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A rapid purification method for calf thymus casein kinase II

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We report here the rapid purification to homogeneity of a cyclic nucleotide-independent protein kinase sensitive to 5,6-dichloro-1-β-D-ribofuranozylbenzimidazole (DRB), identical to the previously described casein kinase II, from lyophilized calf thymus by chromatography on phosphocellulose and Mono-Q FPLC columns.

Casein kinase II; 5,6-Dichloro-1-β-D-ribofuranozylbenzimidazole; Transcription

1. INTRODUCTION

Protein phosphorylation is a widely distributed cellular process in the regulation of several metabolic pathways [1,2]. Several protein kinase activities have been characterized, purified to homogeneity and classified based on their substrate or effector specificity. The cAMP-dependent protein kinases have been well-studied and exert their effect on numerous enzymes and structural proteins by phosphorylation [2]. The cAMP-independent protein kinases are able to phosphorylate acidic proteins such as casein and phosvitin. Casein kinases I and II were distinguished based on their affinity for DEAE-cellulose [2,3] and their differential use of ATP/GTP as substrates. Casein kinase II has been found in all eukaryotic cells and in different cellular compartments including cytoplasm [4], mitochondria [5], membranes [4] and nuclei [6–8]. Although protein phosphorylation in the nucleus is well established, its physiological functions are poorly understood. Experiments with the adenosine analogue DRB have implicated casein kinase II in mRNA biosynthesis [11,14].

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Abbrevations: BSA, bovine serum albumin; DRB, 5,6-dichloro-1-β-D-ribofuranozylbenzimidazole

We report here a short procedure to purify milligram quantities of casein kinase II from calf thymus. This procedure results in high yields and is more suitable for scale-up than previously used methods due to the simpler solubilization procedure.

2. MATERIALS AND METHODS

2.1. *Nucleotides and radiochemicals*

ATP, GTP, CTP and UTP were from Pharmacia. [γ-^{32}P]ATP and [γ-^{32}P]GTP were obtained from Amersham Corporation.

2.2. *Protein substrates*

Partially hydrolyzed and dephosphorylated casein and bovine serum albumin (BSA) were purchased from Sigma.

2.3. *Column chromatography*

Phosphocellulose P-11 was purchased from Whatman. The Mono-Q FPLC column and Q-Sepharose were from Pharmacia. All other reagents were of the highest grade obtainable.

2.4. *Protein kinase assay*

Routine kinase determinations were performed in 20 mM Tris-HCl, pH 7.9, 160 mM KCl or NaCl, 8 mM $MgCl_2$, 0.1 mM dithiothreitol, 20 μM [γ-^{32}P]ATP or [γ-^{32}P]GTP (5000 cpm/pmol) with 2 mg/ml of casein as substrate acceptor in a total volume of 15 μl. The reactions (30°C during 15 min) were spotted on Whatman 3MM paper and washed with 20% trichloroacetic acid. Values were corrected for phosphorylation of proteins contained in the kinase fraction when it exceeded 1% of the activity in the absence of casein.

2.5. *Protein determination*

Protein concentrations were determined by the method of Bradford [9] with BSA as standard.

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2.6. *Enzyme solubilization*

Fresh calf thymus was cleaned, cut into 3 cm^3 pieces and frozen at −70°C within 1–2 h following killing. The frozen tissue was lyophilized and pulverized in a Waring blendor for 10 s at low speed. The protein was then solubilized by addition of 800 ml of buffer A (50 mM Tris-HCl, pH 7.9, 10% glycerol, 0.2 mM dithiothreitol, 0.5 mM EDTA) containing 50 mM KCl, and homogenized, keeping the temperature below 4°C, with three 30-s strokes at high speed in the Waring blendor.

The homogenate was centrifuged at 10000 rpm in a Beckman JA20 centrifuge using a G10 rotor for 20 min at 0°C. An upper compact layer was removed with a plastic spatula, and the supernatant was filtered through two layers of cheesecloth and saved. The pellet was re-extracted in 500 ml of buffer A (50 mM KCl) and spun under the same conditions as above. The KCl concentration of the pooled supernates was adjusted to 550 mM and directly mixed with 200 ml of Q-Sepharose (Fast Flow, Pharmacia), previously equilibrated in buffer A (550 mM NaCl). After 30 min adsorption, the resin was transferred to a syntered glass funnel and the flow-through was collected and used as source of enzyme.

2.7. *Phosphocellulose chromatography*

The Q-Sepharose effluent was diluted with buffer A to a final concentration of 380 mM NaCl, and batch-adsorbed to phosphocellulose (60 ml) previously equilibrated in buffer A (380 mM NaCl). After 30 min, the resin was collected by filtration and washed 4 times with 60-ml aliquots of buffer A (380 mM NaCl). The resin was transferred to a (2.5 × 20 cm) column and the sample was eluted with a 500 ml linear gradient of NaCl (380–900 mM) in buffer A. Protein kinase activity sensitive to DRB eluted at 560 mM NaCl.

