Leucyl- β -naphthylamide-Splitting Enzymes in the Mammalian Endocrine Pancreas

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The proteolytic capacity of the endocrine pancreas in obese-hyperglycaemic mice was evaluated by using the chromogenic substrate L-leucyl- β -naphthylamide (LNA). The analytical sensitivity obtained with this substrate in photometric and fluorimetric assays permitted quantitative determinations of C-N-bond-splitting activity in both crude islet homogenates and electrophoretic fractions thereof. The following observations were made: (1) The rate of LNA cleavage was maximal at about pH 7 in islets as well as in acinar tissue. An apparent K_m of 4×10^{-5} – 6×10^{-5} M was calculated for both the endocrine and exocrine pancreas. (2) The level of LNA-splitting enzyme activity was of the same magnitude in the islets as in the liver, and significantly higher in the islets than in the exocrine pancreas. Starving the animals for 7 days did not affect the enzyme activity levels. (3) Two distinct LNA-splitting enzymes could be separated from the pancreatic islets by means of disc electrophoresis, the most rapidly migrating band representing the highest activity. Though similar electrophoresis patterns were obtained with acinar tissue and liver, only one enzyme could be demonstrated in serum. The data suggest that the β -cells, in addition to being highly specialized for the production of a specific protein, contain a comparatively high capacity for protein catabolism.

Much work has been devoted to the histochemical characterization of the pancreatic islet cells in both mammalian and non-mammalian species. The use of staining techniques, as well as the recent use of sensitive chemical micro-methods, has provided a considerable amount of information about enzymes involved in the metabolism of glucose and adenine nucleotides (Kissane, Lacy, Brolin & Smith, 1964; Lazarow *et al.* 1964; Hellerström, Hellman & Täljedal, 1964; Täljedal, 1967). By contrast, proteinases and peptidases seem to have attracted relatively little interest. In the present study the capacity of mammalian endocrine pancreas for the cleavage of peptide bonds has been evaluated by means of the non-specific substrate LNA.* The use of obese-hyperglycaemic mice made it possible to obtain an almost pure population of β -cells characterized by a high insulin-synthesizing activity (Hellman, 1965).

MATERIALS AND METHODS

Animals and tissue sampling. Five-month-old mice displaying the American variety of the obese-hyperglycaemic syndrome (gene symbol *obob*; Ingalls, Dickie & Snell, 1950)

were divided into three groups: (1) 40 mice of both sexes, given only water for 12 hr. before death; (2) nine female mice, with free access to food and water; (3) ten female mice, given only water for 7 days before death.

After the animals had been decapitated, the pancreases were rapidly dissected out and suspended in 0.25 M-sucrose maintained at 2°. Then 10–20 islets were carefully isolated from the exocrine parenchyma of each pancreas (Hellerström, 1964), pooled and washed in distilled water and homogenized in 100 μ l. of twice-distilled water by means of a Perspex (Lucite) micro-homogenizer. For comparative purposes, small pieces of exocrine pancreas and of liver were similarly homogenized.

Enzyme and protein determinations. Triplicate samples of homogenate (20 μ l. each) were incubated at 37° in 50 μ l. of a solution of LNA (Pabst Laboratories Inc., Milwaukee, Wis., U.S.A.) (1.37 mM) in phosphate buffer (0.2 M; pH 7.1). After 60 min. the reaction was stopped by adding 50 μ l. of 25% (w/v) trichloroacetic acid, and the mixture was then centrifuged at 10000g for 5 min. The liberated naphthylamine was measured in 50 μ l. of the supernatant, essentially as described by Goldbarg & Rutenburg (1958), with a Zeiss PMQII spectrophotometer (580 m μ wavelength) equipped with micro-cells. Protein determinations were performed on triplicate samples of homogenate (10 μ l. each) by the method of Lowry, Rosebrough, Farr & Randall (1951). Bovine serum albumin (fraction 5; Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) was used as

* Abbreviation: LNA, L-leucyl- β -naphthylamide.

Fig. 1. Relationship between the amount of enzyme applied to gels in disc electrophoresis and the LNA-splitting activity recovered after extraction of enzyme-containing gel disks. In this model experiment serum was used as the enzyme source.

reference and the enzyme activities were expressed as moles of substrate split/kg. of protein/hr.

