Occurrence and Properties of a Thermostable Protease Secreted by Sea-Urchin Embryos

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1. A trypsinogen-activating enzyme is distinct from the chorion-dissolving enzyme of the sea-urchin blastula, but is secreted with it. 2. This 'trypsinogenase' does not attack *N*- α -toluene-*p*-sulphonylarginine methyl ester, and is not inhibited by concentrations of di-isopropyl phosphorofluoridate or 7-amino-1-chloro-3-toluene-*p*-sulphonamidoheptan-2-one that are 98% effective against trypsin. It requires a small amount of Ca^{2+} for activity, but is inhibited by more, from as little as 1 mM. 3. Enzymic activity is associated with a wide range of particle weights, some apparently as high as 15×10^6 . The mean particle weight can be decreased by sonication. 4. Fresh trypsinogenase in the higher range of particle weights appears to be remarkably stable to heating, e.g. retaining full activity after boiling for 15 min.

At hatching, the sea-urchin blastula secretes an enzyme or enzymes that dissolve the embryo's protective fertilization membrane, which Anderson (1968) has properly renamed the chorion. The hatching function is proteolytic (Barrett, 1968).

To assay hatching-enzyme preparations with high sensitivity, a method was devised (Barrett, 1970) wherein hatching enzyme converts trypsinogen into active trypsin and the trypsin is then assayed for esterase activity. 'Trypsinogenase' activity was found to the extent of at least 300-fold above background (buffer solution) in all hatching-enzyme preparations tested, but subsequently the chorion-dissolving enzyme and the trypsinogen-activating enzyme were found to be separable by various means. The trypsinogenase activity continued to command attention, however, for two reasons: first, since it may be synthesized in the cleavage or early blastula stages of the echinoid, a period in which the mechanisms and control of protein synthesis in general have been extensively studied (Gross, 1967) but the nature of the individual proteins synthesized has not yet been discovered; secondly, because of anomalous properties of the enzyme that are of biochemical interest, notably its remarkable resistance to heating. Some of these characteristics of the enzyme are the subject of this paper.

MATERIALS AND METHODS

Preparation of enzyme. By methods detailed by Barrett & Angelo (1969), *Strongylocentrotus purpuratus* embryos were raised in Millipore-filtered sea water con-

taining 100 μg each of penicillin and streptomycin/ml until 5% had hatched, then washed and concentrated to a 10% (v/v) suspension for hatching. When hatching was nearly complete the blastulae were centrifuged off at 12000g for 10 min. The supernatant was stored frozen in sea water, and dialysed at 4°C against 1 mM- CaCl_2 -1 mM-glycylglycine, adjusted to pH 8.0 with NaOH, before assay. When necessary, enzyme preparations were concentrated by filtering under 50 lb/in² of N_2 , through a Diaflow membrane UM-10 (Amicon Corp., Lexington, Mass., U.S.A.), which passes salt but retains macromolecules of mol.wt. > 10000.

Enzyme assays. Trypsinogen-activating activity (trypsinogenase) was assayed as detailed in the companion paper (Barrett, 1970). Esterase activity was evaluated with *N*- α -tosylarginine (*N*- α -toluene-*p*-sulphonylarginine) methyl ester (grade I; Mann Research Laboratories Inc., New York, N.Y., U.S.A.) as described by Hummell (1959).

Column separations. Enzyme preparations were fractionated on Sephadex G-200 (Pharmacia AB, Uppsala, Sweden), with a nominal exclusion limit of 200 000 mol.wt., and on Bio-Gel A-15 (Bio Rad Laboratories, Richmond, Calif., U.S.A.), with a nominal separation range of 4×10^4 - 15×10^6 mol.wt. Horse heart cytochrome c, human haemoglobin and γ -globulin and porcine thyroglobulin were purified standards from Mann Research Laboratories Inc. Tobacco mosaic virus was a gift from Dr George Bruening.

Reagents. Di-isopropyl phosphorofluoridate and 7-amino-1-chloro-3-toluene-*p*-sulphonamidoheptan-2-one were from Calbiochem (Los Angeles, Calif., U.S.A.)

RESULTS

Occurrence. By removing chorions from embryos shortly after fertilization (Mazia, Mitchison, Medina

& Harris, 1961) it is possible to raise nude embryos and collect the enzyme as it is secreted, rather than waiting until after the chorion has been digested by the embryo. From several experiments of this sort (results not presented) it is clear that trypsinogenase begins to be secreted at approximately the same time as hatching enzyme proper (as assayed *in vitro* by digestion of chorions; Barrett & Angelo, 1969) but continues to appear in the medium well after all the hatching enzyme has been secreted.

