UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO

Colegio de Ciencias y Humanidades

Unidad Académica de los Ciclos Profesional y de Posgrado

Proyecto Académico de Licenciatura, Maestría y Doctorado en Investigación Biomédica Básica

ESTUDIO ACERCA DE LAS PROTEINASAS DE *Entamoeba histolytica*

Tesis que para obtener el grado de Maestro en Investigación Biomédica Básica

Presenta

PEDRO ULISES G. OSTOA SALOMA.

México, D.F. Enero de 1989

UNAM – Dirección General de Bibliotecas Tesis Digitales Restricciones de uso

ESTE TRABAJO SE REALIZO EN EL INSTITUTO DE FISIOLOGIA CELULAR BAJO LA DIRECCION DEL DR. RUY PEREZ-MONTFORT A QUIEN QUIERO AGRADECER LA OPORTUNIDAD QUE ME HA DADO , ASI COMO SU CONFIANZA, PACIENCIA Y AMISTAD.

INDICE

INTRODUCCION

En la nota preliminar a la bibliografía Mexicana del absceso hepático, que publicara Furnier en 1956, Fernandez del Castillo relata la llegada, en el año de 1611, del austero Don Fray García Guerra, arzobispo de México y Virrey de la Nueva España, quien falleció poco tiempo después de llegar a México, cuando estaban aún frescos los recuerdos de los festejos con los que se le recibió. El Sevillano Mateo Alemán, autor de Guzman de Alfarache, novela picaresca de los últimos años del siglo de oro de la literatura Española e introductor del Quijote a la Nueva España, hizo recuento detallado del mal del Virrey, quien padeció de " .....flaqueza de ánimo, congojas y algún poco de calor demasiado...". Para huir del trajin de la capital se refugió en Tacubaya, donde fue tratado por varios médicos, a pesar de lo cual la fiebre, el dolor en el hígado y el hecho de "..haberse corrompido por la parte interior espantosamente aquel absceso...", obligaron a que un Domingo a las 4 de la tarde abrieran a Su Ilustrísima, quien sobrevivió escasas dos semanas. En la autopsia "... hallaron en la parte cóncava de la punta del hígado, cantidad como de medio huevo, por donde se aliga con las costillas, por las materias que le acudían de aquel lado ya podrido... " (1)

Seguramente, el mal que causó la muerte a Don Fray García Guerra, es el mismo mal que actualmente causa la muerte a aproximadamente, entre 40,000 y 110,000 personas anualmente en el mundo; es el mismo mal que en nuestro país es un problema de salud grave, el mal causado por una de las células procariontes más primitivas, por el Protozoario : Entamoeba histolytica

CICLO DE VIDA

E. histolytica es un protozoario de la clase *Rhizopoda* (Siebold, 1845) y del género *Entamoeba* (Leidy, 1879). Su ciclo de vida es complejo y en él se producen algunos cambios en la morfología del parásito; los más importantes desde el punto de vista médico son el quiste y el trofozoito o forma móvil. Las formas intermedias, el prequiste y el metaquiste, son parte del ciclo biológico. El ciclo comienza con una división binaria, que ha sido confirmada por la cinefotografía; con este método también se ha observado que algunas amibas se dividen de tiempo en tiempo por un mecanismo que recuerda a la conjugación, pero después de algunas divisiones, la motilidad de algunas amibas disminuye y los organismos muestran menos pseudopodos, se hacen redondos y más pequeños, con pérdida del material en su citoplasma. Este estadio se llama *prequiste*, y es seguido inmediatamente por la secreción de una gruesa membrana y de dos o tres divisiones adicionales del núcleo, transformándose en *quiste* nuevamente. En esta etapa las amibas son suceptibles a cambios de temperatura, pero en condiciones adecuadas de humedad pueden sobrevivir por periodos prolongados. Una vez que llegan al estómago, la cápsula del quiste es digerida por el jugo gástrico, el citoplasma se divide tantas veces como existan núcleos, y a veces un número mayor; este estadio se llama metaquiste. Estas pequeñas amibas llegan a la luz intestinal aumentan su tamaño y se vuelven *trofozoitos*, completando el ciclo biológico. (2)

En el estado de trofozoito, y en preparaciones teñidas, la amiba presenta una membrana delgada, el contenido celular claramente se divide en 2 porciones: una zona externa o exoplasma que es más clara y da lugar a seudópodos y una zona interna o endoplasma, finamente granular y con muchas vacuolas. Los estudios en microscopía electrónica demuestran que E. histolytica se caracteriza por la ausencia de la mayoría de los organelos encontrados en las células eucariontes como, por ejemplo, aparato de Golgi, retículo endoplásmico rugoso, mitocondrias, centriolos y microtúbulos. Actualmente , a pesar de la organización simple del parásito no es todavía posible separar aquellos componentes membranosos involucrados en la internalización y degradación de componentes externos, de aquellos relacionados con la síntesis, canalización, concentración, empaquetamiento y liberación de productos extracelulares. (2,3)

Sobre su metabolismo, se sabe que puede sobrevivir a bajas tensiones de oxígeno, aspecto que se confirma por su carencia de mitocondrias. E. histolytica degrada glucosa a piruvato vía la ruta de Embden-Meyerhof. El lactato no es su producto final y la Lactato Deshidrogenasa no ha sido detectada, el pirofosfato inorgánico reemplaza al ATP en reacciones de la glucólisis. La amiba presenta una respiración endógena cuyos productos finales son : acetato, etanol y CO_2 . La respiración se incrementa en presencia de glucosa, galactosa, L-serina y etanol; no hay evidencia de un ciclo de Krebs pero si de una cadena

respiratoria compuesta por acarreadores identificados como flavinas, proteínas no hémicas y proteínas hierro-azufre.(4)

MECANISMOS CITOPATOGENICOS

El estudio de E. histolytica se favoreció por la creación de un medio de cultivo axénico desarrollado por Diamond en 1968 (5) y perfeccionado por él en 1978 (6). A partir de entonces la investigación sobre la amiba se ha intensificado y comprende actualmente varios enfoques entre los que que se encuentran, principalmente , los relacionados a la investigación de los mecanismos citopatogénicos de la enfermedad y la posibilidad de contrarrestar tales mecanismos. El resto de los enfoques comprende el estudio de su biología celular, dado que la amiba, es un organismo que desde ese punto de vista es interesante y puede utilizarse como modelo para el estudio de varios procesos celulares, como por ejemplo la fagocitosis, pinocitosis y todo lo relacionado a la movilización de la membrana . Por su alta movilidad puede ser útil en el estudio del citoesqueleto .

Una de las preguntas fundamentales, que esta relacionada con la comprensión de la amibiasis humana, es la naturaleza de los factores qque provocan que de un estado no dañino (portador sano) la amiba se torne un agresivo invasor (amibiasis invasiva). El resolver esta cuestión no es fácil por el hecho de que la virulencia de la amiba varía ampliamente. Sin embargo se han

establecido algunos parámetros para evaluar la patogenicidad de varias cepas de E. histolytica en comparación con amibas que no son patógenas. De entre estas características destacan las siguientes (7):

1.- Presencia de lectinas en la membrana

2.- " " fosfolipasas en la membrana

3.- Generacion de un ameboporo

4.- Alta tasa de eritrofagocitosis

5.- Aglutinación con Concanavalina A

6.- Produccion de proteinasas

La presencia de adhemibas o lectinas fue reportada por primera vez por Kobiler y Mirelman en 1980 (8) ; estas lectinas pueden ser bloqueadas por oligosacaridos de la N-acetilglucosamina tales como quitriosa o tetraosa. Recientemente ha habido varios reportes sobre lectinas de E. histolytica. Se considera que todas ellas participan en la eritrofagocitosis y la adherencia de la amiba a las células del epitelio intestinal.(9)

Se ha reportado que la amiba, en contacto con la célula blanco, puede romper la membrana celular de esta; este efecto citoletal puede ser provocado por una fosfolipasa que la amiba presenta en su membrana (10,11). Otra posibilidad es que se exponga de manera espontanea en la membrana una proteina que puede formar canales sobre la célula blanco llamado ameboporo; el canal formado podria incrementar la permeabilidad al calcio en la célula blanco, lo que conduciria a una depolimerización de la tubulina y el relajamiento de las uniones intercelulares disociando asi el epitelio (12). Sin embargo, las evidencias que

apoyen esta hipótesis son indirectas. Además surgen multiples preguntas acerca de como el ameboporo es excretado, cual es la señal para llevar a cabo este mecanismo y como la amiba puede evadir su propio ameboporo.

Otro factor son las enzimas proteolíticas, las cuales pueden tener una actividad citotóxica y además pueden actuar sobre la matriz extracelular. (13)

LAS PROTEINASAS COMO MECANISMO CITOPATOGENICO

Las enzimas proteolíticas derivan su nombre por su capacidad para degradar sustratos protéicos. Barrett (14) clasificó a las enzimas proteolíticas en 2 grandes grupos: Exopeptidasas y Endopeptidasas. Las exopeptidasas o peptidasas, remueven o actúan en las regiones terminales de la proteína o el peptido y por lo tanto podemos encontrar 2 tipos: carboxipeptidasas y aminopeptidasas. Las endopeptidasas o proteinasas son aquellas que actúan sobre los enlaces peptídicos que no sean los terminales, su clasificación esta dada sobre la base de su mecanismo catalítico más que por su fuente, especificidad o función. En ese sentido se tienen 5 grupos de endopeptidasas: Proteinasas de Serina, Proteinasas de Cisteina , Proteinasas de Aspártico, Metaloproteinasas y Proteinasas no clasificadas.

Las proteinasas de Serina se caracterizan por la

presencia de una serina reactiva en el sitio activo ; el mecanismo catalítico de estas proteinasas involucra el enlace covalente del sustrato a ese residuo de serina. Las proteinasas de Cisteina contienen un residuo de cisteina que esta involucrado en un complejo covalente con el sustrato. Por su parte las de aspártico contienen 2 residuos de este aminoácido en sus centros activos que estan involucrados en la ruptura del enlace peptídico, en este caso el mecanismo se considera como una catalisis ácido base en lugar de la formación de un complejo covalente enzima sustrato como en las clases anteriores. Las metaloproteinasas contienen iones metálicos en su sitio activo cuyo papel es probablemente mejorar la nucleofilicidad del agua así como polarizar la ruptura del enlace peptídico antes que se dé el ataque nucleofílico a este. La última subclase acomoda a enzimas que no tienen la suficiente pureza como para permitirles una asignación catalítica o que claramente no encajan en algunas de las existentes, ya sea porque no son inhibidas por los inhibidores clásicos o porque pueden pertenecer a una nueva clase catalítica (15).

Investigaciones recientes(16) indican que las proteinasas de E. histolytica son importantes en la necrosis tisular. Actualmente no se tienen datos claros u homogeneos sobre las propiedades de estas proteinasas por lo que muchas preguntas, acerca de cuántas estan presentes y de como ellas contribuyen a la patogenicidad de las amibas, están todavía sin respuesta.

La actividad proteolítica de E. histolytica puede estar

relacionada con la virulencia de varias cepas cultivadas en condiciones axénicas(17-19)

Los primeros reportes acerca de las proteinasas de E. histolytica presentaron un panorama contradictorio sobre el papel de estas en la patogenicidad . Probablemente los resultados obtenidos en estas primeras investigaciones fueron sesgados por la presencia de otros organismos en el medio de cultivo. Reeves (20) encontró que la variedad de enzimas presentes en trofozoitos de cultivos xénicos fue diferente de aquellas encontrados en cultivos axénicos de E. histolytica.

McLaughlin y Faubert en 1977 (21) fueron los primeros en estudiar a las proteinasas de E. histolytica en un medio axénico. Estos investigadores reportaron la actividad de una proteinasa ácida con un peso molecular (p.m.) de 41,000 y un pH óptimo de 3.5 . Además encontraron otra actividad detectada a pH 6 que correspondió a una proteinasa de p.m. de 27,000 . Esta última fue inhibida por iodoacetamida y suero, siendo activada por cisteina y ditiotrietriol (DTT) .

Lushbaugh en 1979 (22) purificó parcialmente a partir de trofozoitos, una citotoxina de p.m. en el rango de los 30,000 que desprendió a células cultivadas en monocapa y las deformó.

Bos en 1979 y 1980 (23,24) confirmó la presencia de esta citotoxina pero obteniendo un p.m. entre 35,000 y 45,000. Además demostró que la actividad citotóxica era activada "in vitro" por agentes reductores y era inhibida por iodoacetamida y suero. Concluyó que este factor puede ser el responsable de la citotoxicidad de la amiba mediada por contacto. El autor correlacionó esta toxina con la proteinasa de bajo p.m. reportada

por McLaughlin y Faubert.

McGowan en 1982 (25) comparó la actividad citotóxica de de 4 cepas de E. histolytica que variaban en virulencia; encontró que habia una correlación directa entre el grado de virulencia y de actividad. Proteinas con p.m. de 59,000 , 66,000, 68,000 y 25,000 exhibieron actividad citotóxica que fue inhibida por suero, leupeptina y aprotinina .La exposisión a agentes reductores no incrementó la actividad de glucosaminidasa, catalasa, lipasa, gelatinasa y caseinasa.

Muñoz y colaboradores en 1982 (26) describiéron una actividad colagenolítica sobre todo hacia colagena tipo I, la cual no fue inhibida por N-etilmaleimida, pero si por cisteina, EDTA y suero; más adelante (19) midieron la actividad colagenolítica de varias cepas de E. histolytica y encontraron una correlación positiva.

Gadasi y Kessler en 1983 (17) reportaron también la presencia de una actividad colagenolítica en E. histolytica, sin embargo esta actividad si se activó con DTT y se inhibió con N-etilmaleimida. Tanto Muñoz como Gadasi mostraron que la actividad de colagenasa estaba relacionada con la membrana plasmática de la amiba. Lushbaugh (27) sugirió que la actividad detectada por Gadasi podría reflejar la presencia de una tiol proteinasa con actividad colagenolítica, en ese sentido habría 2 colagenasas de E. histolytica: una colagenasa típica y especifica (Muñoz) y otra con una actividad colagenolítica inespecifica (Gadasi).

Gadasi y Kobiler en 1983 (18) demostraron una actividad citotóxica de las enzimas proteoliticas al desprender células en

cultivo sobre un sustrato de colagena y fibronectina, responsabilizando a éstas también del redondeamiento de las células

Lushbaugh en 1984 (28) probó la actividad proteolítica y citotoxica de varias cepas de E.histolytica. Los homogenados de las cepas virulentas presentaron una mayor actividad que las menos virulentas. También purificó el factor con ambas actividades y obtuvo una proteina con un p.m. de 24,000 y con una similitud antigénica en todas las cepas. El autor concluyó que la citotoxina es una proteinasa neutra, que es inhibida por suero y que es más activa en trofozoitos de cepas más virulentas. En otro trabajo de ese mismo año (29) Lushbaugh profundizó más su estudio, inhibiendo la actividad citotóxica con p-Cloromercuribenzoato, iodoacetamida, 4-Bromofenacilbromuro (que son agentes que reaccionan con grupos tiol), con leupeptina y antipaina. La actividad se incrementó por agentes reductores. En condiciones ácidas la actividad fue inhibida por pepstatina, EDTA, calcio y mercaptoetanol. El p.m. que se reportó fue de 20,000 con un punto isoeléctrico (p.i.) entre 4 y 5.

Scholze y Werries en 1984 (30) reportaron una proteinasa con una alta actividad en condiciones ligeramente ácidas. Demostraron que es una tiol proteinasa con un p.m. de 21,000 y un p.i. de 4.9. Por el p.m. y por ser tiol proteinasa los autores la calificaron como catepsina B, pero por la actividad sobre algunos sustratos la clasificaron también como catepsina L. Estos autores en 1987 (31) demostraron la especificidad de la enzima utilizando insulina como sustrato. De los resultados que obtuviéron, se concluyó que el sitio de corte de esta proteinasa

esta entre el enlace Gly-Phe y que además es capaz de degradar colagena de una manera distinta a la colagenasa de los vertebrados.

Lushbaugh en 1985 (27) purificó por cromatografía de afinidad lo que el llamó la catepsina B de E. histolytica. El p.m. que reportó es de 16,000. El argumento principal para darle la categoría de catepsina B a su enzima, fue la acción de éste sobre un sustrato reportado como especifico para catepsina B (Z-Arg-Arg-AFC). Por la inhibición que obtiene de la actividad de ésta enzima con iodoacetamida y p-Cloromercuribenzoato la consideró como proteinasa de cisteina. Con esto y los resultados anteriores que ha tenido este autor, concluyó que es la catepsina B de E. histolytica la responsable de la habilidad de los trofozoitos para proliferar e invadir los tejidos, del redondeamiento de las células y su desprendimiento de los cultivos de monocapa "in vitro".

Keene en 1986 (32) aisló una proteinasa de E. histolytica con un peso molecular de 56,000. Consideró que ésta enzima es excretada por la amiba y de hecho hizo su purificación a partir del medio de incubación de los trofozoitos. Le atribuyó un papel en la patogenicidad del parásito por su capacidad de degradar un modelo de matriz extracelular, así como laminina, fibronectina y colagena tipo I. El autor consideró a esta enzima como la principal proteinasa de E. histolytica.

Gitler en 1986 (12) mencionó datos obtenidos por su grupo sobre la purificación, por medio de una cromatografía de afinidad, de tres proteinasas de E. histolytica con p.m. de

32,000, 26,000 y 16,000 con una actividad especifica muy alta. Estos resultados no han sido publicados.

Rosales-Encinas y Rojkind en 1987 (33) reportaron la purificación de la colagenasa de E. histolytica utilizando una columna de afinidad con colagena ; al analizar el eluido de la columna en un gel de electroforesis, encontraron 3 bandas con p.m. de 70,000 , 45,000 , y 27,000 de las cuales el solamente considera la de 70,000 como la colagenasa y las demás como productos de degradación.

En 1988 Luaces y Barrett (34) purificaron por medio de una cromatografía de afinidad, una proteinasa de cisteina con un p.m. de 26,000 a la que le llamaron histolisina. Este resultado se discutió a la luz de los resultados obtenidos por Lushbaugh en torno a la catepsina B amibiana. Se refutó la afirmación de Lushbaugh argumentando que si bien es cierto que el sustrato que utiliza es especifico para catepsina B, no debe ser este el único criterio para adjudicarle esa denominación . El autor reportó características cinéticas de su proteina sobre sustratos sintéticos y concluyó que por los resultados obtenidos su enzima no puede catalogarse como catepsina B . La reportó sin embargo, como la principal causante de la patogenicidad amibiana .

