Zymogen Activation as a Sensitive Enzyme-Amplifying Assay for a Protease with Tryptic Specificity

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(Received 10 October 1969)

A protease, 'trypsinogenase', secreted in small amounts by the sea-urchin blastula, is assayed in two steps as an example of enzyme-amplifying kinetics. In reaction 1 the trypsinogenase catalyses the activation of trypsinogen to trypsin. In reaction 2 the trypsin catalyses the hydrolysis of *N*- α -toluene-*p*-sulphonylarginine methyl ester, at a rate that is linear with trypsinogenase concentration over a 20-fold range. Results are reproducible within a batch of zymogen, but each batch requires a separate standard curve.

In a study of the proteolytic activity secreted by sea-urchin blastulae at the time of hatching, I have encountered in very small amounts an enzymic activity that resembles trypsin in specificity, but does not hydrolyse the common ester substrates for trypsin, at least as followed by spectrophotometry (Hummell, 1959). Fluorimetry would permit even more sensitive monitoring of the reaction (Sardesai & Provido, 1965) but was not available. The need for a very sensitive assay with a non-ester substrate led to the choice of trypsinogen, the inactive zymogen that is converted by the cleavage of a single bond (Lys₍₆₎-Ile₍₇₎) into an *N*-terminal hexapeptide and enzymically active trypsin (Neurath, 1964). Trypsin, the product of the reaction, is in turn assayed in its catalytic capacity as an esterase, providing an amplification factor that may, in theory, go as high as the turnover number (molecular activity) of trypsin. The enzyme has been trivially named echinoid blastula trypsinogenase.

Limited proteolysis is widespread in Nature (Ottesen, 1967), and it has been suggested that enzyme amplifier systems may be of broad significance (Levine, 1966). The present paper is, to my knowledge, the first suggestion of their application as an analytical tool. This scheme is presented as an example of a principle that may have wider utility with conditions tailored to other proteases to be assayed.

MATERIALS AND METHODS

Enzymes. Echinoid blastula trypsinogenase was prepared as hatching enzyme from hatching blastulae of *Strongylocentrotus purpuratus* (Barrett & Angelo, 1969).

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Preparation of trypsinogen. Trypsinogen commercially available from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.) or Calbiochem (Los Angeles, Calif., U.S.A.) is sufficiently low in trypsin background activity to be useful. Trypsinogen, stored at -20°C, was weighed out, dissolved at 0.5 mg/ml in 1 mM-HCl in an ice bath, filtered through a Millipore HA filter (Millipore Corp., Bedford, Mass., U.S.A.) and rapidly dispensed in approx. 0.5 ml quantities into 10 mm $\times$ 75 mm test tubes, previously sterilized in a steam bath for 15 min, heat-dried and cooled. The tubes were all frozen together at -20°C. At 2 min before it was required in the protocol, a tube of trypsinogen was thawed and stored on ice. Samples were withdrawn for a period of up to 10 min and the remainder was discarded to forestall unacceptable build-up of trypsin by autocatalysis.

First reaction: trypsinogen activation. The first reaction mixture was made up of 20 μ l of buffer (100 mM-glycylglycine-3 mM-CaCl₂, adjusted with NaOH to pH 8.0) and 80 μ l of sample dialysed against 1 mM-glycylglycine-1 mM-CaCl₂, adjusted with NaOH to pH 8.0. A series of reaction mixtures was set up in the U-shaped depressions of a disposable Lucite (polymethyl methacrylate) plate (microtitre plate; Cooke Engineering Co., Alexandria, Va., U.S.A.) supported in a water bath at 30°C. Trypsinogen was introduced to start the reaction by means of a 'microdiluter' (Cooke Engineering Co.), a stainless-steel claw that holds 25 μ l by capillary action, and serves both to deliver its contents and to mix them with the enzyme sample and buffer. When the 'microdiluter' was withdrawn, it removed 25 μ l again, so that a 100 μ l reaction mixture remained, but a net dilution of 125/100 had been effected. The 'microdiluters' were washed and flamed between uses.

