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A SIMPLIFIED PROCEDURE FOR THE PURIFICATION OF THE PROTEIN PHOSPHATASE MODULATOR (INHIBITOR-2) FROM RABBIT SKELETAL MUSCLE

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1. Introduction

Two heat-stable proteins have been identified as specific inhibitors of the phosphatase activity [1–11]. Inhibitor-1 requires phosphorylation by the cyclic AMP-dependent protein kinase for its activity [3,4] and has been clearly implicated in the hormonal regulation of glycogen metabolism [12–14]. The inhibitor-2 protein has been purified up to homogeneity from rabbit muscle [10] and liver [7] and its specific role as modulator of the protein phosphatase activity has been described [15,16].

A high M_r multisubstrate protein phosphatase has been extensively purified from rabbit muscle and shown to interconvert to the inactive ATP-Mg-dependent enzyme form (F_C) [17]. The activation of purified F_C-enzyme [18] is brought about by a preincubation with ATP-Mg and F_A, a bifunctional protein also displaying synthase kinase activity [19]. This reversible ATP-Mg-dependent activation was consequently shown to depend upon the presence of inhibitor-2 which we therefore called phosphatase modulator [15,16].

This report describes a simplified and improved procedure for the isolation of the rabbit muscle phosphatase modulator protein.

2. Experimental

Materials and methods were as in [15–20]. The activity of the ATP-Mg-dependent phosphatase was commonly measured after a 10 min preincubation at 30°C with 0.1 mM ATP, 0.5 mM Mg^{2+} and the minimal amount of F_A required for full activation of F_C under these conditions. The phosphatase activity unit is defined as the amount of enzyme which releases 1 nM [^{32}P]phosphate/min at 30°C from ^{32}P-labeled phosphorylase *a* (2 mg/ml). The assay time is 10 min.

Heat-stable phosphatase inhibitor-2 was purified to homogeneity from rabbit muscle according to [10] or by an alternative method, described in section 3. One unit of inhibitor-2 decreased the activity of 20 munits ATP-Mg-dependent phosphatase by 50% when included in the 10 min preincubation of F_C (20 000 U/mg) [17] with F_A and ATP-Mg.

3. Results

A crude extract was prepared from 1 kg fresh rabbit skeletal muscle and directly adsorbed onto DEAE–Sephadex A-50 as in [17]. The resin was washed with 0.2 M NaCl in buffer A (20 mM Tris, 0.5 mM dithiothreitol pH 7.0), packed into a (50 × 30 cm) column and the breakthrough proteins completely eluted with this buffer. The inhibitor-2 activity was then eluted with 0.4 M NaCl in buffer A. The 0.4 M NaCl eluate was concentrated by ammonium sulfate precipitation (the 30–50% saturated ammonium sulfate precipitate was collected), redissolved and dialyzed for 1 h against buffer A. Total volume after dialysis was 5–10 ml (step 1). The 50% ammonium sulfate supernatant fraction was totally devoid of heat-stable phosphatase inhibitor activity.

The dialyzed fraction was heat treated by dropping small portions of it at a time into 5 ml boiling buffer A. The complete mixture was kept for another 5 min in a boiling waterbath. The solution was cooled on ice and the denatured proteins removed by centrif-

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ugation (10 min at 10 000 × g). The denatured proteins were extracted 2 more times with 5 ml buffer A, all the supernatant fractions combined and extensively dialyzed against the same buffer.

The resulting solution was consequently passed over a (1 × 10 cm) column of blue Sepharose CL-6B and the resin extensively washed with 0.1 M NaCl in buffer A. All the inhibitor-2 activity was stuck onto the resin, and was eluted with 0.6 M NaCl in buffer A. The eluate was concentrated by an overnight dialysis against 10% polyethylene glycol 6000 in buffer A (step 2). The preparation was stored frozen at –20°C in small aliquots.

