SPECIFICITY OF FIVE INTRACELLULAR PROTEINASES OF *NEUROSPORA CRASSA*

Dietrich SIEPEN and Maria-Regina KULA

Gesellschaft für Molekularbiologische Forschung mbH, D-3300 Braunschweig-Stöckheim, Mascheroder Weg 1, West Germany

Received 4 March 1976
Revised version received 2 April 1976

1. Introduction

Five intracellular proteolytic enzymes of *Neurospora crassa* are known: an acidic and an alkaline endopeptidase, one carboxypeptidase and two aminopeptidases [1]. The acid proteinase hydrolyzes acid-denatured haemoglobin at pH 3.0. The alkaline proteinase and the carboxypeptidase are serine proteinases which require a sulfhydryl group for activity. The aminopeptidases are both metallo-enzymes and can be inhibited by EDTA.

In order to characterize the enzymes further we investigated the substrate specificity of the five proteinases using the A- and B-chains of oxidized bovine insulin, glucagon and some synthetic substrates. And we examined the reactivation of the EDTA-inactivated aminopeptidases by various metal ions.

2. Materials and methods

Glucagon was purchased from Calbiochem (Lucerne, Switzerland), *N*-benzyloxycarbonyl-L-peptides from Sigma (München, Germany). All other materials were summarized previously [1].

Enzyme activities were determined as described previously [1]. Insulin and glucagon were digested at the optimal pH of each enzyme in ammonium acetate buffer. The separation of peptides was carried out on paper in the first dimension by high voltage electrophoresis at pH 3.5 (50 V/cm) and in the second dimension by chromatography using solvent composed of *n*-butanol, acetic acid, pyridine and water (75 : 15 : 50 : 60, by vol). For evaluation the paper was stained with 0.02% ninhydrin, the peptides were cut out, eluted with 6 N HCl and hydrolyzed at 105°C for 24 h. The amino acid composition of the peptides was determined with an automated amino acid analyzer (Biocal BC 200 equipped with an Infotronics integrator CRS 100 A). Tryptophan containing peptides were detected by staining with Ehrlich's reagent (1% *p*-dimethylaminobenzaldehyde in acetone containing 10% conc. HCl). Since the sequence of insulin and glucagon is known the split bond can be deduced from the amino acid composition. The hydrolyses of peptides by the aminopeptidases was confirmed by chromatography on the amino acid analyzer.

The inactivation of the aminopeptidases was performed as follows: 0.2 ml enzyme solution and 0.02 ml 0.1 M EDTA were mixed and dialyzed for 3 h at 4°C against 0.01 M EDTA in 0.01 M potassium phosphate buffer pH 7.0, subsequently for 15 h against distilled water. For reactivation 0.02 ml of the enzyme solution were incubated with 0.02 ml 0.05 M Tris buffer, pH 8.0, and 0.02 ml 0.1 M Metal-(II)-chloride solution for 30 min at 25°C. From this preincubated solution 0.05 ml were mixed with 0.5 ml substrate in 0.05 M Tris buffer, pH 8.0, and the activity measured.

3. Results and discussion

3.1. *Specificity of the alkaline serine proteinase*

Serine proteinases which are sensitive to diisopropylphosphofluoridate and most active at neutral to alkaline pH, can be divided into at least four groups [2]: trypsin like proteinases, alkaline proteinases, myxobacter α-lytic proteinase and staphylococcal proteinase. As shown in fig.1 the alkaline *N. crassa*

