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REACTION OF 4-METHYLUMBELLIFERYLGUANIDINOBENZOATE WITH PROTEASES IN HUMAN AMNIOTIC FLUID

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ABBREVIATIONS

arginine esterase:	AE
bovine serum albumin:	BSA
cystic fibrosis:	CF
4-methylumbelliferylguanidinobenzoate:	MUGB
(p-nitrophenyl)-p'-guanidinobenzoate-HCl:	NPGB
4-methylumbelliferone:	MU
α_2-mscroglobulin:	α_2M
α-N-benzoyl-L-arginine ethyl ester:	BAEE
soybean trypsin inhibitor:	STI
1-chloro-3-tosylamido-7-amino-2-heptanone:	TLCK
p-hydroxymercuribenzoic acid:	HMB
α-N-benzoyl-DL-arginine-β-naphthylamide:	BANA
N-acetyl-L-tyrosine ethyl ester:	ATEE
trichloroacetic acid:	TCA

SUMMARY

An arginine esterase activity similar to that observed in plasma has been demonstrated in second trimester and term human amniotic fluid. Like plasma, the protease(s) hydrolyzed esters of arginine, were reactive towards 4-methylumbelliferylguanidinobenzoate (MUGB), a sensitive active site titrant of trypsin-like enzymes, and had a pI of 5.1-5.4. The pH optimum for proteolytic activity was 8.0. This protease activity was inhibited by soybean trypsin inhibitor (STI), benzamidine and (p-nitrophenyl)-p'-guanidinobenzoate (NPGB), and was insensitive to 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK) and p-hydroxymercuribenzoic acid (HMB). Upon gel filtration, two MUGB-reactive fractions were observed, one with an apparent molecular weight of 200,000 and the other, 100,000. Both fractions had arginine esterase activity and appeared to be sensitive to inhibition by STI and benzamidine.

The mean MUGB titre value (nmoles of 4-methylumbelliferone released per ml amniotic fluid) for 300 mid-trimester amniotic fluids was 11.40 ± 2.40 nmoles MU/ml. The mean specific activity was 2.36 ± 0.41 nmoles MU/mg protein. Two amniotic fluids from pregnancies which delivered children with cystic fibrosis (CF) were analyzed in blind samples sent from other laboratories. The MU titre values obtained were 4.73 and 4.32 with specific activities of 1.24 and 1.30 respectively. A third was identified in our screening program of amniotic fluids obtained from amniocenteses done for the intrauterine detection of genetic abnormalities. The MU titre value was 5.52 nmoles/ml with a specific activity of 1.34. The specific activities of these fluids when compared to the controls were significantly different ($p<0.001$).

The mean titre value for 23 term amniotic fluids samples was 8.14 ± 1.69 nmoles MU/ml. The mean specific activity was 3.37 ± 0.76 nmoles MU/mg protein. A term amniotic fluid obtained from a woman who delivered a baby with CF showed a markedly reduced level of MUGB reactivity (3.01 nmole/ml). The specific activity was 1.06 which was significantly different from the control term fluids.

The MU titre values and specific activities of amniotic fluids obtained from abnormal pregnancies (such as those with neural tube defects, chromosomal abnormalities and polymorphisms, abortions and stillbirths) and fluids with elevated alphafetoprotein and maternal blood contaminants did not significantly vary from the mean control values (Table 3).

Speculation

The reduced MUGB reactivity of proteases in mid-trimester amniotic fluid from women who subsequently delivered children with cystic fibrosis suggests that these MUGB-reactive proteases may prove useful as a marker for the prenatal diagnosis of cystic fibrosis.

INTRODUCTION

Previous studies have shown that saliva and plasma of patients with cystic fibrosis is deficient in the proteolytic hydrolysis of arginine esters (11-13). Recently, MUGB, a sensitive active site titrant of many serine proteases (3,6, 7) was utilized to demonstrate this deficiency in chloroform and ellagic acid activated as well as catalytically inactive plasma (15,20). Statistically significant differences in the extent of protease-MUGB reactivity were found when plasma of patients with CF, obligate heterozygotes and control samples were compared (15,20). Similar results have been reported when cultuvated human skin fibroblasts were examined (19). These studies suggested that the deficiency in arginine esterase activity in cystic fibrosis might be the result of a reduced number of protease molecules rather than or in addition to decreased catalytic efficiencies.