OBJETIVO

El objetivo del trabajo fue, en primer lugar, contribuir al conocimiento de las proteinasas de E. histolytica . Aportar datos cuantitativos acerca de las clases catalíticas que la amiba presenta así como la importancia de cada clase en la actividad proteolítica del parásito. Desarrollar una nueva metodología para el aislamiento exclusivo de proteinasas utilizando a la amiba como fuente de éstas . Se propuso también , establecer el número de proteinasas que presenta la amiba utilizando la metodología de electroforesis en gel de sustrato y de la misma manera dilucidar la distribución subcelular de estas proteinasas .

Por último, en un intento de hacer una caracterización enzimática a futuro, se desarrolló una metodología para la medición contínua de la actividad proteolítica que pueda sernos útil para el establecimiento de las constantes cinéticas de las proteinasas de E. histolytica

MATERIALES Y METODOS.

La parte correspondiente a materiales y metodos se da en los trabajos respectivos.

RESULTADOS.

Los resultados obtenidos en la realizacion de esta tesis han sido publicados en los siguientes trabajos:

1.- Pérez-Montfort, R. , Ostoa-Saloma, P. , Velazquez-Medina, L. , Montfort, I. and Becker, I. 1987. Catalytic classes of proteinases of Entamoeba histolytica. Mol. Biochem. Parasitol. 26: 87-97.

2.- Ostoa-Saloma, P. , Cabrera, N. , Becker, I. and Pérez-Montfort, R. 1988. Proteinases of Entamoeba histolytica associated with diferent subcellular fractions. Mol. Biochem. Parasitol. En Prensa.

3.- Ostoa-Saloma, P. , Ramirez, J. and Pérez-Montfort, R. 1988. Measurement of casein digestion by a fluorometric method . Anal. Biochem. En Prensa.

Resumen trabajo 1.

En este trabajo se utilizaron varios inhibidores de la actividad proteolítica para determinar las clases catalíticas de las proteinasas de E. histolytica. Además, se reportó un método nuevo para aislar proteinasas utilizando α2-Macroglobulina, que fue probado en un extracto de E. histolytica.

Se reportó que al utilizar inhibidores de la clase catalíica de cisteina entre el 80 y el 90 % de la actividad proteolítica se inhibió por lo que se consideró que ésta es la clase catalítica principal. Cuando se utilizó inhibidores de proteinasas de serina, la actividad se inhibió en un 10-15% lo que la hace ser la 2a clase catalítica en importancia. La actividad al utilizar inhibidores de las proteinasas de aspártico no fue diferente a la de los controles por lo que se consideró inexistente. La presencia de Metaloproteinasas no se determinó con precisión por la variabilidad del comportamiento de los inhibidores utilizados.

Con la técnica de α2-Macroglobulina se lograron aislar varias proteinasas de E. histolytica cuya actividad se comprobó sobre electroforesis en gel de sustrato; se observaron claramente tres zonas de lisis con p.m. de entre 20-30 Kd., 40-55 Kd., 60-80 Kd. Este resultado se comparó con los obtenidos al analizar un extracto de amiba por la técnica de electroforesis en gel de sustrato dando básicamente los mismos resultados, con la

diferencia de que con la tecnica de la α2-Macroglobulina se obtuvo una actividad especifica muy alta demostrando su capacidad como método de aislamiento de proteinasas en un sistema determinado.

Resumen del trabajo 2

Utilizando la técnica de electroforesis en gel de sustrato, modificada con respecto al trabajo 1 y aplicado a un extracto de E. histolytica , se reportó la resolución de 6 zonas de lisis cuyos pesos moleculares se comprenden entre los 20 y los 80 Kd. distinguiéndose claramente tres zonas a las que arbitrariamente se les llamó proteinasas de alto, mediano y bajo p.m. . La resolución de estas zonas varió con la concentración de acrilamida .

Utilizando 2 métodos distintos de fraccionamiento subcelular, asi como otros 2 métodos, también distintos, de separaciòn de proteinas basados en las interaccion hidrofóbica de éstas, se obtuvieron los siguientes resultados:

1.- Las proteinasa(s) de alto p.m. tuvieron propiedades hidrofilicas y por lo tanto fue concordante con el hecho de encontrarla(s) en el citoplasma de la amiba.

2.- Las proteinasas de mediano y bajo p.m. tuvieron propiedades hidrofóbicas . Se localizaron tanto en membranas internas como en membrana plasmática

3.- Las proteinasas de bajo p.m. se encontraron

principalmente en la membrana plasmática y aparentemente pueden existir como proteínas intrínsecas, ya que la actividad en la membrana no se pierde totalmente, al someter a ésta a un tratamiento con sales.

4.- No se consideró que las proteinasas reportadas fueran productos de degradación, dado que los experimentos controles, hechos en presencia de inhibidores de proteinasas, dieron el mismo resultado.

Resumen trabajo 3

En este artículo se reportó un método fluorométrico para medir la actividad proteolítica. La mayoría de los métodos existentes requieren etapas de precipitación, centrifugación y lectura espectrofotométrica de los peptidos generados durante la reacción; este procedimiento lleva por lo tanto bastante tiempo para obtener una curva de digestión enzimática.

El método que se presentó aquí tiene la propiedad de dar trazos continuos aprovechando la característica que tiene la caseina de que, al ser digerida, disminuya su fluorescencia intrinseca continuamente . Este efecto fue proporcional a la concentración de caseina y la velocidad del apagamiento fue proporcional a la concentración de proteinasa . Las proteinasas utilizadas variaron en su clase catalítica y en su pH óptimo de actividad, por lo que se consideró que el método puede ser utilizado por una variedad de proteinasas. La sensibilidad del

método fue bastante aceptable estando por encima de varios métodos tradicionales y sólo por debajo de aquellos métodos que suelen ser demasiado costosos.

Las relaciones lineales que existieron entre el grado de fluorescencia y la concentracion de proteinasa aunado a su alta sensibilidad, hacen del método reportado una herramienta para la determinación de la concentración de proteinasa en una solución determinada. También es un método con el que potencialmente se pueden, mediante un perfeccionamiento, hacer determinaciones cinéticas de las proteinasas a probar.

DISCUSION.

El resultado obtenido, en el sentido de que la clase catalítica que mayoritariamente contribuye a la actividad proteolítica total de E. histolytica es la de cisteína, puede explicarse si consideramos la naturaleza parásita de la amiba, es decir, se sabe que las proteinasas de los mamíferos pertenecen principalmente a la clase catalítica de serina, por lo tanto, los mecanismos de regulación (inhibidores) de la actividad proteolítica del organismo serán principalmente para proteinasas de serina y en menor grado para proteinasas de aspártico, metaloproteinasas o proteinasas de cisteína; por consiguiente si llega un agente extraño que, como en este caso, expresa proteinasas de cisteína, el organismo parasitado no dispondrá de muchos mecanismos de defensa para inhibir esta actividad, produciendo alteraciones que, como con la amiba, conducen a un daño histolítico.

En un modelo de necrosis experimental (16) cuando se abatió la actividad proteolítica de la amiba utilizando inhibidores de la clase catalítica de cisteína, no se observaron los efectos necróticos del parásito. Al inhibir la actividad proteolítica, se puede pensar que los otros factores reportados como responsables del daño amibiano, deben estar en condiciones de actuar, aunque se puede argumentar que los mismos inhibidores por su caracter tóxico, pueden inhibir también la acción de los

otros factores. Sin embargo en el primer trabajo se reportó el porcentaje de inhibición de la actividad proteolítica por parte de la α2-Macroglobulina (≈ 85%) .Cuando se usó este inhibidor fisiológico exclusivo para proteinasas, en el modelo de necrosis experimental se observó una muy pequeña manifestación necrótica que pudo ser debida a el resto de la actividad proteolítica que no se inhibió, más los otros factores reportados como citotóxicos demostrando de cualquier manera que las proteinasas de cisteina contribuyen de manera significativa en la necrosis tisular.

En este primer trabajo se empezó a dilucidar el número de proteinasas que manifiesta la amiba utilizando un extracto crudo de ésta o aislando a las proteinasas por medio de la α2-Macroglobulina. El que en este primer reporte se hayan observado solamente tres grandes zonas de lisis, obedece a las prolongadas incubaciones que recibió el gel de acrilamida después de la separación electroforética antes de superponerlo sobre el gel con el sustrato ; existe la posibilidad de que las proteínas, por la incubación, se hayan difundido y por lo tanto no permitieron una resolución conspicua de las zonas de digestión, de esta manera , tres zonas de lisis muy juntas se observarán como solamente una gran zona.

La mejor resolución de las bandas de digestión, se obtuvó cuando se modificó un poco el método, como se reportó en el trabajo 2. Se cambió el tiempo de incubación y se omitió el proceso de lavado del gel; con estas modificaciones se alcanzó a resolver de manera evidente, al menos 6 zonas de lisis que correspondieron a los p.m. que ya se habian reportado en el trabajo 1, solo que ahora la zona de bajo p.m. se resolvió en

tres bandas de digestión, la zona intermedia en dos y la de alto p.m. se mantuvo como una. Estas zonas de digestión se vieron más claramente separadas cuando se utilizó un porcentaje de acrilamida de 12.5%.

Estas actividades proteolíticas fueron encontradas asociadas a diversos compartimientos subcelulares. Se utilizaron dos métodos distintos para la obtención de los diferentes fracciones subcelulares, uno que utiliza a la lectina Concanavalina A (35) y otro que se basa en la separación de los componentes por su densidad (36). Cuando se utilizó el primer método, llamó la atención la actividad proteolítica en la fracción de membrana plasmática. Se ha reportado (12) que la Concanavalina A puede activar a la amiba provocando la inserción de proteínas en su membrana y en ese sentido se pensó que las proteinasas en la membrana de la amiba no fueran constitutivas, sino que por acción de la Concanavalina A, se insertáran en ese lugar. Cuando se utilizó el segundo método, que no incluye un tratamiento con Concanavalina A, los resultados fueron básicamente los mismos a los del primer método, por lo que se consideró que efectivamente pudiéran existir proteinasas membranales en E. histolytica. Ya anteriormente se habían reportado evidencias indirectas al respecto (17,26), pero no se había reportado el número ni los p.m. de las proteínas responsables de la actividad proteolítica de la membrana plasmática. Este resultado se reforzó cuando se sometió a la membrana plasmática a un tratamiento de alta fuerza iónica, no pudiendose desprender totalmente la actividad proteolítica asociada a dicha membrana; hubo parte de la

asociada a dicha membrana; hubo parte de la actividad que se desprendió por lo que se pensó que pudiéra haber proteinasas periféricas suceptibles de ser excretadas o que pudiéran transitar entre un estado soluble y un estado membranal como se ha demostrado para algunas proteínas del citoesqueleto que por esta propiedad se les llama proteinas anfitrópicas (37).

Estas proteinasas de membrana plasmática de E. histolytica son las principales candidatas para asignarles un papel importante en el efecto necrótico sobre los tejidos animales.

Las membranas internas presentaron , además de las proteinasas detectadas en membrana plasmática, las proteinasas de p.m. intermedio. El que en membranas internas se detecten protenasas de membrana plasmática no es de extrañarse si consideramos la alta movilidad y capacidad de recambio que tiene la membrana plasmática de la amiba. Aley (38) encontró dos clases de vesiculas internas : unas vesículas pequeñas acidificadas que corresponden a lisosómas secundarios y que tienen una tasa de intercambio con el exterior muy lenta, y otras vesículas muy grandes provenientes de endocitosis con material que rápidamente se intercambia con el medio sin sufrir alguna modificación y que generalmente no forman compartimientos acidificados . Se puede pensar que las proteinasas de p .m. intermedio que presentan las membranas internas de la amiba tienen una función digestiva para la amiba más que una participación en la citopatogenicidad del parásito. Sin embargo , Keene, McLaughlin, Bos, McGowan han demostrado la presencia de proteinasas que corresponden a los p.m. encontrados para las

proteinasas de p.m. intermedio . Lo mismo sucede con la proteinasa soluble, su papel en el mecanismo citopatogénico no es claro y sin embargo se han reportado proteinasas de E. histolytica de ese p.m. con participacion en la citopatología amibiana . Estos experimentos se han realizado con las proteinasas puras por lo que probablemente dentro de la amiba tengan funciones ya sea de regulación o digestivas. Por ejemplo, podemos asemejar el efecto con el de la catepsina B que es una proteinasa de cisteina que se encuentra principalmente en los lisosómas hepáticos y, por lo tanto, su función es la de contribuir al metabolismo de las proteinas que ingresan a las célula. Si se aisla la catepsina B y en esas condiciones de pureza se prueba sobre una monocapa epitelial puesta sobre un modelo de matriz extracelular , la catepsina desprenderá a las células y degradará a la matriz extracelular. ¿Podemos decir entonces que la catepsina B es una proteinasa citopatogénica del hepatocito ? Por supuesto que no. Este razonamiento se puede extrapolar hacia las proteinasas de alto y mediano p.m. de la amiba ¿participan en el proceso de necrosis tisular o sólo en el metabolismo de la amiba? La respuesta a esta interrogante no es fácil por lo que se requiere estudiar más a las proteinasas de E. histolytica para dilucidar cuales son las principales responsables de la necrosis tisular.

Para el estudio de las proteinasas de la amiba se requieren herramientas o metodologías que permitan incrementar el conocimiento de éstas. En ese sentido se desarrolló un método novedoso para medir la actividad proteolítica y que puede ser

aplicado a las proteinasas de la amiba . El método se basa en el apagamiento de la fluorescencia, de el(o los) triptofano(s) al encontrarse en ambientes con distinta polaridad.

La caseina es una proteina de la leche que esta compuesta por una mezcla de proteinas hidrófobas solubles denominadas α, ß y k caseina. Cada una de estas caseinas tiene al menos un triptofano que pueda generar la señal mencionada y que al corte por la proteinasa se expone a un ambiente más polar, con el consecuente apagamiento de la fluorescencia . Esto podría explicar la diferencia en la velocidad del apagamiento por parte de las distintas proteinasas empleadas. La quimotripsina que corta entre enlaces donde existen aminoácidos hidrófobos tuvo una velocidad de apagamiento mayor que cualquiera de las otras proteinasas. No se sabe si las tres caseinas contribuyen a el apagamiento de la fluorescencia o es solamente una la que genera el efecto. Esta cuestión queda abierta a posteriores investigaciones . El hecho de que el registro de la actividad es continuo y que la pendiente de la curva cambia conforme se modifica la concentracion de proteasa , sustrato o inhibidor le da a este método un potencial muy grande para hacer análisis cinéticos de las proteinasas estudiadas. El mejorar en ese sentido este método puede ser una buena herramienta para unificar el estudio de la cinética enzimática de las proteinasas y contribuir al mejor conocimiento de las proteinasas de E. histolytica

BIBLIOGRAFIA

1.- Martinez-Palomo, A. La ciencia desde México vol.47 : Las amibas, enemigos invisibles. Fondo de Cultura Economica. 1987

2.- Brandt, H. y Perez-Tamayo, R. Amibiasis. La Prensa Médica Méxicana. 1970.

3.- Martinez-Palomo, A. The biology of Entamoeba histolytica ed.1. New York, John Wiley & Sons.1982.

4.- Weinbach, E.C. 1981. Biochemistry of enteric parasitic protozoa. TIBS. 6(10): 254.

5.- Diamond, L.S. et al. 1968. Techniques of axenic cultivation of Entamoeba histolyticaSchaudin,1903 and E. histolytica-like amoebae. J.Parasitol 54: 1047.

6.- Diamond, L.S. et al. 1978. A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. Trans.R.Soc.Trop.Med.Hyg. 72: 431.

7.- Ravdin, J.I. and R.L. Guerrant. 1982. A review of the parasite cellular mechanism involved the pathogenesis of amebiasis.Rev.Infect.Dis. 4: 1185-1207

8.- Kobiler, D. and D. Mirelman. 1980. Lectin activity in Entamoeba histolytica trophozoites. Infect. Imm. 29: 221

9.- Orozco, E. et al. 1986. Actividad mitogénica y de lectina en mutantes de Entamoeba histolytica deficientes en virulencia. Arch. Invest. Méd. 17 (Supl): 167.

10.- Long-Krug,S.A. et al. 1985. Phospholipase A enzymes of Entamoeba histolytica: description and subcellular localization. J.Infect.Dis. 152: 536.

11.- Lopez-Revilla, R. and Said-Fernandez, S. 1980. Citopathogenicity of Entamoeba histolytica.Hemolityc activity of trophozoites homogenates. Am. J. Trop. Med. Hyg. 29:209.

12.- Gitler, C and D. Mirtelman. 1986. Factors contributing to the pathogenic behavior of Entamoeba histolytica Ann.Rev.Microbiol. 40: 237-61.

13.- McKerrow, J.H. 1987. The role of proteases in the pathogenesis and immune response to parasitic disease. In: Molecular Strategies of Parasitic Invasion. pag:553-557.Alan R.Liss, Inc.

14.- Barrett, A.J. 1977. Introduction to the history and clasification of tissue proteinases. In: Proteinases in Mammalian Cells and Tissues (Barrett, A.J. ed.) pp.1-55, Elsevier North Holland Biomedical Press,Amsterdam.

15.- Bond, J.S. and P.E. Butler. 1987. Intracelular proteases. Ann.Rev.Biochem. 56: 333-64.

16.- Becker,I. et al. 1988. Exp.Mol.Pathol. en prensa.

17- Gadasi, H. and E. Kessler. 1983. Correlation of virulence and collagenilytic activity in Entamoeba histolytica Infect. Inmun. 39: 528-531.

18- Gadasi,H. and D. Kobiler. 1983. Entamoeba histolytica correlation between virulence and content of proteolytic enzymes. Exp.Parasitol 55: 105-110.

19.- Muñoz, M.deL. et al. 1984. Entamoeba histolytica: collagenolytic activity and virulence. J.Protozool. 31: 468-470.

20.- Reeves, R. 1984. Metabolism of Entamoeba histolytica Schaudinn, 1903 . Adv. Parasitol. 54: 1047.

21.- McLaughlin, J. and G. Faubert. 1977. Partial purification and some propierties of a neutral sulfhidryl and an acid proteinase from Entamoeba histolytica.Am.J.Microbiol. 23: 420.

22.- Lushbaugh, W.B. et al. 1979. Isolation of a citotoxin enterotoxin from Entamoeba histolytica. J.Infect.Dis. 139: 9.

