PROTEOLYTIC ACTIVITY IN HUMAN SKIN FIBROBLASTS HARVESTED WITH TRYPSIN AND ITS EFFECT ON MEASUREMENTS OF COLLAGEN HYDROXYLASE ACTIVITIES

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SUMMARY: Lysates of human skin fibroblasts harvested without the use of trypsin do not contain detectable proteolytic activity, but when trypsin is used, lysates may contain activity equal to 10 ng of trypsin/10^7 cells. The amount of cell lysate ordinarily examined for collagen prolyl and lysyl hydroxylase activity is sufficiently small that such amounts of trypsin have no observable effect on the unhydroxylated collagen substrate. Larger amounts of trypsin cause proteolysis of the unhydroxylated collagen substrate and a reduction of both prolyl and lysyl hydroxylation with lysyl hydroxylation more affected at low trypsin concentration than prolyl hydroxylation.

Considerable interest has been generated in measurements of collagen lysyl hydroxylase (E.C. 1.14.11.4) and prolyl hydroxylase (E.C. 1.14.11.2) activities in cultured human skin fibroblasts since the demonstration that cells from subjects with heritable hydroxylysine-deficient collagen are markedly deficient in lysyl hydroxylase activity though prolyl hydroxylase activity is comparable to that present in cells from normal subjects (1,2). In order to quantitate the deficiency and to study the nature of this and other enzymatic defects, hydroxylase activities are usually measured in cultures harvested by brief treatment with trypsin (0.25%) to facilitate cell dispersion and counting for subsequent suspension of the cells at a known density in buffer prior to cell lysis. In the course of studying lysyl hydroxylase activity with a synthetic octapeptide substrate (^{125}I) Tyr-Gly-Ile-Lys-Gly-Ile-Lys-Gly, prepared by solid phase peptide synthesis, we observed that incubation with fibroblast lysate in the absence of cofactors necessary for hydroxylation led to changes in the electrophoretic mobility of the substrate consistent with proteolytic cleavage (3). However, alteration of the peptide was eliminated if the cell lysate was heated briefly in boiling water or if cells were harvested

can occur in the absence of DNA synthesis, we conducted a series of experiments to determine what factors are required for the initiation of swarmer growth. The results of these experiments are presented in this communication.

METHODS AND MATERIALS

Caulobacter crescentus (ATCC 15252) was grown in Peptone-Yeast extract media (PYE) as described by Pointdexter (4). Cultures were grown in a New Brunswick Environmental Shaker at 30°C and 150 r.p.m. and growth was followed using a Klett-Summerson Colorimeter (420 nm filter). Cell counts were made using a Petroff-Hauser counting chamber.

Swarmer cells were isolated from mid-log cultures using a modification of the technique described by Stove and Stanier (5). An initial 15 minute centrifugation at 1,000 x g in an IEC HN-S Swing bucket centrifuge followed by two 10 minute runs yielded swarmer populations of greater than 95% purity. Swarmers were then incubated at 30°C for 60 minutes in the culture media prior to growth studies to insure synchronous growth of the swarmers. Checks on each isolation as well as calculations on the distribution of cell types throughout synchronous growth were performed on negatively-stained cells scanned in a Philips EM200 electron microscope.

Cell fragments used in mixing studies were prepared from washed (3x) stalked cells harvested in their late-log growth phase, disrupted in a French Pressure Cell at 20,000 p.s.i. and treated with DNAse (100μg/ml) for 30 minutes at room temperature. The fragments were then collected by centrifugation for 30 minutes at 27,000 xg, the pellet fractions washed 3x in fresh PYE and stored in PYE for use in the swarmer studies.

Soluble cell envelope factor was released from stalked cell fragments suspended in 0.1M phosphate buffer (pH 7.2) following repeated freeze-thawing (5x) in an acetone-dry ice bath. The cell envelope fraction was stored at 4°C.

RESULTS AND DISCUSSION

During attempts to separate swarmer cells from stalked cells we observed that homogeneous swarmer preparations (95%) were unable to grow unless stalked cells were present (Figure 1). Following the addition of stalked cells, logarithmic growth resumed in the swarmer cell culture after a short lag period. The lack of swarmer growth in the absence of stalked cells suggests that either 1) the medium had become depleted or contained an inhibitor 2) stalked cells secrete a factor required for swarmer growth or 3) contact between stalked and swarmer cells is a prerequisite for swarmer growth. Our experiments lead us to suspect the latter possibility. We have discounted media effects since replenishment of a washed swarmer culture with fresh PYE media or separation of swarmer cells from stalked cells by a

measured in 10 µl of cell lysate under identical conditions and the trypsin concentration was estimated by comparison with the standard curve.

