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Rapid purification of protein phosphatase 2A from mouse brain by microcystin-affinity chromatography

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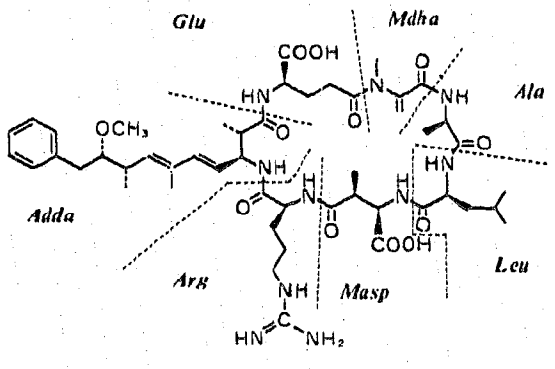
Microcystin LR, which is a monocyclic heptapeptide containing two L-amino acids, leucine and arginine, is a new inhibitor of protein phosphatases 1 and 2A. Microcystin LR-affinity chromatography was used to purify protein phosphatase 2A as a holoenzyme. Five mg of microcystin LR were immobilized to ECH Sepharose 4B by the carbodiimide coupling reaction. Following DEAE-cellulose column chromatography, microcystin-affinity chromatography, as the second step in the procedure, resulted in purification of protein phosphatase 2A in a pure form. The enzyme isolated from mouse brain consisted of two regulatory subunits of 67 kDa and 58 kDa and a catalytic subunit of 41 kDa. Microcystin-affinity chromatography is useful for isolation of protein phosphatase 2A.

Protein phosphatase 2A; Microcystin; Affinity chromatography

1. INTRODUCTION

The recent discovery of potent inhibitors of protein phosphatases 1 and 2A, such as okadaic acid, dinophysistoxin-1, calyculin A, microcystins and nodularin, has revealed that protein phosphatases 1 and 2A are involved in various biological events, such as tumor promotion [1-3], hepatotoxicity [4], regulation of mitosis [5], expression of the AP-1 complex [6] and induction of nuclear factor kB [7]. It was also reported that viral antigens are associated with one catalytic and one regulatory subunit of protein phosphatase 2A [8,9]. We recently reported that okadaic acid binds to a catalytic subunit of protein phosphatase 2A by the photoaffinity labeling of [^{3}H]methyl 7-*O*-(4-azidobenzoyl)okadaate [10]. Although catalytic subunits of protein phosphatases 1 and 2A are intensively studied on the level of molecular biology, regulatory subunits are not well characterized, due to heterogeneity of the enzymes in various cells [11]. Because of the significant functions of protein phosphatases, it is important to study protein phosphatases as holoenzymes. We developed an affinity chromatography using some of these above mentioned inhibitors. Okadaic acid did not give successful results, because its carboxyl group appears to be functionally important [12], whereas microcystin LR (Fig. 1) was found to be useful. Microcystins, including microcystin LR, are monocyclic heptapeptides and are reported to be potent hepatotoxic compounds [13]. Recently, we reported that inhibition of protein phosphatases 1 and 2A by microcystins is related to hepatotoxicity [4]. This paper reports a rapid purification of protein phosphatase 2A as a holoenzyme from a cytosolic fraction of mouse brain by microcystin-affinity chromatography. The purified protein



Microcystin LR

Fig. 1. Structure of microcystin LR, which contains two L-amino acids, leucine and arginine, two D-amino acids, alanine and glutamic acid, methylaspartic acid and methyldehydroalanine and Adda. Adda stands for 3-amino-9-methoxy-10-phenyl-2,6,8-trimethyldeca-4,6-dienoic acid.

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Abbreviations: EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide

phosphatase 2A consisted of two regulatory subunits of 67 kDa and 58 kDa and one catalytic subunit of 41 kDa.

2. MATERIALS AND METHODS

2.1. *Materials*

Microcystin LR, isolated from *Microcystis*, was a generous gift of Dr Wayne W. Carmichael (Wright State University, Dayton, OH, USA). ECH Sepharose 4B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Phosphorylase *b* and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Phosphorylase kinase and inhibitor-2 of protein phosphatase 1 were purified from rabbit skeletal muscle according to the method described previously [14]. Protein kinase C was partially purified from mouse brain by DEAE-cellulose column chromatography.