In these studies, correction for the non-specific hydrolysis of MUGB in crude systems was accomplished by carrying out the reaction in the presence and absence of benzamidine, an efficient competitive inhibitor of trypsin-like enzymes (5,20).

Because of the observations outlined above, and the potential value of amniotic fluid for the intrauterine detection of CF, we have extended these studies to human amniotic fluid (17,18). This study demonstrates the presence of MUGB-reactive proteases in human amniotic fluid obtained during the second trimester, between 14 and 17 weeks gestation, and term, between 35 and 40 weeks gestation. In addition, the MUGB-reactive class of arginine esterase-like proteases in amniotic fluid behaves similar to those described for plasma (11-13,15,20) in their catalytic and isoelectricfocusing properties and molecular weights.

Materials and Methods

MUGB, α-N-benzoyl-L-arginine ethyl ester (BAEE), TLCK, HMB, aldolase (rabbit muscle), blue dextran, casein, cytochrome C, ferritin (horse spleen), lysozyme (egg white), ovalbumin, peroxidase (horseradish), protamine sulfate (Grade I, salmon), soybean trypsin inhibitor (Type 1-S) were from Sigma Chemical Company (21); bovine serum albumin was from Armour Pharmaceutical Company (22). Catalase (beef liver) and benzamidine·HCl were purchased from Aldrich Chemical Company, Inc. (23). NPGB was from Nutritional Biochemical Corporation (24). Bio-Gel A 1.5m (110-200 mesh) and Bio-Gel A 0.5m (100-200 mesh) were from Bio-Rad Laboratories (25). Ampholines were obtained from LKB (26). Rabbit antiserum to human α_2M was purchased from Behring Diagnostics (27). All other chemicals were reagent grade.

Second trimester amniotic fluid was available from amniocenteses performed for the intrauterine detection of genetic abnormalities. An aliquot of each specimen was centrifuged at 3000 rpm for 10 minutes at 4°C to separate the cells and the clear supernatant stored at -20°C if not used immediately. Term fluids were handled accordingly. Gestational dating was calculated from the last menstrual period and confirmed by ultrasound. A total of 300 specimens, from between 35 and 40 weeks gestation were analyzed. The outcome of pregnancy was unknown at the time of MUGB and/or gel analysis.

Procedures for the activation of arginine esterases by treatment with chloroform and ellagic acid and the separation of arginine esterases by isoelectricfocusing have been previously described (12,13) as has the use of the proteolytic inhibitors, STI (110 µg/ml), HMB (0.10 mM and 1.0 mM), TLCK (0.10 mM and 1.0 mM and NPGB (0.10 mM) (13). Proteolytic assays using BAEE were performed as described earlier (11,12). The protamine sulfate assay for proteolytic activity was performed essentially as previously described (13). Amniotic fluid samples were dialyzed for two hours at 4°C against 10 mM Na_2HPO_4, pH 8.3. The reaction mixture contained 1.0 mg dinitrophenylated protamine, 0.05 ml dialyzed amniotic fluid, and either 0.4 mg/ml STI or 67 mM benzamidine (when needed) in either 0.05 ml 0.1 M Na_2HPO_4 and 0.15 M NaCl, pH 6.5-8.5 at increments of half pH units or 0.05 ml 0.1 M veronal·HCl and 0.15 M NaCl, pH 8.0-10.0 at increments of half pH units. After incubation at 37°C for one hour, the reaction was stopped by the addition of 0.10 ml 10% trichloracetic acid (TCA) and centrifuged. Fifty microliters of the supernatant were mixed with 1.0 ml of 0.10 M boric acid-NaOH buffer, pH 9.18 and 0.20 ml fluorescamine (0.1 mg/ml) in dry acetone was added with vigorous mixing. Fluorescence was determined at 390 nm excitation and 470 emission. The proteolytic activity was taken as the difference in fluorescence of an aliquot of the zero time blanks (to which 0.10 ml 10% TCA was added to the initial reaction mixture before incubation at 37°C) and an aliquot taken at the end of the incubation period. The fluorescence was converted to the equivalent of L-arginine used as an arbitrary standard. The amount of proteolytic activity was expressed as micro-equivalents of L-arginine formed per hour milliliter amniotic fluid.

