inhibitor for various studies including cell surface binding, we examined what effect tyrosine modification would have on the inhibitory and protease activities. Two methods were used to modify tyrosine. The first method consisted of tetranitromethane modification. Tyrosine modification with

TABLE V. Effect of Amino Group Modification on Inhibitory and Protease Activities

Experiment	Sialoglycopeptide (ng)	Treatment	[^{35}S]-Methionine incorporated (CPM)	Protein synthesis inhibition (%)	Protease activity (mm)
1	0	—	91,160 ± 1,800	0	0
	180	—	17,320 ± 1,100	81	8
	180	CA, pH 7.0	20,330 ± 3,200	77	7
	180	CA, pH 8.0	91,760 ± 2,830	0	0
2	0	—	69,570 ± 2,100	0	0
	160	Dialyzed	19,480 ± 2,400	72	7
	160	CA, pH 7.0, Dialyzed (pH 5.5)	16,980 ± 4,200	75	8
	160	Ca, pH 8.0, Dialyzed (pH 5.5)	17,200 ± 3,970	75	7

In experiment 1, 3.0 μg of the sialoglycopeptide was dissolved in 400 μl of borate saline buffer (BSB) (ionic strength 0.1, pH 8.5) or PBS (pH 7.0). We added 100 μl of citraconicanhydride (CA) to the above buffer solutions and incubated the solution at 4°C for 30 min. After incubation the samples were dialyzed against HKM buffer overnight at 4°C and 40-μl (180 ng) aliquots were assayed for protein synthesis inhibition and proteolytic activity. The data represent the average ± range of duplicate determinations. In experiment 2, unblocking of the sialoglycopeptide was performed by dialyzing the CA-modified samples overnight against acetate buffer (0.1 M, pH 5.5) at 4°C. The samples were again dialyzed for 24 hr against HKM buffer at 4°C and 40-μl (160 ng) aliquots were assayed for protein synthesis inhibition and proteolytic activity. The data represent the average ± range of triplicate determinations.

tetranitromethane has been shown to be pH dependent [13]; therefore, we have modified the growth inhibitor at pH 7.0 and 8.0. As seen in Table VI modification at pH 7.0 reduced inhibitory and protease activities by 30%. Modification at pH 8.0 completely destroyed both activities. Similar results were obtained when the growth inhibitor was iodinated. The results indicated that when 5 μg of chloramine-T was used in the iodination of growth inhibitor, both activities were lost. The inactivation of the growth inhibitor was not due to the side reaction or oxidation with chloramine-T, because 25 μg chloramine-T alone, had no effect on the activities (data not shown). Although the primary target of iodination is tyrosyl residues, we cannot rule out modification of other reactive groups, such as methionine or histidine, at high levels of chloramine-T (5–25 μg). Together, the chemical modification data has also strongly suggested that the biological inhibitory activity and protease activities are closely related.

DISCUSSION

The principal aim of the work described in this paper was to investigate the new cell surface sialoglycopeptide that we have purified to determine if the proteolytic activity and the biological inhibitory activities were closely related. The results described here demonstrate that when protease activity was lost or reduced, the biological inhibitory activity was also lost or reduced accordingly.

We considered the possibility that the protease activity observed in the inhibitor preparation might be due to the presence of pronase originally added to the intact bovine cerebral cortex cells to release the sialoglycopeptide. This possibility was

TABLE VI. Effect of Tyrosine Group Modification on Inhibitory and Protease Activities

Experiment	Sialoglycopeptide (ng)	Treatment	[^{35}S]-Methionine incorporation (CPM)	Protein synthesis inhibition (%)	Protease activity (mm)
1	0	—	43,930 ± 1,600	0	0
	190	—	12,020 ± 855	72	7
	190	TNM, pH 7.0	20,540 ± 2,400	53	5
	190	TNM, pH 8.0	45,600 ± 2,500	0	0
2	0	—	38,430 ± 1,500	0	0
	160	C-T, 1 μg	16,140 ± 1,400	58	5
	160	C-T, 5 μg	36,490 ± 2,000	5	0
	160	C-T, 25 μg	36,650 ± 2,100	5	0

In experiment 1, 3.0 μg of the sialoglycopeptide was dissolved in PBS or BSB (pH 8.0). We added 25 μl of tetranitromethane (TMN), and incubated the solution at room temperature for 30 min. After incubations the samples were brought to 500 μl with HKM buffer and dialyzed against HKM buffer overnight at 4°C. After dialysis 40 μl (190 ng) of the sialoglycopeptide were assayed for protein synthesis inhibition and proteolytic activity. The data represent the average ± range of duplicate determinations. In experiment 2, the iodination of the sialoglycopeptide preparations (1.0 μg in PBS) was carried out with 62 ng of NaI followed by the addition of various amounts of chloramine-T (C-T) in PBS. The solutions were incubated at room temperature for 1 min followed by quenching the reaction with 200 μl of a saturated tyrosine solution in PBS. The unreacted reagents were removed by gel filtration with a G-25 Sephadex column and the samples were dialyzed against distilled water overnight at 4°C. The dialyzed samples were lyophilized to dryness, resuspended in 250 μl of HKM buffer, and 40-μl (160 ng) aliquots were used to measure protein synthesis inhibition and proteolytic activity. The data represent the average ± range of duplicate determinations.

ruled out because: (1) pronase does not bind to DEAE ion exchange resin at pH 5.0 [20,21]; (2) the isoelectric points of the pronase enzymes range from 5.7–8.5 [22]; (3) pronase is a mixture of serine and metalloproteases [23–25]; and (4) pronase stimulates thymidine incorporation into various cell lines including BALB/C 3T3 [1]. A wide range of protease inhibitors were tested against the protease activity of the growth inhibitor. However, no significant reduction of the protease activity of the sialoglycopeptide inhibitor was found. We cannot, however, rule out the possibility of a false negative due to the nonaccessibility of a catalytic group to the reagents examined. Various amino and carboxy peptidases have been found on the surface of various cells such as lymphocytes, ascites tumor cells, and primary cultured transformed and nontransformed cells [26]. There is, however, no information on the effect of these proteases on cell protein metabolism or growth control.

