Proteases in Cyst Fluid from Human Gross Cyst Breast Disease

Leo Kesner,1 Wanshang Yu, H. Leon Bradlow, Charles W. Breed, and Martin Fleisher

Department of Biochemistry, SUNY-Health Science Center at Brooklyn, Brooklyn, New York 11203 [L. K., W. Y.J; The Rockefeller University, New York, New York 10021 [H. L. B.]; and the Memorial Sloan-Kettering Cancer Center, New York, New York 10021 [C. W. B., M. F.]

ABSTRACT

Cyst fluid from women with gross cystic breast disease was found to contain protease activity when assayed against [14C]albumin. At least six different proteases were detected when the fluid was fractionated by a combination of S-300 Sephacel, hydroxylapatite, and DEAE-Sephacel chromatographic techniques. The distribution of the proteases appeared to be related to the ionic composition of the fluids.

A major protease component, found in both high Na and high K fluids, was isolated. It showed chymotryptic cleavage characteristics against the B-chain of insulin. It was partially inhibited by a1-macroglobulin, N-tosyl-L-phenylalanine chloromethyl ketone, and benzamidine but not by leupeptin, pepstatin, N-tosyl-L-lysine chloromethyl ketone, or a1-protease inhibitor. The protease has an apparent molecular weight of 110,000 with M, 24,000 subunits. This protease may be identical or closely associated with Haagensen's GCDFP-24 progestosterone binding protein which was isolated in a similar manner.

An imbalance between protease and protease inhibitors in cyst fluid may account for gross cyst formation and may be involved in the tumorigenic process. The accumulation of poorly diffusible peptide fragments, as a result of protease activity, would increase the oncotic pressure leading to enlargement of the cyst cavity as water enters to reestablish osmotic equilibrium.

INTRODUCTION

Gross cystic breast disease is the most commonly occurring benign breast disease with a frequency of about 7% in premenopausal women (1). Several reports have indicated a statistical association with the subsequent development of breast carcinoma in these women, with a 2- to 4-fold increased degree of risk (2-5). While the cyst itself is only rarely premalignant, and breast carcinomas are only rarely found at the site of the cysts, a common modality of formation may be involved (1).

The biochemical characteristics of cyst fluid have been studied from a variety of considerations with a view towards understanding the mechanism of cyst formation and its possible role in tumorigenesis. Reports have been published concerning the hormone content (6-8), enzyme levels (9), carcinoembryonic antigen (10), inorganic ion composition (9, 11-12), and protein content (2, 13-19).

A number of unusual proteins have been found in cyst fluid. Some have been studied with respect to their physical characteristics and hormone binding properties, but little is known about their source or biological functions. When the fluids are subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, four major components are seen (17). These were named GCDFP-70, -44, -24, and -15 with the numbers indicating their relative molecular weight size. GCDFP-70 is plasma albumin at concentrations 1/25-1/100 that found in plasma (16, 19). GCDFP-44 is a zinc-containing a2-glycoprotein found at 10 times its concentration in plasma. This protein is also found in thermal sweat. GCDFP-24 accounts for over half the protein in cyst fluid at a concentration 500 times that in plasma.

It is a glycoprotein that can bind progesterone and is also found in colostrum, and in axillary and perianal sweat gland tissue. GCDFP-15 is found in small amounts in the plasma of normal women and larger amounts in the plasma of some women with metastatic breast carcinoma. It is seen in milk and saliva and is associated with tissues containing apocrine glands, as well as the sublingual and submaxillary salivary glands. An immunoassay for this protein has been devised and has been found useful as an histochemical marker for apocrine epithelium (18). Cyst fluid levels of y-glutamyl transpeptidase indicate that it is the richest source in the body of this enzyme (19).

In this paper, we describe a sensitive method for assaying protease activity in cyst fluid and the characterization of one of at least six proteases observed.

MATERIALS AND METHODS

Purification of Protease

Cyst fluid was obtained by aspiration from patients as they presented for treatment of gross cysts. They were stored at -20°C until used. At that time they were thawed and analyzed for electrolytes. An aliquot from each specimen was pooled with others on the basis of their Na/K ratio and it was this mixture, from about 20 specimens, that was used for protease isolation. The method was adapted from the scheme devised by Haagensen et al. (17) for the isolation of the major proteins of cyst fluid.