The final inhibitor-2 preparation has spec. act. 600 000 U/mg protein, which is ~2-times higher than the preparation obtained according to [10], using our assay system. The yield was ~40% (av. 4 preps);

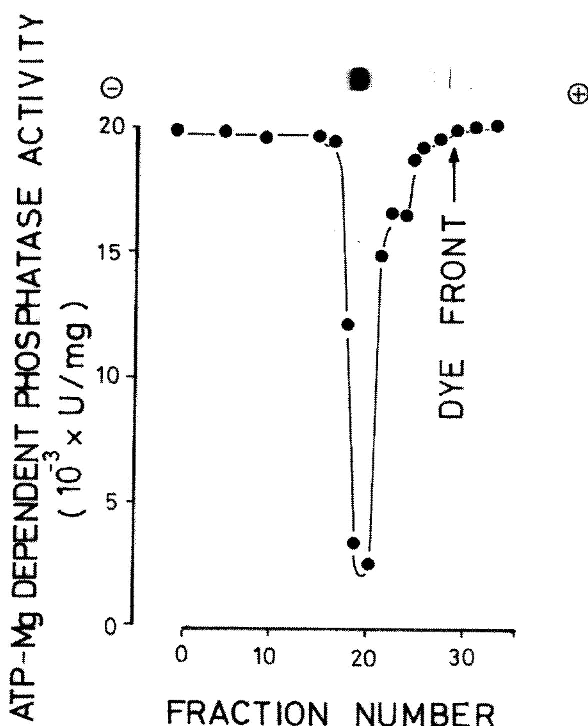

Fig.1. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate: 20 μg inhibitor-2 protein was electrophoresed as in [18]; the 6% polyacrylamide gels were sliced into 2 mm portions, the soluble proteins extracted overnight with 200 μl buffer A, and assayed for inhibitor activity (•) (after a 10-fold dilution) as outlined in section 2. Identical gels were stained for proteins using Coomassie brilliant blue R-250 and destained by diffusion.

Table 1
Purification of the protein phosphatase modulator (inhibitor-2) from rabbit skeletal muscle

	Total protein (mg)	Inhibitor-2 activity		
		Total activity (U × 10^{-3})	Specific activity (U/mg)	Yield (%)
(A)				
Crude extract	600[a]	1000	1600	100
Step 1	14.4[a]	720	50 000	72
Step 2	0.67	400	600 000	40
(B)				
Inhibitor-2 (isolated as in [10])	0.8	200	250 000	20

[a] Amount of proteins in the supernatant fractions after boiling

Specific activities are the average of 4 preparations (A) and 2 preparations for (B)

~0.7 mg pure inhibitor was obtained from 1 kg muscle Both procedures yielded a pure protein as judged by electrophoresis on denaturing gels. The proteins eluted from similar unstained gels, contained all of the inhibitor-2 activity (fig.1). It was noticed that a smaller M_r component (±25 000) also contained some inhibitor activity; this probably represents some breakdown product of inhibitor-2. The purified inhibitor-2 was indistinguishable from the one isolated according to [10] in gel filtration on Biogel P-150 (M_r 55 000) and in sucrose density gradient centrifugation (M_r 17 000) (not shown). Table 1 (A,B) shows the extent of purification of inhibitor-2 using the outlined procedure and compares the pure inhibitor-2 with the one isolated according to [10].

4. Discussion

Our simplified procedure for the isolation of inhibitor-2 has some obvious advantages. The protein can be isolated without elaborate or time consuming steps and performing the heat treatment at a later stage in the purification, allows for a smaller volume sample to be brought to a very fast boil minimizing proteolysis which denatures inhibitor-2. The repeated extractions of the heat-denatured proteins are necessary, since the last 2 extractions yield at least 50% of the recovered activity. The dialysis against 10% polyethyl-

ene glycol is a very convenient way to concentrate since this method is not restricted by a lower protein concentration limit, and the dehydrating agent (M_r 6000) does not enter the dialysis tubing. In contrast to [10], the inhibitory activity was very stable at any stage of the purification and did not require stabilization by Brij 35. The higher specific activities obtained here are in part the result of a more sensitive phosphatase in the assay for inhibitor-2.

The recently discovered stimulatory effect [16] of inhibitor-2 on the ATP-Mg-dependent protein phosphatase activity is incompatible with its definition as a phosphatase inhibitor: the name 'phosphatase modulator' seems more appropriate.

The existence of interconvertible forms of active and inactive (ATP-Mg-dependent-)phosphatase is suggestive for the hormonal regulation of this enzyme. Cyclic AMP-mediated hormones have been shown to decrease the phosphatase activity by producing more phosphorylated inhibitor-1. It is likely that the physiological role of the protein phosphatase modulator (inhibitor-2) is to make the in vivo reactivation of the phosphatase possible by virtue of the F_A and ATP-Mg-dependent activation process.

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