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PURIFICATION AND PROPERTIES OF A NEUTRAL PROTEASE FROM CALF THYMUS NUCLEI

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SUMMARY

1. A neutral protease has been purified from calf thymus nuclei by extraction with 2.5 M NaCl, ethanol fractionation, gel filtration on Sephadex G-75 and incubation at the pH optimum.

2. The pH optimum for deoxyribonucleohistone hydrolysis is 7.8. Hemoglobin, serum albumin and γ-globulin were considerably more resistant to digestion than deoxyribonucleohistone. Hydrolytic degradation of hemoglobin was inhibited by substrate concentrations above 0.5%.

3. The protease is heat labile; its activity was almost completely abolished after heating for 20 min at 60°. The pH stability was studied at 37° and the enzyme was found to be stable over the pH range 5-9. The activity was rapidly lost below pH 3.5. The protease was slightly inhibited by Cd^{2+} and Co^{2+}, and markedly by *p*-chloromercuribenzoate and diisopropyl fluorophosphate. Other metal ions, EDTA, cyanide, cysteine and iodoacetamide had no effect.

INTRODUCTION

In our earlier paper[1] the presence of a neutral protease in calf thymus nuclei was reported. It was concluded that this enzyme is a normal constituent of calf thymus deoxyribonucleoprotein complex[2]. Autolytic breakdown of a purified deoxyribonucleoprotein preparation was observed. The main histone fractions were hydrolyzed at different rates. It is likely that the protease digests nucleoproteins during fractionation procedures, thus affecting the properties of the native nucleoproteins. The binding of the neutral protease to deoxyribonucleic acid was studied[2]; it was shown that the enzyme is a histone-like protein. It is possible that the neutral protease which is capable of degrading histones in the deoxyribonucleoprotein complex could have an important role in the gene expression. The work reported in this paper was undertaken in order to purify and to characterize the neutral protease of calf thymus nuclei.

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MATERIALS AND METHODS

Substrates

Deoxyribonucleoprotein substrate was prepared from calf thymus as reported earlier[1]. A fresh solution of deoxyribonucleoprotein in distilled water was heated for 20 min at 75° to inactivate the proteolytic activity of the substrate preparation. The heated solution was cooled quickly to 0°. Sufficient 5 M NaCl and 0.333 M sodium phosphate–citrate buffer was added, with rapid mixing, to bring the salt and the buffer concentration up to 0.1 M and 0.033 M, respectively.

Hemoglobin was prepared from bovine blood according to the method of ANSON[3].

Bovine serum albumin and bovine γ-globulin were obtained from Nutritional Biochemicals Co.

Assay of the hydrolysis

Samples were incubated at 37° for 20 h. Each incubation mixture contained, in a total volume of 1.2 ml, 10 mg of substrate, 0.033 M sodium phosphate–citrate buffer, and 0.1 M NaCl. Incubations were arrested by the addition of 20% trichloroacetic acid. The suspensions were filtered, and an aliquot of the filtrate was removed for the reaction with Folin–Ciocalteu reagent as previously described[1]. The results are expressed either in absorbance units ($A_{750\ m\mu}$) or in the enzyme units read from a calibration curve according to ANSON[3] (see also ref. 2).

Purification of the enzyme

Nuclei were isolated from 30 g calf thymus tissue according to the method of ALLFREY, MIRSKY AND OSAWA[4]. The packed nuclear sediment was homogenized in an equal volume of ice-cold distilled water using a teflon-pestle homogenizer. 1 vol. of 5 M NaCl and 2 vol. of 2.5 M NaCl were added to the aqueous nuclear homogenate, and the suspension was stirred at 4° for 8 h at low speed. The resulting viscous suspension was centrifuged for 90 min at 30 000 × g, and the sediment was discarded. Deoxyribonucleic acid was precipitated from the supernatant by the addition of 2 vol. of ethanol[5] during rapid mixing. The clear supernatant obtained after centrifugation was dialyzed free of ethanol, and concentrated by lyophilization. This preparation is referred to as the ethanol fraction. Further purification was achieved by gel filtration. The ethanol fraction was diluted with an equal volume of 0.1 M sodium acetate buffer (pH 4.2). The diluted sample was applied to a 2.6 cm × 92 cm Sephadex G-75 column previously equilibrated with 0.1 M sodium acetate buffer (pH 4.2). The enzyme was eluted with the same buffer. Fractions of 3 ml were collected at a flow rate of 6 ml/h. Absorbance at 280 mμ and proteolytic activity of the fractions were measured. Fractions 35–50, which showed the highest activity toward deoxyribonucleoprotein substrate, were pooled. The pH of pooled fractions was then adjusted to 7.8 by adding NaOH and Na_2HPO_4, and the solution was dialyzed for 20 h at 37° against 60 vol. of 0.033 M sodium phosphate-citrate buffer (pH 7.8). The dialyzed solution was used in further characterization studies.