Step 1—Centrifugation. The pooled cyst fluid, which varied in color from light brown to green, was centrifuged at 35,000 x g for 2 h at 4°C. The pellet containing particulate matter was about 10% of the volume, and an oily upper phase was about 4% of the volume. The intermediate aqueous phase was used for the isolation of the proteases.

Step 2—Gel Filtration. All of the chromatographic separations were conducted at 4°C. 10 ml of the aqueous phase was applied to the top of a 1.6- x 33-cm column of Sephacyr S-300 (Pharmacia). The sample was washed in with 2-10 ml volumes of 0.1 M ammonium acetate buffer, pH 6.7, and elution was carried out at 36 ml/h under gravity pressure with 40-12 ml fractions collected. Each tube was tested for absorbance at 280 nm and for protease activity using [14C]albumin as substrate. Those tubes exhibiting significant protease activity were combined and then concentrated on a pressure filter using a cutoff filter with a molecular weight of 10,000 (Spectra/Por). The samples were then subjected to buffer exchange using 0.1 M sodium phosphate buffer at pH 4.8 and then diluted to a volume of 10 ml. The fraction with the greatest protease activity (S2) was then subjected to hydroxylapatite chromatography.

Step 3—Hydroxylapatite Chromatography. The solution was then fractionated on a 1.6- x 30-cm column of hydroxylapatite (Fast Flow, Calbiochem). It was eluted in a stepwise manner with 200 ml of 0.01 M, pH 4.8, phosphate buffer (Fraction A), followed by 100 ml 0.15 M, pH 4.8, phosphate buffer (Fraction B), and 100 ml 0.15 M, pH 8.6, phosphate buffer (Fraction C). Each fraction was concentrated by pressure filtration as described above and the buffer was exchanged with 0.005 M tris, pH 8.0. Fractions A and B, which contained the protease activity, were then subjected to DEAE-Sephacel chromatography.

Step 4—DEAE-Sephacel Chromatography. Fractions A and B were separately subjected to DEAE-Sephacel chromatography on a 1.0- x 25-cm column using 400 ml of a linear gradient generated from 0.005 to 0.5 M Tris buffer, pH 8.0. 10-ml fractions were collected, monitored for protein at 280 nm, and assayed for protease activity. Tubes from the peaks with protease activity were concentrated as described above.

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1To whom requests for reprints should be addressed, at Department of Biochemistry, SUNY-Health Science Center at Brooklyn, 450 Clarkson Avenue, P. O. Box 8, Brooklyn, NY 11203-2098.
Preparation of Radioactive Protein Substrates

Human serum albumin was methylated with ["C"]formaldehyde (20). 50 mg of human serum albumin (Sigma) was dissolved in 5 ml 0.2 M potassium phosphate buffer, pH 7.5, in a screw top vial. NaCNBH₄ was added to make the solution 20 mM. A vial containing 250 μCi ["C"]formaldehyde (NEC) was frozen on dry ice so that the formaldehyde concentrated at the bottom of the vial. The vial was snapped open and diluted with 75 μl of a 1/10 dilution of concentrated cold formaldehyde. This was then added to the protein solution and the mixture was incubated for 4 h at room temperature. The solution was then dialyzed against several changes of distilled water in the cold, until minimal radioactivity was found in the dialysate. The radioactive methylated protein was then divided into a number of vials, each containing 2.5 mg/ml protein, which were stored at -20°C.

Assay for Protease Activity

The assay mixture contained 100 μl of sample, 400 μl of TES² buffer, 15 mM, pH 7.5, and 20 μl of ["C"]albumin equivalent to about 100,000 counts. The mixture was incubated at 37°C for 30 min after which 100 μl of 50% trichloroacetic acid and 100 μl of 5 mg/ml bovine serum albumin were added. The chilled mixture was centrifuged at 3000 rpm for 15 min. A 0.4-ml aliquot of each supernatant was added to 6 ml of Econoscent (National Diagnostics) for counting. Protease samples that released more than 50,000 cpm during the assay procedure were diluted with TES buffer and reassayed. In most cases, a blank determination was made by stopping the reaction at time 0 and subtracting the cpm found from those released after 30 min of incubation. In situations where the solubility of the trichloroacetic acid-precipitated protein may be altered, as in the presence of organic solvents, another method for determining background counts was used. Three different dilutions of the protease are tested under the same conditions. The results are extrapolated back to zero protease content and this value was then subtracted from that obtained in the incubation run. Attempts to find a low molecular weight substrate for a colorimetric assay with commonly used peptide derivatives were generally unsuccessful. However, the thiohennyl esters of leuc-1-lysine and sec-lysine were found to exhibit some activity.³ Protein was determined by the method of Lowry et al. (21) using bovine serum albumin as a standard.