23.- Bos, H.J. 1979. Entamoeba histolytica: Citopathogenicity of intact amebae and cell free extracts; isolation and characterization of an intracellular toxin. Exp.Parasitol 47: 369.

24.- Bos, H.J. et al. 1980. Entamoeba histolytica: Citopathogenicity, including serum effects on contacts dependent and toxin induced lysis of hamster kidney cells monolayer.Exp.Parasitol. 50: 342.

25.- McGowan, K. et al. 1982. Entamoeba histolytica citotoxin purification, characterization strain virulence and protease activity. J.Infect.Dis. 146: 616.

26.- Muñoz, MdeL. et al. 1982. The collagenase of Entamoeba histolytica. J.Exp.Med. 155: 42.

27.- Lushbaugh, W.B. et al. 1985. Purification of Cathepsin B activity of Entamoeba histolytica toxin. Exp.Parasitol 59: 328.

28.- Lushbaugh, W.B. 1984. Relationship of citotoxins of axenicaly cultivated Entamoeba histolytica to virulence. Gastroenterology 86: 1488.

29.- Lushbaugh, W.B. et al. 1984. Proteinase activities of Entamoeba histolytica citotoxin. Gastroenterology. 87:17

30.- Scholze, H. and E. Werries. 1984. A weakly acidic protease has a powerful proteolytic activity in Entamoeba histolytica Mol.Biochem.Parasitol. 11: 293.

31.- Schulte, W. et al. 1987. Specificity of a cysteine proteinase of Entamoeba histolytica towards the α1-CB2 peptide of bovine collagen type I.Mol.Biochem.Parasitol. 25: 39-43.

32- Keene, W. et al. 1986. The major neutral proteinase of Entamoeba histolytica. J.Exp.Med. 163: 536-549.

33.- Luaces, L. and A.J. Barrett. 1988. Affinity purification and biochemical characterization of histolysin, the major cysteine proteinase of Entamoeba histolytica. Biochem.J. 250: 903-909.

34.- Rosales-Encinas, J. and M. Rojkind. 1987. The collagenase of Entamoeba histolytica. Molecular Strategies of Parasite Invasion. pp. 559-566. Alan R. Liss Inc.

35.- Aley, S.B. et al. 1890. Plasma membrane of Entamoeba histolytica . J. Exp. Med. 152: 391-404.

36.- Rosenberg, I. and Gitler, C. 1985. Subcellular fractionation of amoebapore and plasma membrane components of Entamoeba histolytica using self generating Percoll gradients. Mol Boichem. Parasitol 14: 231- 248.

37.- Burn, P. 1988. Amphitropic proteins: a new class of membrane proteins. TIBS. 13: 79.

38.- Aley, S.B. 1981. Composition and dinamycs of the plasma membrane of Entamoeba histolytica. Tesis Doctoral. The Rockefeller University, New York.

Molecular and Biochemical Parasitology, 26 (1987) 87–98
Elsevier

MBP 00871

Catalytic classes of proteinases of *Entamoeba histolytica*

Ruy Perez-Montfort[1], Pedro Ostoa-Saloma[1], Laura Velazquez-Medina[1], Irmgard Montfort[2] and Ingeborg Becker[2]

[1]*Departamento de Microbiología, Instituto de Fisiología Celular, and* [2]*Unidad de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, México D.F., Mexico*

(Received 4 December 1986; accepted 23 June 1987)

Endopeptidase inhibitors were used to determine the catalytic classes of proteinases present in extracts of *Entamoeba histolytica* (strain HM 1:IMSS) axenically grown in vitro. Cysteine proteinases account for most of the proteolytic activity; one or more proteinases with different catalytic mechanisms are also present but could not be unambiguously assigned to a particular catalytic class. Proteinases in amebic lysates were resolved by polyacrylamide gel electrophoresis with sodium dodecyl sulfate. The detergent was exchanged with Triton X-100 and the proteolytic activity in the gels was demonstrated by overlaying it on another gel containing the substrate. Four lysis zones were observed corresponding to molecular weights of 66000, 56000, 40000 and 27000. The first cannot be classified yet, but the last three showed properties consistent with those of cysteine proteinases. Finally, a novel technique is described which uses purified human α-2-macroglobulin to trap, purify and characterize proteases from amebic lysates. The results obtained with this technique confirm those of the overlay technique, since both methods reveal four distinct proteinases in the two different amebic preparations examined.

Key words: *Entamoeba histolytica*; Endopeptidase; Inhibitor; Substrate gel electrophoresis; α-2-Macroglobulin

Introduction

Entamoeba histolytica derives this name from its capacity to destroy tissue. This property has been attributed to different histolytic mechanisms, such as phagocytosis [1], hydrolytic enzymes [2–5], the secretion of toxins [6–8] and the production of proteolytic enzymes, either by the parasite (ref. 4 and references quoted therein, [9–21]), or by the inflammatory cells present at the site of tissue damage [22,23]. The amount of proteolytic enzymes produced by *E. histolytica* has been correlated with the virulence of various strains grown in axenic conditions [24–26]. Several groups have studied the proteases of amebic extracts [4,9,19,24] while others have purified one or more molecular species with proteolytic activity [10–18,20,21]. The main class consists in *E. histolytica* of cysteine proteinases [19]. A proteinase similar to mammalian cathepsin B was purified from *E. histolytica* [10,11,20]. The major neutral proteinase purified from *E. histolytica* is also a cysteine proteinase [21]. The effects of inhibitors [9,10,15,17,19] suggest that *E. histolytica* produces several distinct proteinases and that possibly some of these are not cysteine proteinases. Nevertheless no systematic study of the catalytic classes and/or the total number of different proteins with proteolytic activity produced by the parasite is available.

In this report we describe the use of inhibitors of different catalytic classes of endopeptidases to determine the main families of proteinases in *E.*

Correspondence address: Dr. Ruy Perez-Montfort, Departamento de Microbiología, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70242, 04510 México D.F., Mexico.

Abbreviations: α2m, α-2-macroglobulin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis(β-aminoethylether) *N,N'*-tetraacetic acid; IA, iodoacetamide; NEM, *N*-ethylmaleimide; PAGE, polyacrylamide gel electrophoresis; PHMB, *p*-hydroxymercuribenzoate; PMSF, phenylmethylsulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; Tris, Tris-(hydroxymethyl) aminomethane.

histolytica extracts [27]. We also estimated the number of groups or individual proteolytic enzymes present in amebic lysates and their approximate molecular weight is determined with the use of zymograms. Finally, we report a method for purifying and characterizing molecules with proteolytic activity in complex cellular extracts or lysates with the aid of the universal protease inhibitor α-2-macroglobulin as a molecular trap.

Materials and Methods

E. histolytica cultures. *E. histolytica* strain HM 1:IMSS was a gift from Dr. J. Calderón (Centro de Investigación y de Estudios Avanzados, IPN, México D.F.). Trophozoites were cultured axenically in TYI-S-33 medium [28] at 37°C. Cells were harvested after 72 h in all experiments.

Proteolytic activity. Proteolytic activity was determined by a colorimetric method using azocasein (Sigma Chemical Co., St. Louis, MO) as the substrate [10,16]. Trophozoites were washed thrice with 15 mM potassium phosphate buffer pH 7.5 and 184 mM sodium chloride (PBS-A) [19] by centrifugation at 150 × *g* for 5 min at 10°C. Cells were lysed with 0.5% (v/v) Triton X-100 with or without inhibitors and incubated at 4°C for 1 h. The lysate was centrifuged at 12000 × *g* for 15 min at 4°C and the supernatant was collected. Proteolysis was determined in 40 μl of supernatant, equivalent to 4 × 10^5 trophozoites, incubated with 1.25 mg of substrate at 37°C for different time periods in 500 μl of 50 mM Tris-(hydroxymethyl)aminomethane (Tris), pH 7.5. Proteolytic activity at pH 5 was assayed in a 50 mM acetate buffer. The inhibitors used were *p*-hydroxymercuribenzoate (PHMB), *N*-ethylmaleimide (NEM), iodoacetamide (IA), pepstatin A, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (SBTI), ethylenediaminetetraacetic acid (tetrasodium salt) (EDTA), ethyleneglycol-bis(β-amino-ethylether) *N,N'*-tetraacetic acid (EGTA), leupeptin and human α-2-macroglobulin (α2m). All inhibitors were obtained from Sigma except leupeptin which was obtained from Boehringer (Mannheim) and α2m which was purified from fresh human plasma (see below). Proteolysis was stopped by the addition of 540 μl of 10% trichloroacetic acid and centrifugation at 2000 × *g* for 5 min at room temperature. A_{360nm} of the supernatant was measured. Controls did not release dye spontaneously.

Proteolytic activity of molecules bound by active α2m (see below) on low molecular weight substrates *N*-α-benzoyl-DL-Arg-*p*-nitroanilide, L-Leu-*p*-nitroanilide, *N*-succinyl-L-Ala-L-Ala-L-Ala-*p*-nitroanilide and *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (all from Sigma), was determined in a buffer containing 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid, 0.5 M NaCl, 10% (v/v) dimethyl sulfoxide, pH 7.5 [29] with 1 mM $CaCl_2$. To 2.95 ml of a 1 mM solution of substrate, with or without inhibitors, 50 μl of a 1:5 (v/v) suspension of Sepharose–antibody–α2m–extract molecules (see below) in borate buffered saline (200 mM boric acid and 160 mM sodium chloride, pH 8) was added and incubated at room temperature (24°C) for 24 h or longer. A_{450nm} of the supernatant was measured.

Proteolytic activity of amebic lysates on acid denatured bovine haemoglobin (Sigma Type II) was measured as described by McLaughlin and Faubert [10].

Substrate gel electrophoresis. A procedure similar to the one described by Birkedal-Hansen and Taylor for mammalian collagenase [30] was used. Washed trophozoites were lysed by three freeze-thawing cycles without inhibitors. The lysates were mixed with electrophoresis sample buffer containing sodium dodecyl sulfate (SDS) but no reducing agent (samples were not boiled) and were separated by electrophoresis in 10 or 12.5% polyacrylamide gels (PAGE). Immediately after electrophoresis the gel was incubated in 10 gel volumes of 2.5% Triton X-100 for 90 min at room temperature with gentle stirring; the solution was changed every 30 min. The gel was then washed for a few minutes in distilled water and incubated in 0.1 M Tris-HCl pH 7.4 with 10 mM $CaCl_2$ for 10 min, and immediately laid on top of another gel containing 7.5% acrylamide in 0.075 M Tris-HCl pH 7.4, 0.15 M NaCl and 0.57 mg ml^{-1} of the substrate protein (gelatin from rat tail tendon collagen extracted according to Torre-Blanco and

Alvizouri [31], undenatured rat tail tendon collagen, casein or denatured bovine haemoglobin), but no SDS. In some experiments the gel was cut in strips after the incubation in Triton X-100, put in the Tris buffer with the appropriate inhibitor for 30 min and overlayed on the substrate gel. The gels were placed between glass plates, wrapped in wet tissue paper and incubated in a humid chamber at 37°C for 24 h, and were then fixed and stained in 25% (v/v) isopropanol, 10% (v/v) glacial acetic acid and 0.25% (w/v) Coomassie brilliant blue R-250 (Bio-Rad, Richmond, CA) for 20 h at room temperature, and destained in 10% acetic acid for 40 h also at room temperature. In some cases the protein was incorporated in the first gel and a similar procedure to the one described above was performed on that single gel.

α2m and anti-α2m antibodies. α2m was purified as described by Kurecki et al. [32] from fresh human plasma supplied by Dr. Adela Garcia Lopez (Blood Bank, Instituto Nacional de Pediatria, México D.F.). The activity of α2m was determined by its capacity to inhibit the action of trypsin (Sigma) on casein (Pfanstiehl, Waukegan, IL) [33] and by its protective action from SBTI when trypsin was assayed with the low molecular weight substrate *N-p*-tosyl-L-arginine-methyl ester (Sigma) [33]. Purified α2m was inactivated with 0.4 M ethylamine (Merck, Darmstadt) [34] or by incubation at 37°C for 30 min with a 5-fold molar excess of trypsin. An α2m-Sepharose column was prepared with 10 ml of cyanogen bromide activated Sepharose 4B (Sigma) and 50 mg of pure α2m following the manufacturer's instructions.

Antibodies against purified human α2m were produced in rabbits by a single intramuscular injection of 100 μg of pure α2m with 1.5 ml of Freund's complete adjuvant (Difco, Detroit, MI). Booster injections with 100 μg of α2m in phosphate buffered saline (15 mM sodium pH 7.2 and 150 mM sodium chloride) were given intraperitoneally every 15 days. The animals were bled 8 days after each booster injection starting on the fifth immunization. Antibodies were confirmed by double immunodiffusion and immunoelectrophoresis, and were purified by affinity chromatography with the α2m-Sepharose column. Purified antibodies (10 mg) were coupled to 1 ml of cyanogen bromide activated Sepharose 4B by the procedure described above.

Molecules bound by α2m in amebic lysates. Washed and lysed trophozoites ($2.7–8 \times 10^6$) were resuspended in approximately 1 ml of PBS-A. In some experiments these lysates were iodinated with 1 mCi of ^{125}I (ICN Radiochemicals, Irvine, CA) and 4–6 iodobeads (Pierce Chemical Co., Rockford, IL) at 4°C for 40 min. Free iodine was removed following the method described by Tuszynski et al. [35]. The labeled lysate was separated into four aliquots and kept at 4°C. To three of these aliquots 100 μg of active α2m, ethylamine-inactivated α2m, or trypsin-inactivated α2m were added, respectively. The four aliquots were then placed in a water bath at 37°C for 30 min and cooled again to 4°C. Then 20 μl of the Sepharose-antibody were added to each tube and incubated at least for 2 h at 4°C. The Sepharose beads to which antibody, α2m and ^{125}I-labeled molecules were bound were extensively washed with borate buffered saline until no γ counts above background were detected in the supernatant. The α2m and the molecules bound to it were eluted from the antibody-Sepharose column with 100 μl of 0.5 M acetic acid for at least 2 h at room temperature and mixed with SDS-PAGE sample buffer containing 5% (v/v) 2-mercaptoethanol. The medium was made basic with 2.5 M Tris, the samples were boiled for 2 min and analyzed by SDS-PAGE on 12.5% acrylamide gels [36]. Autoradiography of dried slab gels was performed at −80°C [37] with Kodak XK-1 film and Dupont intensifying screens (Quanta III).

In some experiments cells were lysed with 0.5% Triton X-100 for 1 h at 4°C, labeled with radioactive iodine and then processed as described above. In experiments to determine proteolytic activity α2m was added directly to unlabeled extracts.

Results

Proteolytic activity of amebic extracts. The magnitude of proteolytic activity determined in *E. histolytica* extracts varied in different experiments. Total proteolytic activity against azocasein varied over a 2-fold range. The average

TABLE 1

Effect of different inhibitors on proteolytic activity of amebic extracts[a]

Inhibitor(s)	Class of endopeptidase inhibited	% Inhibition ± S.D. (*n*)		
PHMB (1 mM)	cysteine	92	± 2	(6)
IA (2 mM)	cysteine	92	± 5	(6)
NEM (2 mM)	cysteine	84	± 2	(6)
SBTI (100 μg ml^{-1})	serine	18	± 9	(12)
PMSF (1 mM)	serine	0		(18)
(2 mM)		15	± 5	(12)
Pepstatin A[b] (1 μg ml^{-1})	aspartic	0		(6)
EDTA (2 mM)	metallo	−13	± 1	(2)[c]
(20 mM)		33	± 13	(2)
EGTA (2 mM)	metallo	−17	± 4	(2)[c]
(20 mM)		−22	± 2	(2)[c]
PHMB (1 mM) + SBTI (100 μg ml^{-1})	cysteine + serine	109	± 9	(6)
Leupeptin (50 μM)	cysteine + serine	79	± 1	(6)
α2m (5 mg ml^{-1})	all	77	± 8	(6)

[a] Buffer: 0.05 M Tris-HCl pH 7.5.
[b] Buffer: 0.05 M acetate pH 5.
[c] Activation.

amount of protein per ameba in amebic extracts, as determined by the Biuret reaction, was 2.203 ± 0.231 mg protein per 10^6 amebas (n=67). Variability in activity was also observed in zymograms made with different lysates from the same number of amebas (compare lane B of Fig. 2, lane A of Fig. 3 and see below); nevertheless, the variation coefficient within the same experiment was never more than 8%.

Inhibitors of cysteine proteinases PHMB, NEM and IA reduced activity by approximately 80–90% at a pH of 7.5 (one of the two pH values at which a maximum of proteolytic activity occurs with amebic extracts [9,19]) (Table 1) indicating that the proteolytic activity of amebic extracts is mainly due to cysteine proteinases. Pepstatin A, an inhibitor of aspartic proteinases, had no effect at pH 5 (the other pH at which a maximum of proteolytic activity occurs in amebic extracts [9,19]). PMSF, an inhibitor of serine proteinases, was ineffective at a concentration of 1 mM but partly inhibited proteolysis at a concentration of 2 mM; a similar inhibition was obtained with SBTI at a concentration of 100 μg ml^{-1}. This indicates that part of the proteolytic activity of amebic extracts may be due to serine proteinases. The combination of PHMB and SBTI abolished proteolytic activity completely.

The chelating agents EDTA and EGTA (which inhibit metallo proteinases at concentrations of 1 or 2 mM) had an activating effect at concentrations up to 5 mM (Fig. 1). The activating effect persisted even in the presence of 20 mM EGTA, but EDTA had an inhibiting effect at concentrations beyond 5 mM, reaching a plateau of approximately 30% inhibition at 10 mM. A similar activating effect was also observed in the presence of 1 mM 1,10-phenanthroline (data not shown). Leupeptin and purified human α2m both reduced proteolytic activity by approximately 80%. Using the lysate of 10^6 amebas proteolytic activity on acid denatured haemoglobin at pH 3 was not detected after 3 h at 37°C.

Identification of proteinases in amebic lysates by SDS-PAGE zymograms. As a strategy to identify the main groups of proteinases present in amebic lysates, we separated the molecules contained in these preparations by SDS-PAGE, and tested for the proteolytic activity of the separated molecules by overlaying this gel on a second substrate gel and staining both gels afterwards. Transparent unstained regions on the substrate gel correspond with bands of proteolytic activity in the first gel. Fig. 2 shows some representative results with different ameba concentrations on a gel contain-

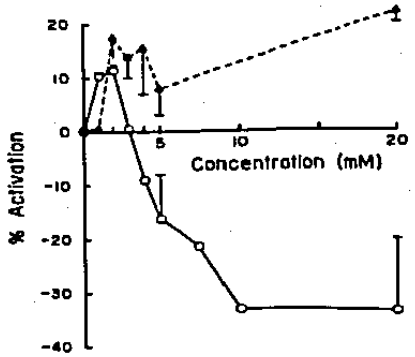

Fig. 1. Effect of EDTA (○) and EGTA (▲) on the proteolytic activity of amebic extracts on azocasein.

