Biochimica et Biophysica Acta, 524 (1978) 418—427

BBA 68463

COMPARISON OF A NITRATE REDUCTASE-INACTIVATING ENZYME FROM THE MAIZE ROOT WITH A PROTEASE FROM YEAST WHICH INACTIVATES TRYPTOPHAN SYNTHASE

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(Received December 9th, 1977)

Summary

A maize root fraction which inactivates nitrate reductase has been shown to have protease activity which can be measured by the hydrolysis of azocasein. This inactivating enzyme was also found to inactivate yeast tryptophan synthase. Yeast proteases A and B, which inactivate this latter enzyme, also gave a specific inactivation of the maize nitrate reductase.

The maize root inactivating enzyme, like yeast protease B, degraded casein, and was inhibited by phenylmethylsulphonyl fluoride. A partially-purified yeast inhibitor prevented catalysis by the yeast proteases and maize root inactivating enzyme, but purified yeast inhibitors were without effect on the latter protein.

The level of nitrate reductase-inactivating activity, and associated azocasein-degrading activity, increased with age of the maize root. Evidence was obtained for a heat stable inhibitor which maintained them in an inactive state, especially in the young root tip cells.

Introduction

The nitrate reductase-inactivating enzyme is a protein fraction that has been isolated from the mature region of the primary root of maize seedlings [1]. While specific for nitrate reductase, among a limited range of maize enzymes that have been tested, it appeared to cause some degradation of casein [1,2]. It was also inhibited by phenylmethylsulphonyl fluoride, an inhibitor of serine-dependent proteases [2]. This maize root inactivating enzyme appeared similar to a tryptophan synthase-inactivating enzyme from *Neurospora* [3] and yeast [4]. In the initial studies on the inactivation of tryptophan synthase in yeast [4] the protease fractions involved were referred to as tryptophan synthase-inactivating enzymes. Two fractions separated on hydroxyapatite were referred

to as Inactivase I and II. It was subsequently shown [5] that they were identical to yeast protease A and B described earlier [6]. A yeast fraction has also been shown to inactivate *Neurospora* tryptophan synthase [7]. This paper reports a comparison of the maize inactivating enzyme with the yeast proteases which inactivate tryptophan synthase.

Materials and Methods

Maize root inactivating enzyme

Nitrate reductase-inactivating enzyme was isolated from the mature root region of 4-day maize seedlings, as previously described [2]. Except where stated, an approx. 40-fold purified sample (CM32 cellulose fraction) was used. Nitrate reductase [2] was a 40% $(NH_4)_2SO_4$ precipitate of the scutella from 3-day maize seedlings dissolved in 25 mM phosphate (pH 7)/1 mM cysteine. 1 unit of nitrate reductase was defined as that amount of enzyme which produces 1 nmol $NO_2^- \cdot h^{-1}$ at 25°C. A suitable aliquot of inactivating enzyme was selected to give a 15—60% inactivation of nitrate reductase in the total 2-h incubation period. Isocitrate lyase was also estimated as described previously [1].

Isolation and assay procedures for yeast enzymes

(1) Tryptophan synthase. Baker's yeast, *Saccharomyces cerevisiae*, was grown on the medium described by Katsunuma et al. [4]. The cells were harvested and suspended in 0.1 M phosphate (pH 7.2)/10^{-2} M EDTA/10^{-4} M pyridoxal phosphate and passed twice through a French press at 2500 lb/in^2. Tryptophan synthase was isolated by the method of Katsunuma et al. [4] to Step 4. This sample was prepared in 0.1 M phosphate (pH 7.2)/10^{-2} M EDTA/5 mM serine/1 mM pyridoxal phosphate and enzyme activity measured as the rate of indole utilization [8]. 1 unit of tryptophan synthase was defined as that amount of enzyme which utilizes 1 nmol indole $\cdot h^{-1}$ at 37° C.