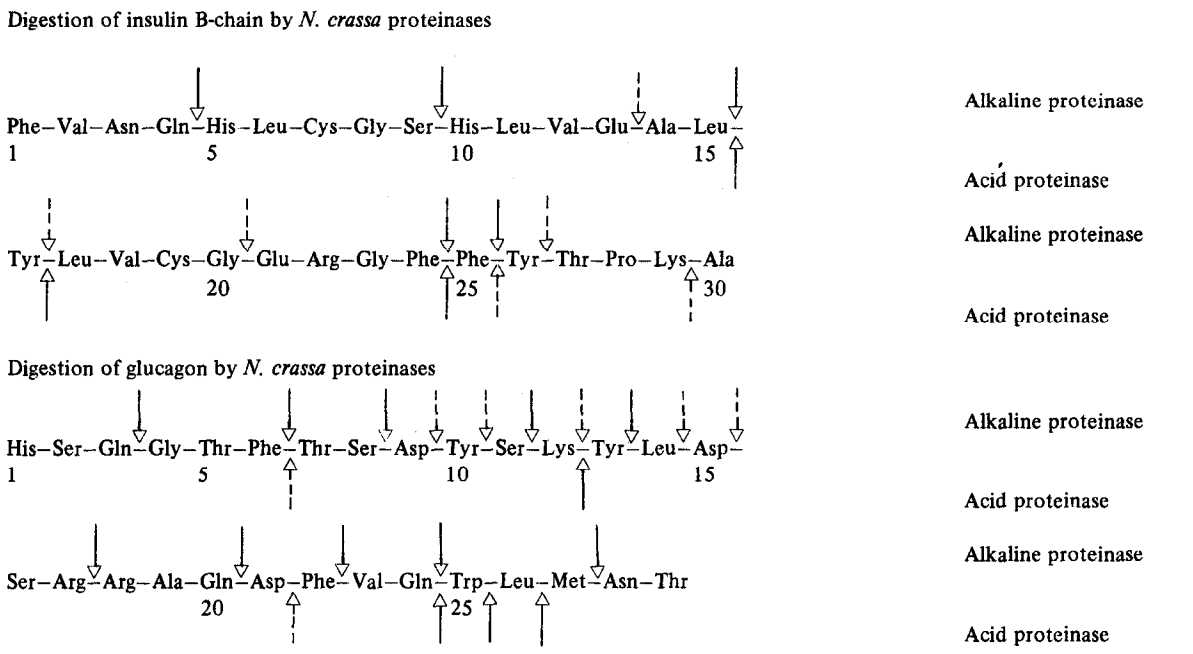

Fig.1. Amino acid sequence of the B-chain of oxidized insulin and of glucagon. The sites of hydrolysis by the alkaline and the acid *N. crassa* proteinase are indicated by arrows, the degree of hydrolysis is greater for solid arrows than for broken ones. Sequence of bovine insulin B-chain and pig glucagon taken from [5].

proteinase belongs to the second group which exhibits specificity for aromatic or hydrophobic residues. The bond Leu–Tyr (15–16) is split much more rapidly than any other bond in the B-chain of oxidized bovine insulin.

3.2. *Specificity of the acid proteinase*

Acid proteinase which are most active at low pH, show specificity against aromatic or bulky amino acid residues in synthetic peptide substrates, but this is not clearly reflected in the hydrolysis of the oxidized insulin B-chain, in which they show considerably broader specifity. The acidic proteinases attack prefentially at Leu–Tyr (15–16), Tyr–Leu (16–17), Phe–Phe (24–25) and Phe–Tyr (25–26) in the B-chain of insulin [2]. The specificity of the *N. crassa* acid proteinase against the insulin B-chain and glucagon is shown in fig.1. The sites of cleavage are quite restricted possibly by secondary interactions.

3.3. *Specificity of the carboxypeptidase*

The carboxypeptidase liberated the first amino acids from the C-terminal end of the B-chain of insulin. The activity includes the liberation of proline. The amino acids Ala, Lys, Pro, Thr, Tyr and Phe were completely removed after 30 min incubation, but even after prolonged incubation no Gly was detected in the digest. Besides *N*-benzoxycarbonyl-L-Ala–L-Phe we tested some other N-blocked dipeptides as substrates of the carboxypeptidase. Table 1 shows the relative rates of hydrolysis. Gly as penultimate residue slows the release of the terminal amino acid quite drastically.

Table 1
Relative rates of hydrolysis of some *N*-benzoxycarbonyl-L-dipeptides by the carboxypeptidase

N-benzoxycarbonyl-L-dipeptide	Rate of hydrolysis (%)
Ala–Phe	100.0
Ala–Val	88.8
Ala–Leu	27.5
Gly–Phe	6.0
Gly–Leu	1.7
Gly–Ala	0

3.4. *Specificity of the aminopeptidase A 1*

We reported already that aminopeptidase A 1 does not hydrolyze the B-chain of insulin. Amongst the other polypeptides tested glucagon was not hydrolyzed. With the A-chain of insulin as substrate the first amino acids starting from the N-terminal end were liberated. The synthetic L-peptides Arg–Leu, Arg–Val, Lys–Leu, Lys–Ala, His–Phe, Phe–Gly–Gly, Gly–Leu–Tyr, Ala–Gly–Gly and Phe–Gly–Phe–Gly were hydrolyzed. At present no explanation can be given why insulin B-chain and glucagon are resistent to the action of aminopeptidase A 1. The relative rates of hydrolysis of some L-amino acid *p*-nitroanilides are shown in table 2. The reactivation of the EDTA-inactivated aminopeptidase A 1 by divalent cations is shown in table 3. The most pronounced reactivation effect was found with Mn^{2+} and Mg^{2+} ions simultaneously present.