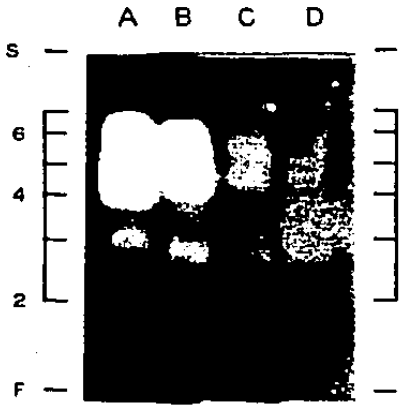

Fig. 2. Zymogram of a gel on which lysates obtained from different numbers of amebas were analyzed. (A) 5×10^5, (B) 2.5×10^5, (C) 1×10^5 and (D) 5×10^4 cells. The substrate gel contained gelatin. The left ordinate scale is the $M_r \times 10^{-4}$. S marks the end of the stacking gel, F is the dye front.

ing gelatin as substrate. Three zones of hydrolysis centering at apparent molecular weights (M_r) of 56000, 40000 and 27000 occur in all cases.

Fig. 3 lane A shows the pattern of gelatin hydrolysis without additions. Two hydrolysis regions are seen, a broad zone between M_r of 90000 and 40000 and another centering at 29000. Dithiothreitol enhances hydrolysis (lane B). PHMB and NEM inhibit most proteinases except a 70 kDa enzyme (lanes C and D), while IA inhibits all activity detectable by this assay (lane E). The lysate incubated with PMSF gave only one lysis zone that was visible at an M_r centering at 60000 (lane F). Pepstatin A somewhat inhibited proteases with lower M_r values (lane G), although it had minimal effects in most other experiments. Lane H shows the effect of 20 mM EDTA which in this case was also inhibitory, but in several other cases this assay showed activation with this chelator or with 1,10-phenanthroline (data not shown). Leupeptin had little inhibiting effect on the higher M_r proteases but inhibited the 29 kDa protease (lane I). 5 min boiling of lysates eliminated proteolysis (data not shown). Thus, our results indicate that amebic lysates analyzed with the aid of substrate gel electrophoresis of gels containing gelatin at pH 7.4 have at least four groups of proteinases. These produce hydrolysis in regions comprehended in the M_r range between 90000 and 27000.

To test the effect of acid pH on the activity, gels on which the lysate corresponding to 10^6 trophozoites was separated were preincubated for 30 min or 3 h in 10 mM acetic acid pH 3. No hydrolysis regions were apparent but, when the haemoglobin was incorporated into the separation gel and this gel was incubated in acid conditions at 37°C for 24 h, we observed two faint lysis zones with M_r of 40000 and 32000 (data not shown). Zymograms using casein and native rat collagen as substrates also showed the hydrolysis zones at M_r values of 56000, 40000 and 27000 (data not shown).

Analysis of molecules in amebic extracts bound by α2m. Active α2m binds most known proteinases but also proteins without proteolytic activity [38]. Inactive α2m only binds nonproteolytic molecules. Thus, molecules bound by active α2m and not bound by inactive α2m should be active proteinases. Fig. 4 shows an autoradiograph of a gel in which the constituents in amebic lysates labeled with radioactive iodine bound by α2m were analyzed. Lane A shows a control in which no α2m was added to an amebic lysate; the anti-α2m antibody on the Sepharose column does not bind any detectable labeled constituent of the lysate. Lane B shows the analysis of the same lysate to which α2m inactivated with ethylamine was

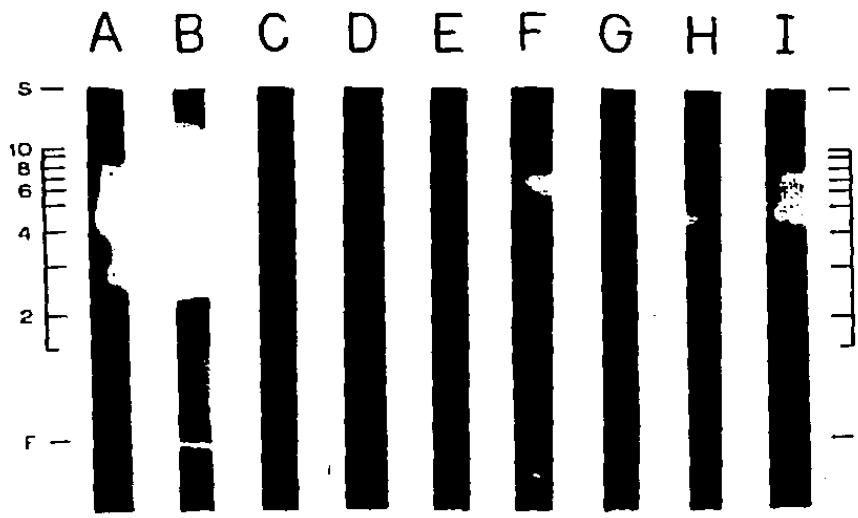

Fig. 3. Zymogram of a gel on which amebic lysates from 2.5×10^5 cells submitted to different treatments were analyzed. Gel strips containing the lysates were incubated for 30 min at room temperature in solutions containing respectively (A) no additions, (B) 4 mM dithiothreitol, (C) 2 mM PHMB, (D) 4 mM NEM, (E) 500 mM IA, (F) 4 mM PMSF, (G) 1 μg ml^{-1} pepstatin A, (H) 20 mM EDTA and (I) 30 μg ml^{-1} leupeptin, and overlayed on the substrate gel containing gelatin. The left ordinate scale is the $M_r \times 10^{-4}$.

added; two prominent bands with M_r values of 180000 and 90000 are apparent. The M_r values of these bands correspond to the α2m monomer and its main breakdown product, which are also visible by Coomassie blue staining on the gel (data not shown) [39]. Both bands are associated with radioactive material resistant to dissociation by SDS and 2-mercaptoethanol. Faint radioactive bands are apparent at M_r values 68000, 60000, 49000, 42000, 39000 and 23000. There is also radioactivity that migrates with the buffer front which may represent molecules of M_r smaller than 10000 and/or labeled lipids. Lane C shows the analysis of the same lysate in the presence of active α2m. Again, bands at 180000 and 90000 are apparent but the relative intensity of both bands differs from that in lane B. The 180 kDa band is more intense than the 90 kDa band. This indicates that, in this preparation, there is either more undegraded α2m monomer, or that it bound covalently more radioactive material, or both [38]. Two faint radioactive bands appear at M_r of 68000 and 60000 and more intense bands at 49000, 42000 and 23000, the last one being the most prominent. Lanes D, E and F reveal what remained bound to the anti-α2m antibody column and was released by treatment with electrophoresis sample buffer and boiling for 2 min, for lanes A, B and C respectively. Lane E shows again the 180 kDa and the 90 kDa bands together with the other radioactive bands seen in lane B. Lane F shows a very intense band corresponding to the undegraded α2m monomer, a very faint band corresponding to the degraded α2m monomer and the other bands seen on lane C. Even though the α2m inactivated with ethylamine did not inhibit the action of trypsin on casein, a substrate of high M_r, and behaved as a fast moving component when analyzed on native pore gradient gels [40], it did behave in a similar way to fully active α2m in this type of experiment, so we decided to test α2m inactivated with trypsin. Fig. 5 shows the autoradiograph of an experiment in which the following preparations were analyzed: lane A, a control with no α2m added, lane B, amebic lysate with α2m inactivated with ethylamine; lane C,

amebic lysate with α2m inactivated with trypsin; and lane D, amebic lysate with active α2m. Because it contained more radioactivity, lane B shows a similar but more intense pattern to the one described previously for lane B of Fig. 4. Lane D is also very similar to lane C in Fig. 4. But both lanes B and D contrast sharply with the pattern observed on lane C. Very faint bands are visible at M_r values of 180000 and 39000 and most of the radioactivity is associated with the 85 kDa breakdown product of α2m.

In experiments to investigate if the molecules bound to α2m were representative of all cell components, including integral membrane proteins, Triton X-100 instead of the freeze-thaw cycles was used to lyse the amebas. The results disclosed the same radioactive bands as in the experiments shown previously and no additional bands were observed.

The trap hypothesis of protease inactivation by α2m states that α2m will inhibit proteolytic activity of proteases towards substrates of high molecular weights and not towards substrates of low molecular weights [38]. To test if some of the molecules associated with the active α2m had proteolytic activity, we prepared unlabeled amebic lysate, submitted it to the usual purification scheme (Materials and Methods) and incubated it with low molecular weight substrates for different proteases. Some of the molecules bound by active α2m had the ability to hydrolyse the substrates *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and Leu-*p*-nitroanilide but not *N*-α-benzoyl-Arg-*p*-nitroanilide or *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide. Boiling the extract 3 min before the purification procedure inhibited this hydrolysis by 62% for *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide and 69% for Leu-*p*-nitroanilide. Pepstatin A (1 μg ml^{-1}), EDTA (2 mM), PMSF (2 mM) and PHMB (1 mM) inhibited the hydrolysis of the

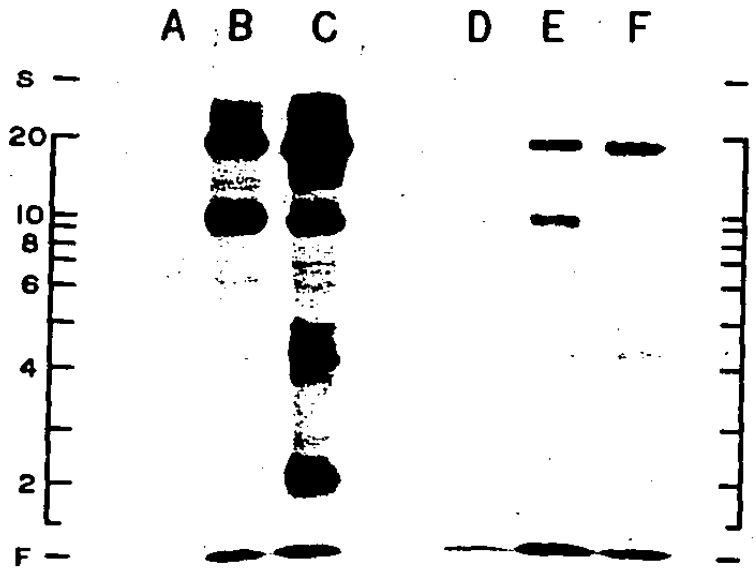

Fig. 4. Autoradiograph of a gel in which radioactive molecules from amebic lysates bound by α2m and anti-α2m antibodies were analyzed. Lanes A, B and C show the eluates obtained with 0.5 M acetic acid of immune precipitates corresponding to pure anti-α2m antibody, ethylamine inactivated α2m and active α2m respectively. Lanes D, E, and F show the eluates obtained by boiling the residual material bound to the anti-α2m-Sepharose columns on lanes A, B, and C respectively with electrophoresis sample buffer. The left ordinate scale is the $M_r \times 10^{-4}$.

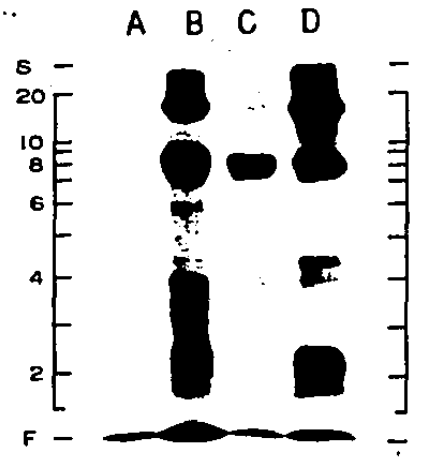

Fig. 5. Autoradiograph of a gel in which radioactive molecules of amebic lysates bound by α2m and anti-α2m antibodies were analyzed. Lane A shows the eluate obtained with 0.5 M acetic acid of the control without α2m. Lanes B, C and D show respectively the eluates from preparations with ethylamine inactivated α2m, trypsin inactivated α2m and active α2m. The left ordinate scale is the $M_r \times 10^{-4}$.

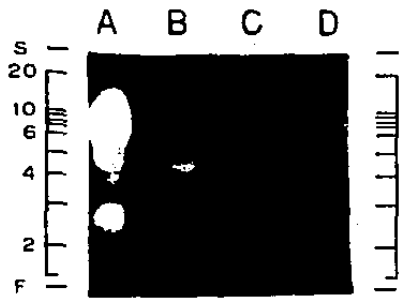

Fig. 6. Zymogram of a gel containing SDS on which an amebic lysate, molecules bound by α2m and by anti-α2m antibodies were analyzed. A lysate from 2.5×10^5 amebas was analyzed on lane A. Lane B contained active α2m and the molecules from the lysate bound by it. Lane C contained trypsin inactivated α2m and the molecules from the lysate bound by it. Lane D contained the molecules from the lysate bound by anti-α2m antibodies. All samples contained 10% (v/v) 2-mercaptoethanol. The substrate gel contained gelatin. The left ordinate scale is the $M_r \times 10^{-4}$.

former substrate by 47% and 67% and the hydrolysis of the latter substrate by 39% and 31% after 24 and 48 h of incubation at 37°C, respectively.

Another experiment combining the technique of the purification of molecules bound by active α2m, followed by a zymogram on a gel containing gelatin (omitting the exchange of SDS with Triton X-100), showed the same hydrolysis regions as did the whole intact lysate. After 24 h of incubating the superimposed gels a broad lysis zone in the M_r range of 150000–50000, another narrower zone immediately below it in the M_r range of 50000–40000 and a third lysis zone in the M_r range of 30000–20000 became apparent in the lane containing the lysate (Fig. 6, lane A). The hydrolysis zones of the preparation corresponding to the purified material were less intense than those of the lysate. The lysis zones appeared but their relative intensity was different from the original extract, the high molecular weight zone was very diminished, the middle molecular weight zone was unchanged and the low molecular weight zone was also diminished, but less than the high molecular weight zone (Fig. 6, lane B). The controls with trypsin inactivated α2m and anti-α2m antibodies showed no lysis zones (Fig. 6, lanes C and D, respectively).

Discussion

The purpose of this study was to try to determine the number of different endopeptidases as well as their catalytic classes in whole extracts and lysates of *E. histolytica*. We also used the universal protease inhibitor α2m as a molecular tool for the purification of molecules with proteolytic activity from those same preparations.

As has been observed before with monoxenic strains [9] and as we report here with axenic *E. histolytica* strain HM 1, extracts and lysates show large variability in their proteolytic activity towards different substrates. This result indicates that *E. histolytica* cultivated in vitro produces different amounts of proteases or proteases with different activities at the end of the logarithmic growth phase. We cannot distinguish between these two possibilities because the average amount of protein per ameba in amebic extracts was constant (see Results). The possibility that other (additional) proteinases are produced is less likely

because the zymograms showed a very constant pattern and we did not observe any additional lysis regions in our experiments. The causes of the variability of the proteolytic activity are unclear but may be related to the heterogeneity of other properties observed in axenic populations of certain *E. histolytica* strains [41].

Experiments with inhibitors for the different catalytic classes of endopeptidases clearly show that most proteolytic activity in *E. histolytica* is due to cysteine proteinases (Table I). Our results agree and confirm those of Avila et al. [19], although we were unable to inhibit all the proteolytic activity using only inhibitors of cysteine proteinases. Complete inhibition was achieved only with a combination of an inhibitor of cysteine proteinases and an inhibitor of serine proteinases. This may suggest that there is more than one catalytic class of proteinases in *E. histolytica*.

According to our results and those of other groups, one catalytic class of endopeptidases that seems to be absent or in very small quantities from these amebic preparations is that of aspartic proteinases. This is based on the observation that proteolytic activity is low below pH 5 and totally inhibited at pH 3 or below (see Fig. 1 in ref. 19, and Fig. 1 in ref. 9 and Results), which is generally the pH range where aspartic endopeptidases are most active, and that pepstatin does not inhibit the proteolytic activity of the preparations (Table I).

The nature of the catalytic class(es) to which non-cysteine proteinases belong is very difficult to determine with certainty because inhibitors are not absolutely specific. For example, it has been reported that PMSF can inhibit certain cysteine proteinases [27] and chelators like EDTA and EGTA are necessary for the activation or stabilization of certain cysteine proteinases [42]. These last observations may help explain the variability and even apparent contradiction of the results shown in Table I and Fig. 3. These results may also be partly explained by the different conditions to which the lysates and extracts have been subjected before proteolytic activity is assayed.

Results with α2m indicate that *E. histolytica* has a small remnant of proteolytic activity that is not inhibited by this protease inhibitor (Table I). The nature of this proteolytic activity is unknown but may be of great interest, since only very few proteinases that are not inhibited by α2m are known. Zymograms showed that there are three main zones of lysis with M_r values centering at 56000, 40000 and 27000 (Fig. 2). Proteolytic activity increased in these regions when the number of amebas was increased, and also in the presence of dithiothreitol and 2-mercaptoethanol, but was partly inhibited by PHMB and NEM and completely by IA (Figs. 2,3 and 6). This behavior is consistent with cysteine proteinases. The combined results of inhibition with several inhibitors in several different experiments, although with somewhat variable results, indicate that some lysis zones particularly in the 60–70 kDa region contain a distinct proteinase or group of proteinases. We cannot assign this group a catalytic class on the basis of our present results. It is noteworthy that no lysis of protein substrates was observed in regions of the gel corresponding to M_r values below 20000 (see below).

In our experiments using human α2m to trap components of amebic preparations, the molecular weights of the radiolabeled molecules bound and released with acid pH, and subsequently denatured by SDS and reduced with 2-mercaptoethanol, are similar to those of the estimated center of the lysis of protein substrates in the zymograms (Figs. 4 and 5). Also, some of the molecules bound by active α2m have the ability to hydrolyse low molecular weight proteolytic substrates; in addition low molecular weight protease inhibitors partially inhibit this hydrolysis and immune precipitates analyzed by the zymogram technique exhibit the same lysis regions as whole amebic lysates. In one experiment in which amebas were lysed by the action of Triton X-100 we observed the same labeled bands. All these experiments indicate that in both amebic preparations (extracts and whole lysates) a representative sample of all or most of the proteinases contained in whole amebas is obtained. We therefore think that *E. histolytica* has three different cysteine proteinases with approximate M_r values of 56000, 40000 and 27000 and one or a small number of other proteinases with an M_r between 60000 and 70000 and with a different catalytic mechanism. Our supposition is based on the fact that three different methodologies with two

types of amebic preparations yield similar and consistent results, making the possibility of observation of experimental artifacts highly unlikely.

