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Characterization of a Digestase Preparation and Its Use to Obtain Primary Cell Cultures

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The action of the preparation Digestase on several protein and synthetic substrates is examined, as well as the effect of a number of proteinase inhibitors on this enzyme preparation. Digestase has been found to exhibit considerable trypsinlike and collagenolytic activity. Experience with the use of Digestase to obtain isolated adenohypophyseal, hepatic, and cardiac cells is described, and some parameters of the functional activity of these cells in the course of their cultivation are evaluated.

Key Words: *Digestase; primary adenohypophyseal, hepatic, and cardiac cell cultures; somatotropic hormone; serum albumin*

Primary monolayer cultures of adenohypophyseal, hepatic, and cardiac cells are widely used as model test systems for evaluating the biological properties of various hormones and bioregulators [4,5,8] among other research purposes. To obtain adenohypophyseal and hepatic cell cultures, use is usually made of trypsin and collagenase, respectively [5,8]. The proteolytic activity of trypsin against hepatic cells has been shown to be so high that it damages their cytoplasmic membranes irreversibly even in very low (0.001%) concentrations [3]. For the isolation of cardiac cells, trypsin or collagenase is employed [13]. At present, there is a distinct need for expanding the range of enzymes that can be utilized to produce primary cell cultures. In this article we describe our experience with the use of Digestase for the isolation of adenohypophyseal, hepatic, and cardiac cells, and also assess some parameters of the functional activity displayed by the cells obtained. Because Digestase is a composite preparation of enzymes with both collagenolytic and trypsinlike activity [11,14], it is used to obtain adenohypophyseal and hepatic cells either alone or in combination with lima bean trypsin inhibitor (LBTI) or with fetal bovine serum (FBS) as an inhibitor of proteolytic activity.

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MATERIALS AND METHODS

Digestase, a composite preparation of collagenolytic proteinases derived from the hepatopancreas of the crab *Paralithodes camtschatica*, was made by the method described in the European patent EP 0402 321 AI.

Proteolytic activity was assayed by *in vitro* hydrolysis of ^{14}C-acetylated acid-insoluble type I collagen [10], type IV collagen [15], casein [12], and hemoglobin [9] and also by hydrolysis of azocasein [12] and insoluble collagen from bovine tendons [16]. Type I collagen was isolated from rat skin [10] and type IV collagen from the bovine

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TABLE 1. Effect of Digestase on Protein and Synthetic Substrates

Enzyme	Activity with protein substrates						Activity with synthetic substrates			
	collagen type I	collagen type IV	casein	hemoglobin	azocasein, arb. units/min×mg	insoluble collagen, Mandl units	BAEE	BTEE	BANA	$Ac(Ala)_3$ NA
	μg substrate/min×mg enzyme						μM substrate/min×mg enzyme			
Result	11	8.6	1060	30	57	900	6.07	0.38	0.013	0.025

Note. A unit of activity corresponds to the amount of enzyme that liberates from collagen peptides equivalent to 1 μM leucine (using ninhydrin reagent) for 5 h at 37°C.

crystalline capsule [15]; casein, azocasein, and insoluble collagen were purchased from Serva and hemoglobin from Sigma. Protein substrates were acetylated *in vitro* with ^{14}C-acetic anhydride as described elsewhere [10].

Amidase and esterase activities were assayed by hydrolysis of N-benzoyl-arginyl ethyl ester (BAEE), N-benzoyl-tyrosyl ethyl ester (BTEE), N-benzoyl-arginyl-*p*-nitroanilide (BANA), and N-acetyl-alanyl-alanyl-alanyl-*p*-nitroanilide ($Ac(Ala)_3NA$) - all from Serva [11].

Digestase was reacted with inhibitors (phenylmethylsulfonyl fluoride, *p*-chloromercuribenzoate, ethylenediamine tetraacetic acid, and dithiothreitol) for 2 h at room temperature. Its activity was assayed by hydrolysis of ^{14}C-acetylated type I collagen.

Lyophilized Digestase was dissolved in the cell culture medium, and the solution was sterilized by passage through a 0.2 μ pore-size filter (Synpor, Czechoslovakia). Cells were isolated from Wistar rats using previously described procedures for isolating adenohypophyseal cells from adult rats [5] and hepatic cells from fetuses at the end of gestation [8]. Cardiac cells were isolated from neonatal Wistar rats. Briefly, ground cardiac tissue was incubated in a collagenase (1 mg/ml) or Digestase (1.75 mg/ml) solution for 30-40 min at 37°C, after which the tissue was dissociated into individual cells by gentle pipetting. The cells were washed free of the enzymes by double centrifugation at 100 g at room temperature. The washed sediment was resuspended in the growth medium (a mixture of medium 199 and Eagle's medium supplemented with 100% FBS) and seeded in 24-well plastic plates (Flow Laboratories) at 1×10^5 or 2×10^5 cells per well. Comparative evaluations of the adenohypophyseal, hepatic, and cardiac cells were carried out on days 4-6 of *in vitro* culture.

