Natural Science Report, Ochanomizu University, Vol. 45, No. 1, 1994 67
お茶の水女子大学 自然科学報告 第45巻 第1号

Purification and Characterization of an Acid Protease from Myxoamoebae of a True Slime Mold, *Physarum polycephalum*

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(Received Apr. 8, 1994)

Summary

In order to compare the characteristics of acid protease(s) in diploid and haploid cells of a true slime mold, *Physarum polycephalum*, an acid protease was purified from the haploid myxoamoebae of *Physarum* by a combination of detergent extraction, acid precipitation, and ion-exchange column chromatographies. The purified enzyme having a molecular weight of 68,000 was composed of two polypeptide chains, 31-kDa heavy chain and 23-kDa light chain. These polypeptides were cross-linked by disulfide bond(s), and the heavy chain contained carbohydrate moiety composed of mannose, glucosamine, fucose and glucose. Optimum pH of the enzyme reaction was 1.7 toward hemoglobin as a substrate. A typical aspartic protease inhibitor, diazoacetyl-D, L-norleucine methyl ester (DAN), inhibited the enzyme activity in the presence of cupric ions, but the enzyme was not sensitive to the other aspartic protease inhibitors, 1, 2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) and pepstatin A. These results indicate that the characteristics of the amoeboid protease is very similar to those of the plasmodial enzyme. In addition, amino acid composition and specific activity of the purified enzyme were also very similar to those of the plasmodial protease.

Key words: acid protease; aspartic protease; myxoamoebae; *Physarum polycephalum*

Introduction

Proteinases are recognized to play various regulatory functions in most organisms. The levels of enzyme proteins are maintained by a balance between the rate of synthesis and enzymatic degradation, and the post-translational regulation of specific enzymes are carried out by proteases [1-9]. Activation of zymogens by proteases is also involved in the process of fertilization, supramolecular assembly, metamorphosis and hormone production [8]. In the course of bacteriophage morphogenesis and spore

formation, proteases may drive sequences of morphological and biochemical changes [10, 11].

During the differentiation of haploid myxoamoebae to diploid plasmodia of *Physarum polycephalum,* the intracellular organization is drastically changed through degradation and re-organization of cellular membranes. Some organells including basal bodies, centrioles and cytoskeletal networks composed of microtubules and microfilaments disappear following plasmodial formation [12]. Some of the intracellular proteases are thought to play crucial roles in these processes.

Previously, we reported the presence of an intracellular acid protease which showed a highly acidic pH optimum in the haploid myxoamoebae of *Physarum polycephalum,* and in the course of differentiation from myxoamoebae into plasmodia, an apparent decrease of its activity in the cell was observed [13]. The enzyme was enriched in the crude membrane fraction and the activity was efficiently extracted with non-ionic detergents [13]. The enzyme was first purified from the haploid myxoamoebae, but a sufficient quantity to investigate the characteristics could not be isolated. As the cultivation of the plasmodia is much easier than that of the myxoamoebae, we purified an acid protease from the plasmodia and investigated its enzymatic and molecular characteristics [14]. The protease was indicated to be a novel type of intracellular acid protease distinct in several properties from ordinary aspartic proteases including cathepsin *D*. As we could get a sufficient amount of the enzyme from myxoamoebae now, to know whether the nature of the acid protease(s) change during the differentiation or not, we compared the nature of the amoeboid enzyme to that of the plasmodial enzyme.

Materials and methods

Organisms. The haploid myxoamoebae of a true slime mold, *Physarum polycephalum,* were cultured on the lawn of bacteria, *Aerobacter aerogenes,* on agar medium in the dark at 24°C as described [15]. The cells were harvested in distilled water, then washed several times with water by low speed centrifugation to remove bacteria.