2.2. *Protein phosphatase assays*

Activity of protein phosphatases 1 and 2A was measured by the release of ^{32}P from ^{32}P-labeled proteins. Phosphorylase *a*, which was phosphorylated by phosphorylase kinase, was used as the substrate for protein phosphatases 1 and 2A, and histone H1, which was phosphorylated by activated protein kinase C with a tumor promoter, teleocidin, was the substrate for protein phosphatase 2A [15]. The reaction mixture (200 μl) for phosphorylase phosphatase included 50 mM Tris-HCl (pH 7.0), 0.1 mM EDTA, 0.1% 2-mercaptoethanol, 5 mM caffeine, 0.12 mg bovine serum albumin and 10 μg of ^{32}P-labeled phosphorylase *a* (20 000 cpm) and was incubated at 30°C for 30 min. The reaction was terminated by addition of 25% trichloroacetic acid. Remaining ^{32}P-labeled phosphorylase *a* on the filter was counted for radioactivity. The reaction mixture (250 μl) for histone H1 phosphatase included the mixture containing 50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 0.1% 2-mercaptoethanol, 0.003% Brij 35, 0.075 mg of bovine serum albumin, 2 mM $MnCl_2$ and 1 μg of ^{32}P-labeled histone H1 in place of ^{32}P-labeled phosphorylase *b*, and was incubated at 30°C for 10 min. One unit of protein phosphatase activity was defined as the release of 1 nmol phosphate/min from the substrates.

2.3. *Preparation of microcystin-affinity chromatography*

Microcystin LR was conjugated with ECH Sepharose 4B by a carbodiimide coupling procedure. Briefly, 5 mg of microcystin LR dissolved in DMSO were combined with 5 ml of ECH Sepharose 4B, suspended in a coupling solution containing 42 mg of EDC, and incubated at 37°C for 6 h. Another 1 ml of ECH Sepharose 4B was then added to the reaction mixture and kept at room temperature overnight. The microcystin LR-coupled Sepharose 4B was equilibrated with buffer A containing 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 2 mM EGTA and 10% glycerol.

2.4. *Purification of protein phosphatase 2A*

Fifty mouse brains were homogenized with buffer A containing 0.25 M sucrose. The supernatant, which was obtained by centrifugation at 100 000 × *g* for 60 min, was subjected to DEAE-cellulose (Whatman DE-52) column chromatography (2.5 × 20 cm) equilibrated with buffer A (Fig. 2). Peak 1 containing protein phosphatase 1 was eluted with 0.1 M NaCl buffer A and the peak 2 containing protein phosphatase 2A, with 0.2 M NaCl buffer A. Protein phosphatase 1 was defined by the evidence that its activity was inhibited by a 100 nM concentration of the inhibitor-2 isolated from rabbit skeletal muscle, as described in section 2.1, and protein phosphatase 2A was also defined by an activity that ^{32}P-labeled histone H1 was dephosphorylated. Each peak containing protein phosphatase 1 or 2A was further subjected to microcystin-affinity chromatography (1.5 × 3.0 cm) equilibrated with buffer A. Proteins were eluted with various concentrations of NaCl.

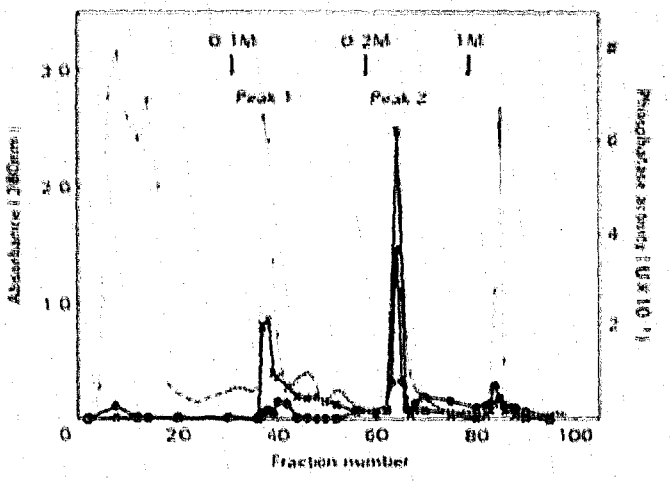

Fig. 2. DEAE-cellulose column chromatography of the supernatant obtained from mouse brain. The activity of phosphorylase phosphatase was measured by 20 μl of each fraction (X) and that of histone H1 phosphatase by 0.5 μl of each fraction (●). The NaCl concentrations were indicated by arrows and absorbance at 280 nm (○) is also shown.

3. RESULTS AND DISCUSSION

The DEAE-cellulose column chromatography of the supernatant obtained from mouse brain by elution with 0.1 M and 0.2 M NaCl buffer A gave two main peaks containing activities of protein phosphatases 1 and 2A. Peak 1 contained the activity catalyzing the dephosphorylation of ^{32}P-labeled phosphorylase *b*, but not histone H1. The activity was completely inhibited by a 100 nM concentration of inhibitor-2 (data not shown). We defined peak 1 as the fraction containing protein phosphatase 1. Peak 2 contained the dephosphorylating activity for ^{32}P-labeled histone H1. These activities were not affected by a 100 nM concentration of inhibitor-2, suggesting that peak 2 contains protein phosphatase 2A.

