Purification of protein phosphatase from hen oviduct

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Two protein phosphatases of 103 and 29 kDa as determined by gel filtration, were purified from hen oviducts. The 103 -kDa phosphatase was purified 7300-fold to near homogeneity and dissociated into two polypeptides in the presence of SDS. Molecular masses of these polypeptides were estimated to be 60 and 38 kDa by SDS-polyacrylamide slab gel electrophoresis using the buffer system of Laemmli, but 68 and 35 kDa using the buffer system of Weber and Osborn. The stoichiometry of these polypeptides was approx 1:1 according to the densitometric analysis of gels at 550 nm. The 29 -kDa phosphatase was purified 2900-fold. Both phosphatases dephosphorylated the α-subunit of phosphorylase kinase more rapidly than the β-subunit.

Protein phosphatase *Hen oviduct* *Purification*

1. INTRODUCTION

Protein phosphatase plays an important role in various regulatory mechanisms in cells and has been purified from a number of mammalian tissues and organs [1,2]. Two avian enzymes have been purified from turkey gizzard smooth muscle [3]. In the previous work an attempt was made to perform partial purification of protein phosphatase from hen oviducts [4]. Hen oviduct is a specific organ which actively synthesizes egg white proteins and the syntheses are regulated by steroid hormones [5]. Recently it has been shown that phosphorylation could be a way to transform steroid hormone receptors [6] and that protein phosphatase may be important in regulating protein synthesis [1,2]. Therefore, it is of interest to investigate protein phosphatase in hen oviducts. Here we describe the purification and properties of two protein phosphatases from egg-laying hen oviducts.

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2. MATERIALS AND METHODS

2.1. *Purification of protein phosphatase*

All operations were carried out at 4°C. Concentration of enzyme solutions was carried out by ultrafiltration with Centriflo CF 25 obtained from Amicon (Danvers, MA). Egg-laying White Leghorn Hen oviducts (527 g) were minced, and homogenized twice for 15 s with an Ultra Turrax homogenizer in 1.4 l of 0.25 M sucrose containing 5 mM Tris-HCl (pH 8.0), 5 mM EDTA and 0.1% (v/v) 2-mercaptoethanol, at top speed. The suspension was further homogenized with a Teflon-glass homogenizer by 5 strokes, and centrifuged at 22000 × *g* for 20 min. The supernatant (1.54 l) collected through 4 layers of gauze was brought to 45% saturation by adding 427 g ammonium sulfate. After stirring for 20 min the resulting precipitate was collected by centrifugation at 22000 × *g* for 20 min. Pellets were dissolved in 0.24 l of 10 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 0.1% 2-mercaptoethanol and 10% (v/v) ethylene glycol (buffer A) and the solution was centrifuged at 100000 × *g* for 1 h. The supernatant collected through 4 layers of gauze was applied onto a phenyl-Sepharose 4B col-

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umn (4 × 19 cm) equilibrated with buffer A containing 0.2 M ammonium sulfate. The column was extensively washed with the following buffers containing the specified concentrations of ammonium sulfate: 0.2 M, 0.5 l; 0.6 M, 1.0 l; 0.2 M, 2.0 l; 0.05 M, 0.3 l. Phosphatase was eluted with 10 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$, 0.1% 2-mercaptoethanol and 30% ethylene glycol (buffer B). Active fractions were combined, concentrated to approx. 20 ml, and dialyzed against 0.5 l of buffer A containing 0.3 M NaCl. The dialysate was applied on a Sephacryl S-300 column (4.1 × 67.5 cm), and eluted with the same buffer (fig.1). Two activity peaks (phosphatase I and II) were separately pooled, concentrated, and dialyzed against buffer B. These two enzyme fractions were further purified by the same method except that Sephacryl S-200 column chromatography in buffer A containing 0.3 M NaCl was added for the purification of phosphatase II. Thus, the purification procedure of the major phosphatase fraction (phosphatase I) will be described below. The dialyzed enzyme was applied on a column (1.5 × 5.7 cm) of Reactive red 120-agarose obtained from Sigma (St.Louis, MO) and the enzyme was eluted with 0.2 l of a linear gradient of 0–0.6 M NaCl, in buffer A. The enzyme activity was eluted at 0.25–0.33 M NaCl (0.27–0.35 M NaCl for phosphatase II). The active fractions were collected, concentrated to 3–5 ml, and dialyzed against 0.5 l of buffer B. The concentrated enzyme was applied on an AH-Sepharose 4B column (1.4 × 3.0 cm) equilibrated with buffer A. The column was washed with 50 ml of buffer A and eluted with 0.1 l of a linear gradient of 0.25–1.0 M NaCl, in buffer A. The enzyme activity eluted at 0.4–0.6 M NaCl (0.3–0.4 M for phosphatase II) was collected, and concentrated to approx. 1 ml. After dialysis against buffer B, the enzyme preparation was stored at 4°C. The enzyme was stable for at least two weeks.

