Catalytic and regulatory properties of the Triton and trypsin forms of the brush border hydrolases

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SUMMARY Amphipathic enzymes, invertase (EC 3.2.1.26), 8-amylase (EC 3.2.1.3), and alkaline phosphatase (EC 3.1.3.1), were purified from the rat small intestinal mucosa as trypsin and Triton forms, the catalytic and regulatory characteristics of which were compared in rats and in *drosophila*. Differences in the catalytic properties of the two enzyme forms were demonstrated, which suggested that the hydrophobic part of the enzyme was involved in maintaining optimal conformation of the catalytic part. Many modifiers have been found to influence the Triton rather than the trypsin form of the enzyme. It is therefore suggested that the hydrophobic sub-units of the enzymes might be involved in transmitting information from the cytoplasm into the external surface of the membrane, the cell in this way regulating the activity of surface enzymes. If this is indeed the case, it is suggested that the hydrophobic part performs functions not only of external but also of internal regulation.

The microvillous membranes of the epithelial cells are widely used as a model for the study of the functions of enzymatically active membranes. This is related to the fact that the brush border surface is that site where a close coupling occurs of the processes of membrane hydrolysis and transport of the formed monomers (Ugolev, 1972). One of the important modes of analysis of the enzymatically active membranes may be a separation of enzymes from the enzyme-membrane complexes by various solubilising agents such as detergents and proteases (Eggermont, 1968; Egorova *et al.*, 1975; Louvard *et al.*, 1975; Sigrist *et al.*, 1975; Louvard *et al.*, 1976; Maroux and Louvard, 1976; Pattus *et al.*, 1976; Egorova *et al.*, 1977).

During the past few years it has been shown in our laboratory that some brush border hydrolases are regulatory enzymes and for a number of them allosteric properties have been demonstrated. The results were discussed in detail in our previous works (Ugolev *et al.*, 1975a,b). We later found the same properties not only for the enzymes incorporated into the natural membranes but also those solubilised by a non-polar detergent, Triton X-100. After trypsin or papain solubilisation, however, the enzymes lost their regulatory properties (Ugolev *et al.*,

1974, 1975a,b; Egorova *et al.*, 1975). These findings made it possible to suggest that the catalytic and regulatory parts of hydrolases are associated with the enterocyte membranes by two subsequent bonds: one sensitive to the action of proteases and another to that of detergent. The regulatory part was assumed to be located between these two bonds (Ugolev, 1972; Egorova *et al.*, 1975; 1977).

As has recently been demonstrated with purified aminopeptidase and maltase (Louvard *et al.*, 1975; 1976; Maroux and Louvard, 1976), the brush border hydrolases of the enterocytes and nephrocytes consist of two parts, a hydrophilic and a hydrophobic part. The hydrophilic (enzymatically active) part of the enzymes was purified after trypsin treatment of the enzyme preparations (the trypsin form). By the action of detergents the enzyme was completely released, including both the hydrophilic and hydrophobic parts of the enzyme (the detergent or Triton form).

The hydrophilic part is believed at present to perform catalytic functions, whereas the role of the hydrophobic part is far less clearly understood. It is suggested that it plays a part in binding an enzyme molecule with the lipid matrix of the membrane (Pattus *et al.*, 1976). It is not yet clear, however, whether this is its only function. As mentioned above, earlier data obtained from our laboratory before the

molecular structure of the microvillous membrane enzymes was recognised suggest that the hydrophobic part may have other functions, including a regulatory one.

The present work is concerned with further study of the functions of the hydrophobic part of enzymes. For this purpose, the catalytic and regulatory properties of the Triton and trypsin forms of carbohydrases (γ -amylase and invertase) and alkaline phosphatase were compared. The experiments were carried out on vertebrates (rats) and invertebrates (*drosophila*) in which the regulatory properties of membrane enzymes were also revealed (Mityushova and Ugolev, 1976). The properties of the Triton form were compared with those of the trypsin form after its complexing with lecithin liposomes. In several instances a comparison was made between the kinetic and regulatory properties of purified enzymes and those of mucosal homogenates and Triton X-100 or protease-solubilised but unpurified enzymes.