The protein phosphatase 2A (2.2 U/1.3 mg of protein) of peak 2 was subjected to microcystin-affinity chromatography. As Fig. 3 shows, most of the inactive proteins were eluted with up to 0.2 M NaCl buffer A.

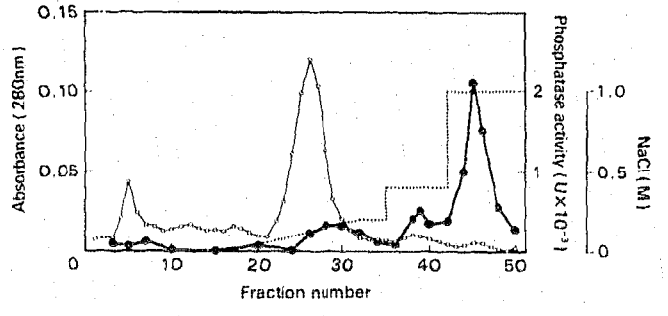

Fig. 3. Microcystin-affinity chromatography of peak 2 (1.3 mg of protein). The activity of protein phosphatase 2A was measured by 20 μl aliquots of each fraction (●). The NaCl concentrations (– – –) and absorbance at 280 nm (○) are shown.

Protein phosphatase 2A was eluted mainly with 1 M NaCl buffer A, but was associated with an additional minor peak of activity, suggesting the presence of heterogeneous protein phosphatase 2A. The SDS-polyacrylamide gel electrophoresis of the fractions eluted with 0.1 M buffer A showed three protein bands of 67 kDa, 58 kDa and 41 kDa (Fig. 4). Protein phosphatase 2A has a molecular mass of 180 kDa, estimated by the gel filtration method, and we assumed that this purified protein phosphatase 2A consists of each of these subunits in a ratio of 1:1.

The protein phosphatase 1 (0.05 U/3.8 mg of protein) of peak 1 was also subjected to microcystin-affinity chromatography. Although protein phosphatase 1 was partly eluted with 0.4 M NaCl buffer A, most of the remaining activity was found in the initial elution, suggesting that over 50% of protein phosphatase 1 did not bind to the microcystin-affinity column (data not shown). The fractions eluted with 0.4 M NaCl buffer A contained protein phosphatase 1 with several contamined proteins (data not shown). These results, that protein phosphatase 1 was eluted with 0.4 M NaCl and protein phosphatase 2A with 1 M MaCl, are in a good agreement with our recent evidence that inhibition of specific [^{3}H]okadaic acid binding by microcystin to protein phosphatase 2A was about 10 times stronger than that to protein phosphatase 1 (unpublished results), and was as strong as that by okadaic acid [4]. Therefore, microcystin-affinity chromatography seems to be suitable for isolation of protein phosphatase 2A rather than that of protein phosphatase 1. Although the results were not here presented, we found that protein phosphatase 2A per se does not bind to ECH Sepharose 4B with buffer A.

The advantage of this microcystin-affinity chromatography was the rapid purification of protein phosphatase 2A in two-step procedures, resulting in obtaining the electrophoretically pure holoenzyme. One disadvantage was the small binding capacity of microcystin-affinity column for the enzymes. We estimated that 3 μg of protein phosphatase 2A maximally binds to 1 mg of microcystin attached to Sepharose 4B. Although microcystin LR was used as a ligand to ECH Sepharose 4B in this experiment, structure/function relationships of microcystins are not well investigated, using several derivatives or other protein phosphatase inhibitors besides okadaic acid. Okadaic acid was conjugated with EAH Sepharose 4B by the carbodiimide reaction between a carboxyl group of okadaic acid and the amino group of EAH Sepharose 4B. Okadaic acid-affinity chromatography did no. give good purification of protein phosphatases. The significance of the carboxyl group of okadaic acid was recently confirmed by a structure/function relationship study of a series of okadaic acid derivatives [12]. Further study on various inhibitors will give more significant clues for developing affinity chromatography of protein phosphatases.

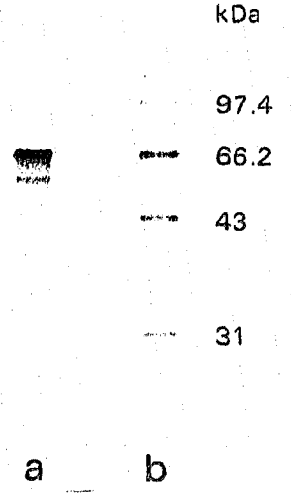

Fig. 4. SDS-Polyacrylamide gel electrophoresis. (a) Three subunits of protein phosphatase 2A, 67 kDa, 58 kDa and 41 kDa. (b) Molecular mass markers.

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