The reaction of MUGB with amniotic fluid proteases was performed according to Jameson et al (6). Twenty µl of amniotic fluid and 20 µl of distilled water or 0.2 M benzamidine was incubated for 2 hours with 20 µl of 0.20 mM MUGB in 0.10 M veronal·HCl, pH 8.3 with 4% dimethylsulfoxide (DMSO) at room temperature in the dark. The reaction was stopped by the addition of 2 ml of the veronal buffer and the amount of MU released was determined fluorometrically using an Aminco-Bowman spectrofluorometer. Excitation was at 365 nm and emission at 450 nm. All titrations were done in triplicate. In order to correct for the spontaneous liberation of MU, blanks containing 20 µl of distilled water, 20 µl distilled water or 67 mM benzamidine and 20 µl of 0.20 mM MUGB in 0.10 M veronal·HCl; pH 8.3 with 4% DMSO were included. In a typical experiment, the fluorescence values obtained when a second trimester amniotic fluid sample was reacted with MUGB under our specific reaction conditions in the absence of benzamidine corresponded to 20.5 nmoles MU per ml and 7.4 nmoles MU per ml in the presence of 67 mM benzamidine·HCl. The difference (13.1 nmoles MU per ml) is taken as the corrected MU titre value for the MUGB-reactive proteases in amniotic fluid.

Since these assays are not conventional rate assays, but are similar to active site titrations in that the enzyme is probably not regenerated following its action on MUGB and release of MU (1-3,7), activity is expressed as MU titre value (nmoles MU per ml amniotic fluid) or as specific activity (nmoles MU per mg protein), and not as MU formed per unit time. Protein was determined according to the method of Lowry et al (8) using BSA as standard.

For molecular weight determinations of the MUGB-reactive proteases in amniotic fluid, five ml of concentrated (x4) amniotic fluid (approximately 18 mg protein per ml) were placed either on a Bio-Gel A 1.5m or Bio-Gel A 0.5m column (1.5 cm x 34 cm) equilibtaed with 10 mM Na_2HPO_4 buffer, pH 7.67. Samples were chromatographed either at 4°C or at room temperature. Blue dextran (M.W. 2,000,000), catalase (M.W. 235,000), hemoglobin (M.W. 66,000) and peroxidase (M.W. 44,000) were run as markers. Seven ml fractions were collected using a Gilson Micro Fractionator (Model FC-80K). Fractions were analyzed for arginine esterase activity in the presence and absence of STI (12,13,15,18) and also for MUGB reactivity. Pooled fractions were concentrated either by dialysis against 60% sucrose or by ultrafiltration at 4°C and tested for cross-reactivity towards antiserum to α_2-macroglobulin by double gel diffusion (9), immunoelectrophoresis and single radial immunodiffusion (16).

Proteases in amniotic fluid were separated by isoelectricfocusing on 5% polyacrylamide gels containing 0.75% ampholine (pH range of 5-7) and were stained for arginine esterase activity using BAEE and alcohol dehydrogenase at pH 9.2 as previously described (12). Parallel gels were immersed in 0.20 mM MUGB in 0.1 M veronal·HCl, pH 8.3 with 4% DMSO for 10 minutes in the dark and were then extensively washed with the veronal buffer.

Data were analyzed for statistical significance by the Student's "t" test.

RESULTS

A number of parameters governing the reaction of MUGB with amniotic fluid proteases were studied. As observed with plasma (15), the fluorescence yield was lower in phosphate and veronal buffers at pH 7.67 than at pH 8.3. Thus, all titrations were carried out at pH 8.3 in 0.1 M veronal·HCl buffers.