The mixtures were incubated at 30°C for 20 min unless specified otherwise.

Salts were reagent grade. Highest grade glycylglycine available from Nutritional Biochemical Corp. (Cleveland, Ohio, U.S.A.), Calbiochem and Cyclo Corp. (Los Angeles, Calif., U.S.A.) was sufficiently contaminated to warrant filtration through a Millipore HA filter before use.

Second reaction: assay of trypsin released from trypsinogen. At the end of the 20 min period, the first reaction mixture was quantitatively transferred by disposable pipette to a microcuvette prepared with 0.9 ml of substrate mixture: 50 mM-sodium glycylglycine, pH 8.0, 10 mM- CaCl_2 , 0.58 mM-*N*- α -tosylarginine (*N*- α -toluene-*p*-sulphonylarginine) methyl ester (Mann Research Laboratories, Inc., New York, N.Y., U.S.A.). The first reaction was stopped by this dilution and competition, and the product, trypsin, proceeded to attack the ester substrate with pseudo-first-order kinetics at a rate proportional to trypsin concentration (Hummell, 1959). The reaction was monitored in a spectrophotometer at 247 nm and 1 unit defined as the amount of trypsinogenase required to give $\Delta E_{247}/\text{min} = 1.00$.

Timing. The protocol must allow time for monitoring the second reaction. A Gilford model 2000 recording spectrophotometer (Gilford Instrument Laboratories, Oberlin, Ohio, U.S.A.) was used to record extinction from four cuvettes alternately. Reactions were started at 1 min intervals, with a 4 min interval after each set of four. Thus trypsinogen was added at times 0, 1, 2, 3, 7, 8, 9, 10, 14 min etc.; the first four reaction mixtures were diluted into *N*- α -tosylarginine methyl ester substrate at 20, 21, 22, and 23 min and monitored until 27 min when the next set was ready.

Precautions for low background trypsin activity. Trypsin adsorbs on glass with remarkable stability to drying and washing (James & Augenstein, 1966). The cuvettes were rinsed with water, soaked for 1 min with commercial bleach (6% sodium hypochlorite) and thoroughly rinsed. Numerous other cleaning agents and oxidants were tried, but left detectable residual trypsin. If glass micropipettes, and test tubes as reaction vessels, were used, they too required washing with bleach. Thus it was found more

convenient to use plastic ware or disposable glassware, and to deliver the trypsinogen with the steel microdiluters, which are ready for re-use after rinsing and briefly flaming.

RESULTS

During the first reaction three processes give rise to the trypsin eventually assayed in the second reaction: (a) exogenous enzyme activates trypsinogen; (b) the trypsin thus liberated proceeds to attack further trypsinogen autocatalytically; (c) the trypsinogen starts out with an unavoidable contamination of trypsin.

The autocatalytic components (b) and (c) impart to the plot of trypsin produced versus time of incubation of the first reaction, a shape that is more or less sigmoidal, depending on the amount of exogenous enzyme assayed. Such plots are shown in Fig. 1, which also demonstrates that this particu-

Fig. 1. Trypsinogen activation by echinoid blastula trypsinogenase: dependence on Ca^{2+} . Tryptic activity (towards *N*- α -tosylarginine methyl ester) was determined after constant amounts of trypsinogenase and trypsinogen were incubated for various periods at pH 8.0 with 16 mM-glycylglycine, and: ●, 0.5 mM- Ca^{2+} ; ■, 1 mM- Ca^{2+} ; ▲, 5 mM- Ca^{2+} ; ○, 10 mM- Ca^{2+} ; □, 10 mM-EDTA.