2.2. *Polyacrylamide gel electrophoresis*

The electrophoresis in the presence of SDS was carried out with 10% and 12.5% polyacrylamide gel slabs according either to [7] or [8]. M_r marker proteins used were phosphorylase *a*, catalase, bovine serum albumin, ovalbumin, lactate dehydrogenase, carbonic anhydrase, α-chymotrypsinogen and soybean trypsin inhibitor. Slabs were

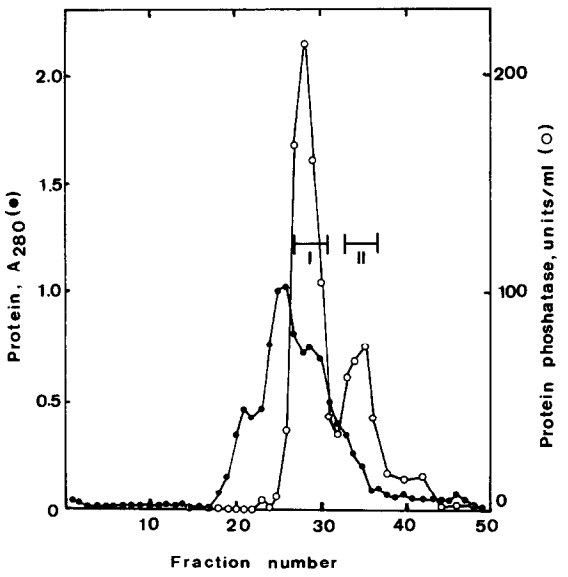

Fig.1. Sephacryl S-300 chromatography of hen oviduct protein phosphatase. Fractions of 17 ml were collected and assayed for phosphatase activity (○) and protein (●). For experimental details see section 2.

stained by Coomassie blue, and subjected to photometric tracing with a Simadzu densitometer, model CS-930 at 550 nm. Non-denaturing polyacrylamide disc gel electrophoresis was carried out at room temperature with 7.5% disc gels containing 0.2 M Tris-HCl (pH 8.5) and 10% ethylene glycol, and an electrophoresis buffer (pH 8.4) containing 0.053 M Tris and 0.053 M boric acid. Ten μg of the enzyme preparation was applied on a disc gel. The gels were sliced into 1.4-mm slices and each slice was extracted with 0.2 ml of buffer A at 4°C for 24 h; 0.1 ml of the extract was used for the assay.

2.3. *Determinations*

Protein was determined by the method of [9]. M_r values of phosphatases I and II were determined by gel filtration with Sephacryl S-200 and S-300 in buffer A containing 0.3 M NaCl. Protein phosphatase activity was assayed with casein as substrate at 37°C for 20 min as in [4]. Alkaline phosphatase activity was measured by the method of [10]. Dephosphorylation of rabbit skeletal muscle phosphorylase kinase was carried out according to [11].

3. RESULTS AND DISCUSSION

Two protein phosphatases, protein phosphatase I and II from hen oviduct, have been purified by affinity chromatography with phenyl-Sepharose 4B and Reactive red 120-agarose [12]. Throughout the entire purification process the protein phosphatase activity was accompanied by the *p*-nitrophenol phosphate hydrolyzing activity. Results are shown in table 1. The major protein phosphatase, phosphatase I, was purified 7300-fold from the homogenate. The molecular mass of the enzyme was estimated to be 103 kDa by gel filtration. The non-denaturing polyacrylamide gel electrophoresis of phosphatase I gave a single protein band (R_f, 0.54), which completely coincided with the phosphatase activity. This enzyme was dissociated into two polypeptides of 60 and 38 kDa as determined by the method of Laemmli. The polypeptide composition of the enzyme is quite similar to that of a protein phosphatase being active against the rabbit reticulocyte protein synthesis initiation factor [2]. However, when the molecular masses of these 60- and 38-kDa polypeptides were determined using the different buffer system of Weber and Osborn, they amounted to 68 and 35 kDa, respectively. Quite similar differences between the molecular masses of phosphatase components which have been found in previous reports [2], are possibly attributed to the electrophoretic system used for the molecular mass determination. The minor protein phosphatase, phosphatase II has been purified 2900-fold from the homogenate. The molecular mass of this enzyme was estimated to be 29 kDa by gel filtration. SDS-PAGE of the final enzyme preparation revealed two protein bands of 38 and 35 kDa as determined by the method of Laemmli. The 38-kDa polypeptide was indistinguishable with the smaller polypeptide of phosphatase I by SDS-PAGE. Both protein phosphatases were activated approx. 1.5-fold with 2 mM $MnCl_2$ but they were not activated with 0–5 mM $MgCl_2$. Higher concentrations of $MgCl_2$ and $MnCl_2$ were inhibitory. Dephosphorylation of the phosphorylase kinase α-subunit by both phosphatases was approx. 5-times faster than that of the β-subunit. When the phenyl-Sepharose 4B fraction was applied to an AH-

Table 1

Purification of hen oviduct protein phosphatase I and II

Fraction	Volume (ml)	Protein (mg)	Total activity (units[a])	Specific activity (units/mg protein)
Crude extract	1540	34000	29000	0.85
Ammonium sulfate	380	4100	33000	8.0
Phenyl-Sepharose 4B	370	300	26000	87
Sephacryl S-300				
I[b]	85	70	14000	200
II[c]	85	31	3500	110
Reactive Red 120-agarose				
I	5.5	7.4	7300	990
II	2.8	1.2	900	750
AH-Sepharose 4B				
I	0.95	0.73	4500	6200
II	1.3	0.26	360	1400
Sephacryl S-200				
I	–	–	–	–
II	1.0	0.044	110	2500

[a] One unit of activity is defined as the amount of enzyme which releases 1 nmol phosphate per min
[b] Phosphatase I
[c] Phosphatase II

Sepharose 4B column, the elution profile of protein phosphatase activity was different from those of the purified enzymes. Therefore, it seems possible that these enzymes may exist as complexes with their substrates in crude enzyme solutions. To investigate physiological roles of the oviduct protein phosphatases in this report, further studies on their substrates seem to be important.

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