Characterization of Protease HD1

The cleavage characteristics of protease HD1 were determined by reaction with the oxidized β-chain of insulin (Sigma). 750 μg of the β-chain was reacted with 100 μg of protease HD1 in 25 mM, pH 7.5 TES buffer in a total volume of 2.25 ml. High-performance liquid chromatographic analysis of the reaction mixture was performed at time 0 and after 4 h of incubation at 37°C. A 40-μl aliquot was chromatographed on a 4.6-× 220-mm, 5-μm RP-18 column (Pierce). Solvent A was 0.1% trifluoroacetic acid in water. Solvent B was 0.1% trifluoroacetic acid in acetonitrile-isopropanol (3:1). The initial solvent composition at time 0 was 80% A/20% B. Flow rate was 1.0 ml/min and a linear gradient was generated over a 75-min period with a final solvent composition of 20% A/80% B. The peptide peaks were detected by monitoring absorbance at 210 nm and they were collected manually. The solvent was evaporated under a stream of nitrogen and the isolated peptides were subjected to automatic sequence analysis (Applied Biosystems).

The molecular weight of protease HD1 was estimated by gel filtration by comparison with proteins of known molecular weight (ferritin, M₁, 440,000; catalase, M₂, 240,000; aldolase, M₃, 158,000; albumin, M₄, 68,000; ovalbumin, M₅, 45,000) on a 1.2 × 33-cm column of Sephacryl S-300. The eluting buffer contained 50 mM potassium phosphate, pH 6.8, plus 100 mM NaCl and 3 mM NaN₃. Electrophoresis of centrifuged cyst fluid and purified fractions to detect subunits was performed on gels made of 10% acrylamide and 0.3% bisacrylamide (17). Prior to application, samples were boiled with an equal volume of 2% SDS containing 1% mercaptoethanol, 40% glycerol, and 0.01% bromphenol blue. Following electrophoresis, the gels were stained with 0.1% Coomassie brilliant blue.

RESULTS

Isolation of a Purified Protease from Pooled Cyst Fluid. Cyst fluids taken from either high K or high Na specimens were pooled and centrifuged at 35,000 × g. Three phases were observed with most of the protease activity found in the colored aqueous phase. The upper lipid layer with about 4% of the volume, accounted for less than 1% of the protease activity. The pellet represented about 10% of the volume. It was found to contain about 10% of the activity after it was washed twice with buffer and then resuspended.

The aqueous phase, when subjected to size exclusion chromatography on a Sephacryl S-300 column, showed five distinct UV absorbing peaks only two of which exhibited protease activity (Fig. 1). The first peak, S1 (tubes 8–9) was turbid and contained lipid material and about 1% of the protein chromatographed. The second protease peak, S2 (tubes 10–13) was tan colored and found to contain 82% of the protein chromatographed after it was concentrated to a 10-ml volume by pressure filtration on a filter with a molecular weight cutoff of 10,000.

Peak S2 was then separated on an hydroxyapatite column. Protease activity was observed in fractions A and B, but not C (Table 1). The fractions containing protease activity were concentrated by pressure filtration and after buffer exchange, separately chromatographed on DEAE-Sephacel columns. All of the protease activity in Fraction A was found in a single peak, HD1 Fig. 2. When fraction B was chromatographed on the DEAE-Sephacel column, four peaks of protease activity were found (HD2-HD5, Fig. 3). When this separation scheme was applied to cyst fluid pooled according to cation content, it was found that high K fluids had most of their protease activity in fraction A. This fraction was chosen for further characterization since it was the most abundant in both cyst fluid types. Peaks HD2-5 were more abundant in the high Na rather than in the high K cyst fluids (data not shown).

