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CLEAVAGE SPECIFICITY OF THE SERINE PROTEINASE FROM RAT LIVER MITOCHONDRIA

Rainer Haas and Peter C. Heinrich, Biochemisches Institut der Universität, 7800 Freiburg, Germany

Rudolf Tesch and Irene Witt, Kinderklinik der Universität, 7800 Freiburg, Germany

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SUMMARY

A new simple fast and reproducible purification procedure for the proteinase from rat liver mitochondria has been worked out. The specificity of cleavage of peptide bonds in glucagon, oxidized A and B chains of insulin and yeast proteinase B inhibitor by the proteinase of the inner mitochondrial membrane has been studied. The proteinase hydrolyzed three peptide bonds in glucagon, Tyr (13) - Leu (14), Trp (25) - Leu (26) and Phe (22) - Val (23) (minor cleavage site); none in the insulin A chain; one in the B chain of insulin, Tyr (16) - Leu (17); and three in the yeast proteinase B inhibitor, Phe (4) - Ile (5), Phe (20) - Leu (21) and Tyr (41) - Thr (42) (minor cleavage site).
Thus, the mitochondrial proteinase cleaves peptide bonds at the carboxyl site of an aromatic amino acid and the amino site of a leucine, isoleucine, threonine or valine. The comparison with chymotrypsin A shows that the mitochondrial proteinase cleaves peptide bonds in a more restricted manner.

INTRODUCTION

Recent studies on histone degrading activities in rat liver have shown that the mitochondrial fraction contains an insoluble proteinase (1). Although the existence of mitochondrial proteinases has been postulated by several authors (2-7), an unambiguous demonstration within mitochondria was presented only recently (8-12). The mitochondrial proteinase has been purified to homogeneity and some of its properties were studied (13). One of the most remarkable properties of the enzyme is its high substrate specificity (13). Therefore, it was of

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interest to examine in greater detail the cleavage specificity of this enzyme. In the present paper the sites of cleavage of glucagon, oxidized insulin A and B chains, and yeast proteinase B inhibitor (14, 15) by the mitochondrial proteinase are presented. Furthermore, a new and simple purification procedure leading to a homogeneous proteinase preparation is described.

MATERIALS AND METHODS

Assay of proteinase activity

Mitochondrial proteinase activity was measured as previously described (13). Protein determinations were carried out according to Lowry et al. (16).

Purification of the proteinase

Mitochondria were prepared from livers of male SIV 50 rats (250-350 g body weight) according to Loewenstein et al. (17). After freezing and thawing twice the mitochondria were subjected to step gradient centrifugation. The discontinuous gradient was formed by consecutive layers of 1.74 M sucrose, 1.6 M sucrose, 1.5 M sucrose and 1.3 M sucrose as detailed by Haas and Heinrich (10). A high density fraction was obtained as a pellet containing the proteinase. To remove concomitant proteins the proteinase containing sediment was washed successively with 0.5 M LiCl in 5 mM Tris/HCl (pH 8), followed by 0.8 % sodium cholate (pH 8) followed by 0.1 M NaCl in 5 mM Tris/HCl (pH 8), and finally by a solution which contained 0.12 M KCl, 5 mM EGTA, 5 mM EDTA in 30 mM Tris/HCl (pH 8). In each solution the high density proteinase fraction was sonified at 100 W for 10 sec, kept on ice for 15 min, and centrifuged at 200 000 x g for 15 min. The solubilization of the proteinase was achieved by incubation of the washed high density fraction in 2 M LiCl, 0.05 % Triton X-100 in 5 mM Tris/HCl (pH 8.5) at 0°C for 1.5 h. After centrifucation as above the supernatant, which contained the solubilized enzyme, was incubated at 37°C for 40 min and dialysed at 0 - 4°C against solubilization medium for at least 8 h.

Incubation of substrates

Two mg of either glucagon or oxidized insulin A or B chain, or yeast proteinase B inhibitor were incubated in a total volume of 0.5 ml of 0.1 M NH_4HCO_3 buffer (pH 8) with 7 µg of proteinase (spec. act. of 11 units/mg) at 25°C for 18 h and lyophilized. Due to the low solubility of glucagon a suspension of the polypeptide was incubated with the proteinase.

Edman degradation

The peptides after proteinase digestion modified with 2-aminonaphthalene-1.5-disulfonic acid as described by Foster et al. (18) were subjected to Edman degradation (Beckman Spinco

Sequencer 890 C) according to the method of Edman and Begg (19) as modified by Hermodson et al. (18). The phenylthiohydantoin-amino acids were identified by gas chromatography, and thin layer chromatography (21). In some cases an amino acid analysis was performed after acid hydrolysis.

RESULTS

The new purification procedure for the mitochondrial proteinase is based on the fact that the proteinase activity found in the high density fraction obtained after sucrose step gradient centrifugation can be solubilized only under high ionic strength conditions. Therefore, the high density fraction was successively treated with a series of salt-buffer solutions, which effectively removed associated proteins.

The solubilization of the proteinase was achieved by the use of 2 M LiCl or 2 M NaCl. It was found that concomitantly solubilized proteins were rapidly digested by the proteinase upon incubation at 37°C. The purification procedure summarized in Table 1 shows that the proteinase of high specific activity is obtained in a yield of 61 %. The purified enzyme exhibited only a single band after SDS-polyacrylamide gel electrophoresis.