With the procedure described here we have been able to confirm the existence of several proteinases isolated by other investigators using more conventional procedures [10–12,15–17,20], but have also been unable to observe others, for example the 16.5 kDa molecule resembling cathepsin B purified by Lushbaugh et al. [18].

Recently Keene et al. purified a neutral cysteine proteinase with a subunit M_r of 56000 [21]. These authors discussed the possibility that this enzyme 'accounts for the thiol proteinase activity of crude extracts and secretions reported previously'. In this report we show that there are at least two additional molecules with smaller molecular weights that also behave as cysteine proteases, confirming observations made by other authors [10–13,15–17,20].

An intriguing finding is that radioactively labeled material is bound by covalent bonds to the intact and the degraded monomer of α2m (see Figs. 4 and 5). The nature of these molecules is unknown and will be subject to further investigation.

A noteworthy coincidence is the correlation of intensity of the radioactive label attached to the 66, 60, 49, 42, and 23 kDa molecules bound to active α2m and the intensity of the corresponding lysis regions in the substrate gel (Fig. 4C, Fig. 5D and Fig. 6B, respectively). This may represent the relative amounts of proteases bound by α2m which are in different proportion to that in the original lysate (Fig. 6A).

Both methods used in the present work have limitations: zymograms can only detect proteinases that are not denatured by SDS and have enough activity on the substrate gel to make their effect visible, and the α2m purification method will only identify proteases that are bound by the inhibitor and labeled by iodination with iodobeads (molecules in the extract can be labeled by a variety of other methods). Both methods have the advantage that they permit an initial estimate of the minimal number of molecules with proteolytic activity in very complex mixtures. This approach allows the identification of proteases and in some cases their classification in the corresponding catalytic class. Such information is useful for further purification of these molecules.

Acknowledgements

This work was supported by a grant from CONACYT (PCSABNA-021715) to R.P.-M. The authors want to thank Drs. R. Perez-Tamayo and R. Kretschmer for careful revision and critical comments on the manuscript and Ms. V. Godinez for typing it.

References

1 Orozco, E., Guarneros, G., Martínez-Palomo, A. and Sánchez, T. (1983) *Entamoeba histolytica*: phagocytosis as a virulence factor. J. Exp. Med. 158, 1511–1521.
2 Jarumilinta, R. and Maegraith, B.G. (1961) Hyaluronidase activity in stock cultures of *Entamoeba histolytica*. Am. J. Trop. Med. 54, 118–127.
3 Nakamura, M. and Goldstein, L. (1957) Occurrence of glutaminase in *Entamoeba histolytica*. Nature 179, 1134.
4 Jarumilinta, R. and Maegraith, B.G. (1969) Enzymes of *Entamoeba histolytica*. Bull. WHO 41, 269–273.
5 Long-Krug, S.A., Fischer, K.J., Hysmith, R.M. and Ravdin, J.I. (1985) Phospholipase a enzymes of *Entamoeba histolytica* description and subcellular localization. J. Infect. Dis. 152, 536–541.
6 Bos, H.J. (1979) *Entamoeba histolytica*: cytopathogenicity of intact amebae and cell-free extracts: isolation and characterization of an intracellular toxin. Exp. Parasitol. 47, 369–377.
7 Lynch, E.C., Rosenberg, I.M. and Gitler, C. (1982) An ion-channel forming protein produced by *Entamoeba histolytica*. EMBO J. 1, 801–804.
8 Young, J. D-E., Young, T.M., Lu, L.P., Unkeless, J.C. and Cohn, Z.A. (1982) Characterization of a membrane pore-forming protein from *Entamoeba histolytica*. J. Exp. Med. 156, 1677–1690.
9 Neal, R.A. (1960) Enzymic proteolysis by *Entamoeba histolytica*. Biochemical characteristics and relationship with invasiveness. Parasitology 50, 531–550.
10 Mc Laughlin, J. and Faubert, G. (1977) Partial purification and some properties of a neutral sulfhydryl and an acid proteinase from *Entamoeba histolytica*. Can. J. Microbiol. 23, 420–425.
11 Lushbaugh, W.B., Kairalla, A.B., Cantey, J.R., Hofbauer, A.F. and Pittman, F.E. (1979) Isolation of a cytotoxin-enterotoxin from *Entamoeba histolytica*. J. Infect. Dis. 139, 9–17.
12 Bos, H. and van den Eijk, A. (1980) Serum inhibited toxicity of *Entamoeba histolytica*. Arch. Invest. Med. (Mex.) 11 (Supl. 1), 135–141.
13 Lushbaugh, W.B., Kairalla, A.B., Hofbauer, A.F., Arnaud, P., Cantey, J.R. and Pittman, F.E. (1981) Inhibi-

tion of *Entamoeba histolytica* cytotoxin by alpha 1 antiprotease and alpha 2 macroglobulin. Am. J. Trop. Med. Hyg. 30, 575–585.
14 Muñoz, M.L., Calderón, J. and Rojkind, M. (1982) The collagenase of *Entamoeba histolytica*. J. Exp. Med. 155, 42–51.
15 McGowan, K., Deneke, C.F., Thorne, G.M. and Gorbach, S.L. (1982) *Entamoeba histolytica* cytotoxin: purification, characterization, strain virulence and protease activity. J. Infect. Dis. 146, 616–625.
16 Scholze, H. and Werries, E. (1984) A weakly acidic protease has a powerful proteolytic activity in *Entamoeba histolytica*. Mol. Biochem. Parasitol. 11, 293–300.
17 Lushbaugh, W.B., Hofbauer, A.F. and Pittman, F.E. (1984) Proteinase activities of *Entamoeba histolytica* cytotoxin. Gastroenterology 87, 17–27.
18 Lushbaugh, W.B., Hofbauer, A.F. and Pittman, F.E. (1985) *Entamoeba histolytica*: purification of cathepsin B. Exp. Parasitol. 59, 328–336.
19 Avila, E., Sánchez-Garza, M. and Calderón, J. (1985) *Entamoeba histolytica* and *E. invadens*: sulfhydryl dependent proteolytic activity. J. Protozool. 32, 163–166.
20 Scholze, H. and Werries, E. (1986) Cysteine proteinase of *Entamoeba histolytica*. I. Partial purification and action on different enzymes. Mol. Biochem. Parasitol. 18, 103–112.
21 Keene, W.E., Petitt, M.G., Allen, S. and McKerrow, J.H. (1986) The major neutral proteinase of *Entamoeba histolytica*. J. Exp. Med. 163, 536–549.
22 Tsutsumi, V., Meno-López, R., Anaya-Velázquez, F. and Martínez-Palomo, A. (1984) Cellular bases of experimental liver abscess formation. Am. J. Pathol. 117, 81–91.
23 Martínez-Palomo, A. (1986) Biology of *Entamoeba histolytica*. In: Amebiasis (Martínez-Palomo, A., ed.), pp. 11–43, Elsevier Science Publishers, Amsterdam.
24 Gadasi, H. and Kobiler, D. (1983) *Entamoeba histolytica*: Correlation between virulence and content of proteolytic enzymes. Exp. Parasitol. 55, 105–110.
25 Gadasi, H. and Kessler, E. (1983) Correlation of virulence and collagenolytic activity in *Entamoeba histolytica*. Infect. Immun. 39, 528–531.
26 Muñoz, M.L., Rojkind, M., Calderón, J., Tanimoto, M., Arias-Negrete, S. and Martínez-Palomo, A. (1984) *Entamoeba histolytica*: Collagenolytic activity and virulence. J. Protozool. 31, 468–470
27 Barrett, A.J. (1977) Introduction to the history and classification of tissue proteinases. In: Proteinases in Mammalian Cells and Tissues (Barret, A.J., ed.), pp. 1–55, Elsevier/North Holland Biomedical Press, Amsterdam.
28 Diamond, L.S., Harlow, D.R. and Cunnick, C.C. (1978) A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans. R. Soc. Trop. Med. Hyg. 72, 431–432.
29 Nakajima, K., Powers, J.C., Ashe, B.M. and Zimmerman, M. (1979) Mapping the extended substrate binding site of cathepsin G and human leukocyte elastase: Studies with peptide substrates related to the α1 protease inhibitor reactive sites. J. Biol. Chem. 254, 4027–4032.
30 Birkedal-Hansen, H. and Taylor, R.E. (1982) Detergent-activation of latent collagenase and resolution of its component molecules. Biochem. Biophys. Res. Commun. 107, 1173–1178.
31 Torre Blanco, A. and Alvizouri, A.M. (In Press) In vitro hydroxylation of proline in the collagen of the cysticercus of *Taenia solium*. Comp. Biochem. Physiol.
32 Kurecki, T., Kress, L.F. and Laskowski, M. (1979) Purification of human plasma α2 macroglobulin and α1 proteinase inhibitor using zinc chelate chromatography. Anal. Biochem. 99, 415–420.
33 Laskowski, M. (1955) Trypsinogen and trypsin. Methods Enzymol. 2, 26–36.
34 Imber, M.J. and Pizzo, S. (1981) Clearance and binding of two electrophoretic 'fast' forms of human α2 macroglobulin. J. Biol. Chem. 256, 8134–8139.
35 Tuszynski, G.P., Knight, L., Piperno, J.R. and Walsh, P.N. (1980) A rapid method for removal of ^{125}I iodide following iodination of protein solutions. Anal. Biochem. 106, 118–122.
36 Ames, G.F.L. (1974) Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. 249, 634–644.
37 Swanstrom, R. and Shank, P.R. (1978) X Ray intensifying screens greatly enhance the detection by autoradiography of radioactive isotopes ^{32}P and ^{125}I. Anal. Biochem. 86, 184–192.
38 James, K. (1980) Alpha2 macroglobulin and its possible importance in immune systems. Trends Biochem. Sci. 5, 43–47.
39 Swenson, R.P. and Howard, J.B. (1979) Structural characterization of human α2 macroglobulin subunits. J. Biol. Chem. 254, 4452–4456.
40 Larsson, L.J., Olson, S.T. and Björk, I. (1985) Kinetics of conformational changes and inactivation of human α2 macroglobulin on reaction with methylamine. Biochemistry 24, 1585–1593.
41 Calderón, J. and Tovar, R. (1986) Loss of susceptibility to complement lysis in *Entamoeba histolytica* HM1 by treatment with human serum. Immunology 58, 467–473.
42 Barrett, A.J. and McDonald, J. (1986) Mammalian Proteases: A Glossary and Bibliography, Vol. 1, Endoproteases, Academic Press, New York.

Molecular and Biochemical Parasitology, (1988)
Elsevier

MBP 01062

Proteinases of *Entamoeba histolytica* associated with different subcellular fractions

Pedro Ostoa-Saloma[1], Nallely Cabrera[1], Ingeborg Becker[2] and Ruy Perez-Montfort[1]

[1]*Departamento de Microbiología, Instituto de Fisiología Celular and* [2]*Unidad de Medicina Experimental, Facultad de Medicina, Universidad Nacional Autónoma de México, 04510 México D.F.*

(Received 29 March 1988; accepted 4 August 1988)

Crude lysates of *Entamoeba histolytica* (strain HM 1:IMSS) analyzed by substrate gel electrophoresis in 12.5% acrylamide separating gels with reducing agents showed six hydrolysis zones with apparent molecular weights of 73000 (high), 45000, 36000 (intermediate), 30000, 26000 and 23000 (low molecular weight proteinases). Amebic lysates fractionated using the procedure of Aley et al. or the procedure of Rosenberg and Gitler and analyzed by the same method show all enzymes in the fractions with the soluble components and only the intermediate and low molecular weight proteinases in the fraction containing internal vesicles or membranes and plasma membrane. Some of these proteinases seem to be integral membrane proteins since they resist treatment with high salt, high urea buffer. All fractions are capable of digesting azocasein. Fractionation of amebic lysates by hydrophobic chromatography using phenyl-Sepharose or phase separation of amebic extracts with Triton X-114 show that proteinases with high, intermediate and low molecular weight behave as hydrophilic proteins while only proteinases of intermediate and low molecular weight behave as hydrophobic proteins. These results suggest that some proteinases are segregated in different compartments of the cell

Key words: *Entamoeba histolytica* Proteinase; Subcellular fraction; Substrate gel electrophoresis; Hydrophobic chromatography; Triton X-114 phase separation

Introduction

The protozoan *Entamoeba histolytica* has several cysteine proteinases capable of degrading proteins of connective tissue [1–3]. Collagenolytic activity of *E. histolytica* against native type I and III human collagen has also been described [4]. Recently we and others have shown that the cysteine proteinases are the main catalytic class of proteolytic enzymes in extracts and lysates of these cells [3,5]. Unpublished results from our laboratories also suggest that these enzymes are involved in the production of tissue necrosis in two different models of acute experimental amebiasis.

The question of which of these amebic proteinases might be involved in tissue damage is important because with better knowledge of such enzymes, strategies may be designed to prevent or circumvent one of the major problems of the disease. Several authors have claimed that *E. histolytica* releases or secretes one or more proteinases to the surrounding medium [6–8], but other authors have not been able to confirm these findings [4,9]. The hypothesis that lytic enzymes are released by the surface active lysosomes beneath the membrane bound vacuoles [10,11] has not been confirmed by more recent observations [12–16]. Another possibility is that proteinases are associated with the plasma membrane of the cell. Muñoz et al. have given indirect evidence that the collagenase of *E. histolytica* is membrane bound

Correspondence address: R. Perez-Montfort, Departamento de Microbiología, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70242, 04510 México D.F., México.

Abbreviations: BBS, borate-buffered saline; PBS-A, phosphate-buffered saline; PBS-A, phosphate-buffered saline for amebas; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PASB, phosphate buffer with ammonium sulfate; TS, Tris-saline buffer.

[4]. Gadasi and Kessler [7] confirmed the association of collagenolytic activity with the plasma membrane fraction, but concluded that the collagenase is not an integral membrane protein since it could be eluted from the membranes by a buffer of high ionic strength.

González-Garza et al. used peroxidase labeled soybean trypsin inhibitor to localize putative proteolytic enzymes in fixed trophozoites obtained from a patient with amebic enteritis. The inhibitor was bound to the plasma membrane and the wall of digestive and intracytoplasmic vacuoles [17]. All these studies are suggestive of an organized distribution of these enzymes within the cell, but there is no thorough study of the subcellular distribution of the proteinases of *E. histolytica*. In this study we report our results of the subcellular distribution of proteinases in crude lysates, that are resistant to sodium dodecyl sulfate (SDS), and visible by substrate gel electrophoresis. Our results show that mainly a low molecular weight group of proteinases is associated with fractions enriched in plasma membranes, while fractions containing soluble components contain high, intermediate and low molecular weight proteinases. Consistently, when hydrophobic and hydrophilic components of amebic lysates are separated by hydrophobic chromatography or Triton X-114 phase separation, mainly low molecular weight proteinases display hydrophobic behavior and proteinases of high, intermediate and low molecular weight behave as hydrophilic proteins.

Materials and Methods

E. histolytica cultures. Trophozoites of *E. histolytica* strain HM 1:IMSS were cultured axenically in BI-S-33 medium [18] at 36.5°C and harvested after 72 h.

Subcellular fractionation. Subcellular fractions of trophozoites were obtained using two different methods. One described by Aley et al. [19] which separates a plasma membrane – Concanavalin A complex from other organelles by differential sedimentation. The other, which uses self generating Percoll gradients to fractionate these cells, was described by Rosenberg and Gitler [8]. Protein was determined, in these last fractions by the procedure described by Schaffner and Weissmann [20]. For some experiments amebas were lysed and fractionated in the presence of 2 mM iodoacetamide or 2.5% (v/v) 2-mercaptoethanol; all lysis and fractionation steps were done on ice or at 4°C.

Proteolytic activity. proteolytic activity was determined by a colorimetric method using azocasein made as described by Starkey [21] ($E^{1\%}_{366} = 29.1$ in 1 N NaOH) as the substrate. The method was very similar to the one we described previously [3] with some modifications. Trophozoites were washed three times with 15 mM potassium phosphate buffer pH 7.5 and 184 mM NaCl (PBS-A) by centrifugation at 150 × *g* at 10°C. The cells were then separated according to the methods of Aley et al. [19] or Rosenberg and Gitler [8] and protein was determined in the fractions obtained. For some experiments some of the subcellular fractions were incubated 1 h at 4°C with 0.5% Triton X-100. Proteolysis was determined by incubating the crude or treated fractions with 1.25 mg of substrate at 37°C for different time periods in 500 µl of 50 mM Tris-(hydroxymethyl)aminomethane (Tris) pH 7.5, containing 5 mM dithiothreitol. Proteolysis was stopped by addition of 25 µl of 100% trichloroacetic acid and centrifugation at 2000 × *g* for 5 min at room temperature. A_{366nm} of the supernatant was measured. Enzyme activity units of hydrolysis of azocasein were measured as described by Starkey [21].

Substrate gel electrophoresis. A procedure very similar to the one we described previously [3] was used, with some modifications. Briefly, samples were mixed with two times concentrated electrophoresis sample buffer containing SDS and 4.5% 2-mercaptoethanol (final concentration) and were not boiled. In some cases the electrophoresis sample buffer contained either 2 or 5 mM iodoacetamide or 1 mM *p*-hydroxymercuribenzoate and no 2-mercaptoethanol. The samples were separated by electrophoresis in 7.5, 10, 12.5 and 15% polyacrylamide gels in the presence of SDS, and sometimes also in the presence of inhibitors. Immediately after electrophoresis the gel was laid on top of another gel containing 7.5% acrylamide

in 7.5 mM Tris-HCl pH 7.4, 150 mM NaCl and 0.57 mg ml^{-1} of gelatin, but no SDS. The gels were placed between glass plates, wrapped in wet tissue paper, and incubated in a humid chamber at 37°C for 3–24 h. Gels that contained inhibitors were either processed as described above or washed four times with 100 ml of a buffer containing 0.1 M Tris-HCl pH 7.4, 150 mM NaCl and 2.5% (v/v) 2-mercaptoethanol and then processed as described above. Staining with Coomassie brilliant blue R-250 was performed as described previously [3]. Experiments with lysates obtained in the presence of 4.5% 2-mercaptoethanol or with lysates incubated without inhibitors at room temperature for various lengths of time were performed in a similar fashion.