Chemicals. DEAE-Sephadex A-50, CM-Sephadex C-50 and Sephadex G-100 were purchased from Pharmacia and hydroxyapatite from Seikagaku Kogyo Co., Tokyo. Bovine hemoglobin and phenylmethylsulfonyl fluoride (PMSF) were obtained from Wako Pure Chemical Inc., Tokyo, diazoacetyl-D, L-norlenucine methyl ester (DAN), 1, 2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP), L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK), *N-p-*

tosyl-L-lysine chloromethyl ketone (TLCK) and soybean trypsin inhibitor were from Sigma. Pepstatin A and leupeptin were from Peptide Institute, Osaka, *N*-ethylmaleimide (NEM) was from Nacarai Tesque, Kyoto, and *p*-hydroxymercuribenzoic acid (pHMB) was from Aldrich. Porcine brain tubulin, rabbit skeletal actin, myosin and phosphorylase b were prepared in our laboratory, and other marker proteins were purchased from Sigma.

Protease assay. The enzyme activity was assayed, unless otherwise specified, at pH 1.7 with hemoglobin as a substrate as described previously [13, 14]. Reaction mixture contained 5 mg of hemoglobin, 50 μmol of HCl-KCl buffer (pH 1.7) and the enzyme in a final volume of 0.5 ml. The mixture was incubated at 37°C for 30 min, and the reaction was stopped by an addition of 0.5 ml of 10% trichloroacetic acid. Then, the mixture was centrifuged at 3,000 rpm for 10 min, and the absorbance at 280 nm of the supernatant was measured. One unit of the enzyme activity is defined as the amount of the enzyme producing an increase in absorbance of 1.0 at 280 nm per an hour. The enzyme was preincubated with the inhibitor and buffer, then the reaction was started by an addition of the substrate in the experiment with inhibitors. Other experimental conditions are described in Tables and Figures.

Protein determination. Protein was determined by the procedure of Lowry *et al.* [16] using a bovine serum albumin as a standard.

Purification of the enzyme. Washed amoebae were homogenized in 20 nm sodium citrate buffer (pH 5.0) containing 0.4% Triton CF-54 and centrifuged at 10,000$\times g$ for 30 min. The supernatant was applied to a column of DEAE-Sephadex A-50 equilibrated with 10 mM sodium citrate (pH 5.0) containing 0.2% Triton CF-54. Adsorbed proteins were eluted with a linear gradient of NaCl, 0–1.0 M, and active fractions were pooled, concentrated and dialyzed against 20 mM sodium citrate buffer (pH 3.0). Then, the precipitate was removed by centrifugation at 10,000$\times g$ for 30 min and the supernatant was dialyzed against 10 mM potassium phosphate buffer (pH 6.8). The dialysate was applied to a column of hydroxyapatite equilibrated with the dialyzing buffer. The adsorbed proteins were eluted with a linear gradient of 20 to 400 mM of the potassium phosphate buffer (pH 6.8), and the active fractions were pooled, dialyzed against 20 mM of the same buffer, then applied to CM-Sephadex A-50 column. The protease activity was not adsorbed, and unadsorbed fraction was pooled, concentrated, dialyzed against 10 mM sodium citrate (pH 5.0). The protease

fraction was then applied to a Sephadex G-100 column equilibrated with the same buffer. Active fraction was pooled, concentrated. This fraction was used in the subsequent experiments.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) under non-denaturing condition was performed in 7.0% polyacrylamide gel in Tris-HCl (pH 9.5) by the method of Davis [17]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12% gel in the presence of 0.1% SDS according to Laemmli [18] with or without 2-mercaptoethanol (2-ME). Protein bands were stained with Coomassie Brilliant Blue (CBB), and carbohydrate-containing bands were stained with periodate-Schiff reagent.

Molecular weight determination. Molecular weight of the enzyme was determined by gel filtration according to Andrews [19] on a Sephadex G-100 column. The calibration of the column was done with catalase (248k), lactate dehydrogenase (132k), phosphorylase b (98k), hemoglobin (65k), ovalbumin (43k), and trypsin inhibitor (21k). The procedure by Laemmli [18] was used for determination of the molecular weights of denatured proteins, and the following protein markers were used: myosin heavy chain (200k), phosphorylase b (98k), bovine serum albumin (BSA, 68k), tubulin (55k), actin (42k), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 35k), carbonic anhydrase (29k), trypsin inhibitor (21k) and cytochrome c (12k).