Electrophoresis. Tissues were homogenized in 5% (v/v) Triton X-100, allowed to stand for 30 min. at 4°, and then centrifuged at 10000g for 5 min. Serum was prepared from the mixed arterial and venous blood obtained from the neck wound at decapitation. The tissue extracts and the serum were subjected to disc electrophoresis on polyacrylamide gels as described by Kleiner & Schram (1966), and the regions of enzyme activity located by incubating the gels in a medium consisting of LNA (1.37 mM), Fast Garnet GBC (E. Gurr Ltd., London, S.W. 14) (0.5 mg./ml.) and tris-maleate buffer, pH 6.0 (100 mM). After slicing the gels into disks of equal sizes (Täljedal, 1966), the enzyme activity in the central, unstained, part of each gel disk was extracted in 50 μl. of tris-maleate buffer, pH 7.2 (100 mM). A portion (5 μl.) of each extract was incubated with 200 μl. of LNA (1.37 mM) dissolved in tris-maleate buffer, pH 7.2 (100 mM), at 37°. After exactly 60 min. the enzyme activity was assayed fluorophotometrically in an Aminco-Bowman micro-photometer with Corning 5860 as primary filter and Wratten 2A as secondary filter (Roth, 1964). As shown in Fig. 1, a linear relationship existed between the amount of enzyme applied to the gels and the activity recorded in the extraction buffer.

RESULTS

Enzyme properties and assay conditions. The rate of cleavage of LNA was highest at about pH 7 in the islets as well as in the acinar tissue (Fig. 2). At this pH value there was a linear relationship between the amount of substrate split and the incubation time for at least 60 min. The cleavage

Fig. 2. Hydrolysis of LNA at different pH values in homogenates of exocrine (a) and endocrine (b) pancreas.

of LNA proceeded as a linear function of the enzyme concentration at least to a 50% consumption of the substrate. The effect of substrate concentration on the enzyme activity is shown in Fig. 3. An apparent K_m of 4×10^{-5} – 6×10^{-5} M was calculated for both the islets and the acinar tissue. The K_m remained the same when islet homogenates were incubated in tris-maleate buffer instead of phosphate buffer.

Enzyme activity levels in tissues. The levels of LNA-splitting enzyme activity in islet homogenates of fed and starved obese-hyperglycaemic mice are given in Table 1. For comparison, corresponding data for the exocrine pancreas and the liver are also given. In fed mice the enzyme activity of the islets was of the same magnitude as that of the liver and higher than that of the acinar tissue ($P < 0.001$). A significantly higher variance was noted for the islets in the animals that had been starved for 7 days compared with the mice that had free access to food. When this was taken into account, no significant difference in mean enzyme activities could be demonstrated between the two groups of mice.

Electrophoresis. Fig. 4 shows that two distinct

LNA-splitting enzymes could be separated from the pancreatic islets by disc electrophoresis. Enzymes with corresponding mobilities were also demonstrated for the exocrine pancreas and the liver. For all the three tissues the most rapidly migrating band represented the highest enzyme activity as revealed by both the appearance of the stained gels and the quantitative determinations performed with extracts of sliced gels (Fig. 5). The impression that the major enzyme in the tissue extracts had

the same mobility as the single one obtained with serum was confirmed by mixing tissue extracts with serum before electrophoresis. The serum enzyme could be demonstrated without pretreatment of the blood, but treatment with Triton X-100 was necessary for obtaining fully mobile enzymes from the tissue homogenates.

DISCUSSION

The chromogenic substrate LNA has been widely used in histochemical and clinical research on aminopeptidase activity. Though it was previously suggested as a substrate for leucine aminopeptidase,

Fig. 3. (a) Relation between the concentration and cleavage of LNA incubated with homogenates of endocrine and exocrine pancreas. In (b) the values have been plotted in a Lineweaver-Burk diagram. ●, Islets in 143 mM-phosphate buffer; ○, islets in 36 mM-tris-maleate buffer; ▲, acinar tissue in 143 mM-phosphate buffer.

Fig. 4. Polyacrylamide gels stained for LNA-splitting enzyme activity after electrophoresis of extracts from pancreatic islets (a) and serum (b). The arrows indicate the positions of enzymes. In both gels the cathodal end is at the top.

