EVIDENCE THAT NEUTRAL PROTEASE FROM CALF THYMUS CHROMATIN IS A SERINE TYPE ENZYME

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1. Introduction

Chromatins from different animal tissues have been shown to contain neutral protease(s) [1,2,3]. The enzyme, when being an integral part of nucleohistone or chromatin complex, degrades only the f1 and f3 histones. Dissociated from the complex it however degrades equally all five histone fractions.

High degree of purification of neutral protease from calf thymus chromatin was achieved by Kurecki and Toczko [4] who also found its mol. wt to be 15 400 ± 1000 and optimum pH of 8.5 towards total histone as substrate. The enzyme isolated by these authors was reported not to be of metal-, thio-, or acidic-type protease.

In the present paper evidence is given to show that neutral protease from calf thymus chromatin is a serine-type enzyme.

2. Materials and methods

2.1 *Isolation and purification of neutral protease*

The enzyme was extracted from chromatin with cold HCl solution and subsequently purified by chromatography on Sephadex G-75 and autolysis followed by rechromatography on Sephadex G-75, as described by Kurecki and Toczko [4]. Further purification was obtained by ion-exchange chromatography on CM Sephadex C-25 instead of the previously used preparative electrophoresis. Enzyme containing fractions after Sephadex G-75 rechromatography were dialyzed in cold room against 50 vol of 0.1 M Tris–HCl buffer (pH 7.1). Protease preparation was then applied to CM Sephadex C-25 column, equilibrated and subsequently washed with 0.1 M Tris–HCl buffer, pH 7.1. The adsorbed protein was eluted step-wise by addition to the buffer NaCl to final concentration 0.15 M and 0.5 M NaCl respectively. The enzyme activity was found in fractions eluted at 0.15 M NaCl concentration (fig.1).

The replacement of preparative electrophoresis by ion-exchange chromatography made the procedure simpler and more efficient (the overall yield of the enzyme was increased from 20% to 40%). The purifi-

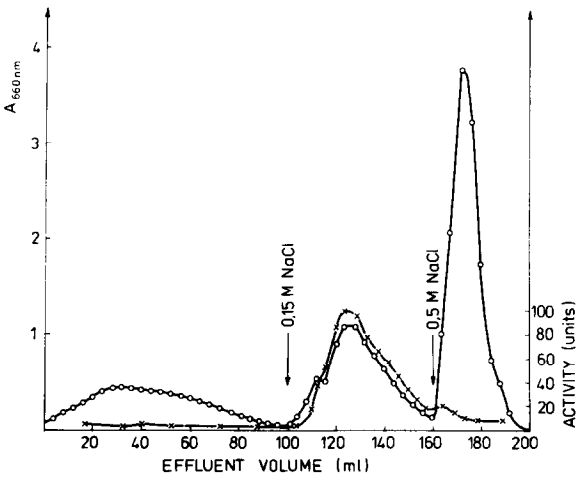

Fig.1. Chromatography on CM Sephadex C-25 column (1.5 × 10 cm). The protein sample (16.2 mg) was applied in 20 ml of 0.1 M Tris–HCl buffer (pH 7.1). Column was washed with the buffer and adsorbed material was eluted with 0.15 M and 0.5 M NaCl as indicated in the diagram. Flow rate 15 ml/hr. Eluate was collected in 4 ml fractions. Active material was combined, dialysed against distilled water and freeze-dried. (o–o–o) protein; (x–x–x) activity.

cation after this step was 1760 times over the chromatin's protein and the specific activity of the enzyme was 67.

2.2. *Determination of enzyme activity*

Activity was assayed as previously described using total histone as substrate [4]. Enzyme unit was defined as the amount of TCA soluble peptides formed, equivalent to 1 μmol of tyrosine.

2.3. *Assay of protease inhibition*

Enzyme was pre-incubated for 10 min at 37°C with the appropriate inhibitor in 0.1 M Tris–HCl buffer, pH 8.0 and the residual activity was measured using total histone (0.5% final concentration) as substrate. Control enzyme samples were pre-incubated without inhibitor.