Amino acid analysis. Analysis of amino acid composition of the enzyme was carried out with an Applied Biosystems model 420A derivatizer connected with a 130A separation system. The PTC-derivatives were identified by a reverse-phase high performance liquid chromatography with a PTC C-18 column (Applied Biosystems). Samples were subjected to gas phase hydrolysis at 165°C for 60 min prior to the derivatization. Half-cytsine was determined as systeic acid after performic acid oxidation followed by HCl hydrolysis [19]. Tryptophan was determined by a spectrophotometric method [20]. Norleucine content of a DAN-modified enzyme was determined after acid hydrolysis by amino acid analysis.

Carbohydrate analysis. The enzyme was hydrolyzed in 5% HCl/methanol, and the resulting methyl glycosides were trimethylsilylated and analyzed by gas liquid chromatography (GLC) on a column of 3% OV-101.

Results

Purification of the Acid Protease from Myxoamoebae of Physarum polycephalum. The purification of the amoeboid enzyme is summarized in Table I, and from 10 g of myxoamoebae, 5.5 mg of the purified enzyme was obtained in a yield of 13.5% with a 17-fold purification. The final step of purification (gel filtration on Sephadex G-100) is shown in Fig. 1, and purified enzyme showed a single band by PAGE under non-denaturing conditions as shown in Fig. 2. The specific activity was 62 units/mg protein, which is almost the same as that of the purified plasmodial enzyme reported before (64 units/mg protein, reference 14).

Table I. Purification of the acid protease from haploid myxoamoebae of *Physarum polycephalum.*

Step	Total protein	Total activity	Specific activity	Yield
Homogenate	680 mg	2516 units	3.7 units/mg	100%
Supernatant	196	1428	7.3	56.8
DEAE-Sephadex	40.8	716	17.5	28.5
Acid precipitation	16.9	510	30.2	20.5
Hydroxyapatite	8.2	455	55.5	18.1
CM-Sephadex	7.0	401	57.3	15.9
Sephadex G-100	5.5	340	61.8	13.5