2.8. *Mono-Q FPLC*

The active fractions were pooled and diluted with buffer A to a concentration of 280 mM NaCl and loaded onto a 1 ml Mono-Q anion-exchange column pre-equilibrated with 280 mM NaCl. After a 20 ml wash, the kinase activity was eluted with a linear gradient (280–500 mM NaCl) in 8 ml of buffer A. The run was performed at 4°C and with a 0.2 ml/min flow rate. The eluate was monitored with an LKB diode array detector and fractions of 0.5 ml were collected. The protein and kinase activity co-eluted at 390 mM NaCl.

2.9. *Gel electrophoresis*

Electrophoresis in 10% acrylamide-SDS gels was as described by Laemmli [10]. Samples were precipitated with 10% trichloroacetic acid, washed once with 3% trichloroacetic acid, twice with acetone and dried for 15 min at 37°C. Dried samples were dissolved in 40 mM Tris-HCl, 2% SDS and 5 mM 2-mercaptoethanol at 100°C during 2 min. The gels were stained with Coomassie brilliant blue R-250 (0.1%) in 50% methanol, 7% acetic acid and destained by diffusion in 50% methanol, 7% acetic acid.

3. RESULTS

3.1. *Purification*

The use of lyophilized calf thymus facilitates pulverization of the tissue in the absence of water, which greatly simplifies initial handling of the large amounts of starting material used in this purification protocol. Differential solubilization of proteins is also achieved without apparent loss of casein kinase II activity. The second extraction of the pellet yielded a significant amount of additional enzyme. About 10% of the total protein is solubilized and although only a fraction of the total kinase activity is initially solubilized by tissue homogenization at low salt concentrations (50 mM KCl), the final yield of casein kinase II per kilogram of thymus is twice that previously reported [11]. The Q-Sepharose batch adsorption step eliminates nucleic acids, which inhibits casein kinase II activity [12] and interfere with the binding of the protein kinase to phosphocellulose and Mono-Q resins. This back-absorption step does not significantly alter the total protein concentration, and the apparent activity of the protein kinase II, which is sensitive to DRB and able to use GTP, increased 2–3-fold after this step (table 1). The filtrate of Q-Sepharose was then mixed in a batch with phosphocellulose P11 and the sample was eluted with a linear salt gradient. The DRB-sensitive protein kinase eluted as a sharp peak at 560 mM NaCl. Specific activity of the protein kinase increased dramatically after the phosphocellulose step. The binding of the protein kinase II to phosphocellulose P11 was probably due more to a substrate-like interaction than to cationic attraction, since it was possible to elute the enzyme from the resin using either GTP or ATP, without an increase of ionic strength. Furthermore, this kinase did not bind to other cationic resins such as CM or S-Sepharose.

Further purification of the kinase by FPLC chromatography on a Mono-Q column revealed the activity as a single peak that coincided (fig.1) with one of the two 280 nm absorbance peaks and showed a typical protein type absorption spectrum (curve 1 in fig.1, inset). The absorption maxima for casein kinase II were observed at 220 nm and 276 nm. The second peak, which was detected in variable amounts in different enzyme preparations, had the spectral characteristics of a nucleic acid (fig.1, inset) and comigrated with tRNA in an agarose gel (not shown). This Mono-Q chromatography step resulted in a very good recovery (79%) and a 15-fold increase in the protein kinase-specific activity. Table 1 summarizes

Table 1

Purification of protein kinase II from calf thymus

	Volume (ml)	Protein (total mg)	Activity (total units)	Specific activity (units/mg)
Homogenate	700	14980	1.2×10^7	0.856×10^3
Q-Sepharose flow-through	950	13965	2.7×10^7	1.9×10^3
Phosphocellulose	65	24.7	1.1×10^7	4.4×10^5
Mono-Q				
fraction no.25	0.5	0.23	0.15×10^7	6.6×10^6
fraction no.26	0.5	0.50	0.33×10^7	6.6×10^6
fraction no.27	0.5	0.37	0.25×10^7	6.8×10^6
fraction no.28	0.5	0.21	0.14×10^7	6.7×10^6
Total		1.32	0.87×10^7	

The purification procedure is described in the text. This preparation utilized 500 g of calf thymus lyophilized. One unit equals 1 pmol of phosphate transferred from [γ-^{32}P]GTP to casein in 30 min at 30°C