 Cleavage Characteristics of Protease HD1. An aliquot of HD1 was reacted with the oxidized β-chain of insulin to determine its cleavage characteristics. Fig. 4 shows the separation of

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² The abbreviation used is: TES, N-tris(hydroxymethyl)aminomethane.
³ We are indebted to Dr. James C. Powers of the Georgia Institute of Technology for these results.
Table 1  Chromatographic partition of protease activity

<table>
<thead>
<tr>
<th>Sample</th>
<th>Protein (mg/ml)</th>
<th>Protease activity (cpm/mg protein x 10^4)</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pooled cyst fluid</td>
<td>34.9</td>
<td>6.85</td>
<td></td>
</tr>
<tr>
<td>S-300 Separation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak S1</td>
<td>0.49</td>
<td>46.8</td>
<td></td>
</tr>
<tr>
<td>Peak S2</td>
<td>28.3</td>
<td>1.89</td>
<td></td>
</tr>
<tr>
<td>Hydroxylapatite separation of S2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction (A)</td>
<td>8.3</td>
<td>17.9</td>
<td></td>
</tr>
<tr>
<td>Fraction (B)</td>
<td>8.4</td>
<td>10.8</td>
<td></td>
</tr>
<tr>
<td>Fraction (C)</td>
<td>12.3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>DEAE separation of (A)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Peak HD1</td>
<td>6.2</td>
<td>73%</td>
<td>119.0</td>
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<tr>
<td>DEAE separation of (B)</td>
<td></td>
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<tr>
<td>Peak HD2</td>
<td>0.62</td>
<td>3.6</td>
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<tr>
<td>Peak HD3</td>
<td>1.16</td>
<td>2.4</td>
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<tr>
<td>Peak HD4</td>
<td>1.17</td>
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<tr>
<td>Peak HD5</td>
<td>1.25</td>
<td>50%</td>
<td>1.2</td>
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</tbody>
</table>

Fig. 2. DEAE-Sephacel chromatography of hydroxylapatite Fraction A. The single peak containing protease activity is labeled HD1.

Fig. 3. DEAE-Sephacel chromatography of hydroxylapatite Fraction B. Four peaks with protease activity are labeled HD2-HD5.

Fig. 4. High-performance liquid chromatographic separation of oxidized insulin β-chain by protease HD1. Separation shows pattern at time 0 and after 4 h of incubation.

Peptides released after 4 h of incubation. The five major peptide peaks were collected and analyzed on an automatic protein sequenator. The cleavage points are shown in Table 2 in comparison with those reported for several cathepsin and chymotryptic enzymes (21–22). The major cleavage points were on the C-terminal side of leucine or phenylalanine, resembling the pattern shown for cathepsin G or chymotrypsin C.

Effect of Potential Inhibitors and Activators of Protease HD1. A variety of substances were added to the assay mixture at a final concentration of 0.1 mg/ml using [14C]albumin as substrate. Those found to have little effect included iodoacetic acid, iodoacetamide, phenylmethylsulfonyl fluoride, leupeptin, pepstatin (in 10% dimethyl sulfoxide), antipain, bestatin, N-tosyl-L-lysine chloromethyl ketone, N-ethylmaleimide, EDTA, α-phenanthaline (in 20% methanol), dithiothreitol, mercaptoethanol, CaCl₂, and α-protease inhibitor. Inhibitory activity was shown by three substances: α1-macroglobulin, (0.5 μM) inhibited 69% of the total activity, and N-tosyl-L-phenylalanine chloromethyl ketone and benzamidine (0.1 mg/ml), inhibited 37% and 18%, respectively.

Molecular Weight of Protease HD1 and Its Subunits. The approximate molecular weight of protease HD1 was determined by gel filtration in comparison with proteins of known molecular weight. It was estimated to be Mw 110,000. When the purified protease was subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis one major and three minor bands were observed. The major band corresponded to protein GCDFP-24 described by Haagensen (17).

DISCUSSION

When human breast cyst fluid samples were incubated with radioactive protein, varying levels of protease activity were observed in all cases. The pooled cyst fluids were fractionated, and at least six different proteases were detected. One of these, HD1, was common to all of the cyst fluids tested and exhibited chymotryptic cleavage characteristics. Many protease inhibitors...
were found to be ineffective against HD1, although it was partially inhibited by α2-macroglobulin, TPCK, and benzamidine.