(2) Yeast protease sample. In this study the yeast fractions isolated by the procedure of Katsunuma et al. [4] to Step 5 will be referred to as yeast protease. This was suspended in 0.1 M phosphate (pH 7)/1 mM mercaptoethanol. For part of the work the yeast sample was fractionated into protease A and B. A commercial sample of baker's yeast supplied by Mauri Bros. and Thompson (Adelaide) was used for isoation of the protease fractions.

Yeast inhibitor

A yeast inhibitor fraction, which inhibits the action of the above proteases on tryptophan synthase, was isolated by the procedure of Ferguson et al. [9]. A commercial sample of yeast was used and the fraction precipitated by treatment with 45—85% (v/v) ethanol was dissolved in 0.1 M phosphate (pH 7)/1 mM mercaptoethanol and dialysed against the same buffer to remove ethanol. A sample of purified protease A inhibitor 1^A [10] and protease B inhibitor [11] were kindly supplied by Dr. Holzer, Biochemisches Institut der Universität Freiburg im Breisgau, G.F.R.

Protease and esterase activity

Standard assay procedures were used for the assay of azocasein-degrading

activity [12], haemoglobin-degrading activity [13], azocoll degrading activity [5], carboxypeptidase activity on *N*-CBz-L-Phe-L-Ala [14] and esterolytic activity on *N*-Ac-Tyr-Et or *N*-Bz-Arg-Et [15]. Protein was determined by the method of Lowry et al. [16] after precipitation with 10% trichloracetic acid.

Results

Inactivation of tryptophan synthase

The partially-purified yeast and maize root samples both inactivated yeast tryptophan synthase (Table I). This inactivation was completely prevented by the yeast inhibitor. Inhibition of both inactivating systems was obtained with phenylmethylsulphonyl fluoride, but that on the mature root was more marked. The time course of the inactivation of tryptophan synthase is illustrated in Fig. 1. During incubation at 25° C, there was only a slight loss of tryptophan synthase activity. Increased inactivation was found with the addition of the yeast and maize inactivating enzyme, a logarithmic decay being observed up to 2 h. Neither the stability of tryptophan synthase nor its rate of inactivation were influenced by the inclusion of 1 mM pyridoxal phosphate in the incubation medium. However, this cofactor at 0.1 mM was always used in the initial yeast extraction medium.

Inactivation of nitrate reductase

Nitrate reductase from the maize scutellum was used as the substrate. When incubated at 25° C, there was a negligible (<10%) decrease in nitrate reductase activity. Any such loss was corrected for in our calculations.

The yeast protease sample promoted an inactivation of nitrate reductase similar to that due to the maize root inactivating enzyme (Table II). In both cases, the yeast inhibitor completely protected nitrate reductase. Phenylmethylsulphonyl fluoride inhibited the action of the maize root inactivating enzyme and gave a high level of inhibition of the yeast protease. The combined action of the yeast and maize sample on nitrate reductase was 80% of that expected from the individual activity of each, additional evidence for a similar mechanism of action.

TABLE I

INACTIVATION OF TRYPTOPHAN SYNTHASE

A yeast sample (3.28 mg protein) with 158 units tryptophan synthase was incubated with the inactivating system for 90 min at 25°C before assay of tryptophan synthase activity (30 min at 37°C). Values for inactivation have been corrected for the slight loss of tryptophan synthase activity that occurs during this period of incubation (see Fig. 1). All samples were partially purified as described and the protein content of each is shown in brackets. PMSF = phenylmethylsulphonyl fluoride.

