DEMONSTRATION OF SPECIFIC AMINOPEPTIDASE INHIBITORS IN *NEUROSPORA CRASSA*

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Received 31 March 1977

1. Introduction

The protein content of cells is regulated by synthesis and degradation. For degradation, specific proteinases are responsible. At present it is not known how many proteinases operate within a living cell to achieve the necessary timing and specificity. In response to the physiological conditions, only certain proteins are attacked and degraded [1,2] or activated [3] by proteolysis. In the regulation of proteinase activities, besides other factors, specific proteinase inhibitors are involved. Proteinase inhibitors from animal cells and plants have been long known and their chemical and physiological properties, as well as primary structure in many cases, are described [4]. Little is known, in comparison, about proteinase inhibitors from microorganisms.

We isolated four proteinase inhibitors from *N. crassa* mutant td_{201} [5] and demonstrated that the alkaline proteinase forms a stable complex with one of the inhibitors [6]. The degradation of tryptophan-synthase from *N. crassa* can be prevented in vitro by the addition of proteinase inhibitors [7]. Similar observations are reported by Holzer and collaborators on tryptophane-synthase from yeast [8]. We investigated further the inhibition pattern of the proteinase inhibitors isolated towards the purified proteinases from *N. crassa* [9] and report here the discovery of specific heat-stable inhibitors of the aminopeptidases from *N. crassa*. Specific inhibitors of endopeptidases are quite common [10,11], but to our knowledge this is the first demonstration of proteinaceous inhibitors directed towards aminopeptidases.

2. Materials and methods

2.1. *Organism*

Proteinases and inhibitors were isolated from *N. crassa* wildtype EM 5256. The strain was cultivated in a fifty liter fermenter (Giovanola, Basel, Switzerland) with Vogel's minimal medium plus 3% sucrose (w/v) at 28°C and constant pH 5.7, otherwise as described in [7]. Growth was followed by observing the partial pressure of oxygen in the culture medium. The mycelia was harvested at the onset of the stationary phase of growth.

2.2. *Enzymes and inhibitors*

The isolation of the proteinases was carried out as described in [9]. The aminopeptidases A1 and A2 used in this study were obtained in homogeneous form, cross contamination was also checked by determining the relative activities of each aminopeptidase toward the substrates L-lysine 4-nitroanilidhydrobromide (Lys-pNA) and L-leucine 4-nitroanilide (Leu-pNA) [12]. In this study Lys-pNA was used as substrate for aminopeptidase A1 and Leu-pNA for A2.

The isolation of the inhibitors followed the methods given in [5]. Inhibitors I1 and I3 correspond to inhibitors called A-1 and A-3 in [5]. The change in abbreviation was necessary to avoid confusion with the aminopeptidases A1 and A2. The molecular weight of inhibitors I1 and I3 from *N. crassa* wildtype EM 5256 was checked by gel-filtration as described in [5] and found to be 10 000 for I1 and 5000 for I3. The inhibitors have not yet been obtained in homogeneous form.

2.3. *Assays*

The activity of proteinases was assayed as described in [9], with the exception of the alkaline proteinase. Alkaline proteinase has been determined by the method of H. Tsai et al. [13]. The same assay conditions as for the proteinases were used to determine the activity of the inhibitors. Before adding substrate, the proteinase to be tested was mixed with inhibitor and preincubated for 5 min at the reaction temperature. A second sample of proteinase without inhibitor was used as control. The inhibition rate i was then determined according to the following equation:

$$i = 1 - \frac{\text{reaction velocity with inhibitor}}{\text{reaction velocity without inhibitor}}$$

2.4. *Control experiments with unspecific protein*

As a control experiment that the inhibitory effect of the still inhomogeneous inhibitor fractions I1 and I3 does not depend on the presence of substrate for aminopeptidases, aminopeptidase activities were measured including the unspecific protein ribonuclease A in the assay system. The activity of the aminopeptidases was not altered up to a concentration of 4 mg ribonuclease A/ml.

3. Results

3.1. *Specifity of inhibitors I1 and I3*

Figure 1 demonstrates the influence of inhibitors I1 and I3 upon the activity of the five intracellular proteinases from *N. crassa*. In a previous report [5] we came to the conclusion that I1 inhibits the acid proteinase. This has to be corrected if we assume the inhibitors from the mutant strain td_{201} and the wildtype EM 5256 to be identical. During the first study the acid proteinase used was still contaminated by aminopeptidases. Our results now show that highly purified acid proteinase is not inhibited by the inhibitors I1 and I3. But these two inhibitors do inhibit the aminopeptidase from *Saccharomyces cerevisiae* (personal communication from H. Tsai).

