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SEPARATION AND PURIFICATION OF MAIZE CASEIN KINASES BY AFFINITY CHROMATOGRAPHY

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SUMMARY

Two groups of protein kinases from maize seedlings are purified on casein-Sepharose and on 5′-AMP-Sepharose columns. Threonine-serine-specific (type TS) casein kinases are eluted with 0.1 *M* potassium chloride while serine-specific (type S) kinases are eluted with 0.2 *M* potassium chloride. Although both groups of kinases are inhibited by heparin, the type TS kinases are inhibited 200 times more strongly than the type S kinases. The type S kinases are eluted as monomeric proteins, while the type TS kinases appear to be oligomeric proteins. Both ATP and GTP are utilized as phosphate donors by type TS kinases, but type S kinases are ATP-specific.

INTRODUCTION

Owing to the growing interest in phosphorylation reactions for the regulation of a variety of cellular processes, it has become necessary to isolate protein kinases and to study their functions at the molecular level. Among the casein kinases, the biological significance of cyclic nucleotide-independent kinases remains very speculative (for a review, see ref. 1). According to Clari et al. [2] two types of cyclic nucleotide-independent casein kinases could be distinguished depending on which amino acids were phosphorylated: serine alone (type S kinases) or both threonine and serine (type TS kinases). For the separation of different types of protein kinases, ion-exchange chromatography was used [2]. In this study, we describe the separation of the two groups of casein kinases by affinity chromatography on casein-Sepharose and on 5′-AMP-Sepharose.

EXPERIMENTAL

The conditions for growing maize seedlings to obtain 62-h-old plants and for their storage was described previously [3].

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Materials

Hydrolyzed and partially dephosphorylated casein, heparin, bovine serum albumin (BSA), 2-mercaptoethanol, Tris, ATP (Sigma, St. Louis, MO, U.S.A.), GTP (Serva, Heidelberg, F.R.G.) and cyanogen bromide (Fluka, Buchs, Switzerland) were of reagent grade. Sepharose 4B and 5'-AMP-Sepharose 4B were obtained as gifts from Pharmacia Fine Chemicals (Uppsala, Sweden) and phosphocellulose P11 was purchased from Whatman Biochemicals (Maidstone, U.K.). Phenylmethylsulphonyl fluoride and glass beads were obtained from Merck Schuchardt (Hohenbrunn bei München, F.R.G.), protein assay dye solution from Bio-Rad Labs. (Richmond, CA, U.S.A.) and inorganic ^{32}P from Amersham International (Amersham, U.K.). Carrier-free [γ-^{32}P]ATP (2.5 Ci/mmol) and [γ-^{32}P]GTP (2.5 Ci/mmol) were prepared according to the procedure of Post and Sen [4]. Radioactive ATP and GTP were devoid of any inorganic phosphate, as determined by the method of Dobersen [5].

Enzyme preparation

Maize seedlings (40 g) were used for the purification of the enzymes. All steps were performed at 4° C. The following buffers were used. Buffer A: 50 m*M* Tris—HCl (pH 7.8) containing 10 m*M* magnesium chloride, 10 m*M* 2-mercaptoethanol and 20% glycerol. Buffer B: composed as buffer A to which 40 m*M* ammonium sulphate was added. Buffer C: 10 m*M* Tris—HCl (pH 7.8) containing 10 m*M* magnesium chloride, 10 m*M* 2-mercaptoethanol and 20% glycerol. Initial extraction of enzymes followed by ammonium sulphate fractionation is described elsewhere [3]. The ammonium sulphate pellet was dissolved in such a quantity of buffer A as to make the conductivity of the resulting solution equal to that of the equilibration buffer B. After centrifugation for 30 min at 18 000 *g*, the supernatant was added to 100 ml of phosphocellulose P11, previously equilibrated with buffer B. The suspension was gently mixed for 90 min and washed four times by decantation, using 500 ml of buffer B each time. The cellulose was than transferred to a column and proteins were eluted with a linear gradient of 0.1—1.5 *M* potassium chloride in buffer A. The elution was performed at 20 ml/h and 4-ml fractions were collected. The protein content of each fraction was determined by the method of Bradford [6] using BSA as standard. Appropriate fractions with enzymatic activity were pooled and concentrated in the Amicon filtration chamber (UM-10 membrane) to about one quarter of the volume. Almost 100% enzymatic activity was recovered in the pooled fractions and about 120-fold purification was achieved. Protein kinase assays were performed as previously described [3]. One unit of kinase activity was defined as the amount of enzyme catalysing the transfer of 1 nmol of the terminal phosphate group from ATP or GTP to a protein substrate (casein) over 20 min at 37° C. Specific activity was defined as U/mg of protein. The partially purified enzymes might be stored at —80° C for an extended period of time. Cyanogen bromide activation of Sepharose 4B and coupling of casein was performed as described earlier [7, 8]. Polyacrylamide gel (10%) electrophoresis (PAGE) was performed in the presence of 0.1% sodium dodecyl sulphate (SDS), as described earlier [9].