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) was performed in 12.5% acrylamide gels as described previously [22]. Gels were stained with silver as described earlier [23] with some modifications. Briefly, gels were fixed by soaking for 1 – 24 h in 50% methanol and 12% acetic acid. The gels were washed 20 min in 20% ethanol and 10% acetic acid. The gels were then soaked for 15 min in the same solution containing 0.3 mg ml^{-1} of freshly dissolved $KMnO_4$. The gels were soaked for 15 min in 0.1% K_2CO_3 and then washed with water for 5 min. A solution of 0.1% $AgNO_3$ was added to the gels and incubated 15 min. The stain was developed with a solution of 2% K_2CO_3 and 0.015% HCHO. Fresh solution was added each time a brown precipitate appeared. Gels were washed three times with water for 1 min and staining was terminated by soaking the gels in 1% acetic acid.

Hydrophobic chromatography. Washed trophozoites (4.7×10^6) were suspended in 5 ml borate buffered saline (BBS; 200 mM boric acid, 160 mM NaCl, pH 8). The cells were lysed by three freeze-thawing cycles and were sonicated thrice for 2 min in an ultrasonic cleaner (model 8852, Cole Parmer, Chicago, IL). The homogenate was centrifuged at 13000 × g for 1 h at 4°C and the supernatant was collected. The volume of the supernatant was adjusted to 20 ml with more BBS and was dialyzed against two changes of 2 l of BBS for 24 h at 4°C. Solid ammonium sulfate was added to the dialysate to a final concentration of 30%, according to Dixon's nomogram [24], and centrifuged at 13000 × g for 1 h at 4°C. The concentration of ammonium sulfate was increased to 70% in the supernatant and the centrifugation repeated. The resulting pellet was resuspended in 10 ml of 10 mM sodium phosphate buffer, pH 6.8 containing 1 M ammonium sulfate (PASB). For hydrophobic chromatography, 9 ml of the suspension were applied to a column (2.5 × 10.0 cm) of phenyl-Sepharose CL-4B (Sigma Chemical Co., St. Louis, MO), previously equilibrated with PASB. The column was washed with 220 ml of PASB without ammonium sulfate and 10 ml fractions were collected. A_{280nm} was determined before eluting with a gradient of 0–25% ethylene glycol in PASB and subsequently with another gradient of 25–50% ethylene glycol (380 ml total volume). The column was washed with 140 ml of 50% ethylene glycol, then with 260 ml of 1% Triton X-100, then with 120 ml water and finally 120 ml with 2% SDS.

Phase separation in Triton X-114. Triton X-114 (octylphenoxy polyethoxyethanol) (lot 23F-0701) was obtained from Sigma and fractionated as described by Bordier [25]. The concentration of Triton in the purified preparations was measured at 275 nm ($E = 1.46 \times 10^3$ M^{-1} cm^{-1}). Washed trophozoites were solubilized in 1% Triton X-114 in Tris-saline (TS) buffer (10 mM Tris, 150 mM NaCl, pH 7.4) containing 0.3 mM *N*-ε-2,4-dinitrophenyl-L-lysine (Sigma) and 0.001% bromophenol blue (BioRad, Richmond, CA) [26] at 0–4°C for 5–60 min. The supernatants were collected in the tubes on ice using prechilled Pasteur pipettes. Supernatants or unfractionated solubilized trophozoites were warmed for 30 min at 37°C and centrifuged at 4200 × g for 5 min at room temperature. The supernatant (yellow aqueous upper phase) was separated from the pellet (blue detergent lower phase). The detergent phase was redissolved in TS to the original volume and incubated for 10 min at 0–4°C (D1). Triton X-114 (with bromophenol blue) was added to a final concentration of 1% to the aqueous phase (A1). The phase separation was repeated on both samples by rewarming and centrifuging as described above. The supernatant of D1 was

discarded and the pellet redissolved in TS (D2) and the pellet of A1 was also discarded and more Triton X-114 was added to the supernatant as described above (A2). Phase separations were repeated up to four times and samples analyzed by substrate gel electrophoresis.

High salt and high urea treatment of membrane and vacuole rich fractions. Amebic lysates were separated following the method of Aley et al. [19]. The fractions containing internal membranes and plasma membrane were frozen and thawed once and 500 μl of high salt, high urea buffer (1 M KCl, 3 M urea, 10 mM phosphate buffer, pH 6.8) were added to remove adsorbed proteins [27]. The fractions were incubated in ice for 45 min and centrifuged at 40 000 × *g* for 60 min. The pellet was washed with 50 mM Tris pH 7.5 and resuspended in 500 μl of that same buffer. The supernatant (200 μl) was desalted in a 1 ml Sephadex G-25 column (Pharmacia, Uppsala) previously equilibrated with 50 mM Tris pH 7.5 [28]. Aliquots of 75 μl were mixed with SDS-PAGE sample buffer and analyzed by substrate gel electrophoresis. The substrate gel was dried between two cellophane sheets and densitometric scans of different lanes were obtained in a DU-50 spectrophotometer (Beckman, Palo Alto, CA).

Results

Substrate gel electrophoresis of proteinases of amebic lysates in acrylamide gels of various concentrations. We have previously shown that amebic lysates show at least three zones of hydrolysis centering at apparent molecular weights (M_r) of 56000, 40000 and 27000 in 10% acrylamide gels in the absence of reducing agents [3]. Hydrolysis is enhanced by dithiothreitol and mercaptoethanol in 10 and 12.5% acrylamide gels and, in these conditions, hydrolysis is seen in regions comprehended in the M_r range between 150000 and 23000 [3]. Trying to characterize better this hydrolysis pattern, we analyzed amebic lysates in polyacrylamide gels with various acrylamide concentrations. Fig. 1 shows the patterns obtained using the lysate equivalent to 2 × 10^5 cells in 7.5, 10, 12.5 and 15% SDS acrylamide gels and coincubating with the gelatin gel for 8 h. Clearly, the acrylamide concentration in the separating gel has a great influence in the resolution of the zones that show hydrolysis of the substrate.

The gel with a 12.5% acrylamide concentration provided the best separation and hydrolysis pattern. It showed hydrolysis regions with M_r centering at 73000, 45000, 36000, 30000, 26000 and 23000. Thus for further studies this concentration of acrylamide was used for separation. The proteinases or groups of proteinases were classified arbitrarily according to their M_r: the 73 kDa molecule(s) as high M_r proteinase, the 45 and 36 kDa molecules as intermediate M_r proteinases and the 30, 26 and 23 kDa molecules as low M_r proteinases.

The hydrolysis pattern seen in Fig. 1 is qualitatively very reproducible. It was observed in lys-

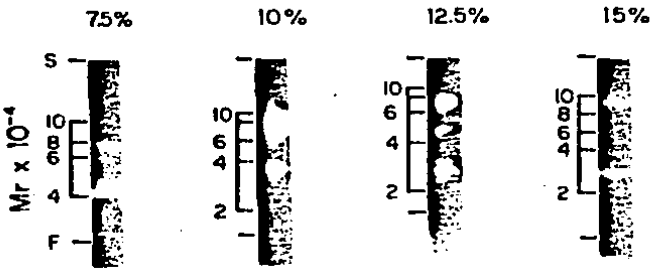

Fig. 1. Zymogram of gels with different acrylamide concentrations on which lysates of 2 × 10^5 amebas were analyzed in the presence of 4.5% (v/v) 2-mercaptoethanol. The substrate gel contained gelatin. Coincubation time of separating and substrate gels was 8 h. S marks the end of the stacking gel, F is the dye front.

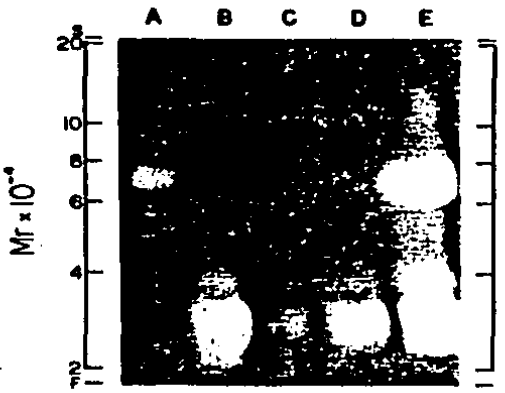

Fig. 2. Zymogram of a gel on which subcellular fractions of amebas were analyzed. The method of Aley et al. was used to fractionate 2×10^8 amebas. (A) Supernate II: soluble components; (B) pellet II: internal membranes; (C) pellet III: non-vesiculated membranes and debris; (D) pellet IV: plasma membrane; and (E) total lysate.

ates obtained and separated electrophoretically in the presence of the inhibitors iodoacetamide or *p*-hydroxymercuribenzoate, after washing out the inhibitor from the separating gel. The same pattern was also observed in lysates obtained in the presence of 4.5% 2-mercaptoethanol, and in lysates that had been incubated up to 8 days at room temperature in the absence of inhibitors or activators (data now shown).

Occasionally, there were quantitative variations in the relative intensity of the individual hydrolysis zones in different preparations, but these variations could not be associated with the age of the culture, the temperature or time of preparation of the enzyme samples, the length of the incubation, freezing and thawing or the presence or absence of inhibitors. We never observed the appearance of new hydrolysis zones and there was no evidence that the pattern varied in a manner that could be explained by degradation of the high M_r proteinases.

Subcellular localization of proteinases. In order to assess the subcellular distribution of the proteinases, amebas were initially separated according to the scheme of isolation of plasma membranes described by Aley et al. [19]. Following this method one obtains four well characterized fractions labeled: supernate II (which contains soluble components); pellet II (which contains internal membranes); pellet IV (which contains plasma membranes) and pellet III (which contains non-vesiculated membranes and debris) (fig. 1 in ref. 19). Substrate gel analysis of these four fractions shows that all groups of proteinases are present in the soluble components (Fig. 2, lane A), but the high M_r proteinase is greatly enriched with respect to the other groups. (Compare with lane E which contains total lysate.) The other three fractions contain the intermediate and low M_r groups of proteinase and lack the high M_r proteinase (lanes B, C and D). These results indicated that a discrete group of proteinases is associated with the membranes, particularly with the plasma membrane, when purified according to this method. This same distribution of hydrolysis zones was observed in experiments in which lysates were obtained, fractionated and analyzed in the presence of iodoacetamide or 2-mercaptoethanol. Since the proteolytic activity seen in the plasma membrane fraction could be due to contamination of this fraction with components of

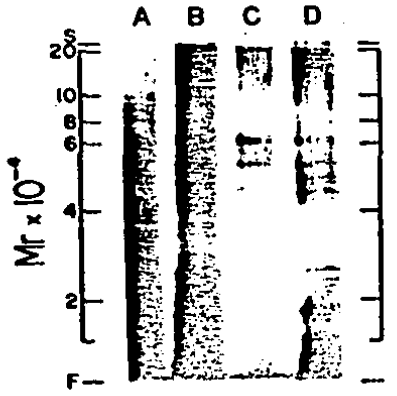

Fig. 3. Comparison of the SDS-PAGE pattern of different subcellular fractions obtained by the method of Aley et al. (A) Supernate II: soluble components; (B) pellet II: internal membranes; (C) pellet III: non-vesiculated membranes and debris; and (D) pellet IV: plasma membrane.

TABLE I

Proteolytic activity of subcellular fractions of *E. histolytica*

Fraction	Reported contents	Activity (proteolytic units/mg protein)
Supernate II	soluble components	1.28
Pellet II	internal membranes	2.51
Pellet IV	plasma membrane	7.66
Pellet III	non-vesiculated membranes and debris	0.55
Total lysate	–	1.90

other fraction(s), we tested this possibility by running SDS-PAGE of the four different fractions and stained the gel with silver. Fig. 3 shows that each of the fractions produces a different band pattern. Since the total protein in the plasma membrane fraction (lane D), as determined by the biuret reaction, was one 24th part of that in the internal membrane fraction (lane B) and five times that in the non-vesiculated membrane and debris fraction (the most likely contaminant, lane C) and all lanes show a different band pattern in the gel, it is very unlikely that the amount and type of proteolytic activity seen in the plasma membrane fraction is due to contaminants. Table I shows the proteolytic activity of the different fractions on azocasein. The plasma membrane fraction has the highest activity per milligram of protein, which is approximately three times that of the internal membrane fraction. Since it has been demonstrated that Concanavalin A may stimulate the release of amebapore and of thiol activated proteinases [8], we decided to use the method described by Rosenberg and Gitler using self-generating Percoll gradients. This could rule out the possibility that the proteinases are associated with the plasma membrane because of the treatment with the lectin in the experiments described above. Fig. 4 shows representative results of the proteolytic activity on azocasein and the protein concentration of the different fractions. There are two peaks of activity, one centering in fraction 9 which is rich in the lysosomal enzymes β-glucosaminidase and acid phosphatase and the highest peak in fraction 14 which is rich in plasma membrane markers [8]. The inset shows a substrate gel electrophoresis of equal volumes of the different fractions obtained. Again, all groups of proteinases are present in fractions 2, 4 and 6 which contain soluble components. Fractions 8, 10 and 12, also show a similar pattern to lane B in Fig. 2 which contains the internal membranes. Fractions 14 and 16 contain mainly the low M_r proteinases and the pattern observed is very similar to that of the plasma membrane fraction obtained by the other method. Fractions 16 and 18 show a loss of two proteinases with M_r of 30000 and 23000. The apparent loss of activity in the substrate gel is due to the sharp decrease in protein concentration in the middle and bottom fractions (see also fig. 1 in ref. 8). Further evidence supporting the membrane association of intermediate and low M_r proteinases was obtained by exposure of fractions containing internal vesicles or plasma membrane to a high salt and high urea buffer. Fig. 5 shows that in both preparations a part of the proteinases behave as integral membrane proteins and another group is loosely bound. Densitometric estimates of the percentage of proteolytic activity associated with the internal membranes (lanes B) and with the plasma membrane (lane E) were 37.9 and 45.5% respectively.

Hydrophobic chromatography. Fig. 6 shows a representative chromatogram of an amebic lysate separated in a column of phenyl-Sepharose. The inset shows a zymogram of gelatin of selected different fractions. The first peak, fractions 5 – 10, represents unbound material and does not contain any detectable proteolytic activity. The first group of proteinases eluted, which is in the last fractions with unbound material, has molecules from the three groups and apparently only one of the low M_r proteinases is detectable in this hydrophilic fraction (pool of fractions 12–18, inset lane A). Subsequently when the ethylene glycol concentration was raised first from 0 to 25% and then to 50% the other proteinases of lower M_r start to elute (fraction 39, inset lane b). The material that remained bound to the column was eluted with 1% Triton X-100 (fraction 90, inset lane C), washed with 120 ml water and finally with 2% SDS. The eluate with 2% SDS did not show any detectable proteolytic activity. In the eluate with 1% Triton X-100 the lysis zone of the pro-

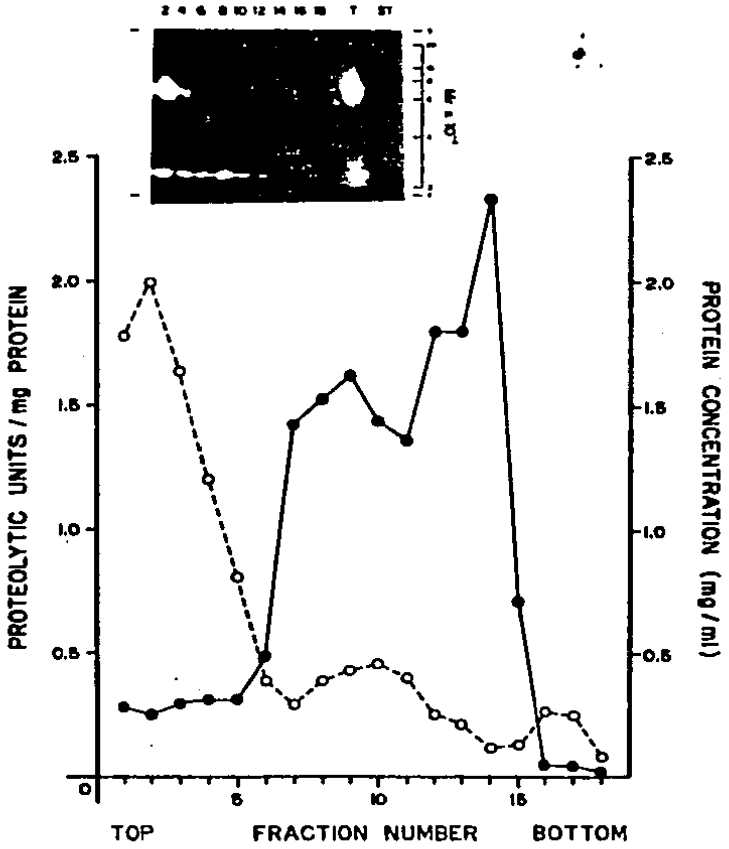

Fig. 4. Subcellular distribution profile of proteolytic activity and protein concentration of *E. histolytica* in Percoll gradients. The inset shows a zymogram of a gel on which equal volumes (25 μl) of different fractions (number above lane) were analyzed. T, total amebic lysate; ST, molecular weight standards; •, proteolytic activity; ○, protein concentration.

teinase with the lowest M_r was more evident. The peak at 280 nm from fraction 80 to fraction 100 is mainly due to the absorbance of the detergent.

Phase separation of amebic lysates. The two phases (aqueous and detergent) obtained by phase separation of a 1% Triton X-114 extracts were analyzed by substrate gel electrophoresis. Fig. 7 shows the distribution of the proteinases during four cycles of separation. High, intermediate and low M_r proteinases remained in the aqueous phase (lanes G, H, I and J corresponding to A1, A2, A3 and A4) while only intermediate and low M_r proteinases partitioned in the detergent phase (lanes C, D, E and F corresponding to D1, D2, D3 and D4). (The meaning of A1 or D1 is explained in Material and Methods.)

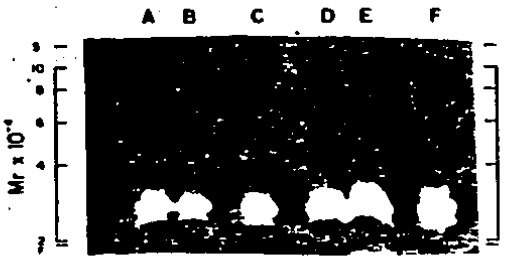

Fig. 5. Zymogram of a gel on which subcellular fractions of amebas treated with high salt, high urea buffer were analyzed. (A) Untreated internal membranes; (B) pellet of treated internal membranes; (C) supernatant of treated internal membranes; (D) untreated plasma membrane; (E) pellet of treated plasma membrane; and (F) supernatant of treated plasma membrane.