TABLE I

PURIFICATION OF NEUTRAL PROTEASE FROM CALF THYMUS NUCLEI

One enzyme unit is defined as the proteolytic activity which liberates per min an amount of split products that give the same absorbance as 1 mequiv of tyrosine.

Purification step	*Total vol. (ml)*	*Total nitrogen (mg)*	*Total activity (units × 10^6)*	*Specific activity (units × 10^6/ mg N)*	*Relative purity*
Nuclear homogenate	22.5	218	698	3.2	1
Ethanol fraction	4.75	32.7	209	6.4	2
Sephadex fraction	42.5	3.95	616	156	49
Dialyzed fraction	49.5	0.63	445	703	220

RESULTS AND DISCUSSION

Purification of the enzyme

Table I shows the results of the purification procedure. It is evident that ethanol treatment considerably impaired the yield of proteolytic activity. Only 15% of the total nitrogen was recovered in the ethanol fraction, indicating that ethanol not only precipitated nucleic acids from the solution of chromatin in 2.5 M NaCl, but also removed a large part of the protein material, including protease. However, at lower concentrations of ethanol, the deoxyribonucleic acid was not completely removed, and subsequently interfered with the chromatographic separation of the enzyme at lower ionic strength. The original chromatin solution in 2.5 M NaCl was very viscous and could be fractionated by gel filtration only if much lower loads were used.

The elution diagram of the ethanol fraction is shown in Fig. 1. All activity was eluted as a single peak. The recovery of nitrogen in the pooled Sephadex fractions was only about 10% (Table I). However, the total activity was greatly increased after gel filtration. It appears as if the enzyme were inhibited by a substance from which it

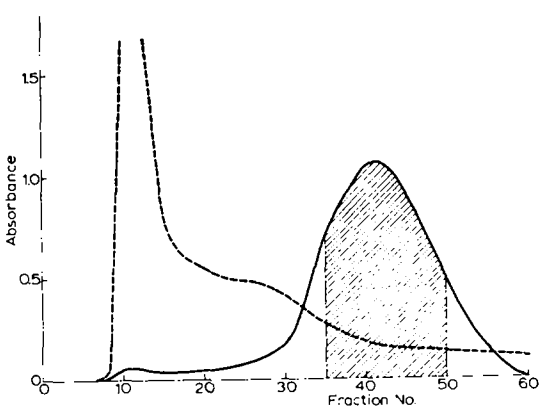

Fig. 1. Sephadex G-75 chromatography of the preparation soluble in 66% ethanol (ethanol fraction). The dialyzed solution, containing 32.7 mg nitrogen in 9.5 ml, was applied to a Sephadex G-75 column (2.6 cm × 92 cm), and the enzyme eluted at 14° with 0.1 M sodium acetate buffer (pH 4.2). Fractions under the shaded area were pooled. --- —·—, $A_{750\ m\mu}$; - — · —, $A_{280\ m\mu}$.

could be separated by chromatography on Sephadex. PASTAN AND ALMQUIST[6] reported the inhibition of rat thyroid protease by a high molecular weight substance present in thyroid homogenate; this inhibitor was separated from the enzyme by filtration on Sephadex G-100 equilibrated in 1 M NaCl.

Since the neutral protease is relatively stable at 37° and pH 7.8, it was possible to obtain a substantial increase in enzyme purity by proteolytic degradation of inactive proteins and removal of hydrolytic products by dialysis. The final preparation was purified 220-fold. The enzyme solution, when kept at −10°, did not lose activity over several weeks of storage.

TABLE II

EFFECT OF INHIBITORS AND ACTIVATORS ON HYDROLYSIS OF DEOXYRIBONUCLEOHISTONE BY THE PURIFIED NEUTRAL PROTEASE

All samples were preincubated for 5 min at 37° with the added reagent. The concentrations of the reagents on preincubation are given in the table. For the assay, 0.4 ml of preincubated solution was mixed with 1.0 ml of 1% deoxyribonucleoprotein substrate.