2.4. *Assay of active sites*

The method of Chase and Show was used with *p*-nitrophenyl-*p'*-guanidinobenzoate hydrochloride (*p*-NPBG) as titrant [5].

2.5. *Determination of protein*

The method of Lowry et al. [6] was used with bovine serum albumin as standard.

3. Results and discussion

In order to obtain further information concerning the nature of the active site of neutral protease from calf thymus chromatin the sensitivity of this enzyme towards four inhibitors of serine type proteases were investigated. The chosen inhibitors were: *p*-NPBG, diisopropyl fluorophosphate (DFP), Kunitz inhibitor from pancreas and soybean trypsin inhibitor. We found that all these agents were potent inhibitors of neutral protease from calf thymus chromatin.

Chase and Show [5] have found that *p*-NPBG reacts stoichiometrically with the serine residue of the active center in serine type proteases, forming a stable acyl derivative. These findings made possible the titration of active molecules in trypsin, plasmin and thrombin preparations [7].

Since *p*-NPBG reacts with neutral protease from calf thymus chromatin, the above method was used by us to determine the amount of active enzyme molecules in chromatin protease preparation. Fig. 2 presents the relation between the amount of protein in enzyme preparation and the amount of liberated *p*-nitrophenol (as the result of enzyme acylation). As can be seen 100 μg of enzyme preparation liberate 0.27×10^{-2} μmol of *p*-nitrophenol from *p*-NPBG. Since the molecular weight of protease, as estimated by the method of gel filtration on Sephadex G-75, was found to be 15 400 ± 1000 [4] the preparation tested con-

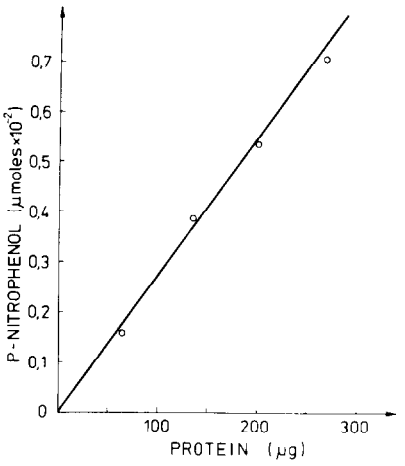

Fig.2. Titration of chromatin protease preparation by *p*-NPBG. The series of enzyme solution (2 ml) containing protein in the range of 67–270 μg/ml in veronal buffer, pH 8.3 (0.1 M) were mixed with 20 μl of *p*-NPBG (0.5×10^{-2} M in dimethyl formamide) and absorbancy at 410 nm was measured spectrophotometricaly within 1 min, at room temperature. For calculations the molar extinction coefficient of *p*-nitrophenol $A_{410\ nm} = 16\ 600$ was assumed [5].

Table 1
Inhibition of protease activity by DFP, Kunitz and soybean trypsin inhibitors

Molar ratio of inhibitor to protease	Inhibition (%)		
	DFP	Kunitz inhibitor	Soybean trypsin inhibitor
0.15	20	13	22
0.30	35	25	–
0.60	62	50	60
1.10	100	80	100
1.90	100	80	96

tained 40% of the active enzyme. This gave the possibility to calculate the enzyme inhibition by other serine protease inhibitors on a molar basis (table 1). The results obtained indicate that neutral protease is fully inhibited by an equimolar amount of DFP. The extent of inhibition of the enzyme by protein type inhibitors at equimolar concentration was found to be 80% for Kunitz inhibitor and approx. 100% for soybean trypsin inhibitor.

The data presented above give strong evidence that neutral protease from calf thymus chromatin belongs to the serine type proteases. The sensitivity of the enzyme towards Kunitz and soybean inhibitors of trypsin allow additionaly to conclude that the structure of the active center in chromatin protease is very similar to that in trypsin.

Neutral protease from calf thymus chromatin studied by us differs in many respects from the neutral protease of rat liver chromatin recently described [8].

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