Inactivating system	Units tryptophan synthase inactivated · h^{-1}
Maize root (34 μg)	38
Yeast (3.4 mg)	34
Maize (34 μg) + yeast inhibitor (1.7 mg)	0
Yeast (3.4 mg) + yeast inhibitor (1.7 mg)	0
Maize (34 μg) + PMSF (1 mM)	12
Yeast (3.4 mg) + PMSF (1 mM)	30

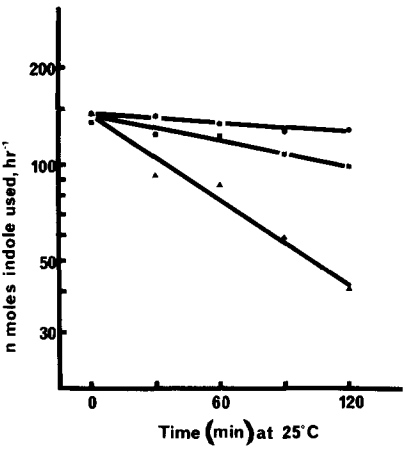

Fig. 1. A yeast tryptophan synthase sample (3.28 mg protein) was incubated either alone (●) or with yeast inactivating enzyme (1.7 mg protein ■) or maize root inactivating enzyme (42 μg protein, ▲). Each sample, in a total volume of 0.4 ml, was incubated for the time shown before assay of tryptophan synthase activity.

TABLE II

INACTIVATION OF NITRATE REDUCTASE

A maize scutella sample (0.38 mg protein) with 69 units nitrate reductase was incubated with the inactivating system for 90 min at 25°C before assay of nitrate reductase (30 min at 25°C).

Inactivating system	Units nitrate reductase inactivated · h^{-1}
Maize root (1.4 μg)	23
Yeast (0.43 mg)	13
Maize root (1.4 μg) + yeast inhibitor (0.43 mg)	0
Yeast (0.43 mg) + yeast inhibitor (0.43 mg)	0
Maize root (1.4 μg) + PMSF (1 mM)	0
Yeast (0.43 mg) + PMSF (1 mM)	4

TABLE III

ACTION OF THE YEAST PROTEASE AND MAIZE ROOT INACTIVATING ENZYME ON MAIZE ISOCITRATE LYASE

The 40—60% $(NH_4)SO_4$ precipitate of the maize scutellum was used as the source of isocitrate lyase. A sample (82 μg protein) was incubated for 1.5 h at 25°C with 20 mM cysteine and 10 mM $MgCl_2$. The sample of yeast inactivating enzyme contained 1.72 mg protein and that of the maize root 2.8 μg. The isocitrate lyase activity at the end of the incubation is shown. The initial activity was 547 nmol glyoxylate produced/h.

Incubation conditions	nmol glyoxylate produced/h
Complete, i.e. with $MgCl_2$ and cysteine	561
minus $MgCl_2$	240
minus cysteine	398
minus $MgCl_2$ and cysteine	223
Complete with yeast inactivating enzyme	477
Complete with maize root inactivating enzyme	534

It was necessary to check that the action of the yeast inactivating enzyme on nitrate reductase was not that of the a general protease. Its action on isocitrate lyase from the maize scutellum was investigated, since it was shown earlier that this enzyme was not inactivated by the maize root inactivating enzyme [1]. In the previous study with isocitrate lyase, a crude extract was used since the enzyme was extremely labile after isolation as a $(NH_4)_2SO_4$ precipitate. It is shown in Table III that this isocitrate lyase fraction was stabilized by the inclusion of $MgCl_2$ and cysteine in the incubation medium. The most significant effect was due to $MgCl_2$. The yeast and maize root inactivating enzyme promoted a slight inactivation of isocitrate lyase. Since the scutella sample had a much higher specific activity for isocitrate lyase than nitrate reductase, the inactivation of the former was tested with a relatively low level of the scutella sample. Under these circumstances, nitrate reductase would have been completely inactivated. Thus, although there was a slight inactivation of isocitrate lyase by the yeast and maize root inactivating enzyme, considerable specificity of action was indicated for nitrate reductase.

Separation of yeast protease A and B and characterization of their action on nitrate reductase

The yeast sample used above was separated, on hydroxyapatite, into two fractions, yeast protease A and B. Both fractions inactivated tryptophan synthase and nitrate reductase (Table IV), but only protease B was sensitive to phenylmethylsulphonyl fluoride.

