Differences in the Degradation of Yeast Phosphofructokinase by Proteinases A and B from Yeast

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Proteinase A from yeast causes a stepwise degradation of yeast phosphofructokinase, resembling the action of subtilisin on this enzyme. Proteinase B, however, exhibits a limited proteolysis similar to the action of α -chymotrypsin.

Partial proteolytic degradation of phosphofructokinase from baker's yeast (*Saccharomyces cerevisiae*) (EC 2.7.1.11) by subtilisin or a mixture of endogenous yeast proteinases is accompanied by activation of the enzyme. Simultaneously the molecular weights of its two types of subunit, α and β , fall from approx. 120000 to 90000, as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Diezel *et al.*, 1973; Taucher *et al.*, 1975). In the yeast cell three different proteinases have been characterized, denoted as proteinases A, B and C (Hata *et al.*, 1967; Lenney & Dalbec, 1967). They have been thoroughly studied and the mechanisms for the regulation of their intracellular activities elucidated. In addition, their significance in inactivation and activation of certain enzymes has been investigated (Holzer *et al.*, 1975). In the present paper the actions of proteinases A and B on yeast phosphofructokinase are reported.

Experimental

Phosphofructokinase was purified from baker's yeast (Stellhefe I, VEB Backhefe, Leipzig, German Democratic Republic) as described by Diezel *et al.* (1973) and Taucher *et al.* (1975); 1 mg of the homogeneous enzyme catalyses the formation of 60 μ mol of fructose 1,6-diphosphate per min at 25°C (Freyer *et al.*, 1974).

Proteinases A (EC 3.4.23.8) and B (EC 3.4.22.9) were prepared by the procedure of Maddox & Hough (1970) from the same yeast strain as phosphofructokinase, but from another stage of growth, designated 'Versandhefe' (from VEB Backhefe). The enzymes were further purified by gel filtration on Sephadex G-75 (100 cm $\times$ 1.6 cm). In the final pure state, proteinase B exhibits a single band on polyacrylamide-gel electrophoresis, whereas proteinase A shows two bands, migrating close together. Their specific activities have been determined as 130 units/mg of protein for proteinase A and 60 units/mg for proteinase B by using azocasein as substrate at pH 7.0

(Taucher *et al.*, 1975). One unit of proteinase activity is defined as the amount of enzyme giving, in the supernatant, an increase in E_{366} of 0.01/min in a 0.5 ml volume, after removal of the precipitated azocasein.

Protein was determined by a modified biuret method (Janatova *et al.*, 1968), with dried bovine serum albumin as standard.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and estimation of molecular weights were performed by the method of Shapiro *et al.* (1967) and Taucher *et al.* (1975).

Phosphofructokinase was dialysed for about 15 h against 0.1 M-sodium phosphate buffer, pH 7.0, containing 5 mM-2-mercaptoethanol and 1 mM-EDTA, and was then diluted to 5 mg of protein/ml. After dialysis against sodium phosphate buffer of equal composition, the respective proteinase was added to phosphofructokinase and the mixture was incubated at 20°C. At appropriate time-intervals, samples were removed; proteolysis in these samples was stopped by rapid cooling to -25°C, and they were kept frozen at this temperature. Denaturation was carried out at 100°C for 5 min in 1% (w/v) sodium dodecyl sulphate.

Results

When phosphofructokinase was incubated with a small amount of proteinase A (8 units of proteinase/mg of phosphofructokinase), the activity of the target enzyme remained constant for about 2 h and then decreased to about 20% of the original activity within the next few hours (Fig. 1). Because of the stability of phosphofructokinase activity in the control, this loss may be attributed to the effect of proteinase A on the enzyme. To correlate this change in activity with alterations in the subunit composition, sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out. As shown in Plate 1(a), 2 h after addition of proteinase A the subunits α and β (mol.wt. approx. 120000) were almost completely degraded to the species α' and β' (mol.wt. approx.

Fig. 1. Time-course of phosphofructokinase activity in the presence of proteinases A and B and α -chymotrypsin

After dialysis (see the Experimental section), the enzyme was incubated at 20°C with 8 units of proteinase/mg of phosphofructokinase. ■, Without proteinase; ●, +proteinase A; ○, +proteinase B; □, + α -chymotrypsin. The activity is given relative to the control.