There is a close relationship between biological inhibition and protease activity (Fig. 5). Incubation of the bovine sialoglycopeptide below pH 3 and above pH 9.0 reduced or completely destroyed both activities. Acid-inactivated inhibitor could be reactivated by dialysis and both the biological inhibitory and protease activities were restored (Table II). The kinetics of heat inactivation of protease activity parallels that of inhibitory activity, and 50% reduction of both activities were observed after 20 min. To determine whether the carbohydrate moiety or polypeptide backbone were involved in the protease or inhibitory activities, we examined both activities of the deglycosylated inhibitor and the CNBr-treated inhibitor. We found that the carbohydrate does not play an important role in both activities although the polypeptide backbone was essential for both activities (Tables III and IV).

The role of carbohydrates in glycoproteins are complex. It has been shown that glycoproteins are resistant to proteolysis. Sharon and Lis [27] suggested that sialic acid residues are especially effective in preventing degradation of the polypeptide backbone due to their negative charge. In addition to protecting against proteolytic degradation, Fujisawa et al [28] showed that carbohydrates are not necessary for the biological activity of interferon but are required for preserving the native structure.

Modification of amino groups with citraconic anhydride at pH 7.0 slightly reduced the protease and inhibitory activities while modification at pH 8.5 completely destroyed both activities. Unblocking of the modified protein completely restored both activities (Table V). In addition, modification of tyrosyl residues by tetranitromethane or iodination also indicated that tyrosyl groups are essential for protease and biological activities (Table VI).

Various soluble inhibitors from the conditioned media of cultured cells [29] or membrane-associated inhibitors [5,8] have been isolated. There is, to date, no information on the presence or absence of protease activities of these growth inhibitors. The absence of information concerning protease activity of the growth inhibitors might be due to the generally held thought that protease activities are only associated with growth promoting activities.

One of the more dramatic changes after neoplastic transformation has been an increase in secreted and cell-surface-bound proteases. High levels of neutral peptidase, aminopeptidase, and collagenase activities have been found at the peripheries of invasive tumors [30]. Transformed cells are known to release plasminogen activator that converts serum plasminogen to plasmin. Rifkin et al [31] have proposed that high levels of plasminogen activator are related to the transformed cells; however, Mott et al [32] have demonstrated high levels of plasminogen activator in many

normal tissues. The protease that we have isolated from bovine cerebral cortex cell surfaces has been shown to be distinct from plasminogen activator because it is not a serine protease and it also inhibits cell protein synthesis and division rather than stimulates cell proliferation [9].

The exact relationship between the protease activity and the inhibitory activity of bovine sialoglycopeptide remains unclear. Carney and Cunningham [33] have demonstrated that an interaction of thrombin with chick embryo cells is sufficient for stimulation of cell division. Preliminary data obtained in our laboratory has indicated that the inhibitor binds in a specific manner to the target cell surface and may not be internalized. These results, which are similar to those of Carney and Cunningham [33], suggest that an interaction of the inhibitor with sensitive target cell surfaces may be sufficient for biological activity.

Various growth factors have been shown to have protease activity. For example, esteropeptidase activity has been associated with epithelial growth factor and mesenchymal growth factor [34]. However, the protease activity and the growth promoting activity have been shown to reside on different subunits. We have no evidence that the bovine sialoglycopeptide, in its biologically active form, has subunit structure (Fig. 2). Gunderson et al [35] recently have isolated a growth inhibitory factor from mouse sera that has serine type protease activity. They have demonstrated, however, that the protease activity is necessary for inhibitory activity, and that PMSF inhibited both activities. This serine protease, therefore, clearly is not related to the bovine sialoglycopeptide.

Proteases play a key role in the regulation of many biological processes. They can serve to create rapid and permanent changes that can either turn on or turn off biological reactions. Proteases can turn on biological reactions by converting an inactive form of a protein to an active form in a process called zymogen activation. Zymogen activation is involved in the activation of proteolytic enzymes, hormones, and growth factors such as EGF. Proteolytic enzymes have also been shown to be directly involved in the stimulation of cell growth of normal cells. Furthermore, the presence or absence of protease inhibitors provides an additional level for control mechanisms of cellular processes. Baker et al [36] have recently isolated a protease inhibitor, protease-Nexin, from human fibroblasts that mediated the growth regulatory activities of thrombin and plasminogen activator. It is possible that the bovine sialoglycopeptide reacts with the target cell by a similar mechanism. Studies are currently in progress to investigate the interaction of the sialoglycopeptide inhibitor with the target cell and to determine if the biologically active form of the inhibitor may involve a complex with a Nexin-like substance.

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