Methods

TISSUE AND ENZYME PREPARATIONS

The preparation of the mucosal homogenates of the rat small intestine and solubilisation of the enzymes were performed using a routine technique, previously described in detail (Eggermont, 1968; Ugolev *et al.*, 1975b).

Drosophila melanogaster valvae of CS strain

were also used in our experiments. Valvae were collected during their release from the medium and rinsed twice in cold Ringer solution. The intestine was extracted through the proximal end of a valve. A segment corresponding to the middle intestine was isolated. For the study of enzymatic activities a homogenate containing 40 intestines in 1 ml Ringer solution was used. All the procedures were performed cold. In some experiments the activity of a quarter of the middle intestine of one valve was investigated.

The Triton and trypsin forms of enzymes were extracted from the intestinal mucosal homogenates of Wistar rats (body weight 180-200g) using a modification of the method of Dahlqvist and Thomson (1963) after preliminary solubilisation by Triton X-100 (0.4% V/V, Schuchardt, Munich) or trypsin (mg/ml of homogenate, Spopha, Czechoslovakia) respectively. In order to obtain purified mucosal preparations the small intestines from 100 animals were used. Figure 1 shows the distribution of intestinal carbohydrases after chromatography using DEAE-cellulose. A complexing of purified carbohydrases with lecithin liposomes (0.52% emulsion of this phospholipid was used) was produced by stirring the enzyme-liposome mixture at 4° for 24 hours (Koltushkina and Ugolev, 1975; Egorova *et al.*, 1977).

ANALYTICAL METHODS

Invertase activity was determined by an increase in

Fig. 1 Distribution of intestinal carbohydrases after chromatography using DEAE-cellulose. a: γ -amylase; i: invertase; m: maltase; B: concentration gradient of Na-phosphate buffer (M).

free hexoses using a modification of the method of Nelson (Ugolev and Iezuitova, 1969). Sucrose at a concentration of $1.1-23.2 \times 10^{-2} \text{ M}$ served as substrate. γ -amylase activity was determined by an increase in glucose by the modified method of Dahlquist (1964). The concentration of soluble starch varied within the limits $1.2-40.0 \text{ g/l}$. Alkaline phosphatase activity was defined using sodium p -nitrophenyl phosphate (0.6 mM) as substrate. Tributyrine (6.6 mM) and β -phenyl- α -alanine (2 and 20 mM) were used as the modifiers of alkaline phosphatase activity. The effects of the modifiers were given in percentage of inhibition or activation with respect to the activity measured in the absence of the modifiers and taken as 100% . Protein was determined by measuring the absorbance (at 290 nm). Sucrose and starch solutions were prepared on K^+ phosphate buffer ($\text{pH } 7.0$) and sodium p -nitrophenyl phosphate in Ringer solution. The kinetic characteristics of γ -amylase and invertase (K_m and V) were determined using Lineweaver-Burk plot. The pH function was investigated in the range of $4.0-9.0$. Incubation was carried out at 37° for rats and at 30° for *drosophila*. Various periods of incubation (10 to 30 minutes) were used.

Results

KINETIC CHARACTERISTICS OF TRITON AND TRYPSIN FORMS OF ENZYMES IN RATS

K_m and V

Comparison of the kinetic characteristics (K_m and V) of γ -amylase and invertase purified preparations showed definite distinctions between the Triton and trypsin forms of the enzymes. V for the Triton form of invertase (Fig. 2) was found to be higher ($0.33 \pm 0.02 \text{ mM/min}$) than for the trypsin form ($0.25 \pm 0.02 \text{ mM/min}$); K_m was lower ($9.1 \pm 0.6 \times 10^{-2} \text{ M}$ compared with $12.5 \pm 1.2 \times 10^{-2} \text{ M}$), $P < 0.05$. As regards

γ -amylase, K_m for the Triton form was found to be lower than for the trypsin form, as in the case of invertase. The enzyme activity of the Triton form at low substrate concentrations, however, was higher and at high concentrations was lower than for the trypsin form of the enzyme. V for the Triton form was also lower than for the trypsin form of the enzyme.