3.2. *Dissociation in vitro of the aminopeptidase-inhibitor complexes*

All four proteinase inhibitor complexes investigated are temperature sensitive (table 1). The difference in stability of the complexes (A2–I1) and (A2–I3) is obvious.

At pH 3, loss of aminopeptidase activity is largely prevented when the inhibitors I1 or I3 are present. This protection is not due to a degradation of inhibitors by aminopeptidases, but must be attributed to a gain of stability of the aminopeptidases in complex with an inhibitor. If the activity of aminopeptidase is destroyed by heating for 5 min at 95°C, after incubation for 24 h at 4°C the inhibitor can be recovered without loss. This is true for all experiments over the pH-range from 3–8. The pH-stability of the complex (A1–I1)

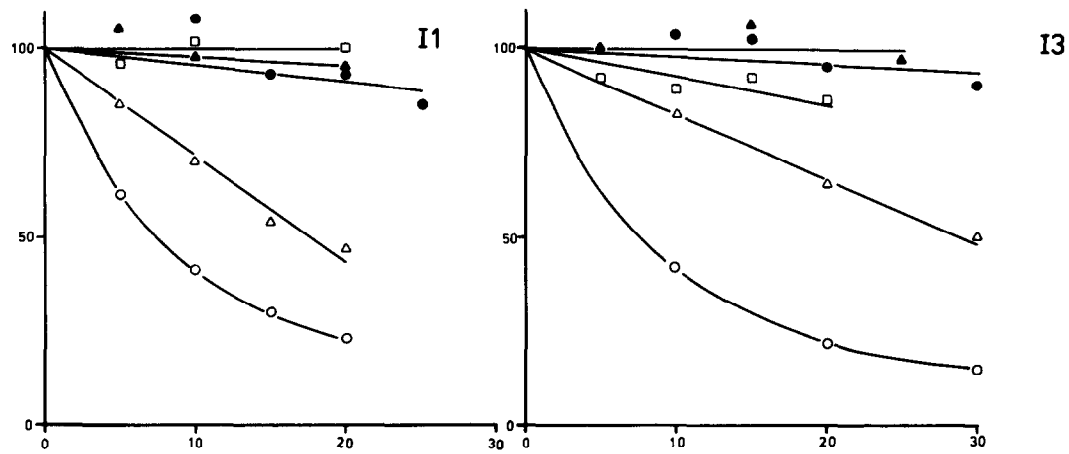

Fig.1. Inhibition effect of inhibitors I1 and I3 against the five intracellular proteinases from *N. crassa*. (*x*-axis) Amount of inhibitor ($\times 10^{-3}$ ml). (*y*-axis) Inhibition rate i in percent. (△——△) Aminopeptidase A1 (EC 3.4.11) 10 mU. (○——○) Aminopeptidase A2 (EC 3.4.11) 10 mU. (□——□) Carboxypeptidase (EC 3.4.12) 1.6 mU. (▲——▲) Acid proteinase (EC 3.4.23) 17 mU. (●——●) Serine proteinase (EC 3.4.21) synonym: alkaline proteinase, 16 mU with I1 and 23 mU with I3.

Table 1
Influence of temperature upon the aminopeptidase–inhibitor complexes

Temp.(°C)	A1[a]	(A1–I1)[b]	(A1–I3)[b]	A2[a]	(A2–I1)[b]	(A2–I3)[b]
30	100	70	74	100	62	77
40	118	54	57	137	55	64
50	76	40	28	89	35	0
60	36	0	0	48	30	0

[a]Free aminopeptidase activity relative to activity at 30°C
[b]Inhibition rate – % free aminopeptidase activity

Inhibitor plus proteinase and proteinase alone were incubated for 5 min at the indicated reaction temperature. The tests were started by adding substrate solution of the same temperature as the reaction temperature. Amount of enzyme: 10 mU A1 (substrate: 10 mM Lys-pNA) and 13 mU A2 (substrate: 0.5 mM Leu-pNA).

shows a maximum around pH 6 while the pH-stability of the complexes (A1–I3) and (A2–I3) is almost constant in the pH-range from 4–8 (table 2). Table 3 demonstrates that the complexes involving aminopeptidase A2 are more stable against the deforming influence of urea than the complexes involving aminopeptidase A1. Uncomplexed aminopeptidase A2 is also more resistant to urea than A1.

