AN IMPROVED METHOD FOR THE PURIFICATION OF cAMP-DEPENDENT PROTEIN KINASE FROM RABBIT MUSCLE USING HYDROPHOBIC CHROMATOGRAPHY

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Received 3 December 1976
Revised version received 30 December 1976

1. Introduction

cAMP-Dependent protein kinases are ubiquitous key enzymes of metabolic control. Complete purifications of protein kinase holoenzyme, only described in a few cases [1,2,14] are based on pure classical procedures which are laborious and often result in low yields. With the necessity to provide larger quantities for physical investigations (ESR spectroscopy studies on ligand binding, cf. ref. [3] and manuscript in preparation), we developed a procedure for the rabbit muscle enzyme, which is rather time-saving (three chromatographic steps lasting about 50 h) and results in a high yield (up to 20% of the content of the crude extract). In this procedure all dialysis steps are avoided and hydrophobic chromatography proved to be rather effective.

2. Materials and methods

[γ^{32}-P]ATP and [8-^{3}H]cAMP were purchased from Amersham Buchler GmbH. DEAE-cellulose DE 52 was from Whatman Inc., hydroxylapatite (gel HTP) from Bio-Rad Laboratories and Sepharose 4B from Pharmacia Fine Chemicals. The *n*-alkyl amines were obtained from Merck-Schuchardt. Histone was prepared according to Johns [4]. Protein kinase activity was assayed by the filter paper method as described by Beavo et al. [1]. Tritiated cAMP binding was determined by the method of Gill and Garren [6]. Protein concentrations were determined by the method of Lowry [7].

For preparation of the hydrophobic gels, Sepharose 4B was activated according to March et al. [8]. Two hundred milliliters Sepharose 4B and 50 g CNBr were used. For the reaction with alkylamines the activated gel was suspended in an equal volume of cold 0.2 M $NaCO_3$ buffer of pH 9 and divided into several portions of 20 ml. To each portion 160 mmol of alkylamine, dissolved in 60 ml 40% Dioxane/H_2O (v/v) and adjusted to pH 9 with concentrated HCl, were added. The coupling reaction was carried out for 16 h at 4°C. The gels were washed with 10 vol of each of the following solutions: distilled water, 0.1 $NaHCO_3$ of pH 9, 0.05 N NaOH, water, 0.1 M acetic acid and water.

The following buffer (buffer A) was frequently used: 5 mM MES (2-*N*-morpholino ethane sulfonic acid) of pH 6.5 and 15 mM mercaptoethanol.

3. Results

3.1. *DEAE-cellulose chromatography*

The first step for purification of the protein kinase was performed as described by Beavo et al. [1] through the chromatography on DE 52 cellulose, starting with three rabbits (about 2.5 kg of muscles).

3.2. *Hydrophobic chromatography*

The homologous series of alkyl–Sepharoses [9,10] prepared was tested with respect to their capability to absorb protein kinase. The enzyme fractions obtained at the lower NaCl concentration (peak I, [1]) from DEAE-cellulose chromatography, dialysed versus buffer A, were used. Desorption was performed with