The level of plasma albumin found in cyst fluid has been very variable. Haagensen (17), reported that GCDFP-70 was immunologically identical to plasma albumin, but exists at concentrations 25- to 100-fold lower than that found in plasma. He attributed this low level to a high degree of impermeability of the cyst fluid well to plasma transudate. Yap et al. (16) also studied cyst fluid proteins and reported that their concentrations were very variable and could differ by up to 15-fold. Albumin levels in 96 cyst fluids ranged from 0.2 to 4.2 g/liter. Concentrations were very variable and could differ by up to 15-fold. Albumin levels in 96 cyst fluids ranged from 0.2 to 4.2 g/liter. The level of plasma albumin found in cyst fluid has been very variable. Haagensen (17), reported that GCDFP-70 was immunologically identical to plasma albumin, but exists at concentrations 25- to 100-fold lower than that found in plasma. He attributed this low level to a high degree of impermeability of the cyst fluid well to plasma transudate. Yap et al. (16) also studied cyst fluid proteins and reported that their concentrations were very variable and could differ by up to 15-fold. Albumin levels in 96 cyst fluids ranged from 0.2 to 4.2 g/liter. Concentrations were very variable and could differ by up to 15-fold. Albumin levels in 96 cyst fluids ranged from 0.2 to 4.2 g/liter.

Attempts to discover the origin of cyst fluid based upon analysis of their protein content assumed that the capsule is relatively impermeable and that the proteins found are stable, once they are secreted into the capsule. In view of our discovery of the presence of active proteases in the cyst fluid, it is likely that those proteins found were either recently secreted into the cyst capsule, or are nondiffusible remnant peptides with bonds which are resistant to further protease action. It is thus important to reexamine the variable levels of albumin and all other cyst fluid proteins in previous studies in terms of possible losses and structure modification due to protease activity.

If the osmotic pressure within a small, relatively impermeable cyst was isosmotic initially, protease action would create additional peptide fragments resulting in increased oncotic pressure. This would cause an enlargement of the cyst cavity as water entered to reestablish osmotic equilibrium. Thus the rate of cyst fluid formation and enlargement may result from an imbalance between protease and protease inhibitors in the cyst fluid. In human plasma, at least eight different proteinase inhibitors have been identified which constitute 10% of all the circulating protein. While α1-protease inhibitor did not protect against HD1, α2-macroglobulin was very effective. The large molecular weight of α2-macroglobulin (Mr, 720,000) however, may impede its passage into the cyst capsule. This simple mechanistic explanation of how cysts enlarge does not address the question of the origin of the proteases.

It appears as if a 17-fold increase in specific activity has been achieved through the purification procedure when the values for HD1 and original cyst fluid are compared (Table 1). However, many problems can be anticipated if specific activities are compared by the methods used in this study. When large amounts of other potential substrates are present in the incubation mixture, as in the original cyst fluid and fraction S2, the radioactive albumin is effectively diluted and the apparent specific activity would appear to be lower than expected. There is also the possibility that protease inhibitors are present in the mixture which may then be separated away at some stage of the purification process, which would also lead to an apparent increase in specific activity.

Several lines of evidence point to the possibility that protease HD1 and GCDFP-24 are the same substance or coelute together. When purified GCDFP-24, a gift from Dr. Haagensen, was tested for protease activity, it was found to have essentially the same specific activity as HD1 (data not shown). Double immunodiffusion revealed that a polyclonal antibody prepared against GCDFP-24 by Dr. Haagensen, cross-reacted with HD1, HD2, HD3, HD4, and HD5.

HD1 and GCDFP-24 are isolated under similar conditions and have similar molecular weights. The small differences (Mr, 110,000 versus 120,000) can easily be accounted for by minor differences in experimental technique. Submit molecular weights are also similar, about Mr, 24,000.

There is both direct and indirect evidence that proteases are involved in the cancer process. Epidemiological evidence points to the fact that populations which consume large amounts of seeds, containing protease inhibitors, have lower incidence of breast, prostate and colon cancer (25). Protease inhibitors have been shown to block tumor promotion in mouse skin (26) and to reduce the incidence of breast cancer in irradiated mice (27). Metastasizing cells have been shown to produce a variety of proteolytic enzymes (28). Breast tumor explants have been shown to release up to 11 times more cathepsin B than normal breast tissue explants. Other studies have shown that hydrolytic enzymes on the surfaces of malignant cells greatly influence their growth properties and invasive potential (29–30). From these considerations and in view of the increased incidence of breast cancer among women who have had gross cystic breast disease, we must consider the possibility that the cyst fluid proteases may be involved in both the etiology of cyst formation and the tumorigenic process.

REFERENCES

PROTEASES IN HUMAN BREAST CYST FLUID


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