Standardization of Assay Conditions

Amniotic fluid specimens contain activity hydrolyzing MUGB. This reaction was complete in one hour (Figure 1). The liberation of MU was proportional to amniotic fluid concentration over a two hour period and independent of MUGB concentration (0.025 mM-0.20 mM). This activity towards MUGB was lost when amniotic fluid samples were boiled.

Proteolytic Inhibitors and MUGB Reactivity

The reaction of amniotic fluid with MUGB was inhibited by the presence of 67 mM benzamidine (Table 1) and NPGB (0.10 mM), suggesting that MUGB was reacting at the active site(s) of these enzymes. In contrast, there was no inhibition of the reaction of MUGB with amniotic fluid samples per-incubated with TLCK (0.10 mM-1.0 mM) or HMB (0.10 mM-1.0 mM).

Effect of Centrifugation and Dialysis on MUGB Reactivity

Centrifugation of the amniotic fluid specimens at 3000 rpm for 10 minutes at 4°C had no effect on the level of MU liberated. Aliquots of both control and

centrifuged samples were found to react to the same extent with MUGB with little or no change in mg protein/ml or specific activity.

Dialysis of amniotic fluid specimens against 10 mM Na_2HPO_4, pH 7.67 for four hours at 4°C had no effect both on the amount of protein per sample (mg/ml) or the extent of MUGB-protease reactivity.

Stability of the MUGB-Reactive Species

The MUGB-reactive proteases in amniotic fluid have been found to be stable when stored at -20°C, following repeated freeze-thaws, and when kept either at 4°C on ice or room temperature (approximately 22°C) for the periods examined (up to 72 hours).

Hydrolysis of Arginine Substrates by Amniotic Fluid Proteases

When proteolytic activity was investigated in amniotic fluid specimens, an activity was observed similar to that described in plasma (15,18) but at a very much reduced level. Amniotic fluid samples were found to have small levels of BAEE hydrolytic activities (0.04-0.90 µmoles BAEE hydrolyzed/hr/ml amniotic fluid) using a spectrophotometric assay. When proteolytic activity was assayed using dinitrophenylated protamine sulfate (13), the range of values was 0.05-0.09 (in µ equivalents of arginine formed/hr/ml amniotic fluid). Although this activity is low, the values were reproducible within 5%. The activity was linear over a two hour incubation and proportional to amniotic fluid concentration. The initial rate of hydrolysis of protamine sulfate as a function of pH is given in Figure 2. Maximal proteolytic activity and inhibition by both STI (0.4 mg/ml) and benzamidine (67 mM) occurred at pH 8.0. At this pH, proteolytic activity was inhibited approximately 50% with benzamidine and by approximately 30% with STI.

Effect of Chloroform and Ellagic Acid Treatment on Amniotic Fluid Proteases

In contrast to plasma which has negligible catalytic activity towards BAEE and other classical substrates such as α-N-benzoyl-DL-arginine-β-naphthylamide (BANA) and N-acetyl-L-tyrosine ethyl ester (ATEE) until activated with chloroform and ellagic acid (3 mg/100 ml distilled water), similar treatment of amniotic fluid samples had no effect on increasing the low levels of BAEE or protamine sulfate catalysis observed prior to this treatment. The extent of MUGB reactivity remained the same for both controls (untreated) and chloroform and ellagic acid treated samples, similar to the observation in plasma (15,18).

Isoelectricfocusing of Amniotic Fluid Samples

Isoelectricfocusing experiments of amniotic fluid samples between pH 5-7 on 5% polyacrylamide gels and subsequent staining with MUGB (0.20 mM) yielded at least two fluorescent bands of activity, with a pI of 5.1-5.4. Parallel gels could also be stained for arginine esterase activity using BAEE and alcohol dehydrogenase at pH 9.2 similar to that reported for plasma (12,15). The MUGB-reactive bands and BAEE-alcohol dehydrogenase activity bands were found to be in identical locations in parallel gels. The gel patterns obtained from both second trimester and term amniotic fluids appeared similar.