RESULTS

Lysates of ten different fibroblast strains suspended at 0.1 - 1 x 10^8 cells/ml were assayed for trypsin activity with gelatin substrate. Over 50% of the lysates of cells harvested with trypsin contained detectable proteolytic activity though no activity was found in cells harvested without the use of trypsin. Activity in the lysates indicated trypsin concentrations up to 10 ng/10^7 cells. Assay of esterase activity with TAME was found to be an unreliable method of measurement since the trypsin concentrations estimated by reaction with this substrate were invariably significantly higher than indicated by the more sensitive and specific gelatin assay. Although lysates of some cells contained trypsin activity, other lysates of the same cells did not. Addition of a five-fold excess of soybean trypsin inhibitor to cell lysates, or directly to assay mixtures, did not result in measurable increase in prolyl or lysyl hydroxylation. Addition of soybean trypsin inhibitor at this concentration to assay mixtures of cell lysates without detectable trypsin activity had no affect on prolyl or lysyl hydroxylation but phenylmethanesulfonyl fluoride (1mM) in the assay mixture produced 65% inhibition of prolyl hydroxylation and 85% inhibition of lysyl hydroxylation. The mechanism of this inhibition has not yet been investigated.

Preincubation of protocollagen substrate (150,000 dpm) of either 3,4 (^{3}H) proline or (^{3}H) (G) lysine labeled substrates with 1 µg of trypsin (in a total volume of 0.11 ml) at 25°C for one hour, followed by addition of a five-fold excess of soybean trypsin inhibitor prior to assays with the substrates, indicated that under these conditions these substrates are susceptible to trypsin cleavage because lysyl and prolyl hydroxylation were inhibited 85-90%. Varying amounts of trypsin were then added to assay mixtures for prolyl and lysyl hydroxylases and these studies showed that lysyl hydroxylation was affect-

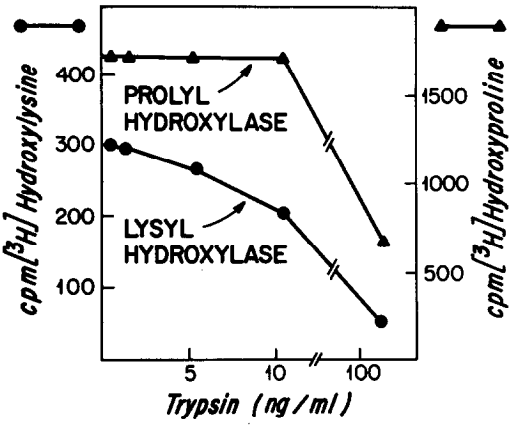

Figure 1: Effect of trypsin on prolyl and lysyl hydroxylation of protocollagen substrate

Assay mixtures containing 100 µM α-ketoglutarate, 100 µM ferrous sulfate, 500 µM ascorbic acid. 0.5 mg of catalase, 100 µM dithiothreitol, 2 mg of bovine serum albumin, 20 µl of cell lysate (5 x 10^7 cells/ml) harvested without trypsin, approximately 1.5 x 10^6 dpm of protocollagen substrate and the indicated quantities of trypsin in a total volume of 1 ml of 50mM Tris-HCl (pH 7.2) were incubated for one hour at 37°C.

ed to a greater extent and at lower trypsin concentrations than prolyl hydroxylation (Figure 1). However, it should be noted that the highest trypsin concentration found in our lysates (10 ng/10^7 cells) would not appreciably reduce lysyl hydroxylation since the amount of lysate usually assayed (10–25 µl of lysate from cells suspended at 0.1 - 1 x 10^8 cells/ml of lysing buffer (2)) is that from 0.1 - 2.5 x 10^6 cells and the resultant trypsin concentration in the assay mixture would be at most 2.5 ng/ml.

Assay of varying amounts of a lysate containing trypsin activity (40 ng/ml) indicated that prolyl hydroxylation increased linearly with the amount of lysate added, but lysyl hydroxylation did not (Figure 2a). The non-linearity of lysyl hydroxylation with increased quantities of cell lysate was not observed in the case of a lysate of cells harvested without trypsin (Figure 2b).