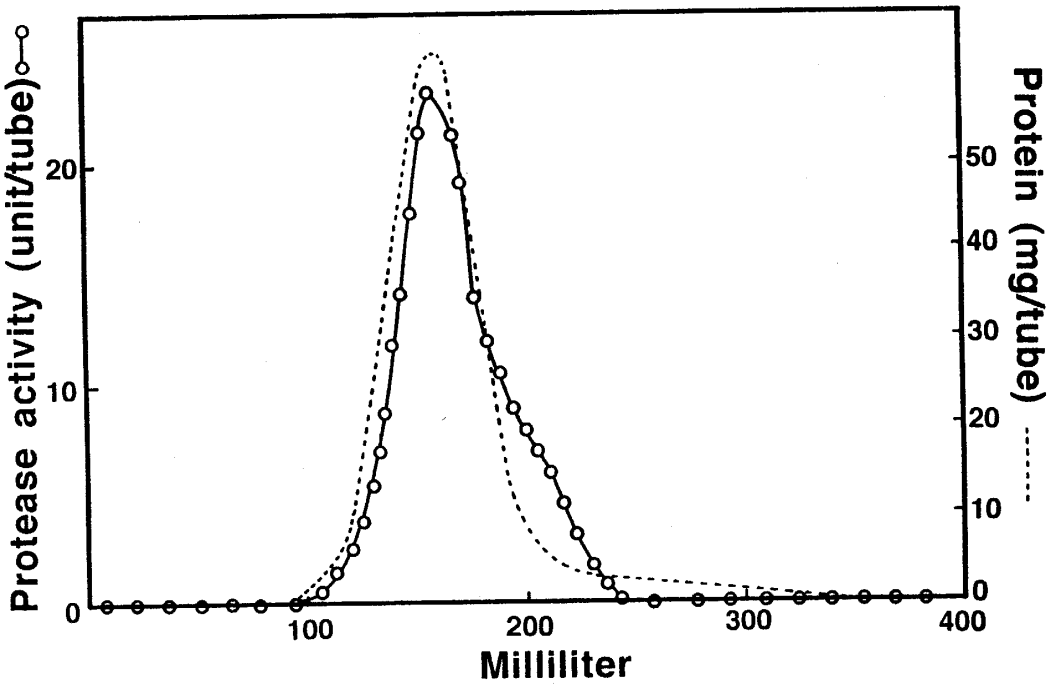

Fig. 1. Chromatographic profile of the amoeboid acid protease on Sephadex G-100 column. An active fraction after CM-Sephadex C-50 was applied on a column (1.25×72 cm) of Sephadex G-100. Fraction size, 2.0 ml.

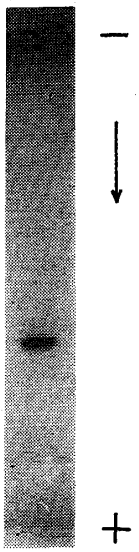

Fig. 2. PAGE of the acid protease under non-denaturing conditions.

Optimum pH and Stability. The optimal pH for hydrolysis of hemoglobin was 1.7 (Fig. 3), and the enzyme was relatively stable in the pH range of 3.0–6.0 (data not shown). But it was less stable at higher pH value.

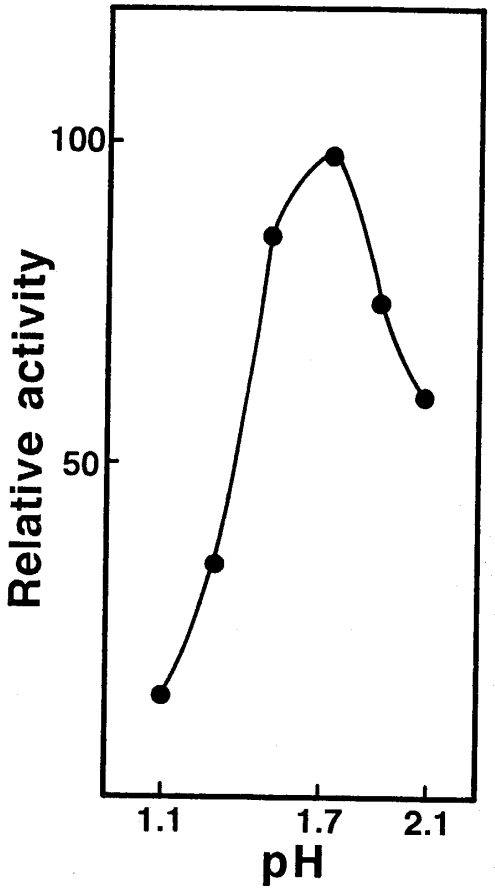

Fig. 3. pH Dependence of the hydrolytic activity of the acid protease. The maximum activity is shown as 100%.

Molecular Weight Determination. The molecular weight of the enzyme was estimated to be 68,000 on Sephadex G-100 gel filtration (Fig. 4). But on SDS-PAGE in 12% gel, a molecular weight of 54,000 was obtained under non-reducing conditions (Fig. 5 & 6). Under reducing conditions, two bands corresponding to molecular weights of 31,000 (heavy chain) and 23,000 (light chain) were obtained (Fig. 5 & 6). These results show that