Discussion

In this study we have determined with more accuracy the number and M_r of SDS-resistant proteinases reported in our previous work [3]. We have also attempted to establish the subcellular distribution of these molecules as well as their hydrophobicity or hydrophilicity. By introducing several minor modifications in the substrate gel electrophoresis method we used previously such as changing the acrylamide concentration in the separating gel, omitting the exchange of SDS with Triton X-100 after electrophoresis, including a reducing agent in the electrophoresis sample buffer and varying the time of contact between the substrate gel and the separating gel, a better resolution of the lysis zones was obtained (Fig. 1). In general, good standard conditions were applying the lysate equivalent to 2×10^5 amebae per

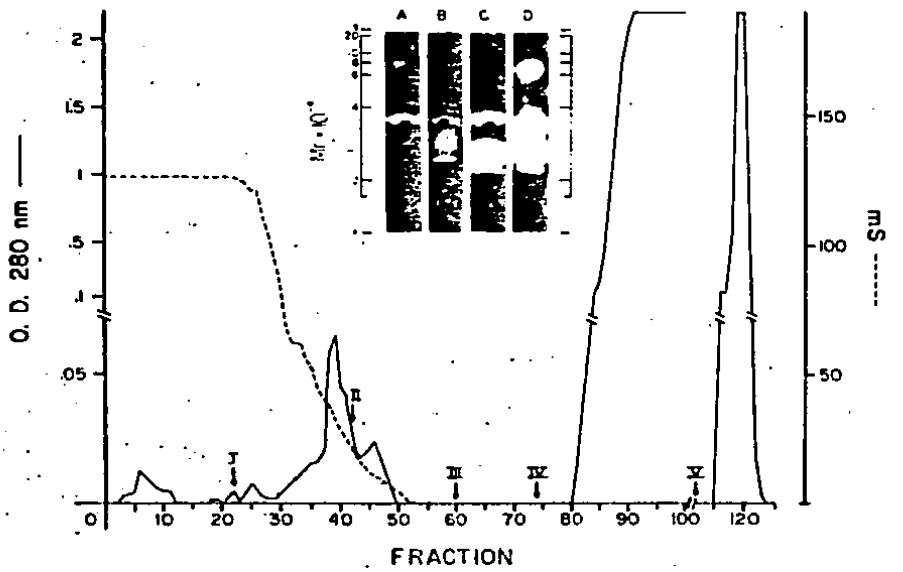

Fig. 6. Chromatographic separation of an amebic extract in a phenyl-Sepharose column. Unbound sample (flow through arrow I) was collected initially. Bound proteins were eluted with a linear gradient of 0–25% ethylene glycol (arrow I) and subsequently with another linear gradient of 25–50% ethylene glycol (arrow II). The column was then washed with 50% ethylene glycol (arrow III), 1% Triton X-100 (arrow IV) and finally with 2% SDS (arrow V). Fraction volume was 10 ml. The dashed line shows the conductance. The inset shows a zymogram of a gel on which different fractions of the column eluate were analyzed. (A) Pool of fractions 12–18; (B) fraction 39; (C) fraction 90; and (D) total amebic lysate.

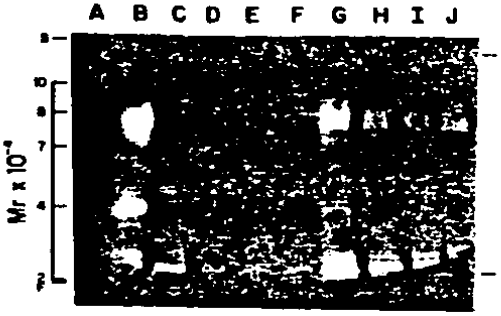

Fig. 7. Zymogram of a gel on which aqueous and detergent phases of amebic extracts obtained with Triton X-114 were analyzed. (A) Molecular weight standards; (B) total amebic lysate; (C – F) D1, D2, D3 and D4 respectively; (G – J) A1, A2, A3 and A4 respectively (see Materials and Methods).

lane and 8 h coincubation of the separating and substrate gel. In these conditions six lysis zones were apparent, and were best observed with the 12.5% acrylamide separating gel. (The 15% acrylamide separating gel showed comparable results when the amount of lysates was equivalent to more than twice the amount stated above.) The lysis zones occurred at M_r of 80000–60000, 56000–45000, 40000–35000, 33000–29000, 30000–25000 and 23000. The results indicate that the number of proteinases that can be detected by substrate gel electrophoresis in *E. histolytica* strain HM 1 is at least six and not four as we had previously reported [3]. We think this pattern reflects an initial distribution of proteinase activity in intact amebae for the following reasons: (a) the same pattern was observed in lysates obtained in the presence of inhibitors or activators of cysteine proteinases, (b) a very similar distribution of lysis zones or radioactive bands was obtained when the proteinases were purified using α_2-macroglobulin [3], and (c) several authors have purified high, intermediate and low M_r proteinases from lysates of *E. histolytica* [1,29–31]. Further support comes from experiments designed to test the stability of these proteinases to autodigestion. The observed pattern did not change in crude lysates incubated for 8 days at room temperature in the absence of inhibitors. Also, the distribution of the proteolytic activity associated to different hydrolysis zones in different cell compartments, in the presence of inhibitors or an activator of cysteine proteinases, and in solvents of different polarity was entirely reproducible.

We now identify the 80–70 kDa proteinase with the 66 kDa molecule of unknown catalytic class we reported [3]. Recent experiments have confirmed our previous finding that this hydrolysis zone is partially inhibited by *p*-hydroxymercuribenzoate (2 mM) and completely by higher concentrations of iodoacetamide (> 5 mM).

The wide band with M_r centering at 27000 in our previous study resolved into three bands with mean M_r of 30000, 26000, and 23000. This discovery was the result of the modifications to the method of substrate gel electrophoresis mentioned above. These hydrolysis zone as well as the ones caused by the intermediate M_r proteinases are inhibited by cysteine proteinase inhibitors. The results obtained by two different methods of subcellular fractionation clearly show that the high M_r proteinase(s) is (are) exclusively in the soluble fraction of the cells and that the fractions containing internal vesicles or membranes or plasma membrane contain only intermediate and low M_r proteinases. The soluble fractions of the cells also contain some intermediate and low M_r proteinases. It cannot be established if these molecules are identical, similar or different to those in the membrane fractions. This will be the subject of further investigation. Our results suggest that the 73 kDa proteinase(s) is (are) the most hydrophilic of these molecules and that the 23 kDa proteinase(s) the most hydrophobic. This last observation is supported because it is more evident when the hydrophobicity of the buffers is highest and it tends to disappear from the aqueous phase following Triton X-114 phase separation.

It is currently thought that *E. histolytica* is constantly turning over its plasma membrane [29], interchanging its outer membrane with fresh internal membrane. Our results support this hypothesis because they suggest that the proteinase composition of the internal vesicles and internal membranes is similar to that of the plasma membrane. The postulate that at least a subpopulation of intermediate and low M_r proteinases are integral membrane proteins is supported by their resistance to elute from membranes in high salt,

high urea buffers and by their hydrophobic nature demonstrated by two different methods. Proteinases are associated with the membrane even if cells are not stimulated by Concanavalin A (Fig. 4). In earlier studies Muñoz et al. [4] suggested that collagenase activity appeared to be located on the plasma membranes, because direct contact of the amebae with collagen films was required for digestion. Gadasi and Kessler [7] separated subcellular fractions using the method of Aley et al. [19] to localize collagenolytic activity. They reported that the cytosol, internal membrane and plasma membrane fraction had collagenolytic activity, with the last fraction having significantly more activity than the others. These results are in agreement with our proteolytic assay for nonspecific proteinases (Table I and Fig. 4). On the other hand, both the 56 kDa cysteine proteinase purified by Keene et al. [1] and the 21 kDa cysteine proteinase purified by Scholze and Werries [30] have been shown to digest native collagen. We have also shown by substrate gel electrophoresis using native rat collagen as substrate that proteinases in all three categories of M_r we have described in amebic lysates are capable of hydrolysing collagen [3]. We have confirmed these results using amebas fractionated with the method of Aley et al. and native human liver collagen as the substrate. The hydrolysis pattern obtained is very similar to that shown in Fig. 2 (data not shown).

These results taken together with the results in this study strongly suggest that an important part of the membrane bound collagenolytic activity of *E. histolytica* may be due to these proteinases.

Acknowledgements

This work was supported in part by grants from the Subsecretaría de Regulación Sanitaria y Desarrollo, Secretaría de Salud, México and from CONACYT to R.P.-M. The authors would like to thank Drs. A. Gómez-Puyou, I. Montfort and R. Pérez-Tamayo for careful revision of the manuscript, Ms. V. Godinez for secretarial help and Mr. José Avilés for the photographic work.

References

1 Keene, W.E., Pettit, M.G., Allen, S. and McKerrow, J.H. (1986) The major neutral proteinase of *Entamoeba histolytica*. J. Exp. Med. 163, 536–549.
2 Schulte, W., Scholze, H. and Werries, E. (1987) Specificity of a cysteine proteinase of *Entamoeba histolytica* towards the α1-CB2 peptide of bovine collagen type I. Mol. Biochem. Parasitol. 25, 39–43.
3 Perez-Montfort, R., Ostoa-Saloma, P., Velázquez-Medina, L., Montfort, I. and Becker, I. (1987) Catalytic classes of proteinases of *Entamoeba histolytica*. Mol. Biochem. Parasitol. 26, 87–97.
4 Muñoz, M.L., Calderón, J. and Rojkind, M. (1982) The collagenase of *Entamoeba histolytica*. J. Exp. Med. 155, 42–51.
5 Avila, E., Sánchez-Garza, M. and Calderón, J. (1985) *Entamoeba histolytica* and *E. invadens*: sulfhydryl dependent proteolytic activity. J. Protozool. 32, 163–166.
6 Petri, W.A. and Ravdin, J.I. (1987) Cytopathogenicity of *Entamoeba histolytica*. The role of amebic adherence and contact-dependent cytolysis in pathogenesis. Eur. J. Epidemiol. 3, 123–136.
7 Gadasi, H. and Kessler, E. (1983) Correlation of virulence and collagenolytic activity in *Entamoeba histolytica*. Infect. Immun. 39, 528–531.
8 Rosenberg, I. and Gitler, C. (1985) Subcellular fractionation of amoebapore and plasma membrane components of *Entamoeba histolytica* using self generating Percoll gradients. Mol. Biochem. Parasitol. 14, 231–248.
9 Neal, R.A. (1960) Enzymic proteolysis by *Entamoeba histolytica*: biochemical characteristics and relationship with invasiveness. Parasitology 50, 531–550.
10 Eaton, R.D.P., Meerovitch, E. and Costeron, J.W. (1969) A surface-active lysosome in *Entamoeba histolytica*. Trans. R. Soc. Trop. Med. Hyg. 63, 678–680.
11 Eaton, R.D.P., Meerovitch, E. and Costeron, J.W. (1970) The functional morphology of pathogenicity in *Entamoeba histolytica*. Ann. Trop. Med. Parasitol. 64, 299–304.
12 El-Hashimi, W. and Pittman, F. (1970) Ultrastructure of *Entamoeba histolytica* trophozoites obtained from the colon and from in vitro cultures. Am. J. Trop. Med. Hyg. 19, 215–226.
13 Griffin, J.L. and Juniper, K. (1971) Ultrastructure of *Entamoeba histolytica* from human amebic dysentery. Arch. Pathol. 21, 271–280.
14 Griffin, J.L. (1972) Human amebic dysentery: Electron microscopy of *Entamoeba histolytica* contacting, ingesting and digesting inflammatory cells. Am. J. Trop. Med. Hyg. 21, 895–906.
15 Deas, J.E. and Miller, J.H. (1977) Plasmalemmal modifications of *Entamoeba histolytica* in vivo. J. Parasitol. 63, 25–31.
16 González-Robles, A. and Martínez-Palomo, A. (1983) Scanning electron microscopy of attached trophozoites of pathogenic *Entamoeba histolytica* J. Protozool. 30, 692–700.
17 González-Garza, M.T., Arellano-Blanco, J. and Gómez-Estrada, H. (1977) Marcaje citoquímico de las enzimas

proteolíticas de *Entamoeba histolytica*. Arch. Invest. Med. (Mex.) 8, 139–144.
18 Diamond, L.S., Harlow, D.R. and Cunnick, C.C. (1978) A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. Trans. R. Soc. Trop. Med. Hyg. 72, 431–432.
19 Aley, S.B., Scott, W.A. and Cohn, Z.A. (1980) Plasma membrane of *Entamoeba histolytica*. J. Exp. Med. 152, 391–404.
20 Schaffner, W. and Weissmann, C. (1973) A rapid, sensitive, and specific method for the determination of protein in dilute solution. Anal. Biochem. 56, 502–514.
21 Starkey, P.M. (1977) Elastase and cathepsin G; the serine proteinases of human neutrophil leukocytes and spleen. In: Proteinases in Mammalian Cells and Tissues (Barrett, A.J., ed.), pp. 57–89, Elsevier/North Holland Biomedical Press, Amsterdam.
22 Ames, G.F.-L. (1974) Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. J. Biol. Chem. 249, 634–644.
23 Merrill, C.R., Dunau, M.L. and Goldman, D. (1981) A rapid sensitive silverstain for polypeptides in polyacrylamide gels. Anal. Biochem. 110, 201–207.
24 Dixon, M. (1953) A nomogram for ammonium sulphate solutions. Biochem. J. 54, 457–458.
25 Bordier, C. (1981) Phase separation of integral membrane proteins in Triton X-114 solution. J. Biol. Chem. 256, 1604–1607.
26 Alcaraz, G., Kinet, J.-P., Kumar, N., Wank, S.A. and Metzger, H. (1984) Phase separation of the receptor for immunoglobulin E and its subunits in Triton X-114. J. Biol. Chem. 259, 14922–14927.
27 Stevenson, K.B., Nauseef, W.M. and Clark, R.A. (1987) The neutrophil glycoprotein Mo1 is an integral membrane protein of plasma membranes and specific granules. J. Immunol. 139, 3759–3763.
28 Tuszynski, G.P., Knight, L., Piperno, J.R. and Walsh, P.N. (1980) A rapid method for removal of ^{125}I iodide following iodination of protein solutions. Anal. Biochem. 106, 118–122.
29 Gitler, C. and Mirelman, D. (1986) Factors contributing to the pathogenic behavior of *Entamoeba histolytica*. Annu. Rev. Microbiol. 40, 237–261.
30 Scholze, H. and Werries, E. (1984) A weakly acidic protease has a powerful proteolytic activity in *Entamoeba histolytica*. Mol. Biochem. Parasitol. 11, 293–300.
31 Rosales-Encina, J.L. and Rojkind, M. (1987) The collagenase of *Entamoeba histolytica*. In: Molecular Strategies of Parasitic Invasion (Agabian, N., Goodman, H. and Nogucira, N., eds.), pp. 559–566, Alan R. Liss, Inc., New York.

Measurement of Casein Digestion by a Fluorometric Method[1]

Pedro Ostoa-Saloma, Jorge Ramírez, and Ruy Perez-Montfort
Departamento de Microbiología, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-242, 04510 México D. F., México

Received June 30, 1988

A qualitative and quantitative method to assay proteolytic degradation of casein with a spectrofluorometer was developed. Proteolysis produced by different pure or mixed proteinases in a pH range 2 to 7.4 quenches the fluorescence emitted at a wavelength of 350 nm by casein excited at 300 nm in less than 5 min. This method is very sensitive, fast, and requires minimal sample preparation. Proteinases that do not generate peptides appropriate for fluorescence quenching cannot be detected with this assay and proteinases with intrinsic fluorescence may require special adjustments of the spectrofluorometer. This method monitors the disappearance of intact substrate proteins continuously, omitting the separation step necessary in other methods to measure product peptides.

Casein has long been used as a substrate for proteolytic assays (1), because it is susceptible to the action of most proteases (2). It is a mixture of soluble hydrophobic milk proteins. Commercial casein is composed mainly of genetic variants of α_s-, β-, and κ-caseins (3).

Most proteolytic assays using casein as the substrate use the protein mixture or colored derivatives dissolved in an aqueous buffer (1,2,4,5). The substrate is cleaved by the proteolytic enzyme under certain defined conditions and the reaction is arrested by precipitation of the undigested proteins with powerful denaturing agents such as trichloroacetic acid, phosphotungstic acid, and HCl, or perchloric acid. The precipitated proteins are separated from the soluble peptides by centrifugation, filtration, or other means, and the extent of hydrolysis is then determined with a spectrophotometer or colorimeter in the solution containing the soluble peptides (2). This procedure is easy to perform and has been used extensively with many variations. In this article we report a method that, using a fluorometer, simplifies the proteolytic assay even further. It is very fast (because it omits the precipitation of undigested casein and the subsequent separation step), has a high sensitivity, and can be used with a variety of pure proteinases or complex mixtures of them. It also permits the continuous measurement of hydrolysis of a protein substrate. Even though the kinetics of casein hydrolysis by proteinases should be interpreted with great caution, this method theoretically allows a much better estimate of the initial velocity of the reaction and also permits a better determination of the change of the activity of the enzyme in time than do existing assays. The data obtained with this assay can also be used for quantitative calculations of enzyme and/or substrate concentration.

MATERIALS AND METHODS

Casein. Bovine milk casein was obtained from Sigma Chemical Co. (St. Louis, MO) and from Pfannstiehl Laboratories, Inc. (Waukegan, IL). A stock solution was prepared by dissolving 1 g of casein in 20 ml of 200 mM boric acid and 160 mM NaCl, pH 8, by heating in a boiling water bath for 1 h. The solubilized casein was cooled to room temperature, centrifuged at 12,000*g* for 30 min, and passed through a 0.45-μm filter (Millipore Corp., Bedford, MA). Protein was determined in the filtrate according to Lowry *et al.* (6). For digestion with pepsin, 60 mg of casein was dissolved by stirring in 10 ml of 0.75% lactic acid in 30 mM HCl (7).

Proteinases and inhibitors. Trypsin, soybean trypsin inhibitor, papain, pepsin, protease from *Streptomyces griseus* type XIV (pronase), and collagenase from *Clostridium histolyticum* type VII were all obtained from Sigma. α-Chymotrypsin was obtained from Worthington Biochemical Co. (Freehold, NJ). All proteinases, ex-

[1] Supported in part by grants from the Subsecretaría de Regulación Sanitaria y Desarrollo, Secretaría de Salud, and CONACyT, México to R.P.-M.