90000) (for nomenclature of the subunits and their degradation products, see Taucher *et al.*, 1975). In addition, two bands with mol.wt. 45000 and 35000 became visible. Later, the bands of mol.wt. approx. 90000 disappeared and a rather stable band of mol.wt. 80000 became dominant. At the time when the enzyme activity was about 20% of the original value, the ratio of the subunits with mol.wt. 90000 to the sum of the other degradation products did not exceed 15%.

In the course of incubation of phosphofructokinase with proteinase B (8 units of proteinase/mg of phosphofructokinase), a temporary activation of up to 140% occurred, followed by a slow decrease in activity (Fig. 1). A similar transient activation was observed if phosphofructokinase was incubated with α -chymotrypsin.

It is evident from Plate 1(b) that activation is accompanied by conversion of the subunits α and β into subunits α' and β' . The slow inactivation of the enzyme that occurs later may be correlated with the degradation of the α' and β' subunits to a product with mol.wt. 80000. The degradation pattern of phosphofructokinase by α -chymotrypsin is similar to that of proteinase B.

To demonstrate that the actions of proteinase A and proteinase B on the subunits of phosphofructokinase are really qualitatively different, larger amounts of both proteinases were added to phosphofructokinase [Plates 1(c) and 1(d)]. Within 2h, 50 units of proteinase A/mg of phosphofructokinase caused almost complete digestion of the enzyme.

Proteinase B, however, when added in the same amount to phosphofructokinase, gave rise to the formation of the α' and β' fragments, which are stable for several hours. Finally, a band with mol.wt. 80000 became detectable.

Discussion

Clearly, proteinases A and B exhibit qualitatively different degradation patterns of yeast phosphofructokinase. Similar degradation profiles are obtained when proteinase A is compared with subtilisin (Taucher *et al.*, 1975) and proteinase B is compared with α -chymotrypsin. As for subtilisin, the attack of phosphofructokinase by proteinase A may be characterized by a stepwise conversion of the α and β subunits first into α' and β' fragments and then into fragments with mol.wt. 80000, 45000 and 35000. In contrast, the degradation of these subunits by proteinase B and by α -chymotrypsin attains a plateau at the formation of α' and β' fragments.

Differences between the actions of proteinase A and B also become apparent if the activity of phosphofructokinase is followed during the course of incubation. As shown by Taucher *et al.* (1975), the conversion of the native enzyme into the form composed of subunits of mol.wt. 90000 is accompanied by an increase in molecular activity. The constancy of enzyme activity in the first phase of incubation with proteinase A evidently results from the counterbalance of activation and inactivation, which occur simultaneously. At higher proportions of phosphofructokinase to proteinase A and shorter periods of incubation a transient activation becomes apparent, resembling the action of subtilisin on this enzyme. The significant increase in activity caused by proteinase B and α -chymotrypsin can be correlated with the formation of α' and β' subunits.

Holzer (1975a) found an increase in phosphofructokinase activity in crude extracts after addition of proteinase A, and a decrease by proteinase B. The exactly opposite actions of the two enzymes described in his paper might originate from the different experimental conditions.

In the light of recent conceptions for the role of intracellular proteinases in the yeast cell and their interactions with specific inhibitors, which have been summarized in a review by Holzer (1975b), the different actions of the two proteinases on phosphofructokinase may be of interest. Although the regulatory significance of phosphofructokinase activation by limited proteolysis remains to be established, the two proteinases might be involved in the turnover of the phosphofructokinase protein during the life cycle of the yeast cell. In fact, the different proteolytic activities in yeast depend on the growth state of the cell (Saheki & Holzer, 1975).

EXPLANATION OF PLATE I

Degradation patterns of yeast phosphofructokinase during incubation with proteinases A and B evaluated by means of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

The samples of phosphofructokinase were dialysed and diluted as described in the Experimental section. The enzyme was then incubated with 8 units of proteinase (a and b) or 50 units of proteinase (c and d) per mg of phosphofructokinase at 20°C. After the indicated times, samples were removed and the proteolytic processes were stopped by rapid freezing to -25°C. The enzyme was denatured with 1% (w/v) sodium dodecyl sulphate and 1% (w/v) 2-mercaptoethanol at 100°C for 5 min. For polyacrylamide-gel electrophoresis (7% w/v, Cyanogum 41), 20 µg of protein was applied. Electrophoresis was carried out for 5 h at 8 mA/gel tube. Even when phosphofructokinase was treated with 50 units of proteinase/mg, no proteinase band could be detected after staining with Coomassie Brilliant Blue G-250.

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