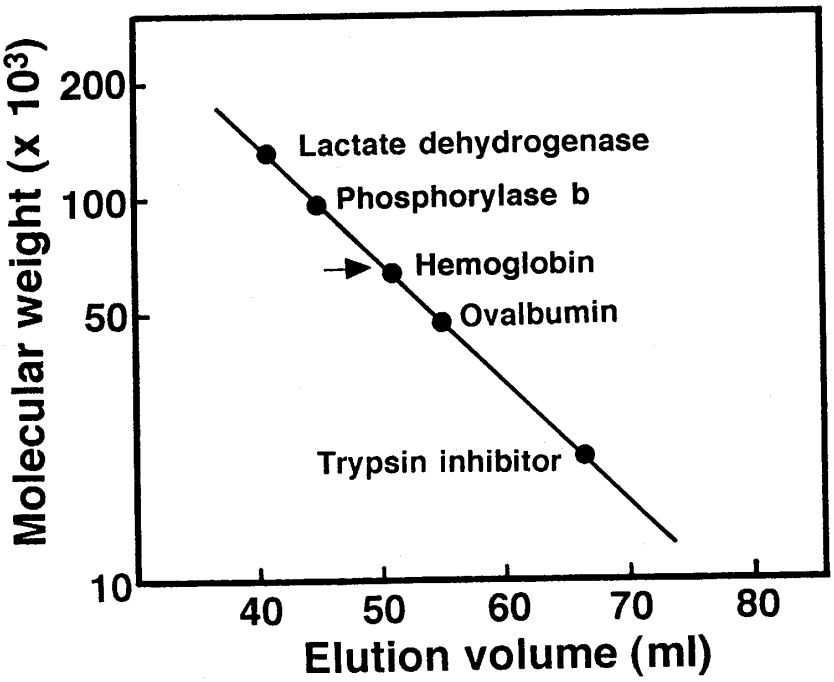

Fig. 4. Molecular weight determination of the acid protease by Sephadex G-100 gel filtratin. The position of the enzyme in the Sephadex G-100 column chromatography is indicated by the arrow.

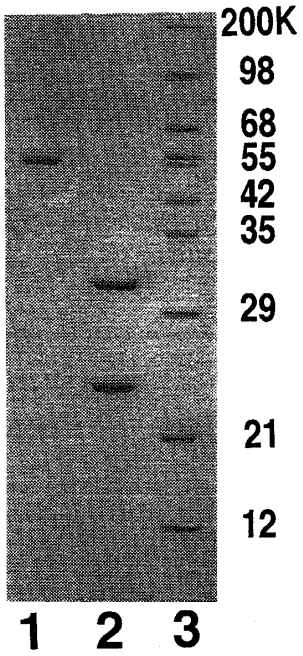

Fig. 5. SDS-PAGE of the acid protease. Lane 1, SDS-PAGE was performed under nonreducing conditions. Lane 2, under reducing conditions. Lane 3, molecular weight markers: myosin (200 k), phosphorylase b (98 k), bovine serum albumin (BSA, 68 k), tubulin (55 k), actin (42 k), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 35 k), carbonic anhydrase (29 k), trypsin inhibitor (21 k), and cytochrome c (12 k).

the enzyme has a two-chain structure cross-linked by interchain disulfide bond(s). This molecular feature is very similar to that of plasmodial one [14].