Differences from trypsin. The conversion of trypsinogen into trypsin has been effected by various means (Abrams & Jacobsen, 1951; Awad & Wilcox, 1963; Gabeloteau & Desnuelle, 1960; Yamashina, 1956), all involving the hydrolysis of the Lys₍₆₎-Ile₍₇₎ bond of trypsinogen, by an enzyme with the same specificity of attack as trypsin itself has. An obvious hypothesis is that the echinoid trypsinogenase is essentially another naturally occurring trypsin. With the substrate of choice for spectrophotometry, *N*- α -tosylarginine methyl ester (Hummell, 1959), however, no trypsin-like esterase activity can be demonstrated. On a weight basis, rate of attack on *N*- α -tosylarginine methyl ester by the crude trypsinogenase enzyme can be set at lower than 10^{-4} times that of purified trypsin. A fluorimetric modification of the method (Sardesai & Provido, 1965) might be used for even greater sensitivity. The enzyme is similarly inactive against *N*- α -benzoylarginine naphthylamide.

The active site of trypsin is well characterized, and two irreversible inhibitors are particularly useful as a diagnostic: di-isopropyl phosphorofluoridate attacks a seryl residue (Balls & Jansen, 1952) and 7-amino-1-chloro-3-toluene-*p*-sulphonamidoheptan-2-one attacks a histidyl residue (Shaw, Mares-Guia & Cohen, 1965), both of which are involved in the active site. Results demonstrating that each of these inhibitors is powerless against

trypsinogenase, under conditions by which trypsin is quantitatively inhibited, are presented in Tables 1 and 2.

Sensitivity to metal ions. In the presence of 0.1 mM-Ca²⁺, other bivalent cations were supplied in tenfold excess in the first reaction of the assay. Fe²⁺, Co²⁺, Hg²⁺ and Zn²⁺ inhibited the trypsinogenase by at least 70%, whereas Mn²⁺, Sr²⁺, Mg²⁺, Ba²⁺ and Cu²⁺ did not inhibit. It was established that the effects were on the trypsinogenase activity, not, after tenfold dilution, on the activity of the trypsin produced. In fact, trypsin is insensitive to 1 mM concentrations of Fe²⁺, Co²⁺ or Hg²⁺ but sensitive to Cu²⁺ (Green, Gladner, Cunningham & Neurath, 1952).

Resistance to boiling. The finding that freshly prepared trypsinogenase is highly resistant to heating was startling, but it has been confirmed many times. The enzyme retains a significant amount of activity after 1 h in a boiling-water bath. Thus it appears to be more stable than the bacterial protease thermolysin (Endo, 1962).

The notion that apparent stability was a function of renaturation in the cold, after denaturation at boiling temperatures, has been tested as far as ordinary techniques allow. After boiling for 15 min, trypsinogenase can be diluted with cold buffer, pipetted into the reaction vessel and trypsinogen added to start the reaction, all within 15 s. Its activity is essentially the same immediately after boiling as that before boiling or after a 60 min period in the cold allowed for renaturation. If renaturation is the mechanism, it must occur very rapidly.

A different explanation was suggested by the observation that, after many cycles of storing in the

Table 1. *Effect of di-isopropyl phosphorofluoridate on trypsinogenase and trypsin*

Enzyme preparations were assayed for trypsinogenase activity (Barrett, 1970) or esterase activity (towards *N*- α -tosylarginine methyl ester; Hummell, 1959) after incubation at 25°C for 30 min in the presence or absence of 0.05 mM-di-isopropyl phosphorofluoridate (DFP), followed by exhaustive dialysis in the cold. Units of trypsinogenase activity are defined by Barrett (1970).

Enzyme	DFP	Trypsinogenase activity (milliunits)	Esterase activity (μ mol/min)
Trypsinogenase	—	78	
	+	85	
Trypsin	—		22.4
	+		0.3

Table 2. *Effect of 7-amino-1-chloro-3-toluene-*p*-sulphonamidoheptan-2-one on trypsinogenase and trypsin*

Enzyme preparations were assayed for trypsinogenase activity (Barrett, 1970) or esterase activity (towards *N*- α -tosylarginine methyl ester; Hummell, 1959) after incubation at 26°C for 60 min in the presence or absence of 1 mM-7-amino-1-chloro-3-toluene-*p*-sulphonamidoheptan-2-one (TLCK, 'tosyl-lysine chloromethyl ketone'), followed by exhaustive dialysis in the cold.