Fractionation of Second Trimester and Term Amniotic Fluids on Bio-Gel A 0.5m Columns

Two MUGB-reactive peaks were obtained when either a second trimester or term amniotic fluid samples were chromatographed on Bio-Gel A 0.5m columns. One peak had a molecular weight of approximately 200,000 and the other approximately 100,000 (Figure 3). The fractions from each peak were pooled and concentrated either by ultrafiltration or against 60% sucrose at 4°C. Proteolytic activity towards protamine was observed for each peak. This activity was inhibited by both STI and benzamidine by approximately 50%. There was no evidence of α_2-macroglobulin in either of the two concentrated peaks when evaluated by double gel diffusion, immunoelectrophoresis, and single radial immunodiffusion.

Quantitation of Second Trimester and Term Amniotic Fluid with MUGB

Three hundred amniotic fluids were analyzed for MUGB reactivity (mean ± one standard deviation). The control mean MUGB titre value (nmoles MU/ml) obtained was 11.40 ± 2.40 range of 6.50-20.20 (Table 2). The mean protein concentration was 4.79 ± 1.12 mg/ml (2.29-8.83); the mean specific activity was 2.36 ± 0.41 (1.80-3.79). Two mid-trimester fluids from pregnancies which delivered children with cystic fibrosis were identified out of a group of coded samples sent from other laboratories. The MU titre values obtained were 4.73 and 4.32 nmoles MU/ml with specific activities of 1.24 and 1.30 respectively. A third was identified following screening of amniotic fluids from amniocentesis performed at our institution for the prenatal detection of genetic abnormalities. The MU titre value for this fluid was 5.52 nmoles/ml with a specific activity of 1.34. The specific activity values for the three CF fluids are significantly different from the controls ($p<0.001$).

Twenty-three amniotic fluids (from normal pregnancies in which the outcome of delivery was known) were analyzed for MUGB reactivity (Table 2). The mean MU titre value (nmoles MU/ml) obtained was 8.14 ± 1.69, range of 4.98-10.94. The mean protein concentration for these samples was 2.52 ± 0.68 mg/ml (1.59-4.11); the mean specific activity was 3.37 ± 0.76 (2.05-4.83). A term amniotic fluid obtained from a woman who delivered a child with CF was found to have a markedly reduced MU titre value (3.01) and specific activity (1.06) when compared to the control term fluids ($p<0.001$).

Mixing experiments were performed with second trimester and term amniotic fluids; expected MU titre levels were obtained.

MUGB reactivity has also been documented in mid-trimester amniotic fluids from pregnancies which were abnormal or were contaminated with maternal blood (Table 3). The MU titre values and specific activities were not significantly different from the control samples.

DISCUSSION

These data indicate that amniotic fluid contains MUGB-reactive proteases which appear to have certain properties in common with the arginine esterases in plasma (12,13,15,20). The presence of material in amniotic fluid which behaves like a proteolytic enzyme(s) is suggested by the following: a) hydrolytic activity towards the L-arginine substrates, b) susceptibility to protease inhibitors, c) responsibeness to changes in pH, d) resolution of hydrolytic activity following isoelectricfocusing, and e) susceptibility to boiling. More specifically, the proteases in amniotic fluid were found to hydrolyze both BAEE and dinitrophenylated protamine, were inhibited by benzamidine, a competitive inhibitor of serine proteases (5,20), NPGB, an active site titrant of trypsin (4, 20), and STI, a sensitive inhibitor of arginine esterases (11-13,15,20). There was no inhibition by either TLCK, a chloroketone analog of L-lysine which inhibits many proteolytic enzymes such as thrombin and plasmin by a stoichiometric reaction at the active site(s), or HMB, a thiol group reagent and a known inhibitor of thiol proteases such as cathepsin B (13). The pH optimum for maximal proteolytic activity was pH 8.0; maximal inhibition of protease activity was found at this pH with 50% inhibition by benzamidine and approximately 30% inhibition by STI. These amniotic fluid proteases have a pI of 5.1-5.4 upon isoelectricfocusing. All of these observations suggest that the MUGB-reactive proteases found in amniotic fluid are similar to those described for plasma (Table 4).