RESULTS

The enzymatic preparation obtained after phosphocellulose column chromatography was dialysed overnight against 2 l of buffer C, and divided into two equal parts. One part of the partially purified enzyme was applied to a 4-ml column of 5'-AMP-Sepharose, previously equilibrated with buffer C. After applying the sample, the column was washed with ca. 40 ml of buffer C until the protein concentration in the washing was lower than 0.1 μg/ml. The elution of the enzymes was performed in a stepwise fashion at a flow-rate of 12 ml/h, and 2-ml fractions were collected.

The first peak of casein kinase activity (named fraction 1) was eluted with 0.1 *M* potassium chloride in buffer C (Fig. 1).

After elution with 20 ml of the above buffer, the column was treated with 20 ml of 0.2 *M* potassium chloride in buffer C. A second peak of casein kinase (named fraction 2) was eluted at this higher ionic strength (Fig. 1). During further elution with higher salt concentration (0.6 *M* potassium chloride in buffer C followed by 1 *M* potassium chloride in the same buffer), no enzymatic activity was observed.

The second half of the original dialysate was chromatographed on a casein-Sepharose column under the same conditions as above. Two different activities were separated (fractions 1 and 2) at 0.1 *M* potassium chloride in buffer C and at 0.2 *M* potassium chloride in buffer C, respectively (Fig. 2).

The purification and elution profiles of the two protein kinases obtained by the two affinity absorbents described above were similar (Table I). Purification

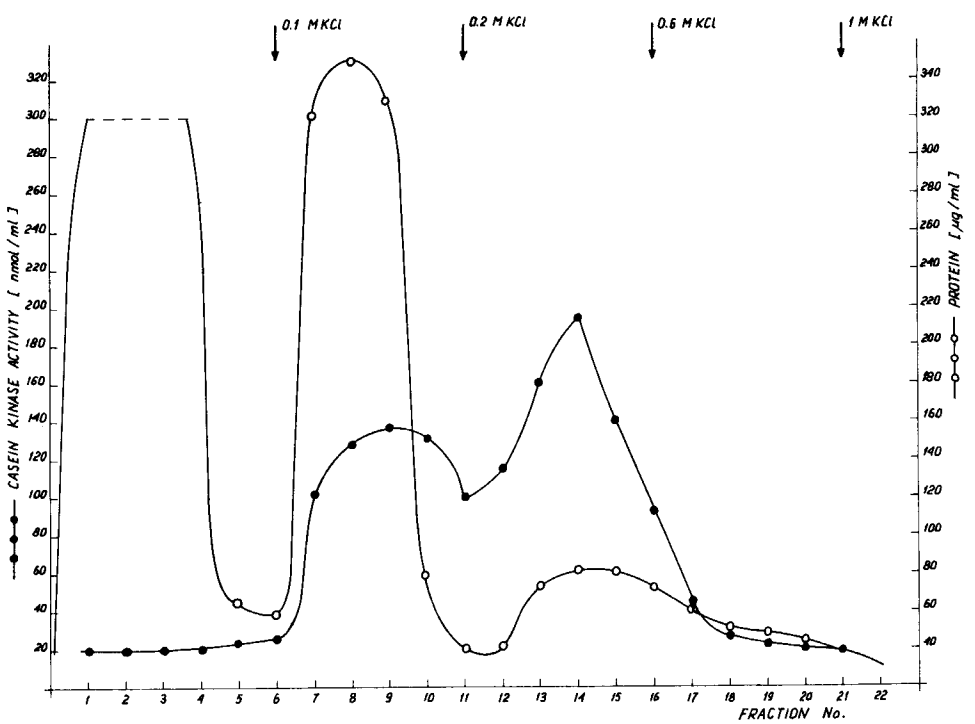

Fig. 1. Elution profile of casein kinases from 5'-AMP-Sepharose column.