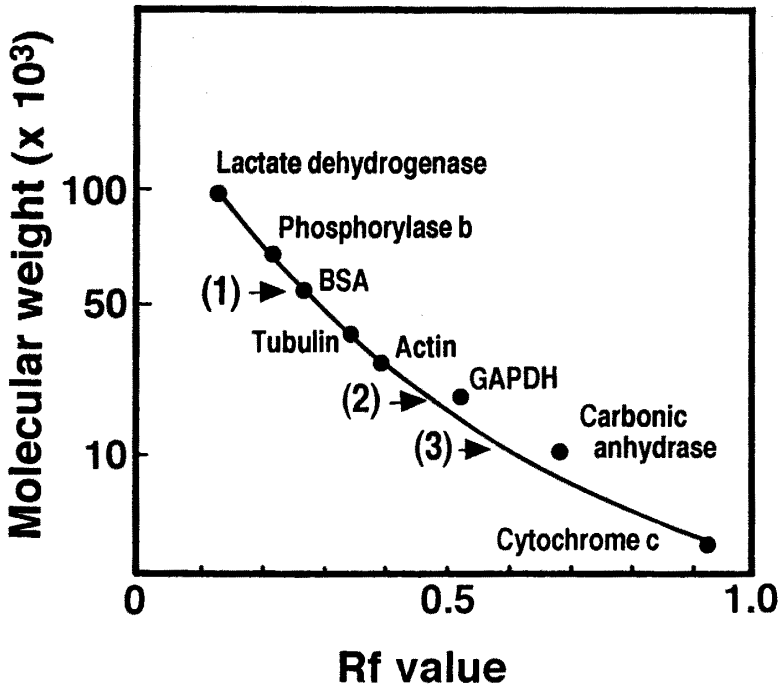

Fig. 6. Molecular weight determination by SDS-PAGE. The arrow (1) indicates the position of the amoeboid acid protease in SDS-PAGE under nonreducing conditions, and arrows (2) and (3) indicate those of the separate polypeptides in SDS-PAGE under reducing conditions.

Amino Acid Analysis. Table II shows the amino acid composition of the acid protease from myxoamoebae, and compared to that of plasmodial enzyme. A relatively high contents of proline and phenylalanine and low content of basic residues are notable, and these are very similar to those of plasmodial enzyme [14].

Carbohydrate Moiety of The Enzyme. The heavy chain (31k) of the enzyme gave a positive stain with the periodate-Schiff reagent after SDS-PAGE under reducing conditions, but the light chain (23k) was not stained (data not shown). From this, the enzyme is considered to be a glycoprotein with the carbohydrate moiety linked to the heavy chain. The carbohydrate analysis showed it cotained mannose, glucosamine, fucose and glucose (Table III). Plasmodial enzyme contained 7 residues of mannose, 5 residues of glucosamine, and 1 residue each of fucose and glucose per one molecule of the native enzyme as reported before [14].

Table II. Amino acid composition of the acid protease.

Amino acid	Residues/mol	Nearest integer
Half-cystine	10.4	10
Aspartic acid	55.4	55
Threonine	43.7	44
Serine	40.6	41
Glutamic acid	45.2	45
Proline	45.3	45
Glycine	41.0	41
Alanine	40.4	40
Valine	35.7	36
Methionine	7.0	7
Isoleucine	19.9	20
Leucine	39.1	39
Tyrosine	16.5	17
Phenylalanine	29.0	29
Lysine	19.5	20
Histidine	6.2	6
Arginine	4.5	5
Tryptophan	7.2	7
Total		507

Table III. Carbohydrate composition of the acid protease.

Carbohydrate	mol/mol protein
Mannose	6
Glucosamine	4
Fucose	1
Glucose	1

Effects of Protease Inhibitors on The Enzyme Reaction. The effects of various inhibitors for proteases on the amoeboid protease activity were examined. Only DAN, a typical aspartic protease inhibitor, inhibited the enzyme in the presence of cupric ions (Fig. 7). Other inhibitors tested, including EPNP [21] and pepstatin A [22], which are known as typical aspartic protease inhibitors, showed no inhibitory effects on the enzyme activity (Table IV). The plasmodial enzyme also showed the same sensitivity to the inhibitors [14].

Table IV. Effects of various protease inhibitors on the enzyme activity.

Inhibitor	Concentration	Relative activity (%)
None		100
DAN[a]	42 μg/ml	7
Pepstation A	1 mM	96
EPNP[b]	500 μg/ml	94
Leupeptin	1 mM	96
Soybean trypsin inhibitor	10 μg/ml	99
PMSF	1 mM	93
	10 mM	87
TPCK	1 mM	83
	10 mM	79
TLCK	1 mM	90
	10 mM	86
pHMB	1 mM	95
	10 mM	90
NEM	1 mM	97
	10 mM	93

[a]The protease was preincubated for 1 h in the presence of 0.13 mM $CuSO_4$ under the coditions in the legend to Fig. 7.
[b]The protease (54 μg) was preincubated wit 500 μg of EPNP in 1.0 ml of 50 mM sodium acetate (pH 4.6) at 25°C for 72 h. Under these conditions, porcine pepsin was 95% inactivated.

