ON THE STOICHIOMETRY AND REVERSIBILITY OF INTERACTION BETWEEN *NEUROSPORA* PROTEASE I AND ITS INHIBITOR

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1. Introduction

The proteinaceous protease inhibitors of animal and plant origins have been extensively studied in the past decades [1–3]. Yet, little attention has been paid to the occurence of such inhibitors in microorganisms. Recently we have shown that there exists in *Neurospora crassa* a group of protease inhibitors which preferentially inhibit the proteolytic enzymes from the same organism [4]. The findings of the coexistence of proteases and inhibitors and their specific interactions in microorganisms led us to the postulate, that the interplay of proteases with their cognate inhibitors may play an important role in the regulation of intracellular proteolytic activity [4,5]. With an aim to understanding the mechanism of this control process, efforts were made to isolate and characterize a protease–inhibitor complex from *Neurospora*. In this report we present results showing the stoichiometric and reversible nature of the interaction between Protease I and its cognate inhibitor A-2.

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2. Materials and methods

2.1. *Materials*

Protease I, inhibitor A-2 and tryptophan synthase were prepared from *N. crassa* as described previously [4,6].

2.2. *Organisms*

The *N. crassa* strain used in these studies was the tryp 3 mutant td_{201} (Fungal Genetic Stock Center No. 702). The stock was maintained on agar slants containing Vogel's minimal medium supplemented with 150 mg L-tryptophan per liter [7]. The cells were grown under the same conditions as described previously [6], except that tryptophan enriched media were used.

2.3. *Assay of protease and inhibitor activity*

The proteolytic activity toward casein was measured by the spectrophotometric method of Kunitz [8] as modified by Kunimitzu and Yasunobu [9]. One unit of protease is defined as that amount of enzyme which causes the increase of one A_{280}-unit per minute at 37°C. The inhibitor activity was determined by monitoring the protection of tryptophan synthase against proteolytic inactivation as described previously [4]. One unit of inhibitor is defined as the equivalent amount of inhibitor which inhibits one unit of protease I.

3. Results and discussion

3.1. *Isolation of the protease–inhibitor complex*

The endogenous protease–inhibitor complex can be partially isolated if the pH of the buffers used is kept above 7. The isolation was carried out essentially as described previously for *Neurospora* proteases [4], except that the crude extract was made in 0.01 M phosphate buffer, pH 8.0, and the subsequent fractionation on the DEAE-cellulose column was conducted at pH 7.2. Two proteolytically active peaks were detected after the column was developed with a linear gradient from 0–0.2 M sodium chloride (fig. 1). The activity of the second peak (P2, fig. 1) which was hardly observed under the low pH conditions [4] can be greatly stimulated by 4 M urea, by incubation with 0.1 M citrate buffer pH 4 or by extensive dialysis. Since such a phenomenon has not been observed with purified protease I, it may be attributed to a protease–inhibitor complex similar to that observed in yeast [5,10]. After heat treatment (90°C, 5 min) the proteolytic activity of the complex was completely destroyed, while a heat stable material remained in the supernatant that was active in the inhibition of purified protease I. This material was identified as inhibitor A-2 by its chromatographic behavior on a SP-Sephadex column and its molecular weight (approximatly 10 000 daltons). The protease–inhibitor complex could be purified further by gelfiltration on a Sephadex G-100 column at pH 8.0 retaining the properties described above.