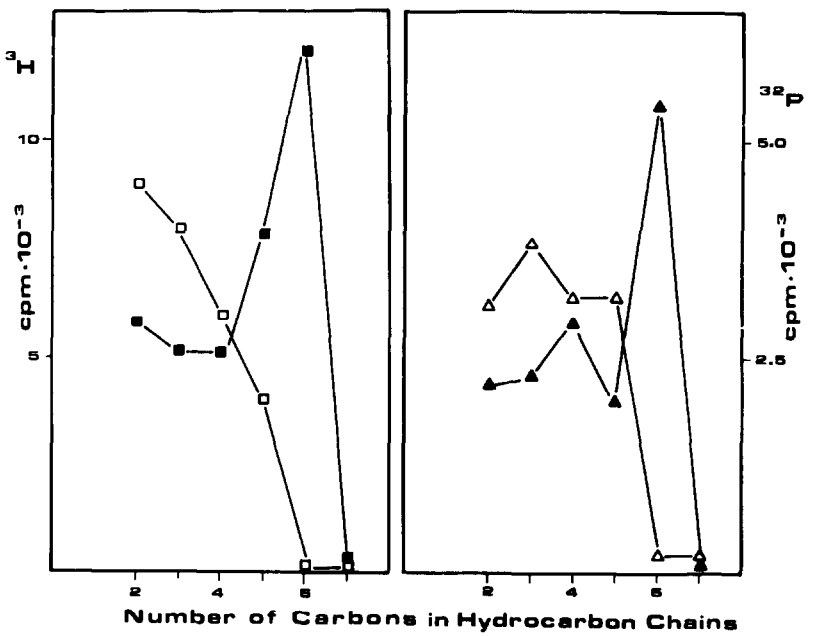

Fig.1. Adsorption and desorption of protein kinase on different alkyl–Sepharoses. 0.85 mg protein from the cellulose DE 52 column (in about 1 ml of buffer A) were applied onto alkyl–Sepharose micro columns of 1 ml bed volume and washed through with another 2 ml of buffer A (empty marks). Desorption was performed with 2 ml of 0.4 M NaCl in buffer A (filled marks). Binding activity (□,■) was determined according to ref. [6] after incubation for 15 min in a total vol. of 0.2 ml of 20 mM potassium phosphate of pH 6.9, 4 mM magnesium acetate and 12 μM [^{3}H]cAMP (2700–3100 cpm/pmol). Phosphotransferase activity (△,▲) was carried out according to ref. [1] after incubation with histone for 2 min in a total volume of 0.1 ml of 25 mM MES of pH 6.9, 4 mM magnesium acetate, 2 μM cAMP, 0.25 mM EGTA, 0.2 mM [γ-^{32}P]ATP (10 000–50 000 cpm/nmol). The filter papers were counted in water using the Cerenkov irradiation.

0.4 M NaCl in buffer A. The results shown in fig.1 indicate that the Sepharose with the 6-carbon ligand is best suited for adsorption and desorption of the kinase. Protein kinase activity could not be eluted even with high NaCl concentrations from heptyl–Sepharose.

For the preparative hydrophobic step active fractions from the DEAE-cellulose column (peak I) were pooled, diluted with 4 vols of cold distilled water (total volume approx. 1.2 liters), 300 ml hexyl–Sepharose were added and the slurry was stirred for 15 min. The gel was then collected in a funnel washed with 1.5 liters of 9 mM NaCl in buffer A, resuspended in 200 ml of the same buffer and poured into a column tube. The column was washed with 20 mM NaCl in buffer A, until no protein was detectable in the eluate. Then a linear gradient of 20–300 mM NaCl in buffer A was applied (fig.2). The gel could be regenerated by brief exposure (15 min) to 3 M guanidinium hydrochloride and subsequent washing with distilled water, it was stored in 5 mM sodium acetate (pH 5) containing 0.02% NaN_3.

3.3. *Hydroxylapatite chromatography*

The active fractions from the hydrophobic column were directly applied to a column packed with hydroxylapatite equilibrated in 20 mM potassium phosphate (pH 6.7), containing 15 mM mercaptoethanol. The column was washed with 110 mM potassium phosphate buffer, pH 6.7, containing 15 mM mercaptoethanol, until the eluate was free of protein, and a linear gradient from 110–300 mM potassium phosphate containing 15 mM mercaptoethanol was applied (fig.3). The fractions containing protein kinase activity (about 150 ml) were collected and concentrated to a final volume of about 10 ml using Serva Hollow-fibers SHF 36.

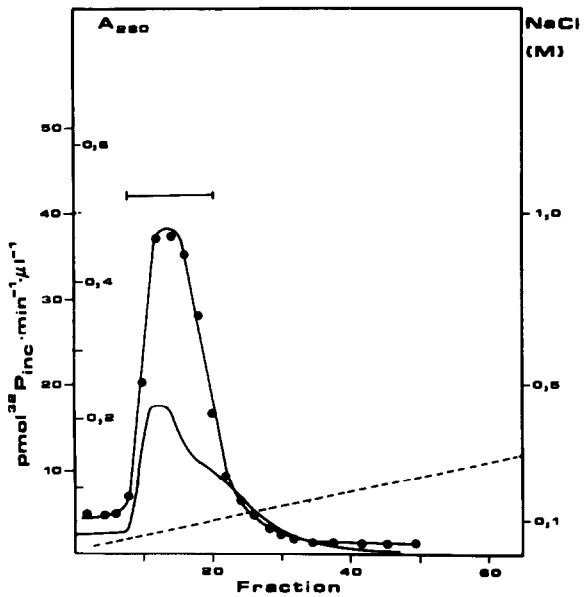

Fig.2. Chromatography of protein kinase on hexyl–Sepharose. 5.1 g protein were applied onto a column of 11 × 5 cm, a 2 × 1 liter gradient was used and fractions of 30 ml were collected, for details see text. Phosphotransferase activity (•) was determined as described in the legends of fig.1; full lines A_{280}, (- - - -) concentration of NaCl in buffer A, (⊢——⊣) fractions collected for further purification.