Prolactin and somatotropic hormone (STH) concentrations in the adenohypophyseal cell cultures and serum albumin concentrations in the hepatic cell culture were determined by homologous radioimmunoassays developed in our laboratory [1,2,6]. Crystalline trypsin was purchased from Spofa and type I collagenase, LBTI, dibutyryl-cAMP, and dexamethasone from Sigma.

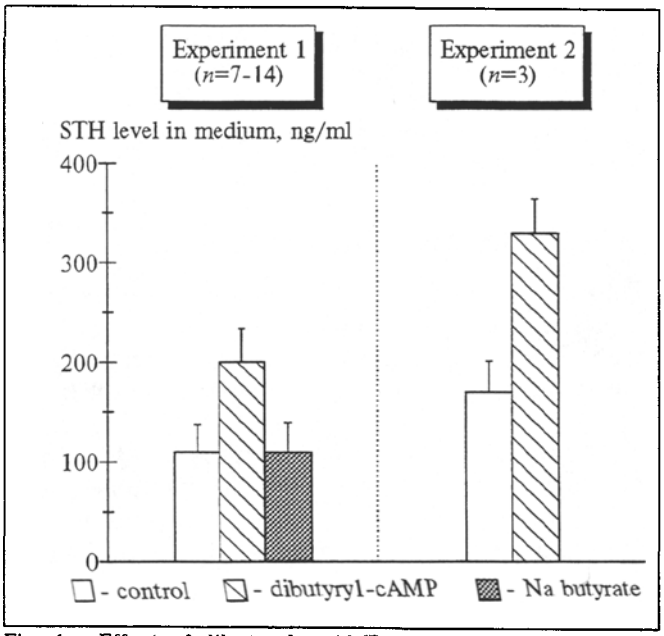

Fig. 1. Effect of dibutyryl-cAMP and sodium butyrate (2.5 mmol/liter each) on STH secretion in primary cultures of adenohypophyseal cells obtained using trypsin (exp. 1) or Digestase (exp. 2). Incubation time 2.5-3 h. One asterisk: $p<0.01$; two asterisks: $p<0.001$.

RESULTS

The assays of Digestase for its reaction with protein substrates showed that it digested the soluble type I and IV collagens, insoluble tendon collagen and also casein, azocasein, and hemoglobin, the

TABLE 2. Effect of Inhibitors on Collagenolytic Activity of Digestase

Inhibitor	Activity, %
Phenylmethylsulfonyl fluoride	59
p-Chloromercuribenzoate	90
Ethylenediamine tetraacetic acid	110
Dithiothreitol	108

Note. Acetylated type I ^{14}C-collagen was used as the substrate.

caseinolytic activity being the highest (Table 1). The data on the digestion of the synthetic peptides indicated that Digestase contains primarily trypsin-like - esterase and amidase - activity (digestion of BAEE and BANA); its chemotrypsinlike (digestion of BTEE) and elastase (digestion of $Ac(Ala)_3Na$) activity was lower.

The assays of inhibitors of proteinases of various classes for their effects on the collagenolytic activity of Digestase (Table 2) confirmed the results obtained with hydrolysis of substrates and showed that the preparation contains, in the main, serine collagenolytic proteases (the effect of phenylmethylsulfonyl fluoride), which agrees with the reported data on collagenolytic proteinases from crab hepatopancreas [7,11,14]. In addition, Digestase possesses a thiol-dependent activity, as was indicated by its inhibition by *p*-chloromercuribenzoate and its activation by ethylenediamine tetraacetic acid and dithiothreitol (Table 2).

To obtain isolated adenohypophyseal cells, Digestase was used in a concentration of 2.5 mg/ml, which gave a cell yield comparable to that produced by trypsin (about 1×10^6 cells per gland from an adult female rat). With lower concentrations (0.5-1.0 mg/ml), the yield of adenohypophyseal cells decreased to 3×10^5 - 5×10^5 cells per adenohypophysis. In assays using a combination of Digestase and LBTI (or FBS), the yield of adenohypophyseal cells and basal prolactin secretion in the cultures (Table 3) were approximately half those obtained with Digestase alone.

The study of the functional activity of the cells obtained with Digestase (at concentrations of 0.5, 1.0, or 2.5 mg/ml) showed that STH secretion in the cultures did not differ much from that in a cell culture obtained using trypsin, regardless of whether the incubation time was long (48 h) or short (2.5-3 h) (Table 4 and Fig. 1). Dibutyryl-cAMP increased STH secretion in cultures obtained with Digestase approximately to the same extent as in those obtained with trypsin (Fig. 1). Somatotrophs appear to tolerate well mechanoenzymatic treatment using trypsin or Digestase. Sodium butyrate did not alter STH secretion (Fig. 1)