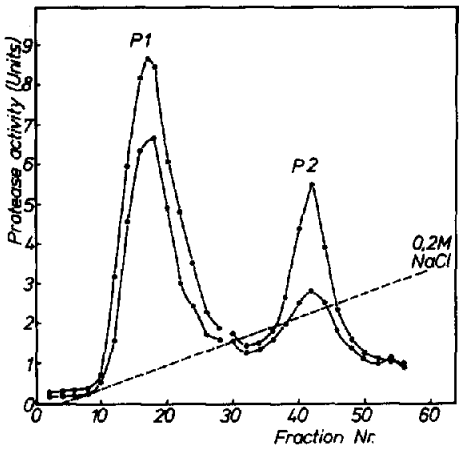

Fig. 1. The fractionation of the protease–inhibitor complex by column chromatography. A column (2.5 × 40 cm) of DEAE-cellulose was equilibrated with 0.01 M phosphate buffer pH 7.2. Extracts were prepared as described in the text. The column was developed with a linear gradient of 0–0.2 M sodium chloride in 0.01 M phosphate buffer, pH 7.2. Proteolytic activity was assayed in the presence (•——•) and absence (○——○) of 2 M urea.

3.2. *Dissociation in vitro of the protease–inhibitor complex*

The effect of urea on the dissociation of the protease–inhibitor complex was studied in detail in parallel with the reconstituted complex. As shown in table 1, 4 M urea was proved to be most effective for activation of the protease in both isolated and reconstituted systems. Since no similar effect was observed with purified protease I (table 1), we concluded that the activation is due to the dissociation of the protease–inhibitor complex. However, it should be noted that in high concentrations of urea (e.g. above 6 M) the enzyme activity decreases. The latter may be accounted for by the denaturation of the enzyme through the action of urea.

Furthermore, when the isolated protease–inhibitor complex was incubated in 0.1 M citrate or phosphate buffer of different pH at 4°C for 48 hr, dissociation of the complex was detectable at pH-values

Table 1
The effect of urea on the proteolytic activity of protease I and the protease–inhibitor complex

Concentration of urea (M)	Proteolytic activity (mU)[a]		
	Protease I	Isolated protease–inhibitor complex	Reconstituted protease–inhibitor complex[b]
0	32.0	11.0	7.5
2	37.0	21.0	15.7
4	44.0	32.3	22.0
6	21.5	8.6	1.6
8	8.4	4.2	1.7

[a] Proteolytic activity was determined at pH 7.2 in the presence of various concentrations of urea after 10 min preincubation at 37°C in 0.1 M phosphate buffer and urea as indicated.

[b] The complex was reconstituted by mixing purified protease I (32 mU) and inhibitor A-2 (24.5 mU) in 0.1 M phosphate buffer pH 7.2.

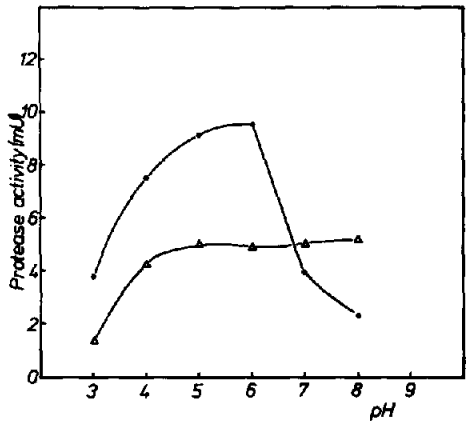

Fig. 2. The effect of pH on the dissociation of the protease–inhibitor complex. Protease I and the isolated complex were incubated with 0.1 M citrate buffer (pH 3–6) and 0.1 M phosphate buffer (pH 7–8) at 4°C for 48 hr. Thereafter the proteolytic activity was assayed at pH 7.2 as usual (△——△ protease I, ○——○ protease–inhibitor complex).