Fig. 2. Trypsinogen activation by trypsinogenase: dependence on amount of enzyme. (a) Three separate determinations (○, △, and □) done with tubes of trypsinogen from batch (a); (b) three determinations of the same enzyme done with tubes of trypsinogen from batch (b).

lar protease requires Ca^{2+} , in very low concentration. Inhibition by EDTA is reversible by dialysis against 1mM-calcium chloride. The amount of product obtained is not strictly linear with time, and thus the standard incubation time of 20min has been chosen somewhat arbitrarily.

Interacting with the other reactants from the beginning, the small tryptic contaminant present in the substrate trypsinogen has a considerable influence on the end results. Results are reproducible within a batch of trypsinogen tubes made up and frozen together, but different batches give somewhat different curves. Thus Fig. 2(a) reports three series of trypsinogenase concentrations assayed with the same batch of trypsinogen, and allows an estimate of reproducibility. For comparison, Fig. 2(b) shows a similar plot for the same enzyme assayed with trypsinogen tubes from a different batch. The curves are typical of many I have obtained. They differ significantly, but share the following characteristics: (a) the plot does not run through the origin, but intersects the abscissa (i.e. very small amounts of enzyme are not detected); (b) linearity is observed over at least a 20-fold concentration of enzyme before response levels off at high concentrations; (c) with the low concentration of trypsinogen used here as substrate (0.1mg/ml) background production of trypsin is negligible, too little to break down *N*- α -tosyl-arginine methyl ester at a rate of 0.001 E_{247} unit/min.

DISCUSSION

The reactions occurring in the trypsinogen-activation step are complex and interrelated and the quantities of enzyme determined have so far been expressed only in operational terms. An estimate of the actual number of bonds broken by trypsinogenase would require evaluation of the contributions of at least the autocatalytic component, and trypsin autolysis. Assuming the simple scheme:

where k_1 and k_2 are the pseudo-zero-order rate constants for reactions 1 and 2, and R_3 is the complex rate of reaction 3, it may be possible to evaluate the contributions of reactions 2 and 3, from reaction mixtures without trypsinogenase. With these data in hand it is possible in principle to add trypsinogenase to the system and derive k_1 , from the expression:

$$\frac{d[\text{trypsin}]}{dt} = k_1[\text{trypsinogenase}] + k_2[\text{trypsin}] - R_3$$

In practice, however, the determinations demand high precision to be useful and analysis becomes

very cumbersome. The calculations have not been pursued.

The peculiar inhibition of echinoid blastula trypsinogenase by Ca^{2+} , starting at concentrations as low as 1mM, is a drawback, which probably considerably limits sensitivity. At higher Ca^{2+} concentrations the trypsin produced would be more stable (Desnuelle & Gabelotau, 1957), a clear advantage to be considered in devising similar assays for other enzymes of tryptic specificity.

Within the restriction imposed by Ca^{2+} , however, the use of trypsinogen as a substrate has allowed the quantitative assay of an enzyme activity that I have not succeeded in demonstrating by other means.

As increasing numbers of enzymes resembling trypsin are investigated, more variations from the basic plan of tryptic specificity are recognized (De Villez & Buschlen, 1967; Ekfors, Malmiharju, Reikkinen & Hopsu-Havu, 1967; De Villez & Johnson, 1968; Stambaugh & Buckley, 1968). An enzyme resembling trypsinogenase to the extent that it is active at neutral pH and has peptidase activity without esterase activity has been implicated in human immunological vascular damage (Janoff & Zeligs, 1968). Particularly in the probing of the low protease activities that effect intracellular turnover, other candidates for assays deriving sensitivity from the amplification principle may be found.

I thank Professor D. Mazia for encouragement and support of the work, which was begun in his laboratory at Berkeley, and Miss M. A. Omori and Mr D. J. Klingborg for excellent technical assistance in the later stages at Davis. Support was from Postdoctoral Fellowship GM 13944 and Grant HD-03479 from the National Institutes of Health, U.S. Public Health Service.

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