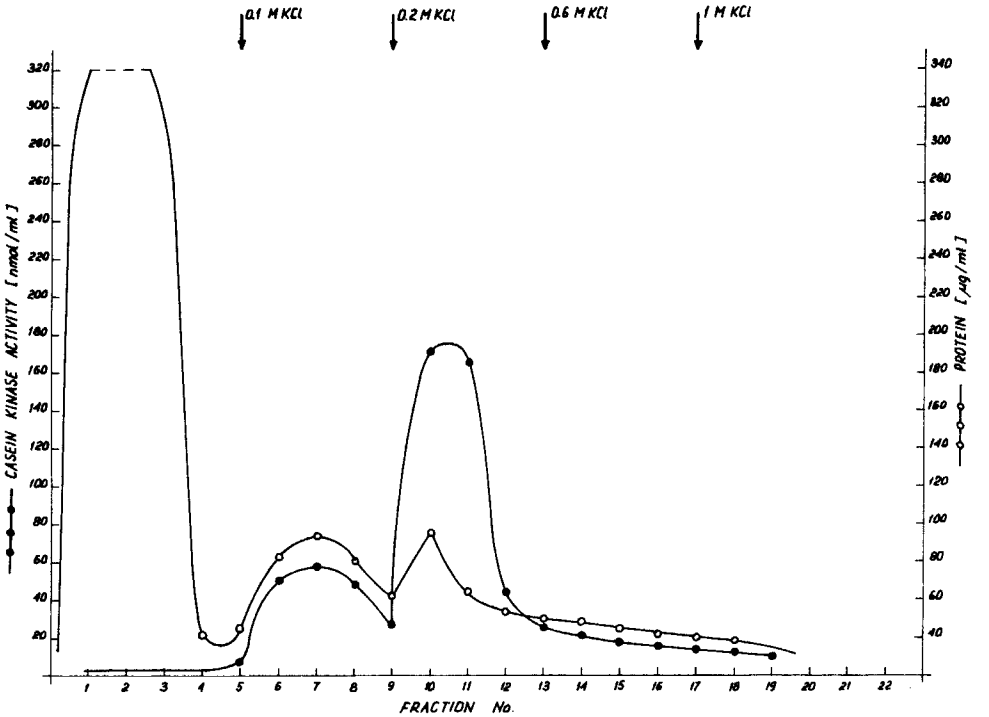

Fig. 2. Elution profile of casein kinases from casein-Sepharose column.

TABLE I

SEPARATION OF CASEIN KINASES BY AFFINITY CHROMATOGRAPHY

Column	Total		Purification factor related to	
	Protein (mg)	Activity (U)	Eluate from P-cellulose	Initial homogenate
5'-AMP-Sepharose				
Fraction 1	1.3	439	1.9	218
Fraction 2	0.4	535	7.5	891
Casein-Sepharose				
Fraction 1	1.1	486	2.5	284
Fraction 2	0.3	415	7.8	918

of the two kinases (fractions 1 and 2) by these methods was about two- and eight-fold, respectively, with ca. 40% recovery. One has to take into consideration that the overall purification of the two enzymes (fractions 1 and 2) with respect to the initial homogenate was about 250- and 900-fold, respectively.

Increasing concentrations of heparin were investigated for their effect on the enzymatic activities of fractions 1 and 2. The results, shown in Fig. 3, indicate that fraction 1 was strongly inhibited by heparin (50% of enzymatic activity inhibition, I_{50}, at 0.2 µg/ml). For inhibition of the kinase activity of fraction 2, a 200-times higher concentration of heparin was needed (I_{50} at 40 µg/ml). Moreover, the two activities displayed sharp contrast in the phosphorylation of

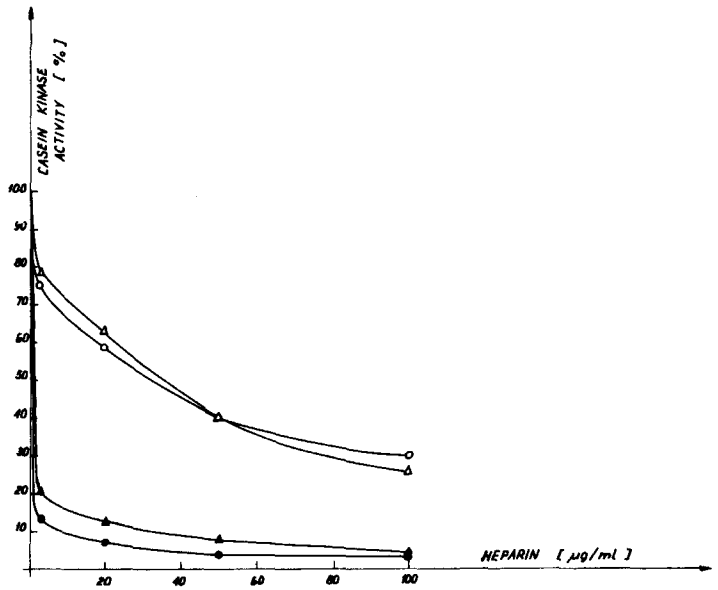

Fig. 3. Effect of increasing concentrations of heparin on the casein kinases activity: (▲) fraction 1 eluted from 5'-AMP-Sepharose; (△) fraction 2 eluted from 5'-AMP-Sepharose; (●) fraction 1 eluted from casein-Sepharose; (○) fraction 2 eluted from casein-Sepharose.

amino acids of the substrate. Amino acid hydrolysis followed by paper chromatography indicated that fraction 1 was able to phosphorylate serine and threonine within the casein molecule, whereas serine was exclusively phosphorylated by fraction 2 (data not shown). Fraction 1 of the protein kinase can utilize both ATP and GTP as phosphate donors. Using casein as an acceptor, the kinase activity with GTP as substrate was approx. 70% of that with ATP. In contrast to fraction 1, fraction 2 preferentially utilized ATP over GTP (7% of phosphorus incorporation with GTP) (Table II).