Reagent	*Concn. (mM)*	*% of initial activity*
None	--	100
$CaCl_2$	10	87
$MgCl_2$	1	103
$CdCl_2$	1	79
$CoCl_2$	1	84
$HgCl_2$	1	92
$MnCl_2$	1	104
$ZnCl_2$	1	93
$CuCl_2$	1	94
$Pb(CH_3COO)_2$	1	100
$Al(NO_3)_3$	1	97
EDTA	1	98
NaCN	1	97
Cysteine	1	86
Iodoacetamide	1	95
p-Chloromercuribenzoate	1	67
DFP	0.5	29

Properties of the purified neutral protease

pH optimum. The pH optima for the hydrolysis of four different protein substrates are shown in Fig. 2. It is evident that the deoxyribonucleoprotein substrate is much more susceptible to the attack of the neutral nuclear protease than are serum albumin, hemoglobin and γ-globulin. Slow degradation of hemoglobin by the neutral enzyme is striking because the same substrate is most rapidly hydrolyzed by the acid proteolytic enzymes of calf thymus[7]. The optimal pH for proteolytic digestion of deoxyribonucleoprotein substrate is 7.8. This pH optimum is the same as that reported for the "crude" enzyme in the homogenate of calf thymus nuclei[1]. The highest hydrolytic rate with hemoglobin and serum albumin was observed at pH 8.2 and 7.8, respectively, whereas γ-globulin was very resistant to hydrolysis over the whole pH range 6–8.

Effect of substrate concentration. Fig. 3 shows the dependence of the hydrolysis rate on the concentration of deoxyribonucleoprotein and hemoglobin substrates,

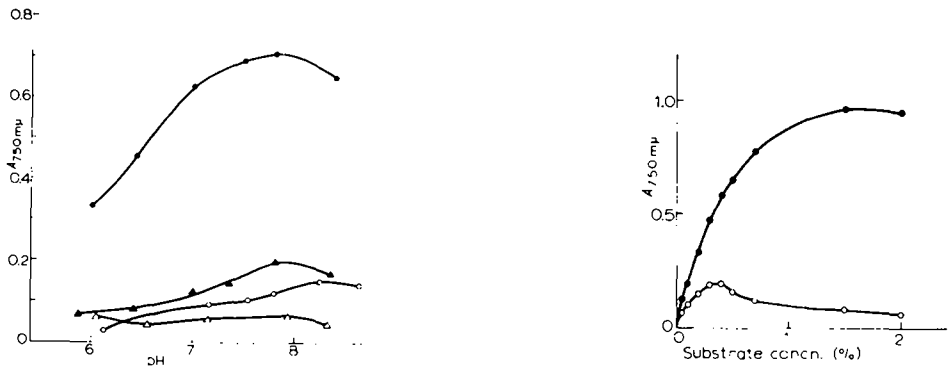

Fig. 2. Effect of pH on the activity of neutral protease from calf thymus nuclei. 10 mg substrate in 1.2 ml 0.033 M sodium phosphate–citrate buffer containing 0.1 M NaCl. ●, calf-thymus deoxyribonucleoprotein; ○, bovine hemoglobin; ▲, bovine serum albumin; △, bovine γ-globulin.

Fig. 3. Influence of substrate concentration on the activity of calf thymus neutral protease. 0.033 M sodium phosphate–citrate buffer (pH 7.8) containing 0.1 M NaCl. ●, calf thymus deoxyribonucleoprotein; ○, bovine hemoglobin.

respectively. Strong inhibition of the reaction rate by increasing substrate concentrations (above 0.5%) was observed with hemoglobin. This inhibition is of interest because the usual hemoglobin concentration for the protease assay is 2%.

When the experimental points obtained at lower substrate concentration were plotted according to the method of Lineweaver and Burk (Fig. 4), the Michaelis constants for hydrolysis of both substrates were derived: for deoxyribonucleohistone 0.75%, and for hemoglobin 0.10%.

Effect of pH on stability. The influence of pH on the stability of the neutral protease at 37° was measured, and the results are presented in Fig. 5. The activity was rapidly decreased below pH 3.5. The enzyme was considerably stable in the pH range 5–9. At neutral pH there was a slight loss of activity with the minimum at pH 7.8.

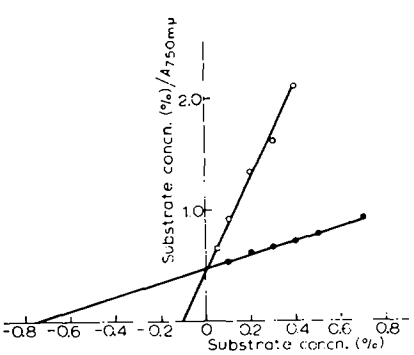

Fig. 4. Lineweaver-Burk plot for the hydrolysis of calf thymus deoxyribonucleoprotein (●) and bovine hemoglobin (○) by the purified neutral protease. Experimental conditions as for Fig. 3.