0003-2697/89 $3.00

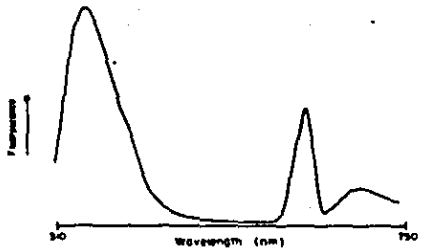

FIG. 1. Fluorescence emission spectrum of casein. A solution containing 1 mg of casein in 2 ml of 50 mM Tris–HCl, pH 7.4, was excited at 300 nm and the emission spectrum between 310 and 750 nm recorded.

cept pepsin, and the inhibitor were dissolved at a concentration of 1 mg/ml in 50 mM Tris–HCl, pH 7.4; the papain solution also contained 1% (v/v) 2-mercaptoethanol. Pepsin was dissolved at the same concentration in 10 mM HCl.

A complex mixture of proteinases was obtained using a lysate of washed trophozoites of the protozoan *Entamoeba histolytica* as previously described (8). An amount equivalent to 4×10^5 cells was used in all experiments.

Fluorometric measurements. All fluorometric measurements were made using a spectrofluorometer Model SMC 210 from SLM Instruments, Inc. (Urbana-Champaign, IL) with a magnetic stirrer, connected to a chart recorder, Model Electronik 196, from Honeywell (Fort Washington, PA).

The stock solution of casein was diluted to a concentration of 0.5 mg/ml with 50 mM Tris–HCl, pH 7.4, with 1% 2-mercaptoethanol for papain digestion or, for pepsin digestion, with 20 mM KCl–HCl, pH 2. A quartz cuvette containing 2 ml of this mixture was placed in the sample holder of the spectrofluorometer. The casein solution was stirred constantly and excited with light at a wavelength of 300 nm; fluorescent emission was recorded at various wavelengths. After obtaining basal readings with intact casein, 10 μg of a proteinase was added, and the fluorescence spectrum was obtained after 2 h at room temperature or the fluorescence, at a wavelength of 350 nm, was recorded immediately.

RESULTS

The fluorescence emission spectrum (310–750 nm) of casein excited at 300 nm showed three peaks at wavelengths of 350, 595, and 671 nm (Fig. 1). Published fluorescence spectra (9) and spectra in 50% dimethyl sulfoxide of individual amino acids (data not shown) suggest that the peak at 350 nm is due mainly to Trp and that there is only a minimal contribution from Tyr and Phe in the proteins. Depending on the fluorescence properties of the solutions which contain the proteinases to be tested, any one of the three peaks may be chosen to assay for proteolytic activity. We chose the peak at 350 nm because initial experiments with the peaks at the other wavelengths showed complex changes in the fluorescence. For example, in assays varying the concentration of a pure proteinase, changes in the fluorescence could start with fluorescence enhancement and lead to fluorescence quenching at higher concentrations. By testing the peak at 350 nm, consistent quenching of fluorescence upon proteinase addition was observed, so this peak was used in all subsequent assays.

Figure 2 shows the fluorescence emission spectra of casein between wavelengths of 310 and 460 nm before (upper tracings) and 120 min after the addition of different proteinases (lower tracings). It is evident that all proteinases that digested the substrate produced fluorescence quenching of the peak at 350 nm.

The fact that quenching was associated with protein digestion is supported by the experiment shown in Fig. 3. Here trypsin was added to casein, measuring fluorescence at 300–350 nm; fluorescence was quenched very rapidly, and it reached a plateau in 3 min.

(That the observed quenching of fluorescence is not due to a loss of turbidity was ascertained by measuring the optical density of a similar casein solution. There was no change in the absorbance at 520 nm for 5 min after the trypsin had been added to the casein. In addition, the casein solution was very transparent and not turbid at all.)

The fluorescence quenching could be stopped by the addition of soybean trypsin inhibitor, and the inhibition of quenching was proportional to the amount of inhibitor added. An amount of inhibitor equivalent to the amount of trypsin added completely blocked the quenching of fluorescence. If trypsin inhibitor was added to the casein solution before the proteinase, virtually no quenching occurred, and the fluorescence did not change

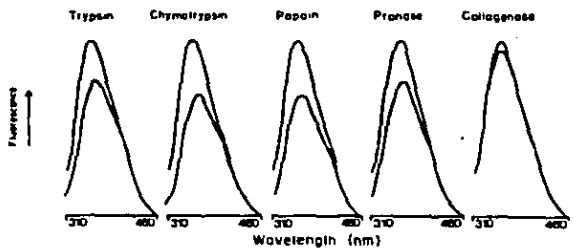

FIG. 2. Fluorescence emission spectra of different casein solutions excited at 300 nm before and after proteinase addition. Spectra between 310 and 460 nm of solutions of casein were recorded initially (upper tracings). Each enzyme (10 μg) was added in 10 μl buffer and incubated for 2 h at room temperature (25°C) and the spectra were recorded again (lower tracings).

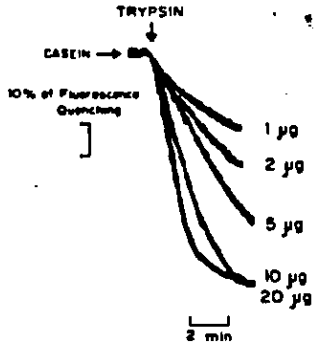

FIG. 3. Fluorescence emission at 350 nm of different casein solutions excited at 300 nm and treated with 1, 2, 5, 10, and 20 µg of trypsin, respectively.

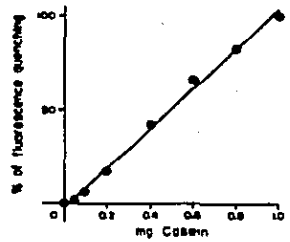

FIG. 5. Linear correlation of maximum percentage of fluorescence quenching and quantity of substrate. Maximum percentage of fluorescence quenching was defined as the point where there was a distinct change in the slope of the tracing (see, e.g., Fig. 6). The equation of the line calculated using linear regression was $y = 105.583x - 2.136$; $r = 0.997$.

if trypsin was added afterward to the mixture. Figure 4 shows that the rate of quenching was proportional to the amount of proteinase added to the system and Fig. 5 indicates that the maximum percentage of fluorescence quenching showed a linear correlation with the substrate concentration in the range 0.05 to 1 mg. Figure 6 shows that the relationship between the velocity of quenching and the concentration of different proteinases was linear in a range 2 to 50 µg for papain, pepsin, trypsin, and chymotrypsin.

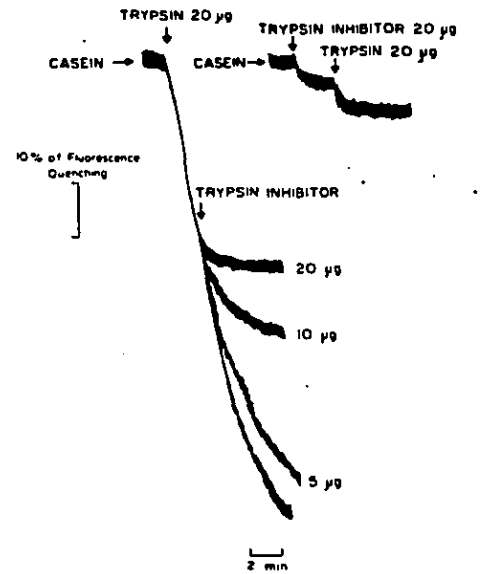

FIG. 4. Fluorescence emission at 350 nm of different casein solutions excited at 300 nm and treated with 20 µg of trypsin and subsequently with 5, 10, and 20 µg of trypsin inhibitor. The right side tracing shows addition of the enzyme and the inhibitor in inverse order.

The digestion of casein by pepsin at pH 2 quenched its fluorescence in a manner similar to that shown for trypsin. No fluorescence quenching was observed with pepsin at pH 7.4. The lysate of *E. histolytica* containing a complex mixture of proteases also quenched the fluorescence of the casein solution at pH 7.4 (data not shown).

DISCUSSION

The fluorometric method of casein digestion detection presented in this article offers many of the same advantages of traditional methods that use the same substrate protein and measure the sum of product peptides. It works in a pH range just as wide as that of the other methods and the substrate protein contains just as many different peptide bonds accessible to the proteinases. One disadvantage is that not every hydrolytic cleavage will give rise to a signal and that the size of the signal will probably depend on how near the cleavage site of the proteinase is to the relevant Trp (see below). In this assay chymotrypsin and papain generated better signal changes (higher quenching) per weight of protein than did trypsin and pronase (Fig. 2). Collagenase, which does not generate peptides in a conventional casein digestion assay, did not quench the fluorescence of the casein solution. This assay is qualitatively completely reproducible. For quantitative assays it is necessary to define only the maximum level (%) of fluorescence quenching every time a series of experiments is made. This value can be used to correlate fluorescence quenching with the exact amount of degraded casein, if the substrate concentra-

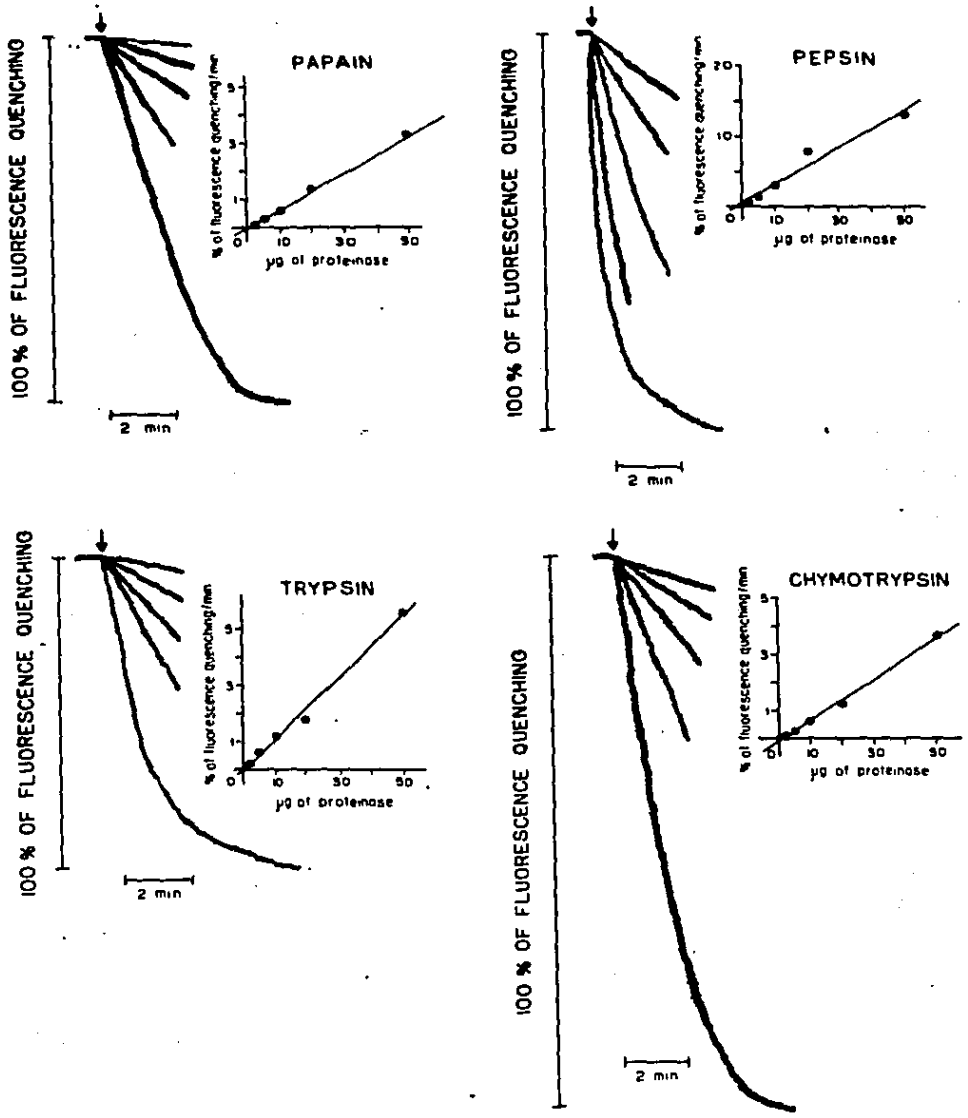

FIG. 6. Linear correlation of fluorescence quenching velocity and concentration of various proteinases. The arrows mark the time of proteinase addition. The tracings with increasing negative slope correspond to fluorescence quenching at 350 nm after addition of 2, 5, 10, 20, and 50 μg of proteinase, respectively. The linear regression equations calculated for the lines shown in the insets were papain, $y = 0.061x + 0.005$, $r = 0.998$; pepsin, $y = 0.267x + 0.640$, $r = 0.981$; trypsin, $y = 0.110x - 0.037$, $r = 0.995$; and chymotrypsin, $y = 0.075x - 0.077$, $r = 0.998$.

tion is known with precision, or with the amount of proteinase added. Unknown values can be interpolated linearly at least for the quantities shown in Figs. 5 and 6. The detection limits for different proteinases may vary depending on the fluorescence quenching generated by the particular proteolytic cleavage. Our estimate for trypsin under the conditions we used is the detection of 0.1 μg of enzyme in 3 min of incubation time. Comparison with other reported methods using unmodified casein as substrate indicate that this method has the following advantages: (a) it is approximately 50 times as sensitive and 6 times faster (10); (b) it is just as sensitive as other methods using protein substrates derivatized with fluorescent probes (10), but derivatization is not necessary and there are no photolabile fluorophores; (c) it is very practical, since it requires little effort in preparing the substrate and the proteinase(s) and avoids all separation steps of the generated peptides; (d) the signal generated can be measured in 5 min so that the time needed for one or many tests is relatively short; and (e) the only equipment necessary is a fluorometer with a stirrer and a chart recorder.

Stirring was necessary to obtain a reproducible and constant rate of fluorescence quenching, since in experiments without stirring this rate varied. A stirrer can be adapted to almost all spectrofluorometers that do not have one by using a rheostat, an electric motor, and a magnet. We also found that the volume of the sample and the form of the magnetic stirring bar affected the end results; in our system a volume of 2 ml and a cell stirrer gave the best results.

One advantage of this method over traditional methods, using casein as substrate, is that it can monitor continuously the proteolytic degradation of a protein. In this way more data about the reaction are obtained using less reagents and time. Also, as previously mentioned, taking all due precautions in the interpretation, it allows a better estimate of the initial velocity and the continuous monitoring of the change in enzyme activity in time.

One possible limitation of this method might be interference in fluorescence quenching by proteolytic enzymes that have a high intrinsic fluorescence. Theoretically, this can be avoided by making the appropriate adjustments to the basal fluorescence generated separately by the proteinase. Using a crude lysate of *E. histolytica*, which contains a complex mixture of proteases and shows some intrinsic fluorescence, this was successfully accomplished.

It has been previously shown that the fluorescent emission at 348 nm of proteins excited at wavelengths between 295 and 305 nm is due mainly to the Trp residues (9). To emit fluorescence the Trp has to be in a hydrophobic medium, as is the case for most of the residues of this amino acid in the sequences reported for the α_{s1}-, βA^2-, and κ-B-caseins (11–13). We assume that many of the Trp residues are in a similar environment in all the caseins that are components of commercial casein. Proteolysis of these proteins must generate peptides in which the polarity of the environment around certain Trp residues increases with respect to that in the intact proteins. This change in the polarity of the milieu surrounding these Trp residues probably produces the observed fluorescence quenching.

Finally, we state some considerations on the contribution of this method to general aspects of proteinase assays using proteins as substrates. Theoretically hydrolysis of peptide bonds can be detected measuring (i) the decrease of the intact protein used as substrate, (ii) the increase in free carboxylic or amino groups, and (iii) the increase of peptide products. For the first case basically two types of methods have been described which are (a) the loss of turbidity when a protein is solubilized by proteolysis and (b) the loss of enzyme activity (10). Our assay falls into this category but introduces another method for measuring the disappearance of the intact substrate protein. It measures proteolysis as a function of the change of the environment of an aromatic amino acid.

ACKNOWLEDGMENT

The authors thank Dr. Antonio Peña for helpful suggestions and for careful revision of the manuscript.

REFERENCES

1. Laskowski, M. (1955) *in* Methods in Enzymology (Colowick, S. P., and Kaplan, N. O., Eds.), Vol. 2, pp. 26–36, Academic Press, New York.
2. Walter, H. E. (1984) *in* Methods of Enzymatic Analysis (Bergmeyer, H. U., Ed.), Vol. 5, pp. 270–277, Verlag Chemie, Weinheim.
3. Scott, T. A., and Brewer, M. (1983) Concise Encyclopedia of Biochemistry, pp. 276–277, de Gruyter, Berlin.
4. Charney, J., and Tomarelli, R. M. (1947) *J. Biol. Chem.* **171**, 501–505.
5. Starkey, P. M. (1977) *in* Proteinases in Mammalian Cells and Tissues (Barrett, A. J., Ed.), pp. 57–89, Elsevier–North Holland, Amsterdam.
6. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
7. Umezawa, H., and Aoyagi, T. (1977) *in* Proteinases in Mammalian Cells and Tissues (Barrett, A. J., Ed.), pp. 637–662, Elsevier–North Holland Biomedical Press, Amsterdam.
8. Perez-Montfort, R., Ostoa-Saloma, P., Velazquez-Medina, L., Montfort, I., and Becker, I. (1987) *Mol. Biochem. Parasitol.* **26**, 87–97.
9. Lakowicz, J. R. (1983) Principles of Fluorescence Spectroscopy, pp. 341–382, Plenum, New York.
10. Wunderwald, P. (1984) *in* Methods of Enzymatic Analysis (Bergmeyer, H. U., Ed.), Vol. 5, pp. 258–270, Verlag Chemie, Weinheim.
11. Mercier, J. C., Grosclaude, F., and Ribadeau-Dumas, B. (1971) *Eur. J. Biochem.* **23**, 41–51.
12. Ribadeau-Dumas, B., Brignon, G., Grosclaude, F., and Mercier, J. C. (1972) *Eur. J. Biochem.* **25**, 505–514.
13. Mercier, J. C., Brignon, G., and Ribadeau-Dumas, B. (1973) *Eur. J. Biochem.* **35**, 222–235.