Thus, a conversion of the Triton form of enzymes into the trypsin form—in other words, a separation of the hydrophobic part—leads to changes in the catalytic properties of enzymes. The hydrophobic part appears to play an essential role in a maintenance of optimal conformation of the catalytic (hydrophilic) part of enzymes.

A complexing of the trypsin form of invertase with liposomes—that is, binding of an enzyme with the lipid membrane—was earlier shown (Koltushkina and Ugolev, 1975) to decrease K_m and increase V . For γ -amylase a decrease in K_m and insignificant change in V were observed.

The differences in the kinetic characteristics between the trypsin form of carbohydrases and its complexes with liposomes were similar to those found between the trypsin and Triton forms of enzymes.

pH-function

Figure 3 shows pH-function for the Triton and trypsin forms of invertase. In both cases the maximum of invertase activity was at $\text{pH } 6.5$. However, as can be seen, these curves differ significantly. Thus, the activity for the Triton form at $\text{pH } 5.0$ accounted for 83% , while that for the trypsin form accounted for no more than 27% of the whole. As a whole, a zone of high activity for the Triton form of the enzyme was wider than for the trypsin form. Our previous studies on the pH-function of invertase, γ -amylase, and maltase demonstrated that the zone of high activity for the enzyme-liposome complexes

Fig. 2 Determination of K_m and V of invertase of the rat small intestine. A: effect of substrate concentration on the rate of hydrolysis; abscissa: concentration of sucrose (in M); ordinate: the rate of hydrolysis in μM per minute. B: Lineweaver-Burk plot. Abscissa: reciprocal to the concentration of sucrose; ordinate: reciprocal to the rate of hydrolysis. Solid line: the Triton form of the enzyme; dashed line: the trypsin form of the enzyme.

Fig. 3 pH function of invertase of the rat small intestine. Abscissa: pH-value; ordinate: activity (in % of the maximum taken as 100%). Solid line: the Triton form of the enzyme; dashed line: the trypsin form of the enzyme.

was much greater than for the trypsin form of the enzymes. Thus, phospholipid-bounded carbohydrases are more resistant to acid and alkaline denaturation. These findings are well consistent with the results of other investigators which showed that ligands are capable of stabilising and optimising a conformation of the enzyme (Koltushkina and Ugolev, 1975). The hydrophobic part binding the hydrophilic part with the membrane may affect the properties of the hydrophilic part in the same direction as does the membrane.

REGULATORY PROPERTIES OF TRITON AND TRYPSIN FORMS OF ENZYMES IN RATS

Figure 4 shows the effects of two modifiers (tributyrine and phenylalanine) on the activity of the Triton and trypsin forms of alkaline phosphatase. The modifiers significantly inhibited the activity of the Triton form of the enzyme (27% for tributyrine and 17% and 37% for 2 and 20 mM of phenylalanine, respectively, $P < 0.05$). The activity in control was $1.04 \pm 0.09 \mu\text{M}/\text{min}/\text{mg}$ of protein. Similar inhibition of the alkaline phosphatase activity by modifiers was earlier observed using mucosal homogenates and Triton X-100 solubilised enzyme (Ugolev *et al.*, 1975b). In contrast, the activity of the trypsin form (1.24 ± 0.02 in control) did not change significantly in the presence of tributyrine (1.26 ± 0.09) and 2 mM of phenylalanine (1.17 ± 0.03). Considerable inhibition (27%, $P < 0.05$) was observed only in the presence of 20 mM of phenylalanine. However, in this case the level of inhibition was significantly lower when compared with the Triton form of the

Fig. 4 The effect of tributyrine and phenylalanine on the Triton and trypsin forms of alkaline phosphatase activity of the rat small intestine. White bars: the Triton form; hatched bars: the trypsin form. Vertical line: inhibition (+) (stimulation) (in %). 1: tributyrine, 2: 2 mM phenylalanine, 3: 20 mM phenylalanine.

enzyme. Thus, regulatory properties seem to depend on a mode of solubilisation preceding a purification. Analogous results were obtained from the study of the regulatory properties of γ -amylase (Fig. 5).