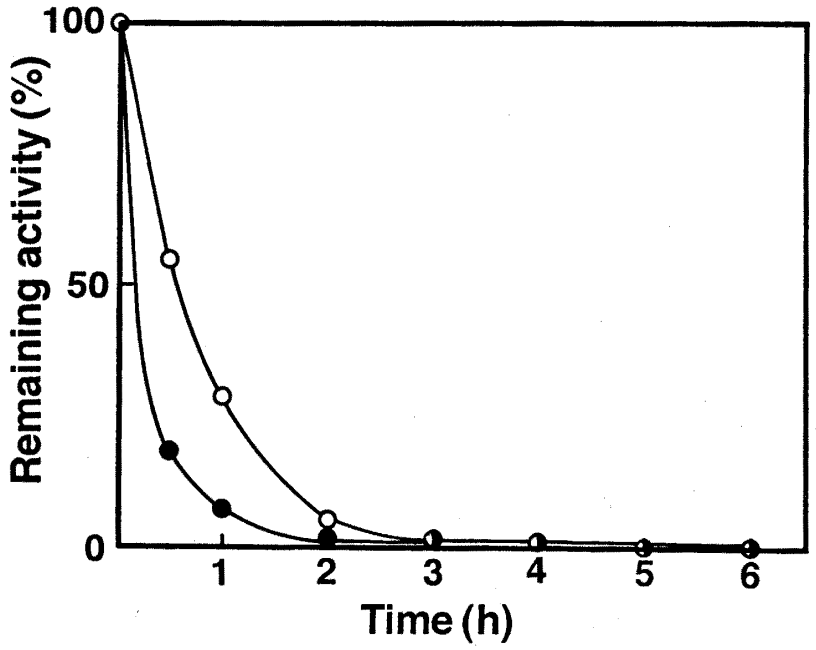

Fig. 7. Inhibition of the acid protease by DAN. The protein (54 μg) and DAN (42 μg) were incubated with 0.2 mM $CuSO_4$ at 24°C in 0.05 M sodium phosphate buffer (pH 6.0, final volume, 1.0 ml). Aliquots of 0.1 ml were taken at the indicated times and the protease activity was assayed. The molar ratio of enzyme : DAN : Cu(II) was 1 : 200 : 200. ○, Amoeboid acid protease; ●, porcine pepsin. DAN-modified enzyme was also used for the determination of the extent of norleucine incorporation.

Discussion

An intracellular acid protease showing a highly acidic pH optimum was purified from haploid myxoamoebae of *Physarum polycephalum* and compared its characteristics to those of the enzyme isolated from diploid plasmodia of *Physarum*. The enzyme had several unique characteristics, relatively high molecular weight and two-chain structure composed of two different subunits. The enzyme protein is considered to be initially synthesized as a single chain precursor and processed like other aspartic proteases. The investigation on the mechanism of this processing from a precursor protein to mature two-chain form is now under way in our laboratory.

The carbohydrate composition was slightly different from that of plasmodial enzyme. But relatively high content of glucosamine in both enzymes indicates that the both enzymes contain *N*-linked carbohydrate chains in their heavy subunits.

The sensitivity to protease inhibitors is very similar to that of plasmodial one. Among the inhibitors tested, only DAN inhibits the activity of the enzyme. The inhibition by DAN and simultaneous incorporation of norleucine into the enzyme protein indicate that the protease has at least one essential carboxyl group at its active site.

As these characteristics of the protease from myxoamoebae are very similar to those of the enzyme from plasmodia, these enzymes are considered to be identical. We first reported the apparent change of the activity of the acidic protease during differentiation from myxoamoebae to plasmodia, and we wanted to know whether these enzymes are identical or not. From above findings, the acid protease changed quantitatively but not qualitatively during the differentiation of *Physarum*.

The role of this protease is not yet understood, so, it is necessary to elucidate the biological significance of this enzyme in *Physarum* cells.

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