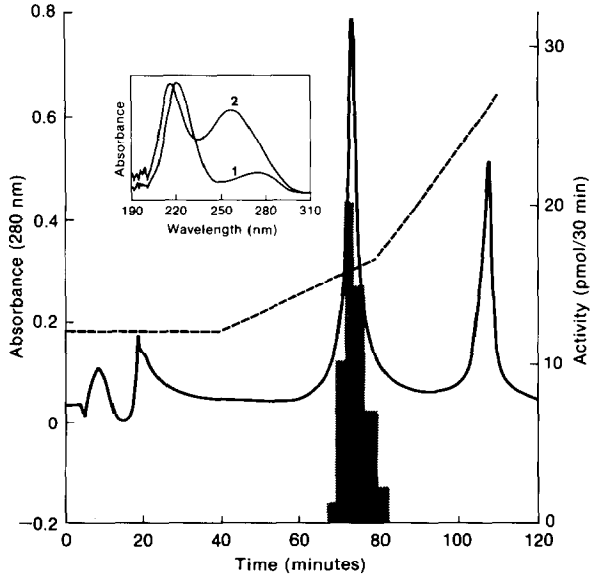

Fig.1. Chromatography of protein kinase in Mono-Q FPLC. The kinase activity was eluted with a linear gradient of NaCl (280–500 mM) [– – –]. The eluate was monitored with a diode array detector (——). The 280 nm relative absorbance unit corresponds to a full-scale deflection = 0.639. The flow rate was 0.2 ml/min and fractions of 0.5 ml were collected. All fractions were assayed for protein kinase activity as described in section 2 (shaded area). Inset, spectrum from the absorbance peaks. (1) Protein kinase spectrum, with maxima at 222 nm and 274 nm. (2) Second absorbance peak spectrum, with maxima at 217 nm and 257 nm.

the purification of casein kinase II from calf thymus. The protein and kinase activity profiles are symmetrical and coincident, suggesting that the protein is homogeneous. Thus, only two chromatographic steps are required for purification of the DRB-sensitive kinase to homogeneity. A 33% yield was obtained, taking the Q-Sepharose flowthrough as 100%.

3.2. *Native and subunit molecular mass*

The enzyme eluted from a calibrated HPLC sizing column, at a position corresponding to a molecular mass of 170 kDa (not shown), when the elution profiles from the HPLC column were plotted against the molecular mass of known protein standards.

Fig.2 shows the results of the electrophoretic analysis of the fractions obtained during purification. After the last chromatographic step, the homogenous enzyme consisted of three major polypeptides of 44 kDa (α), 37 kDa (α') and 27 kDa (β). The stoichiometry of the polypeptides was estimated as 1:0.9:2 by densitometry (assuming equal binding of Coomassie blue to all subunits). The relative ratio of the 44 kDa and 37 kDa varied among preparations, suggesting that proteolysis might have contributed to increased amounts of the 37 kDa subunit.

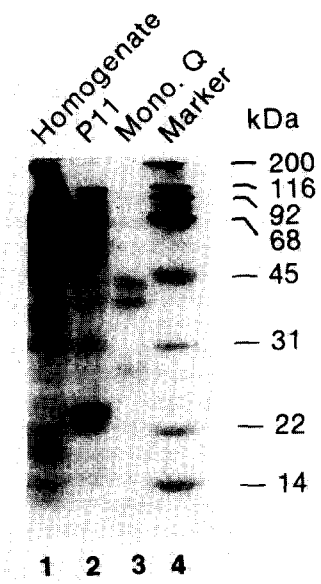

Fig.2. SDS-polyacrylamide gel electrophoretic analysis calf thymus protein kinase purification. Lanes: 1, calf thymus homogenate (40 μg); 2, phosphocellulose pool (40 μg); 3, fraction no.25 from the Mono-Q column (6 μg); 4, protein molecular mass standards. Samples were run on a 10% discontinuous SDS-slab gel according to the method of Laemmli [10] and stained with Coomassie blue.

4. DISCUSSION

The results presented in this report show that large amounts of homogenous protein kinase II can be purified from calf thymus using the rapid purification procedure described. The primary advantage of this method over other procedures [11] is that it avoids the need for extraction at high salt concentrations, which not only solubilizes more protein kinase, but also several other protein contaminants that are then difficult to separate from the enzyme. Moreover, the small homogenization volumes used here for lyophilized tissue allow the processing of much larger quantities. This procedure yields 2–3 mg of homogenous protein kinase/kg of calf thymus in a short period. The efficiency of the phosphocellulose chromatographic step (230-fold purification), the elution of the kinase from this resin with ATP or GTP, and the failure to bind other cationic resins, suggest that the binding of the enzyme to P11 is highly specific, similar to affinity chromatographic steps. The purified protein kinase obtained appears to be indistinguishable with regard to subunit composition and biochemical properties from that described in previous reports [11,13].

Casein kinase II has been found in all eukaryotic cells from yeast to man, and is highly conserved with respect to structure and general enzymatic requirements [13–21]. Immunological similarities between the *Drosophila* and the bovine enzyme have also been reported [22].

The results of our line of work strongly suggest a link between the DRB-induced inhibition of RNA polymerase II transcription and that of casein kinase II. The DRB inhibition curves of in vivo and in vitro RNA polymerase II transcription [11,23] are almost identical to the curves of DRB inhibition of the kinase reaction catalyzed by the human and/or pure calf thymus protein kinase described here. This calf thymus protein kinase, which has been purified based on its sensitivity to DRB and ability to use GTP as a phosphate donor, appears similar to the partially purified DRB-sensitive protein kinase from transcriptionally active HeLa cell extracts [23].

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