DISCUSSION

In other studies we found that lysates of human skin fibroblasts harvested

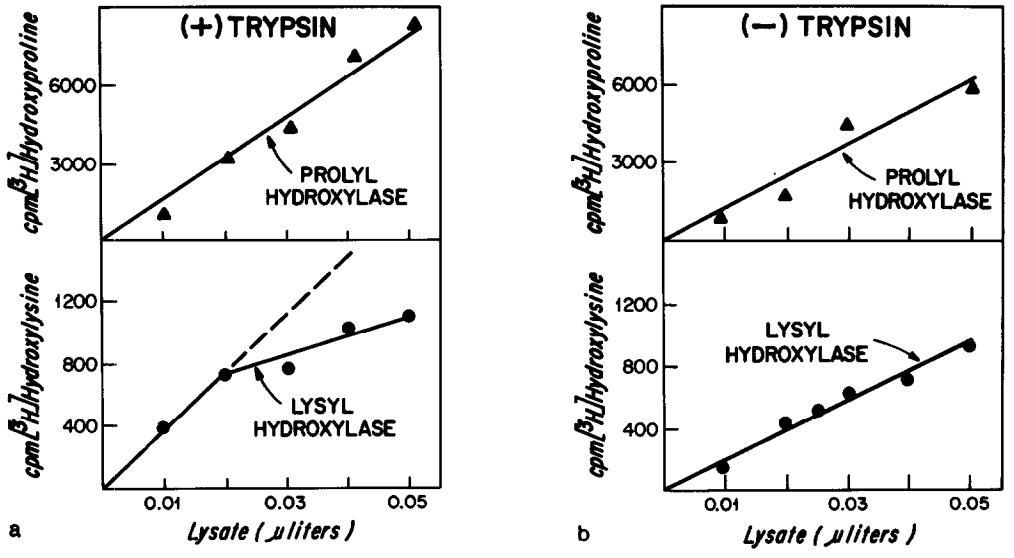

Figure 2: Prolyl and lysyl hydroxylation of protocollagen by lysates of cells harvested with and without trypsin.

Replicate cultures of normal control cells were harvested either by incubation with 0.25% trypsin for 15 min or by scraping cells off culture dishes with a teflon policeman. Cells harvested by either method were suspended in the same volume of buffer prior to lysing by sonication. Cells harvested without trypsin could not be accurately counted and some mechanical losses occured and it is estimated that the cell density is 20-30% lower than in lysate of cells harvested with trypsin. The lysate of the cells harvested with trypsin contained 40 ng of trypsin per ml. Protocollagen substrates (1.5×10^6 dpm) were incubated for 35 min with the indicated amounts of lysates.
a) Cells harvested with trypsin; b) cells harvested without the use of trypsin.

with trypsin contained protease activity active against a lysine-containing octapeptide (3) and the present experiments confirmed that there is a high probability that protease activity attributable to trypsin will be present in the lysate. It has usually been assumed that trypsin would be inactivated by components of fetal calf serum in the medium used to dilute the trypsin solution and that the enzyme would be subsequently removed by repeated washing of the cell pellet with phosphate buffered saline. However, it appears that this treatment may not always completely eliminate trypsin from the cell pellet, possibly because the enzyme is in an intracellular location, or firmly bound to the cell membrane. This phenomenon of retention of trypsin activity may

be characteristic of other cells since trypsin activity has also been found in endothelial cells harvested with trypsin (8). The amount of trypsin retained by the cell pellet is very small; the maximum detected amount was 10 ng/10^7 cells. Although such amounts of trypsin were unacceptable in assays with the octapeptide substrate (3), studies of the effects of various amounts of added trypsin in assays with protocollagen substrate indicated that the maximum amount of trypsin found would have no detectable effect on the hydroxylations since ordinarily very small volumes of cell lysates are assayed. This conclusion is supported by our finding that the addition of soybean trypsin inhibitor to assay mixtures or to cell lysates prior to addition to assay mixtures did not result in detectable increases in prolyl or lysyl hydroxylation. However, if larger volumes (over 20 µl) of cell lysate containing trypsin at a concentration of 40 ng/ml was used, the amount of lysyl hydroxylation did not increase linearly. Hydroxylation of prolyl residues in protocollagen substrate is less sensitive to trypsin at these concentrations and the amount of prolyl hydroxylation increased in a linear manner with increasing amounts of lysate.

We found that cell lysates from subjects with lysyl hydroxylase deficient collagen previously reported (1,2) had levels of collagen lysyl hydroxylase activity less than 15% of control whether or not cultured cells were harvested with trypsin. However, these studies indicate the advisability of measuring protease activity in cells harvested with trypsin if a substrate susceptible to proteolysis is to be used in assays for any type of enzyme activity. Since high trypsin concentrations inhibit both prolyl and lysyl hydroxylations, measurement of proteolytic activity in tissue homogenates prior to measurements of collagen lysyl and prolyl hydroxylase activities is advisable in order that any effects on the protocollagen substrate might be assessed.

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