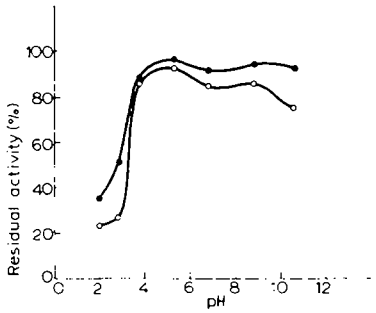

Fig. 5. pH stability of the purified neutral protease at 37°. The enzyme was incubated in 6.67 mM sodium phosphate-citrate buffers of pH 2.90–8.85, and in 0.02 M HCl (pH 1.95); pH values of 9.90 and 10.65 were obtained by addition of Na_2HPO_4 and NaOH. Residual activity was determined by measuring the hydrolysis of deoxyribonucleoprotein substrate at pH 7.80. ●, 2 h incubation; ○, 20 h incubation.

Since this pH value is also the pH optimum of the protease, the observed minimum in stability at neutral pH appears to be due to autolytic degradation of the enzyme. At pH values above 10, the activity was depressed by alkaline denaturation. Little difference was found between the curves showing the recovered activity after 2 h and 20 h incubation. It seems that either a fraction of the enzyme is considerably stable even at pH 2, or that the acid-denatured enzyme retains some of its activity. It is evident that the protease of calf thymus nuclei differs from the neutral enzyme found in calf bone marrow, which was very unstable even at −25° (see ref. 8).

Heat stability. The activity of the purified enzyme preparation which was heated for 20 min at pH 7.8 was rapidly lost at 60°. It is evident that the purified enzyme is more heat-labile than the "crude" preparation containing nucleohistones[1], in which the activity was completely depressed by 20 min heating at temperatures above 80°. It appears that nucleohistones can partially protect the neutral protease against thermal inactivation.

Effect of inhibitors and activators. Purified neutral protease was incubated with deoxyribonucleohistone substrate in the presence of various compounds suspected of producing possible inhibition or activation of the proteolytic activity. Metal ions Ca^{2+}, Mg^{2+}, Hg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Pb^{2+} and Al^{3+} were without effect at 1 mM concen-

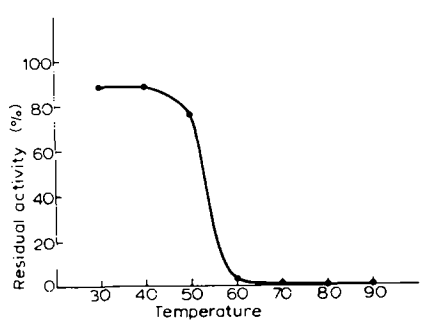

Fig. 6. Thermal stability of the purified calf-thymus neutral protease. The enzyme was incubated for 20 min in 16.67 mM sodium phosphate-citrate buffer (pH 7.80). The remaining activity was determined at pH 7.80 using deoxyribonucleoprotein substrate.

trations. The hydrolysis of deoxyribonucleohistone was only slightly impaired in the presence of Cd^{2+} and Co^{2+}. EDTA, cyanide, cysteine and iodoacetamide had no significant effect. The activity was markedly decreased by *p*-chloromercuribenzoate at 1 mM and by DFP at 0.5 mM. It has been reported by PHILLIPS AND JOHNS[9] that histone preparations obtained from calf thymus were degraded in alkaline solution, and that this effect was almost abolished by 1 mM DFP. It appears that the proteolytic digestion of histones was due to the presence of the neutral protease which is described in this paper. DFP inhibition of proteolytic activity was reported with the neutral proteases extracted from rat thyroid mast cells[6], skin[10] and submandibular gland[11]. This property, which seems to be in common with neutral proteases of animal tissues, suggests that the hydroxyl groups of serine are involved in their active centers.

The nuclear protease of calf thymus may have an important role in nucleohistone metabolism. The pronounced susceptibility of histones to the attack of the neutral protease, which was supposed to be a normal constituent of chromatin[2], is hardly compatible with the low turnover of histones in the cell nuclei[12]. The observed activation of the enzyme by gel filtration indicates that there was some latent activity in the crude extract. Further experiments are necessary to investigate whether the neutral protease exists in the intact nuclei either as a precursor similar to proenzymes of the gastrointestinal proteases, or in an inactive form reversibly bound to competitive inhibitors.

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