Fig. 5 The effect of tributyrine on the γ -amylase activity of the rat small intestine. Vertical line: inhibition (in %). 1: homogenate; 2: solubilisation by trypsin; 3: the trypsin form of the enzyme; 4: solubilisation by Triton X-100.

Thus, inhibition of homogenate γ -amylase by tributyrine was 50% and after Triton X-100 solubilisation it reduced insignificantly. However, solubilisation by trypsin without subsequent purification resulted in a loss of sensitivity of γ -amylase to tributyrine. Modifiers also had no effect on the trypsin forms of γ -amylase.

Thus, the study of the regulatory properties of the brush border hydrolases demonstrated that modifiers influence both the activity of the membrane-bound enzymes and Triton X-100-solubilised enzymes and that of the Triton form of purified enzymes. Conversely, trypsin or papain-solubilised enzymes and the trypsin form of the enzymes displayed much less sensitivity to the action of modifiers and in some instances lost it completely. Therefore the regulatory properties are peculiar only to the entire enzyme, including the hydrophilic and hydrophobic parts. A separation of the latter results in a deprivation of the enzyme's regulatory properties.

Regulatory properties of alkaline phosphatase of middle intestine in drosophila

Figure 6 presents data showing the effect of tributyrine on the alkaline phosphatase activity of the middle intestine in *drosophila melanogaster*. The preparations used were intestinal homogenates, Triton X-100, and trypsin-treated homogenates. As can be seen, the enzyme activity of mucosal homo-

genates markedly decreased in the presence of tributyrine. The inhibition in this case was 21.0%. After Triton X-100 treatment the activity of the enzyme also reduced under the action of tributyrine (18.4% of inhibition). By contrast, when affected by trypsin the alkaline phosphatase activity was not inhibited in the presence of tributyrine.

Thus, during the study of intestinal alkaline phosphatase in *drosophila* we could observe effects similar to those obtained with the rat intestinal enzymes. In both cases the enzymes of homogenates and those solubilised by Triton X-100 exhibited regulatory properties, while solubilisation by trypsin led to a loss of sensitivity to the action of a modifier.

Discussion

The results obtained suggest that solubilisation by proteases leads to a separation of the catalytic and regulatory parts of an enzyme molecule, whereas after solubilisation by Triton X-100 its amphipathic structure persists. In other words, catalytic sub-units are solubilised by proteases and the entire enzyme, including regulatory sub-units, is released by detergents. This finding is supported by the circumstance that after papain treatment of the brush border fractions the molecular weights of a number of enzymes are smaller than those after treatment by Triton X-100.

In terms of the hypothesis on the enzyme-transport ensemble (Ugolev, 1972), Maroux and Louvard

Fig. 6 The effect of tributyrine on the alkaline phosphatase activity of the middle intestine in *drosophila*. Vertical line: activity in the presence of tributyrine (in %). The activity of homogenates in the absence of tributyrine (control) is taken as 100%. 1: untreated homogenate; 2: Triton X-100-treated homogenate; 3: papain-treated homogenate.

Fig. 7 Model of the amphipathic structure and regulatory properties of the microvillous enzymes. M: membrane; 1: the hydrophilic part of an enzyme with the active centre; 2, 3: the hydrophobic part of the enzyme.

suggested that the hydrophobic parts not only play a role in the binding of hydrolases with the membranes but also participate in transport processes (Louvard *et al.*, 1975). In one of their latest works the authors (Louvard *et al.*, 1976) reported that the hydrophobic sub-unit of the enzyme is, at least partially, located on the internal side of the membrane. Taking into account our data on the regulatory functions of the hydrophobic part of the enzyme, the hydrophobic sub-units might be expected to be involved in a transmission of information from the cytoplasm into the external surface of the membrane. In this way the cell regulates the activity of surface enzymes (Fig. 7). If this be the case, the hydrophobic part may perform functions not only of external but also of internal regulation.

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