Table 1. LNA-splitting enzyme activity in the endocrine and exocrine pancreas and the liver from starved and fed obese-hyperglycaemic mice

Activities are expressed as moles of naphthylamine liberated/kg. of protein/hr., and are given as mean values $\pm$ S.E.M. with the numbers of animals in parentheses.

Animals	LNA-splitting activity		
	Islets	Acinar tissue	Liver
Fed (9)	0.74 $\pm$ 0.04	0.51 $\pm$ 0.02	0.83 $\pm$ 0.06
Starved (10)	1.19 $\pm$ 0.16	0.60 $\pm$ 0.05	0.80 $\pm$ 0.06

Fig. 5. Relative distribution of LNA-splitting enzyme activity in the polyacrylamide gel after electrophoresis of an extract from pancreatic islets. The peak corresponds to the most intense enzyme band as revealed by the preceding staining. The slowly migrating enzyme was of too low activity to be detected by the quantitative assay. The cathodal end is to the left.

recent investigations have demonstrated that LNA acts as a rather unspecific substrate for several C-N-bond-splitting enzymes including a group of newly discovered peptidases (Patterson, Hsiao & Keppel, 1963; Sylvén & Snellman, 1964). Though the natural substrate for these peptidases is unknown, the LNA-splitting activity seems to parallel those of other proteinases and peptidases (Sylvén & Bois-Svensson, 1964). Since the liberation of naphthylamine may be conveniently recorded with adequate sensitivity on a micro scale, LNA was considered an advantageous substrate for evaluating the proteolytic capacity of mammalian β -cells.

LNA-splitting enzymes have been demonstrated histochemically in the endocrine pancreas of several species. A weak reaction was found in the islets of man, monkey and rat (Burstone & Folk, 1956), whereas a very strong reaction was noted in the α -cell islets of duck (Hellerström, 1963). No difference in staining intensity could be observed between the β -cells and the exocrine pancreas in obese-hyperglycaemic mice (Hellerström & Hellman, 1963). However, the demonstration of a poor correlation between histochemical and biochemical estimations of LNA hydrolysis (Sylvén & Bois-Svensson, 1964) makes it difficult to evaluate these results and stresses the importance of quantitative information.

The present experimental design had the advantage of using a fairly pure population of β -cells

(Hellman, 1965) and the data clearly demonstrate the presence of high LNA-splitting activities in this material. Since the exocrine pancreas is known as a rich source of proteolytic enzymes, it should be emphasized that each islet was washed before homogenization and that the enzyme activity was in fact higher in the endocrine than in the exocrine parenchyma. The observation of a rather high rate of LNA hydrolysis in the islets of obese-hyperglycaemic mice conforms well with the previous demonstration of high dipeptidase activities in this tissue (Hellerström & Hellman, 1963). It also suggests that the β -cells are characterized, not only by a high specialization for protein synthesis, but also by a comparatively great capacity for protein catabolism. The place of this metabolic feature in the ultimate picture of β -cell metabolism cannot be predicted. However, there are data that suggest that the activity of proteolytic enzymes is related to the rate of cytoplasmic protein synthesis (Rotschild & Junqueira, 1951; Greenbaum & Greenwood, 1954; Ellis, Sewell & Skinner, 1956).

A study of enzyme kinetics revealed the same pH optima and apparent K_m for the enzymes of endocrine and exocrine pancreatic origin. Two molecular forms of LNA-splitting enzyme could be separated from all solid tissues studied, the most rapidly migrating fraction containing the highest activity. The fact that the latter enzyme had the same mobility as the single band obtained with serum raised the question whether or not the rich vascularization of the islets may have contributed to the observed differences between the endocrine and exocrine pancreas. This possibility is, however, contradicted by the observation that pretreatment with Triton X-100 was a prerequisite for obtaining fully mobile enzymes from the tissues, whereas the serum enzyme was readily mobile without such treatment. The mobilizing action of Triton X-100 indicates that the LNA-splitting enzyme activity resides in a particulate fraction of the β -cells, which is consistent with the suggestion that a major part of the LNA-splitting enzyme activity is lysosomal in nature (Sylvén & Bois-Svensson, 1964).

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