Glucagon, insulin A and B chain and yeast-proteinase B inhibitor (14,15) have been used for the elucidation of the cleavage specificity of the mitochondrial proteinase. After incubation at 25°C for 18 h the digestion products were analyzed by Edman degradation. No separation of the peptide fragments was carried out. Since the amino acid sequences of all the substrates are known, it was possible to determine the cleavage sites in the various polypeptides from the amino acid sequences found after several Edman degradation steps.

The most susceptible bonds in the substrates used are demon-

Table 1 Purification of the mitochondrial proteinase

	Purification step	Volume (ml)	Total protein (mg)	Total activity (units)[a]	Specific activity (units/mg)	Purification factor (-fold)	Yield (%)
1.	Mitochondria	300	4 830	n.m.[b]	-	-	-
2.	High density inner membrane fraction	9	10.60	9	0.85	1	100
3.	High density inner membrane fraction, recentrifuged	3	3.10	8.98	2.90	3.4	99.8
4.	High density inner membrane fraction, washed	5	2.3	8.4	3.65	4.3	93.3
5.	Proteinase solubilized in 2 M LiCl	5	0.25	5.5	22.0	25.9	61

(a) 1 unit = 1 μmol N-acetyl-L-tyrosine ethyl ester split per minute at 30°C under assay conditions (see Materials and Methods)

(b) n.m. = not measurable, no activity detectable

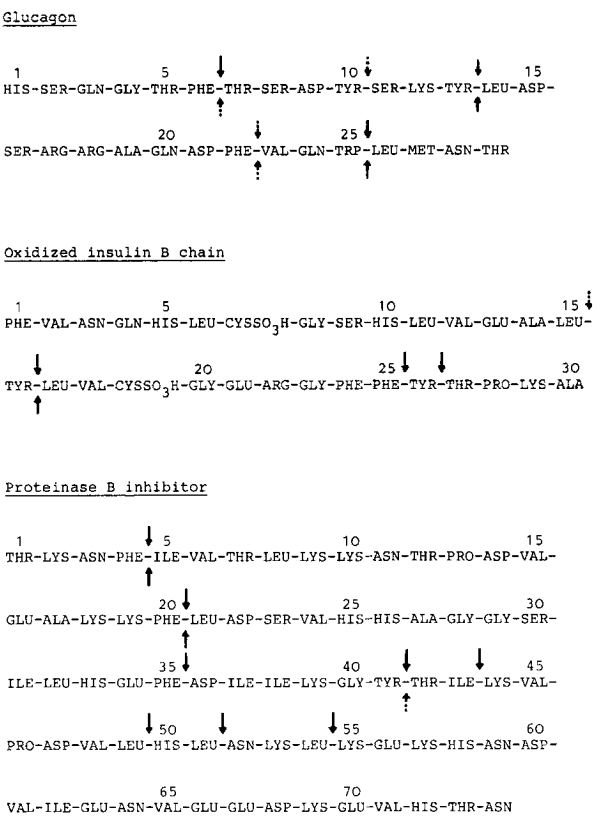

Fig. 1 Major (↑) and minor (⇡) sites of cleavage of glucagon, oxidized insulin B chain and yeast proteinase B inhibitor by the mitochondrial proteinase. For comparison the cleavage sites observed with chymotrypsin A are given (↓, ⇣) (22-24, 14).

strated by arrows and minor cleavage sites by broken arrows (Fig. 1). For comparison the cleavage sites obtained after digestion with chymotrypsin A are presented. It can be seen that the mitochondrial proteinase produces fewer peptide fragments, indicating a higher specificity. As shown in Fig. 1 the peptide bonds to be cleaved require an aromatic amino acid (Phe, Tyr, Trp) at the carboxyl site of the peptide bond and a leucine, isoleucine, threonine or valine at the amino site.

DISCUSSION

Compared to the isolation steps previously described (13) the purification method of the proteinase presented in this paper is rapid, simpler, and more reproducible. With the purification procedure described it is possible to obtain highly purified proteinase in mg quantities in only 2 days.

As previously shown (13) the proteinase has a pH optimum of around 8 and a molecular weight of 24 000. Serine and histidine are found in the active site of the enzyme, soybean trypsin inhibitor, phenylmethanesulfonyl fluoride, di-isopropyl phosphofluoridate, and L-1-tosylamido-2-phenylethylchloromethyl ketone are powerful inhibitors.

The "group-specific" proteinase isolated from rat liver by Katunuma and coworkers (25) exhibits essentially the same properties, particularly since these authors have redetermined the molecular weight (26) of the enzyme and also found histones to be degraded (12). On the other hand, we found that apo-ornithine aminotransferase from rat liver is inactivated by our purified mitochondrial proteinase. Thus, it is likely that the "group-specific" proteinase and the enzyme isolated in our laboratory are identical. However, the finding that histones, nonhistone chromosomal proteins, a variety of inner mitochondrial membrane and matrix proteins (Haas, unpublished results), glucagon, oxidized insulin B chain and proteinase B inhibitor from yeast are degraded by the proteinase, contradicts to the concept of "group-specificity" for the enzyme.

Our previous studies on the mitochondrial proteinase have revealed chymotrypsin-like properties (13). From the results of the present investigation it became clear that the mito-

chondrial proteinase cleaves peptide bonds between an aromatic amino acid and an adjacent leucine, isoleucine, threonine or valine whereas chymotrypsin splits almost all peptide bonds at the carboxyl end of aromatic amino acids. Thus, the cleavage specificity of the proteinase from rat liver mitochondria is much higher than that of chymotrypsin. The physiological function of the mitochondrial proteinase remains to be elucidated.

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