Enzyme	TLCK	Trypsinogenase activity (milliunits)	Esterase activity (μ mol/min)
Trypsinogenase	—	97	
	+	83	
Trypsinogenase	—	225	
	+	169	
Trypsin	—		82.0
	+		1.5

Fig. 1. Gel exclusion chromatography on a column (2.5 cm $\times$ 40 cm) of agarose (Bio-Gel A-15) of samples (2 ml) of trypsinogenase in 20 mM-tris-10 mM- CaCl_2 -0.5 M-NaCl-1 mM- NaN_3 buffer, pH 8.0. Fractions (3.0 ml) were collected. (a) Fresh trypsinogenase preparation: $\bullet$, enzyme activity (units and assay as in Barrett, 1970); $\square$, E_{280} . Molecular-weight markers: v, tobacco mosaic virus; t, thyroglobulin; g, γ -globulin; h, haemoglobin; c, cytochrome c. (b) Trypsinogenase preparation: after sonication for five 1 min periods in a test tube in an ice bath with a Biosonik II micro-probe at maximum power: $\bullet$, enzyme activity; $\square$, E_{280} .

freezer and thawing for use, some of the heat-stability was lost. Further findings supporting the separability of the enzyme and its anomalous stability are presented below.

Particle size and heterogeneity. To examine the homogeneity of the trypsinogenase activity and obtain a rough estimate of molecular weight the enzyme was subjected to gel exclusion chromatography on Sephadex G-200. Most of the trypsinogenase activity was found in the void volume, suggesting a molecular weight higher than 200 000. To confirm and extend this conclusion, the enzyme was chromatographed on Bio-Gel A-15. Fig. 1(a) shows that the particle size may be very large; some of the activity appears in the excluded volume, so that like the tobacco mosaic virus used for calibration its particle weight exceeds 15×10^6 . The range of sizes is also very wide; some of the activity is eluted near the position for cytochrome c, which marks the

Table 3. Heat-stability of fractions of trypsinogenase separated on an agarose column

Fractions were assayed before and after boiling in a sealed tube for 15 min. A rough estimation of particle weight corresponding to the position of the fraction is based on the calibration curve plotted in Fig. 1(a).

Fraction no. ...	22	30	37
Estimated particle weight	$>15 \times 10^6$	4×10^6	1×10^6
Trypsinogenase activity, fresh	180	58	35
Trypsinogenase activity, after boiling	176	55	4.0
Heat resistance	98%	95%	11%

lower limit of separation efficiency at mol.wt. 40 000. If the virus and cytochrome c are assumed to mark the boundaries of the separation interval and are plotted at the limits of particle weight designated by the manufacturer, then Fig. 1(a) shows that the elution volumes of the calibrating proteins are linearly related to the logarithms of the particle weights, without the anomalies observed with Sephadex (Andrews, 1965).

A sample of the same enzyme preparation was subjected to prolonged sonication. Fig. 1(b) shows that the activity of the sonicated enzyme is still associated with a very wide range of particle sizes, but peak activity has moved considerably, to a position corresponding roughly to only 4×10^6 molecular weight.

On the hypothesis that the anomalous stability of the trypsinogenase was correlated with its state of aggregation, fractions from a separation similar to that of Fig. 1(a) were selected and boiled. The results in Table 3 give an indication, which remains to be confirmed, that the lower-molecular-weight enzyme is normally heat-labile, whereas heat-stability is correlated with the higher size range.

DISCUSSION

A trypsinogen-activating principle is secreted by sea-urchin blastulae, beginning contemporaneously with the secretion of hatching enzyme, but continuing well after the hatching period. Since in all cases where it has been investigated the conversion of bovine trypsinogen into trypsin requires the hydrolysis of the Lys₍₆₎-Ile₍₇₎ peptide bond (Neurath, 1964), it is assumed tentatively that the trypsinogenase must be capable of attacking at least peptide bonds involving the carboxyl group of basic amino acids, i.e. with the same specificity of attack as trypsin. However, the enzyme is distinguished from trypsin in that it is devoid of esterase activity, and evidence is presented above that it is resistant to the inhibitors to which trypsin is characteristically sensitive.

The remarkable stability of trypsinogenase to high temperature invites further investigation. Failure to show increased activity with recovery time mitigates against the mechanism of renaturation after reversible heat denaturation, but cannot exclude it. The discovery by gel exclusion chromatography that the enzyme units are either aggregated into a large variety of different multimers or bound to other matter, in a broad continuum of sizes, suggests that binding confers protection against denaturation, perhaps in a rather extraordinary example of stabilization by substrate. Freezing-and-thawing cycles would serve to break down the agglomerates, with resultant loss of the protective effect. As a third alternative, it is conceivable that boiling simultaneously destroys active sites and uncovers new ones, at rates that, fortuitously, balance.

Perhaps the area of greatest ignorance about the enzyme is its possible physiological role, since it has been investigated only with the highly artificial zymogen substrate. Its action is completely inhibited by the concentration of Ca^{2+} present in the sea water where it is found (Barrett, 1970). It can be assumed that this effect is a function of its interaction with the particular macromolecular substrate, but its activity on other substrates remains to be explored.

By using trypsinogen activation as an assay tool, and the anomalous heat-stability as a powerful first step, the way should now be open to purify the enzyme in large enough quantities to detect its activity in more nearly physiological conditions.

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