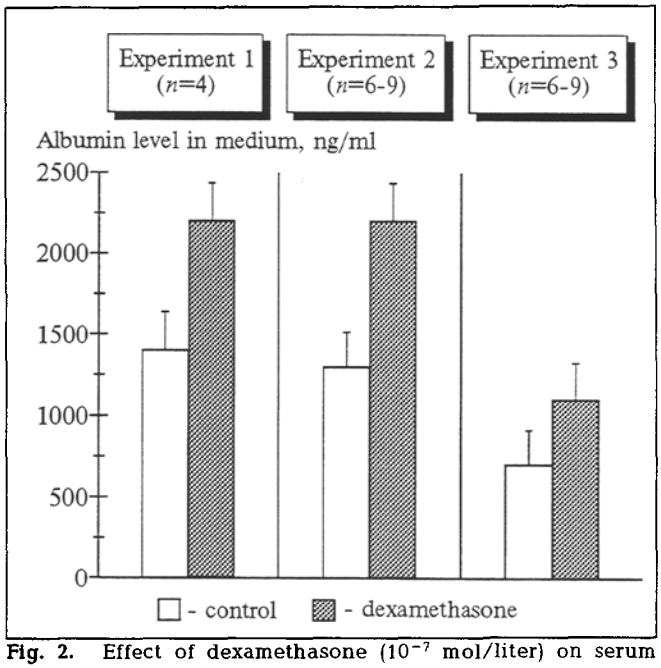

Fig. 2. Effect of dexamethasone (10^{-7} mol/liter) on serum albumin production in primary cultures of hepatic cells obtained using collagenase (exp. 1), collagen + LBTI (exp. 2), or Digestase + LBTI (exp. 3). Incubation time 24 h. In all cases the difference from the respective control is significant at $p<0.01$.

and the effect of dibutyryl-cAMP was therefore not due to the butyrate which can be released upon hydrolysis of this cAMP analog during incubation.

In the tests with fetal hepatic cells, Digestase or collagenase (0.25 mg/ml each) was used in combination with LBTI (0.25 mg/ml). Both combinations produced approximately equal yields of isolated hepatic cells (90×10^6 cells per liver). Previously, when collagenase was used alone without LBTI, not more than 50×10^6 cells per fetal rat liver could be recovered. Intravital light microscopy did not reveal any substantial morphological difference between the cells obtained with the combination of Digestase + LBTI or collagenase + LBTI. However, when cells seeded at the same density (0.8×10^6 cells per well) were cultured, a proportion of the cells obtained using Digestase was found to have been lost during changes of the culture medium. This was probably due to the fact that Digestase is a composite preparation contain-

TABLE 3. Prolactin Secretion in Cultures of Adenohypophyseal Cells Obtained Using Digestase in Combination with LBTI or FBS ($M\pm m$)

Group	Prolactin in medium, ng/ml	*n*	*p*
Digestase (2.5 mg/ml)	3839±87.3	4	–
Digestase + LBTI (1 mg/ml)	2151±232	4	0.001
Digestase + FBS (20%)	1603±84.7	3	0.001

Note. Here and in Table 4: incubation time 48 h.

TABLE 4. Basal STH Secretion in Cultures of Adenohypophyseal Cells Obtained Using Trypsin or Digestase ($M \pm m$)

Enzyme	Concentration, mg/ml	STH in medium, ng/ml	n
Trypsin	2.5	4935±627	7
Digestase	2.5	4760±564	4
Digestase	1.0	4546±722	5
Digestase	0.5	4604±942	5

Note. $p<0.05$ in all cases.

ing a set of proteolytic enzymes capable, even in the presence of LBTI, of altering the surface membrane structures responsible for the cell-substrate interaction. It was also found that treatment of the collagenase preparations with LBTI resulted in an increased yield of cells from the tissue without any alteration in their functional activity. The collagenase preparations used appear to have contained trypsinlike proteinases that killed some cells in the course of their isolation from the liver.

As shown in Fig. 2, the basal level of serum albumin production in the culture of hepatic cells obtained with Digestase in combination with LBTI was approximately two times lower, apparently because a proportion of the cells had been lost when the culture medium was changed. If so, then the functional activity of cultured hepatic cells obtained with Digestase may be considered not to differ much from that in cultures of hepatic cells obtained with collagenase. This is confirmed by our observation that dexamethasone increased albumin production to similar extents (by 66-74%) in both types of hepatic cell culture.

In the tests with neonatal rat hearts, the cell yield using collagenase was about $(5\text{-}6)\times10^6$ cells per heart. In the case of Digestase, the yield was lower, $(2\text{-}4)\times10^6$ cell per heart, but, as shown by intravital light microscopy, the cells obtained using these two enzymes virtually did not differ in morphology.

The present experiments enable us to recommend using Digestase to obtain primary cultures of hepatic cells (in combination with LBTI), cardiac cells, and (with certain reservations) adenohypophyseal cells. In all cases, Digestase acted on the tissues in the same way as did trypsin and collagenase when used to obtain isolated cells. The results of our studies indicate that Digestase is also suitable for use in the mechanoenzymatic treatment of other tissues in order to obtain primary cell cultures and then to study their functional activity.

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