Fig.4. SDS–polyacryamide electrophoresis of the purified protein kinase, performed according to ref. [13]. Molecular weight values of 38 000 and 48 000 for C and R, respectively, are found, which are in accordance with those reported by Dills et al. [15].

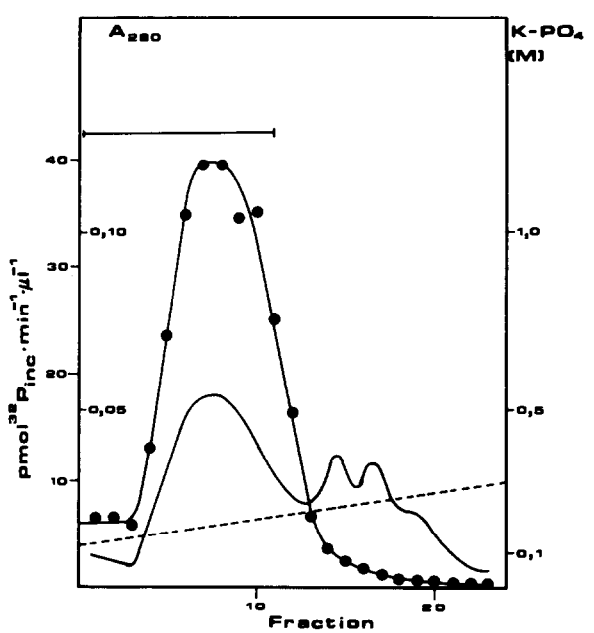

Fig.3. Chromatography of protein kinase on hydroxylapatite. The active fractions collected from the hexyl–Sepharose column (fig.2) were applied onto a column of 5 × 2.5 cm; a 2 × 250 ml gradient was used and fraction of 25 ml were collected, for details see text. Phosphotransferase activity (•) was determined as described in the legends of fig.1; full lines, A_{280}, (- - - -) concentration of potassium phosphate, (⊢——⊣) fractions collected.

3.4. *Properties of the purified enzyme*

The enzyme was stored between 0°C and 4°C and was stable for at least one month with respect to its phosphotransferase activity, cAMP binding, ATP binding and its pattern in SDS–gel electrophoresis. Table 1 shows the degree of purification and the yield for the three chromatographic steps on the basis of cAMP binding. The phosphotransferase activity obtained for the kinase was in the range of 1.5 µmol ^{32}P (from ATP into histone)/mg protein and min (in the presence of

Table 1
Data from the purification of the protein kinase

Step	Protein	cAMP binding		Degree of purification	Yield
		Units	Spec. act.		
	(mg)	(nmol)	(nmol/mg)	–	(%)
Crude extract	138 000	566	0.0041	–	100
Cellulose DE 52	5100	311	0.061	15	55
Hexyl–Sepharose	95	173	1.82	444	31
Hydroxyl-apatite	7	81	11.6	2830	14

cAMP). cAMP Stimulated the phosphotransferase activity very sensitively, up to 100-fold (at high enzyme concentrations [11]). The specific cAMP binding activity listed in table 1 of 11.6 nmol/mg protein is very near to the calculated value of 12.5 nmol/mg for the pure enzyme assuming a kinase of mol. wt of 160 000 [11]. The analytical SDS electrophoresis, shown in fig.4, reveals a rather pure enzyme exhibiting the two bands of both subunits.

4. Conclusions

The great advantage of the present procedure is the elimination of all time-wasting steps like dialysis and intermediate protein concentration. The yields were in the range of 12–18% of the crude extract. The data for the single steps (table 1) reveal that hydrophobic chromatography is rather effective resulting in an increase of the degree of purification by a factor of about 30 with the relative reduction in the yield of only 45% (related to the amount at the step before). The capacity of the hydrophobic gel was found to be rather high, we used a column bed of only 300 ml for the amount of protein indicated in table 1.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Wa 91/7/8) and the Fonds der Chemischen Industrie.

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