There are, however, several differences between amniotic fluid and plasma proteases. It has been well-established that the plasma arginine esterases are in "zymogen" form (catalytically inactive, but MUGB-reactive), and require treatment with chloroform and ellagic acid for expression of hydrolytic activity towards BAEE and other classical substrates (12,13,15,20). All attempts to increase the relatively low levels of proteolytic activity in amniotic fluid by similar activation procedures were unsuccessful. Two molecular weight MUGB-reactive species were isolated upon gel filtration, one with a molecular weight of approximately 200,000, and the other, approximately 100,000. In contrast, plasma contains only one MUGB-reactive peak (15,20) similar to the lower molecular weight species found in amniotic fluid. Amniotic fluid more closely resembled unactivated plasma rather than activated plasma in that α_2-macroglobulin could not be detected in the MUGB-reactive fractions by several immunological techniques. The failure to detect α_2-macroglobulin may reflect the extremely low levels in amniotic fluid (3 µg/ml amniotic fluid vs. 3 mg/ml plasma).

The reaction of MUGB with the amniotic fluid proteases was proportional to amniotic fluid concentration, independent of MUGB concentration, and gave a burst of MU at the early stages of the reaction (Figure 1). These characteristics are similar to the criteria established for "active site titration" of pure enzymes (1,6). Because of the evidence that MUGB is reacting as a titrant, the data are expressed as nmoles of MU formed/ml amniotic fluid and nmoles of MU formed/mg protein rather than as nmoles of MU formed/ml amniotic fluid/unit time. In addition, the activity apparently requires active sites as boiled samples lost their MUGB reactivity. The identical migration (pI) of MUGB-reactive and BAEE-reactive material upon isoelectricfocusing as well as the correlation of proteolytic activity and MUGB reactivity upon fractionation by gel filtration strongly suggest that this active site titrant is directly measuring the levels of arginine esterase(s) in amniotic fluid.

Both second trimester and term amniotic fluids were similar in their catalytic activities towards the arginine ester substrates and in their reactivity towards MUGB. They differed in the level of nmoles MU formed/ml in that the second trimester fluids gave a higher mean MU titre value, 11.40 nm/ml vs. 8.14 nm/ml although the total protein was lower in the term fluids. Isoelectricfocusing experiments have indicated that the pI is the same, 5.1-5.4, for both second trimester and term fluid arginine esterase-MUGB reactive proteases, and the gel banding patterns tended to resemble plasma for both BAEE hydrolytic activity and MUGB reactivity.

There are several sources from which enzymes may be derived in amniotic fluid such as fetal urine, fetal saliva, release upon destruction of sloughed cells, and maternal transudates. It cannot be determined for these experiments which combination or specific source is the major contributor of the amniotic fluid proteases which react with MUGB (2,17,18).

These observations, in addition to suggesting a potential marker for CF in utero, also have relevance to the pathogenesis of the disease. A reduced level of proteolytic activity of the type described here is a generalized phenomenon associated with CF since saliva (11), plasma (12,13,15,20) and cultivated skin fibroblasts (19) from patients with CF manifest this aberration. We have postulated earlier that the depression of proteolytic activity results in an elevation of basic polypeptides (14) which have an enhancing effect on the UDP-galactose:glycoprotein galactosyl transferase activity resulting in increased synthesis of glycoproteins and secretion (10). The increased levels of glycoproteins in mucus secretion may cause the formation of mucus plugs upon chelation with calcium. Therefore, it is possible to construct a hyopthetical model for the pathogenesis of CF starting from a generalized deficiency of proteolytic activity (14).

CONCLUSION

Based on previous work documenting the reduced levels of MUGB-reactive material in plasma (15,20) and cultivated fibroblasts (19), we are now attempting to document the potential usefullness of MUGB-reactive material in amniotic fluid as a method for the prenatal diagnosis of CF (17,18). Examinations of three mid-trimester and one term amniotic fluid specimens obtained from women who delivered babies with cystic fibrosis have indicated a significantly reduced level of MUGB-reactive material when compared to controls ($p<0.001$) suggesting that the amniotic fluid protease activity may indeed be of primarily fetal origin and may also be useful as a marker for the prenatal detection of cystic fibrosis. Additional studies with more samples will be required to confirm this observation.