The electrophoretic pattern on SDS—PAGE has shown differences between fractions 1 and 2. Fraction 2 exhibited one protein band at molecular weight (M_r) ca. 30 000, whereas the electrophoretic pattern of fraction 1 was complex, with multiple bands of markedly higher molecular weight (Fig. 4).

TABLE II

DONOR PHOSPHATE SPECIFICITY OF SEPARATED CASEIN KINASES

Column	Specific activity (U/mg)		GTP/ATP
	ATP	GTP	
5'-AMP-Sepharose			
Fraction 1	358	247	0.69
Fraction 2	1238	74	0.06
Casein-Sepharose			
Fraction 1	386	278	0.72
Fraction 2	1368	109	0.08

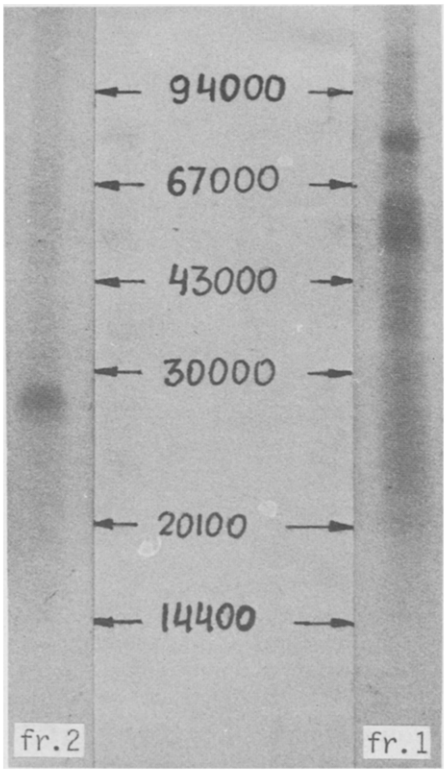

Fig. 4. SDS–polyacrylamide gel electrophoresis of kinases eluted from casein-Sepharose column.

The results obtained concerning separation and identification of casein kinase fractions were found to be reproducible in six performed experiments.

DISCUSSION

The most common application of affinity chromatography is the biospecific purification of proteins. This technique can also be used for separation of different classes of the same enzyme, by exploiting their differential affinity for substrates, substrate analogues and inhibitors. Recently, we reported separation of casein from histone kinases by use of a heparin-agarose column [10]. Here, we present conditions that permit separation of two classes of casein kinases (fractions 1 and 2), both on casein-Sepharose and on 5′-AMP-Sepharose columns.

Heparin proves to be 200-times more effective for inhibition of fraction 1 than of fraction 2 casein kinases from maize seedlings. Heparin specifically inhibits the type TS casein kinase, a cyclic nucleotide-independent protein kinase S [11]. Fraction 1 casein kinase utilizes either ATP or GTP as phosphate donors. Casein kinase from fraction 2 exclusively uses ATP for the protein phosphorylation. Hathaway and Traugh [1] have shown that one property characteristic for the type S kinase is an absolute specificity for ATP as the phosphate donor. Type TS casein kinase utilizes ATP or GTP as the phosphate

donor. Our fraction 1 kinase phosphorylates serine and threonine residues, whereas our fraction 2 kinase modifies only serine residues. Further evidence for the separate identities of fraction 1 and fraction 2 kinases is presented by the observation of their distinctive SDS–electrophoretic patterns. Fraction 2 kinase migrates as a single protein band with an M_r of ca. 30 000. Type S casein kinases from other sources display a single band of similar molecular weight [1]. The electrophoretic patterns of fraction 1 revealed multiple bands with markedly higher molecular weight, but it has not yet been resolved which of these bands represents kinase or its subunits.

The above data allow us to suggest that fractions 1 and 2 represent type TS and S casein kinases, respectively. The increased affinity of fraction 2 kinase for immobilized AMP may be a reflection of its absolute specificity for ATP as a phosphate donor. Similarly, the known clustering of serine residues in an acidic surface region of casein [12] may account for the increased affinity of serine-specific fraction 2 (relative to fraction 1) for the immobilized protein substrate. In this case, known differences in specificities toward enzyme substrates have been utilized to separate two distinct subgroups of maize casein kinases.

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