In the characterization of the maize root inactivating enzyme [2] the main purification was achieved by chromatography on CM-cellulose (CM32). On a column equilibrated with 10 mM acetate (pH 5), the maize root inactivating enzyme was recovered in a single fraction eluted with 10 column volumes of the initial buffer plus 50 mM NaCl. When the protease A and B fractions (isolated from hydroxyapatite) were each chromatographed on CM-cellulose, they were recovered in the same fraction as the maize root inactivating enzyme. However, in contrast to the latter enzyme, there was a considerable loss of activity of yeast protease during dialysis and the subsequent chromatography step, especially in the case of protease B. The maize root inactivating enzyme lost no activity during incubation at pH 6, 7 and 8 at 25° C for 30 min. Data in

TABLE IV

ACTION OF PROTEASE A AND PROTEASE B FROM YEAST ON TRYPTOPHAN SYNTHASE AND NITRATE REDUCTASE

The yeast proteases A and B were separated by hydroxyapatite column chromatography and the assay procedures for tryptophan synthase and nitrate reductase inactivation are described in Tables I and II.

	Protein (mg/ml)	PMSF (1 mM)	Units tryptophan synthase inactivated ($h^{-1} \cdot ml^{-1}$)	Units nitrate reductase inactivated ($h^{-1} \cdot ml^{-1}$)
Protease A	4.36	—	580	95
	4.36	+	575	90
Protease B	0.17	—	410	130
	0.17	+	105	20

TABLE V

NITRATE REDUCTASE INACTIVATING ACTIVITY AND ASSOCIATED PROTEASE ACTIVITY

The primary root (minus 0—2 cm trip region) of 4 day maize seedlings was used and extracted with 0.05 M phosphate (pH 7.5)/5 · 10^{-3} M cysteine and 5 · 10^{-4} M EDTA (4 vols.: 1 g tissue fresh weight). The CM-cellulose fraction was eluted with 50 mM NaCl added to 10 mM acetate (pH 5), the initial column buffer. The enzyme activities given are referred to in the following units: nitrate reductase inactivation, units nitrate reductase inactivated per h; azocasein degradation, A_{440} units per h; haemoglobin degradation, μmol amino acid released per h; carboxypeptidase, μmol alanine released per h.

Fraction	Volume (ml)	Protein (mg)	Total activity			
			Nitrate reductase inactivation	Azocasein degradation	Haemoglobin degradation	Carboxy-peptidase activity
27 000 × *g* supernatant	82	36	7 320	16.9	77	185
40—65% $(NH_4)_2SO_4$ precipitate	12	25	6 288	7.1	56	74
pH 4 supernatant	18	8	13 644	26.6	39	54
CM-cellulose fraction	32	1.6	10 930	22.2	12	20

the literature [5] indicates that the yeast proteases are more labile, especially at pH 6 (protease B) and pH 8 (protease A). On hydroxyapatite, the maize root inactivating enzyme was eluted as one species at approx. 100 mM phosphate.

Proteolytic activity associated with the nitrate reductase-inactiving enzyme in the maize root

The main purification steps used for the isolation of the maize root inactivating enzyme are shown in Table V. An activation occurred during pH 4 treatment and a final 34-fold purification resulted. The recovery and purification of the azocasein-degrading activity is similar to that of the nitrate reductase-inactivating activity and an activation is again observed. There is apparently additional azocasein-degrading activity in the crude extract, which is not associated with the inactivating enzyme and is not recovered in the fraction precipitated by 40—65% saturation with $(NH_4)_2SO_4$. In the isolation procedure for the inactivating enzyme there is no correlation with haemoglobin-degrading or carboxypeptidase activity and no activation of these two species. A low recovery of each was obtained in the CM-cellulose fraction where all the nitrate reductase-inactivating and associated azocasein-degrading activity was found. The latter activities were inhibited by 1 mM phenylmethylsulphonyl fluoride, but 0.1 mM *p*-chloromercuribenzoate was without effect.