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Table 1. Effect of Four Proteolytic Inhibitors on the Reaction of MUGB with Amniotic Fluid.

Inhibitor		Total MU titre value*	Corrected MU titre value*
-		37.71	15.23
Benzamidine	(67 mM)	15.23	15.23
NPGB	(0.1 mM)	15.68	15.65
TLCK	(1.0 mM)	32.78	15.56
HMB	(1.0 mM)	35.35	14.43

*nmoles MU/ml amniotic fluid

The indicated concentrations of the four inhibitors are final concentrations in a total reaction volume of 0.080 ml. The control reaction mixture contained 20 µl of amniotic fluid, 40 µl distilled water and 20 µl of 0.20 mM MUGB in 0.1 M veronal·HCl, pH 8.3 with 4% DMSO. Reactions carried out in the absence of 67 mM benzamidine contained 20 µl of amniotic fluid pre-incubated with 20 µl of the inhibitor (NPGB, TLCK or HMB), 20 µl of distilled water and 20 µl of 0.20 mM MUGB. Reactions carried out in the presence of 67 mM benzamidine contained 20 µl amniotic fluid pre-incubated with 20 µl of an inhibitor (NPGB, TLCK or HMB) for 10 minutes to which was added 20 µl benzamidine and 20 µl of 0.20 mM MUGB. Appropriate blanks not containing amniotic fluid were included to correct for the spontaneous liberation of MU.

Table 2. MU titre Values and Specific Activities of Mid-trimester and Term Control and CF Amniotic Fluids.

	nmoles MU/ml*	Specific Activity*
Midtrimester:		
Controls (N=300)	11.40 ± 2.40	2.36 ± 0.41
CF m	4.32	1.30
CF m	4.73	1.24
CF m	5.52	1.34
Term:		
Controls (N=23)	8.14 ± 1.69	3.37 ± 0.76
CF t	3.01	1.06

* Mean ± S.D.

m mid-trimester amniotic fluid

t term amniotic fluid

See Materials and Methods section for assay conditions.

Table 3. MUGB Reactivity in Mid-trimester Amniotic Fluids Obtained from Control and Abnormal Pregnancies.

Outcome	Number	nmoles MU/ml*	Specific Activity*
Control	300	11.40 ± 2.40	2.36 ± 0.41
neural tube defect	5	11.03 ± 0.65	2.54 ± 0.27
chromosomal abnormality	8	12.40 ± 3.06	2.45 ± 0.23
abortions-stillbirths	4	12.05 ± 0.75	2.27 ± 0.13
elevated α-fetoprotein	5	12.54 ± 1.85	2.37 ± 0.21
chromosomal polymorphisms	12	10.73 ± 1.96	2.39 ± 0.37
maternal blood contamination	15	12.36 ± 2.39	2.25 ± 0.39

* Mean ± S.D.

See Materials and Methods section for assay conditions.

Table 4. Properties of MUGB-reactive Proteases Common to Plasma and Amniotic Fluid.

	Plasma	Amniotic fluid
MU titre value nmoles MU/ml	127.2 ± 23.1*	11.4 ± 2.4*
Specific activity (nmoles MU/ml/mg protein)	1.82**	2.36 ± 0.4
Molecular weight	90,000	200,000 100,000
Substrates hydrolyzed	BAEE, BANA, TAME, Protamine sulfate	BAEE, BANA, TAME, Protamine sulfate
pH optimum	7.6	8.0
Inhibitors	STI, Benzamidine·HCl NPGB	STI, Benzamidine·HCl NPGB
Compounds without an inhibiting effect	HMB, TLCK	HMB, TLCK
Isoelectricpoint (pI)	5.1-5.4	5.1-5.4

* Mean ± S.D.

** Based on an average 70 mg protein/ml plasma

For details see Discussion section.