3.3. *Kinetic plots*

Kinetic plots are important for the characterisation of inhibitors, even if no quantitative data can be determined at present. When the four possible aminopeptidase–inhibitor complexes between A1, A2 and I1, I3 were measured at various substrate concentrations it can be seen that the inhibition rate of the complexes (A2–I1) and (A2–I3) is independent of the substrate concentration, while the inhibition rate of the complexes involving A1 are dependant from substrate concentration. A plot of the reciprocial inhibition rate towards the relative inhibitor concentration gives for (A2–I1) the picture of a non-competitive inhibition (fig.2). Complex (A1–I1) appears to be competitive or mixed-inhibition type (fig.3), while

Table 2
pH-Stability of the aminopeptidase–inhibitor complexes

pH	A1[a]	(A1–I1)[b]	(A1–I3)[b]	A2[a]	(A2–I1)[b]	(A2–I3)[b]
4.0	71	14	79	65	69	77
5.0	87	34	81	75	68	80
6.0	94	55	84	95	56	79
7.0	100	31	74	100	42	75
8.0	75	10	75	100	40	75

[a]Free aminopeptidase activity relative to activity at pH 7.0
[b]Inhibition rate – % free aminopeptidase activity

Aminopeptidases and aminopeptidases mixed with inhibitor were incubated for 24 h at 4°C in phosphate–citrate buffer according to McIlvan. The samples are transferred to a cuvette and the reaction was started by adding substrate. The test, pH 8.0, was shifted not more than 0.4 units to lower values. As the difference of activity at pH 7.5 and pH 8.0 for the aminopeptidases A1 and A2 is not more than 6%, changes in the relative activity related to the difference in pH were therefore neglected. The amount of inhibitor added in the tests gave an inhibition rate of 70–80%, at pH 8.0, without preincubation. Amount of enzyme: 10 mU A1 (substrate: 10 mM Lys-pNA) and 13 mU A2 (substrate: 0.5 mM Leu-pNA).

Table 3
Influence of urea on the aminopeptidase–inhibitor complexes

Urea (mM)	A1	(A1–I1)	(A1–I3)	A2	(A2–I1)	(A2–I3)
0.0	100	48	68	100	64	63
0.4	64	46	17	100	60	63
0.8	36	12	12	81	50	58
1.2	24	0	0	52	23	55
1.6	16	0	0	31	20	44
2.0	10	0	0	10	17	0

[a]Free aminopeptidase activity relative to activity at 0 mM urea
[b]Inhibition rate – % free aminopeptidase activity

The substrate solutions were made up with urea in certain concentrations. The tests were conducted as usual. All urea concentrations are corresponding to the final concentration during the assay. Amount of enzyme: 13 mU A1 with I1 and 16 mU A1 with I3 (substrate: 10 mM Lys-pNA) and 12 mU A2 with I1 and 5 mU A2 with I3 (substrate: 0.5 mM Leu-pNA). The final substrate concentration were 5 mM Lys-pNA and 0.5 mM Leu-pNA, respectively.