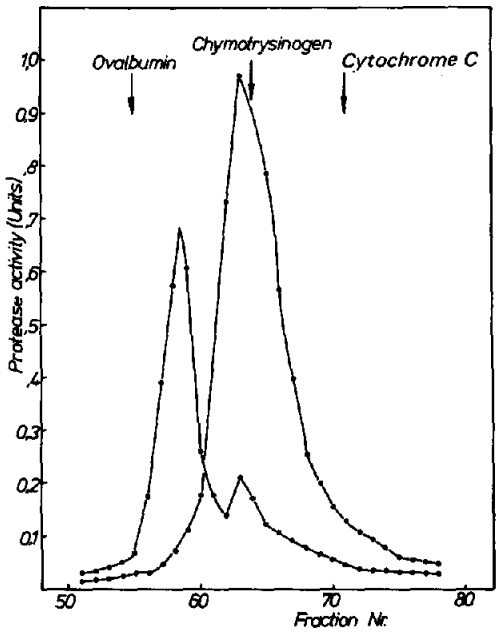

Fig. 3. Gel-filtration of protease I and the protease–inhibitor complex. A Sephadex G-100 column (1 × 90 cm) was equilibrated and developed with 0.1 M phosphate buffer pH 8.0. The protease I and the reconstituted protease–inhibitor complex (see text) were chromatographed separately, but under otherwise identical conditions. The flow rate of the column was 10 ml/hr. Fractions of 2 ml each were collected. Proteolytic activity was measured in the presence of 2 M urea (○——○ protease I, ●——● protease–inhibitor complex).

below 7 (fig. 2). At very low pH-values (e.g. below pH 4) the protease itself is inactivated.

3.3. *The stoichiometry of formation of the protease–inhibitor complex*

The stochiometry of the complex was studied with purified protease I and inhibitor A-2. Both protease (4 units) and inhibitor (3 units) were mixed in 0.1 M phosphate buffer pH 8.0 and kept at 4°C for 3 hr. The mixture was then loaded onto a column of Sephadex G-100. Fig. 3 depicts an elution profile of the proteolytic activity, which was assayed after activation with urea. As expected the mobility of the complex in the column is faster than the protease alone. The molecular weight of the complex was estimated to be about 35 000 daltons by the method of Whitaker [11]. This is in good agreement with the sum of the

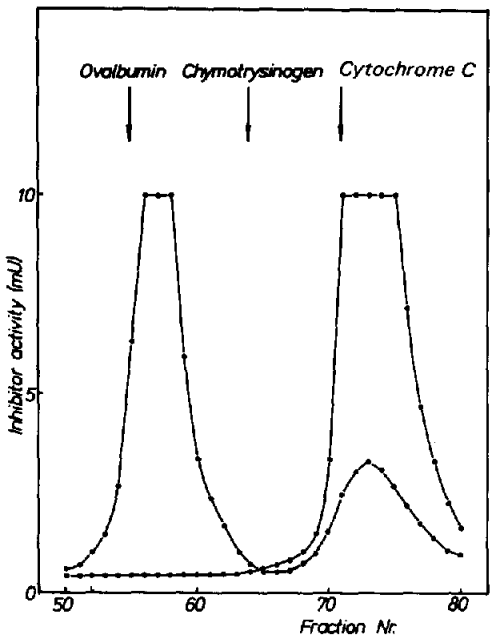

Fig. 4. Gel-filtration of inhibitor A-2 and the inhibitor–protease complex. The inhibitor–protease complex was reconstituted by mixing purified protease I (54 mU) and inhibitor A-2 (60 mU) in 1 ml 0.1 M phosphate buffer pH 8.0 at 4°C for 3 hr. Free inhibitor A-2 and the complex were chromatographed separately under the same conditions as described in fig. 3. Inhibitor activity associated with the complex was determined after heat dissociation of the complex (90°C, 5 min) (○——○ free inhibitor A-2, ●——● inhibitor–protease complex).

molecular weights of protease I (mol. wt. 24 000) and inhibitor A-2 (mol. wt. 10 000) [4] and suggests a molar ratio of 1:1 in the formation of the complex. In a reverse experiment using excess inhibitor we analysed the effluent of the Sephadex G-100 column for inhibitor activity. As shown in fig. 4, the chromatographic mobility of inhibitor A-2 in the complex is very different from that of the free inhibitor, corresponding also to a molecular weight of approximatly 35 000 daltons. The fact that the active inhibitor can be recovered from the complex in good yield after heat dissociation further suggests that the interaction between inhibitor and protease is a reversible process.

Acknowledgements

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