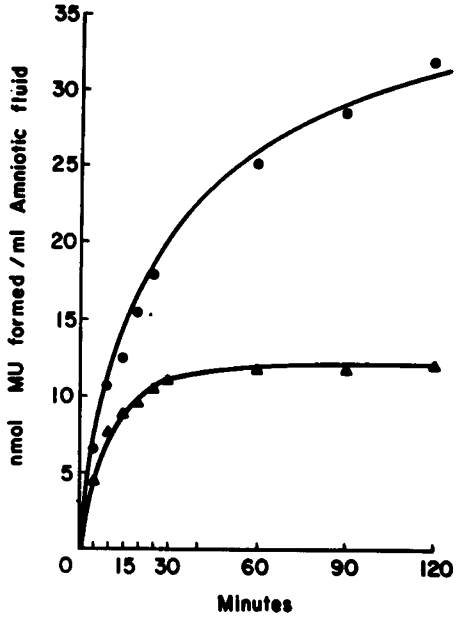

Figure 1.

Reaction of Amniotic Fluid Proteases with MUGB.

Time course of the reaction between MUGB and proteases in amniotic fluid in the absence of (●—●—●) and in the presence of (▲▲▲▲) benzamidine. The difference between the two curves is the contribution of the non-specific hydrolysis of MUGB to the reaction. See Materials and Methods section for assay conditions.

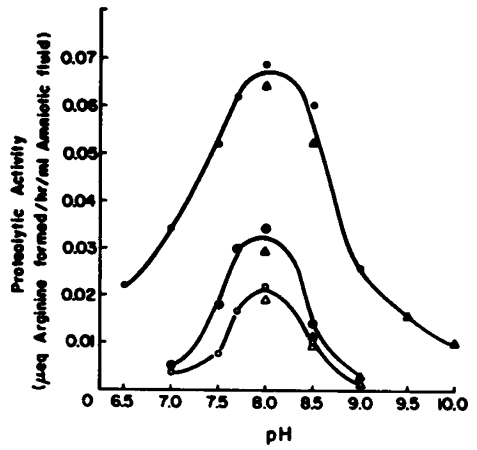

Figure 2.

Proteolytic Activity in Amniotic Fluid as a Function of pH.

pH profile of protamine sulfate activity was obtained using two buffer systems, 0.10 M Na_2HPO_4 and 0.10 M veronal·HCl. Circles represent values obtained with the phosphate buffers, triangles with veronal buffers. Upper curve represents values obtained in the absence of inhibitors, i.e., total proteolytic activity. Middle curve represents proteolytic activity inhibited by benzamidine (67 mM) and lower curve represents proteolytic activity inhibited by STI (4 mg/ml). See Materials and Methods section for assay conditions.

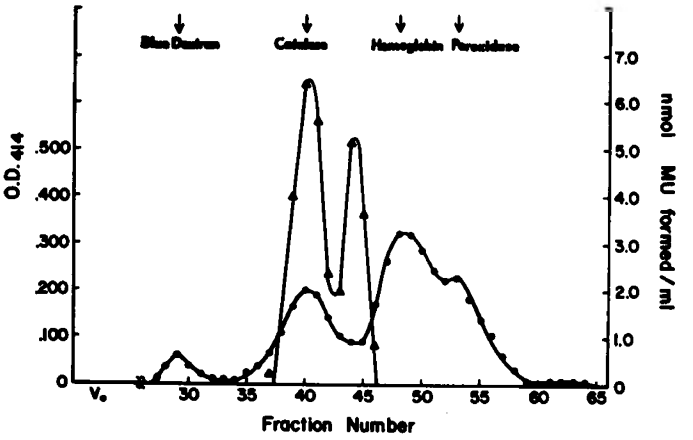

Figure 3.

Elution Profile of Second Trimester Amniotic Fluid Obtained From Bio-Gel A 0.5m Chromatography.

Five ml of concentrated amniotic fluid (approximately 18 mg protein/ml) were applied to a Bio-Gel A 0.5m column. Each fraction was tested for MUGB-reactive proteases (▲▲▲). The OD_{414} profile (●—●—●) of the molecular weight markers eluted from the column is given; the peaks from left to right are: blue dextran (M.W. 2,000,000), catalase (M.W. 235,000), hemoglobin (M.W. 66,000) and peroxidase (M.W. 44,000).

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