Table 2
Relative rates of hydrolysis of some L-amino acid *p*-nitroanilides by the aminopeptidases

L-amino acid *p*-nitroanilide	Rate of hydrolysis (%)		c (mol/l)
	Aminopeptidase		
	A 1	A 2	
Lys	100.0	68.0	0.010
Ala	26.3	31.0	0.010
Leu	19.1	100.0	>0.001
Phe	2.3	5.0	>0.001
Pro	1.4	5.5	0.010
Tyr	1.1	6.2	>0.001
Gly	0.5	8.9	>0.001
Cys	0.3	1.0	>0.001
Glu	0.1	0	>0.001

c= >0.001 means a saturated solution of substrate in 0.1 M potassium phosphate buffer pH 8.0 at 25°C.

Table 3
Reactivation of the EDTA-inactivated aminopeptidases by divalent cations

	Activity (%)	
	Aminopeptidase	
	A1	A2
Aminopeptidase + H_2O	100.0	100.0
Aminopeptidase + EDTA	0	0
Aminopeptidase + EDTA + Mn^{2+}	55.3	8.6
Ca^{2+}	42.7	58.6
Mg^{2+}	17.6	37.7
Co^{2+}	14.2	25.0
Zn^{2+}	0	0
Cd^{2+}	0	0
Ni^{2+}	0	0
$Mn^{2+} + Zn^{2+}$	0	0
$Ca^{2+} + Zn^{2+}$	0	0
$Mn^{2+} + Ca^{2+}$	67.1	24.1
$Mn^{2+} + Mg^{2+}$	82.2	29.6
$Mn^{2+} + Co^{2+}$	44.4	21.0
$Ca^{2+} + Co^{2+}$	36.0	27.8
$Mg^{2+} + Co^{2+}$	43.6	30.8
$Ca^{2+} + Mg^{2+}$	70.4	58.6

3.5. *Specificity of the aminopeptidase A 2*

Aminopeptidase A 2 exhibited a broad specificity and could hydrolyze the first N-terminal amino acids of the oxidized A- and B-chain of insulin, glucagon and the same L-peptides as tested with the aminopeptidase A 1. The relative rates of hydrolysis of some L-amino acid *p*-nitroanilides are shown in table 2. The reactivation of the EDTA-inactivated aminopeptidase A 2 by divalent cations is summarized in table 3. The greatest reactivation was effected by Ca^{2+} ions, also in the presence of Mg^{2+} ions.

Both *N. crassa* endopeptidases could be well classified: one as a typical alkaline serine proteinase and the other as a pepsin-like acid proteinase. The specificity of the *N. crassa* carboxypeptidase is quite similar to the yeast carboxypeptidase (yeast proteinase C) [3] which also liberates proline from a polypeptide chain. Aminopeptidase A 2 shows a very broad specificity which is similar to that of the classic leucine aminopeptidase. In contrast A 1 shows a very narrow specificity which must be further characterized. The metallo-enzyme character of both aminopeptidases was confirmed by reactivation of the EDTA-inactivated enzymes by Mn^{2+} and Mg^{2+} respectively by Ca^{2+} ions. The metal ions in the native enzyme must not be the same as shown for the classic leucine aminopeptidase which contains Zn^{2+} in the native state, but can be activated by Mg^{2+} and Mn^{2+} [4]. Since both *N. crassa* aminopeptidases are stable during dialysis, the metal ions in the native enzymes were not removed during isolation.

Acknowledgements

This work has been supported within the Biotechnology Program of the Bundesministerium für Forschung und Technology der Bundesrepublik Deutschland.

References

[1] Siepen, D., Yu, P. H. and Kula, M.-R. (1975) Eur. J. Biochem. 56, 271–281.
[2] Morihara, K. (1974) in: Advances in Enzymology (Alton Meister, ed.) Vol. 41 pp. 179–243, John Wiley and Sons Inc. New York, London, Sydney, Toronto.
[3] Hayashi, R., Moore, S. and Stein, W. H. (1973) J. Biol. Chem. 248, 2296–2302.
[4] Himmelhoch, S. R. (1969) Arch. Biochem. Biophys. 134, 597–602.
[5] Atlas of Protein Sequence and Structure (1972) (Dayhoff, M. O. Ed.) National Biomedical Research Foundation, Silver Spring.