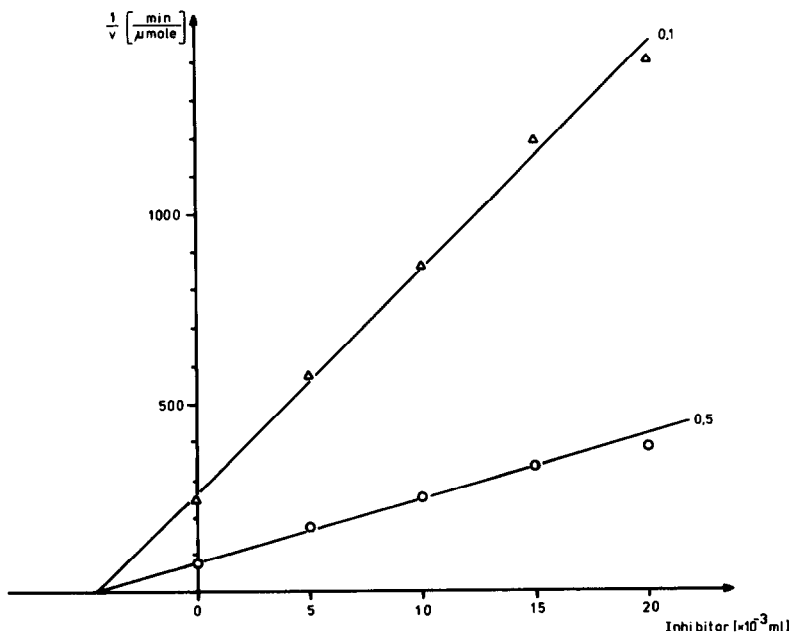

Fig. 2. Plot of reciprocal velocity versus relative inhibitor concentration. Reciprocal velocity is plotted against inhibitor concentration for the complex (A2–I1). Amount of enzyme: 10 mU aminopeptidase A2. (○——○) Substrate concentration 0.5 mM Leu-pNA. (△——△) Substrate concentration 0.1 mM Leu-pNA.

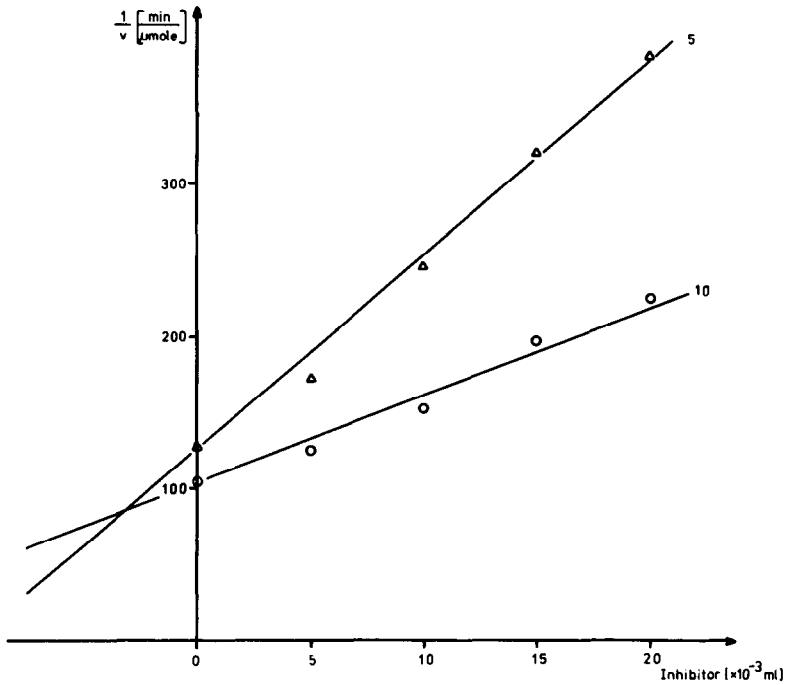

Fig.3. Plot of reciprocal velocity versus relative inhibitor concentration. Reciprocal velocity is plotted against inhibitor concentration for the complex (A1–I1). Amount of enzyme: 9 mU aminopeptidase A1. (o——o) Substrate concentration 10 mM Lys-pNA. (△——△) Substrate concentration 5 mM Lys-pNA.

for the complexes (A2–I3) and (A1–I3) a non-linear relation between the reciprocal inhibition rate and the relative inhibitor concentration is observed.

4. Discussion

We have shown that in *N. crassa* specific inhibitors directed towards the two aminopeptidases are present. From their physical and chemical characteristics it appears that these inhibitors named I1 and I3 are identical to the inhibitors A-1 and A-3 described earlier [5].

The control of the two aminopeptidase activities in *N. crassa* by the inhibitors may have a different mechanism on the molecular level. If one assumes a crude model of an enzyme, with an active centre embracing all the areas involved in catalysis, then it follows that the aminopeptidase A1 binds the inhibitors I1 and I3 at the active centre, as the inhibition rate is directly dependent on substrate concentration. The aminopeptidase A2 seems to have a separate inhibitor binding site remote from the active centre, as the inhibition rate is found independent of substrate concentration. The deviation from linearity between the reciprocal inhibition rate and the relative inhibitor concentration involving the inhibitor I3 can be explained by the assumption that the aminopeptidases bind more than one molecule of the inhibitor I3. In this context one has to recall that the molecular weight of I3 is just half of I1. The possibility exists that I1 is composed out of two I3 molecules. That I3 is derived by cleavage of I1 is also not ruled out.

There are clearly demonstrable differences between the four proteinase–inhibitor complexes with regard to the influence of temperature, pH and urea and to the kinetic plots. Besides the compartmentation in the cell [14,15], the physical and chemical differences of the complexes may offer the cell a possibility to regulate the aminopeptidase activities. The mechanism of this regulation remains to be investigated further.

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