A further comparison of the maize root and yeast proteases

Unlike yeast protease B, the maize root inactivating enzyme had no esterolytic activity on *N*-Ac-Tyr-Et or *N*-Bz-Arg-Et and does not act on azocoll. The maize root inactivating enzyme was also shown to be inhibited by a partially purified yeast inhibitor sample. However, it was later found not to be inhibited by the purified protease A inhibitor 1^A [10] or the protease B inhibitor 1^B [11]. Up to 10 μg of each inhibitor was tested with 14 μg maize inactivating enzyme and found to have no effect. The inactivating enzyme was pre-

TABLE VI

DISTRIBUTION OF NITRATE REDUCTASE-INACTIVATING AND AZOCASEIN-DEGRADING ACTIVITY ALONG THE MAIZE ROOT

The primary root of 3 day maize seedings was sectioned and extracted as in Table V. Nitrate reductase-inactivating and azocasein-degrading activity were determined on the 40—60% $(NH_4)_2SO_4$ precipitate of each sample. Each root sample was sectioned from 100 seedlings and data expressed per root.

Root sample (distance from tip, cm)	Fresh weight (mg)	Nitrate reductase inactivation (units nitrate reductase inactivated per h)	Azocasein degradation (A_{440} units per h)
0—1	12.5	0	0
1—2	14.6	0.8	0
2—3	14.2	1.8	0.07
3—4	14.9	2.5	0.11
4—5	15.1	1.9	0.19
5—6	14.0	2.2	0.22

incubated with each inhibitor for 10 min at 25° C before addition of nitrate reductase. Another yeast enzyme which is sensitive to yeast protease B is fructose 1,6-bisphosphatase [17]. It was found in the present study that the maize inactivating enzyme also inactivated this yeast enzyme and its action was again prevented by phenylmethylsulphonyl fluoride.

Influence of root age on the activity of the inactivating enzyme

No nitrate reductase-inactivating activity was detected in the young root tip (0—1 cm) region of primary roots (Table VI). Activity was detected in the next root fraction and increased with distance from the tip. There is a similar pattern for increase in azocasein-degrading activity with age of the root. It has previously been observed that nitrate reductase from the root tip is much more stable in crude extracts than from the mature root region [18].

When the root tip sample was treated at pH 4, nitrate reductase-inactivating and azocasein-degrading activity was discovered (Table VII). Both activities were sensitive to phenylmethylsulphonyl fluoride and the nitrate reductase-

TABLE VII

DEMONSTRATION OF A NITRATE REDUCTASE INACTIVATING ENZYME IN THE MAIZE ROOT TIP

The root tip (0—1 cm) of the primary root of 3-day maize seedlings was used. The extraction, fractionation procedure and enzyme activity units are as in Table V. The inhibition of nitrate reductase inactivation was tested with mature root inactivating enzyme.

Fraction	Protein (mg)	Total activity		Inhibition of nitrate reductase inactivation (%)
		Nitrate reductase inactivation	Azocasein degradation	
40—60% $(NH_4)_2SO_4$ precipitate	12.0	0	0	80
pH 4 supernatant	4.0	860	0.82	34
CM-cellulose fraction	0.2	620	—	33

inactivating activity was shown to chromatograph on CM-cellulose as for the mature root species. It was also established that the original fraction of the root tip (which had no inactivating activity) had inhibitory activity against the mature root inactivating enzyme. This inhibitory component was not destroyed by 10 min in a boiling water bath. After pH 4 treatment of the root tip sample (which released the nitrate reductase-inactivating activity) there was a decrease in the inhibitor activity.

In crude extracts of the yeast cell, where high levels of an inhibitor mask the proteases (especially protease B), incubation at pH 5 resulted in release of protease activity [7,19]. When crude extracts of the maize root tip, maize scutella or spinach leaf were incubated at pH 5 for 18 h at 0 and 25°C (±1% Ca^{2+}), no consistent enhancement of nitrate reductase-inactivating activity was observed.

Discussion

Two tryptophan synthase-inactivating enzymes described by Katsunuma et al. [4] were later shown by Saheki and Holzer [5] to correspond to protease A and protease B respectively. These had been described earlier by Lenney [6]. Evidence is presented in this paper which suggests that a component of the maize root, first characterised as a nitrate reductase inactivating enzyme [1], also appears to be a protease. Both the yeast and maize root protease have been shown to be highly specific, the maize root species only inactivating nitrate reductase [1] and the yeast proteases attacking tryptophan synthase and a limited group of other enzymes [20]. All three proteases have also been characterised by hydrolysis of one of the following non-physiological substrates: haemoglobin (protease A), azocoll (protease B) and casein or azocasein (maize root protease).

In the present study, it was shown that the maize root protease inactivates tryptophan synthase from yeast and that both yeast proteases act on the maize nitrate reductase and again appear to be specific. While this may suggest a similarity in the mechanism of action of the proteases, nitrate reductase and tryptophan synthase could have some structural feature which makes them susceptible to such proteases. It has been shown in animal cells [21] that the half life of a range of enzymes is correlated with their size and charge, the larger molecules with acidic polypeptides being most labile in vivo. It has also been established in both animal and bacterial cells that catabolic rates of proteins correlate well with their relative sensitivity to a variety of proteases in vitro [21]. Nitrate reductase is a relatively large molecule with a molecular weight of about 2×10^5 and an isoelectric point in the acidic pH range, two fractions of the spinach leaf enzyme being identified at pH 3.5 and 4.9 by isoelectric focussing [22]. It has been shown for *E. coli* [23] that, in the tetrameric enzyme tryptophan synthase, one type of subunit is much more susceptible to degradation. Tryptophan synthase, like nitrate reductase, is also very labile in vitro [3,24].

The maize root protease (molecular weight 44 000), although approximately the same size as yeast protease A (40 000), is more similar to the smaller yeast protease B (32 000). Like the maize protease it is inhibited by phenylmethyl-

sulphonyl fluoride [5], degrades casein [25] and has a pH optimum in the neutral to alkaline pH range (5.5 on tryptophan synthase [25] and 9 on casein [26], whereas protease A is an acid protease. Unlike protease B, the maize protease is not inhibited by *p*-chloromercuribenzoate and has no esterolytic activity. All three proteases appear to have similar charge properties as indicated by their chromatography on CM-cellulose at pH 5. While a partially purified yeast inhibitor fraction acted as inhibitor to the maize root protease, pure yeast protease inhibitors were without effect. A protease from *Neurospora* [3], which also inactivates tryptophan synthase, has similar characteristics to the maize root protease and yeast protease B.

An inhibitor of the protease in maize root appears to be dissociated from it by treatment at pH 4. This may explain the activation of nitrate reductase-inactivating and azocasein-degrading activity during the pH 4 step in the purification procedure and the demonstration of an active form (presumably enzyme · inhibitor complex) of the protease in the young root tip cells. The inhibitor, like that in the yeast cell, is heat stable. No activation of haemoglobin-degrading or carboxypeptidase activity in the maize root was detected. In crude extracts of yeast, protease B was recovered in a largely inactive state and incubation at pH 4.8 and 25°C gave a 50-fold increase in tryptophan synthase-inactivating activity [10]. The activation of protease B was mediated by the inactivation of its inhibitor by protease A. A similar incubation of crude plant extracts gave no consistent activation of nitrate reductase-inactivating activity.

The level of nitrate reductase-inactivating and azocasein-degrading activity increased with the age of the root. It remains to be determined how the level of the inhibitor changes, but, in yeast, an increase in the level of protease with age is accompanied by an increase in inhibitor level [27].

It is considered that yeast proteases have a role in enzyme inactivation in the cell and are involved in a process of catabolite inactivation [28]. One can only speculate on a function for the maize root protease, but there is evidence that nitrate reductase has a higher rate of degradation in older root cells, where higher levels of protease have been demonstrated [29,30].

Acknowledgements

The author wishes to thank Miss Glenys Witford for her skilled technical assistance and Mr. John Shannon for assistance with some of the protease assays. The work was supported by a grant from the Australian Research Grants Committee.

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