Isolation and Preliminary Characterization of a Casein Kinase from Cauliflower Nuclei

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ABSTRACT

A casein-type protein kinase has been isolated from cauliflower (Brassica oleracea var. botrytis) nuclei and purified to a specific activity of 23,000 units/mg of protein (1 unit is defined as the transfer of 1 pmol of [32P]ATP to substrate per minute at 25°C). The enzyme has a molecular weight of approximately 39,000 as judged by sucrose density gradient sedimentation. The casein kinase requires ATP as the phosphate donor and will phosphorylate casein and phosvitin, but not histones. The enzyme activity is not affected by cAMP or cGMP. The casein kinase appears to be analogous to casein kinases described in other plant and animal systems.

MATERIALS AND METHODS

Buffers. Buffer A contained 20 mM MES (pH 6.0), 20 mM MgCl2, 20 mM KCl, 0.25 mM sucrose, 10 mM 2-mercaptoethanol, and 40% (v/v) glycerol. Buffer B contained 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 10 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 1% (v/v) dimethylsulfoxide, 0.1% (v/v) Triton X-100, and 30% (v/v) glycerol. Buffer O contained 50 mM Tris-HCl (pH 8.0), 10 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 1% (v/v) dimethylsulfoxide.

Casein Kinase Assays. γ-[32-P]ATP was prepared enzymically (18). Unless otherwise stated, casein kinase assays contained in a final volume of 0.2 ml, 100 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 10 mM DTT, 25 μM ATP (75–150 cpm/pmol) and 500 μg/ml casein (Sigma). Casein kinase assays were initiated with 5 μl of casein kinase preparation, incubated for 30 min at 28°C, and terminated with 3 ml of cold 10% (w/v) trichloroacetic acid containing 10 mM Na-pyrophosphate. Precipitates were collected on GF/A glass fiber discs (Whatman) washed with 15 ml of cold 10% (w/v) trichloroacetic acid followed by 3 ml of 95% ethanol, and dried. Radioactivity was determined in a toluene-based scintillator. Assays were performed in triplicate and values corrected for nonspecific [32P]Pi trapped during precipitation (typically 80–150 cpm/assay). A unit of casein kinase is defined as the amount of enzyme that catalyzes the transfer of 1 pmol of [32P]Pi from γ-[32P]ATP to casein/min at 28°C.

Casein Kinase Solubilization and Gel Filtration. Solubilization and gel exclusion chromatography follow the techniques described by Guilfoyle (6) for the isolation of RNA polymerase from cauliflower. A nucleus-enriched fraction was prepared from cauliflower inflorescence with buffer A as described by Chen et al. (2). Protein kinase was solubilized from the nuclear fraction by shearing the preparation in buffer B containing 0.5 mM ammonium sulfate with a polytron (Brinkmann Instruments) followed by sonication with a Branson microtip sonicator. The solubilized casein kinase was recovered in the supernatant after centrifugation for 60 min at 50,000 rpm (Spinco type 60 rotor). The supernatant was diluted to 100 mM ammonium sulfate with buffer B. After recentrifugation (60 min, 50,000 rpm) the casein kinase in the supernatant was precipitated by the gradual addition of solid ammonium sulfate (0.35 g/ml) and stirring for 30 min. The precipitate was recovered by centrifugation (60 min, 55,000 rpm) and suspended in 40 ml of buffer B containing 500 mM ammonium sulfate.

The soluble casein kinase was loaded onto a column (90 × 2.5 cm) of agarose A-1.5m (Bio-Rad) equilibrated with buffer B containing 500 mM ammonium sulfate and eluted with the same buffer at a flow rate of 0.25 ml/min. Five-ml fractions were collected, aliquots assayed for casein kinase activity, and the peak fractions were pooled and precipitated with ammonium sulfate as described above.

Ion Exchange Chromatography. DEAE-cellulose (Whatman) and phosphocellulose (Whatman) were prepared as described previously (4). The ammonium sulfate precipitate from the agarose...
fractionation was suspended in buffer B containing 25 mM ammonium sulfate and loaded onto a column (30 x 1.5 cm) of DEAE-cellulose equilibrated in the same buffer. The column was washed with 1 column volume of buffer C containing 25 mM ammonium sulfate. Essentially none of the casein kinase activity is retained on the column under these conditions. Elution of the DEAE-cellulose column with 25 to 400 mM ammonium sulfate gradient did not elute any additional casein kinase activity.

After passage over DEAE-cellulose, the casein kinase was loaded directly onto a column (30 x 1.5 cm) of phosphocellulose equilibrated in buffer C containing 25 mM ammonium sulfate. After the column was washed with 2 column volumes of buffer C containing 25 mM ammonium sulfate, casein kinase was eluted with a 25 to 400 mM ammonium sulfate gradient. The volume of the gradient was 2 column volumes and 1.5-ml fractions were collected. Casein kinase eluted from 50 to 150 mM ammonium sulfate. Peak fractions were concentrated by ammonium sulfate precipitation as described above.

Sucrose Gradient Sedimentation. The ammonium sulfate precipitate after phosphocellulose was dissolved in buffer C containing 250 mM ammonium sulfate and loaded onto 5 to 20% (v/v) isokinetic sucrose gradients in buffer C containing 250 mM ammonium sulfate. The gradients were centrifuged for 16 hr at 48,000 rpm in a Beckman SW 50.1 rotor. Gradients were fractionated and aliquots of each fraction assayed for casein kinase activity. By the sedimentation of BSA (included as an internal standard in one gradient), the mol wt was calculated according to Martin and Ames (14).

Ammonium Sulfate and Protein Determinations. Ammonium sulfate concentrations were determined with a Bausch & Lomb refractometer. Protein was determined according to Lowry et al. (13) after the samples were precipitated with cold 10% (v/v) trichloroacetic acid.

RESULTS

Casein Kinase Purification. Cauliflower inflorescence is a rich source of casein kinase from higher plants. The nucleus-enriched fraction from which the casein kinase was solubilized contains approximately equal amounts of RNA polymerase I and II activity (6). Both casein kinase and RNA polymerase activities are solubilized quantitatively by the procedure described. Casein kinase was initially resolved from RNA polymerase by agarose A-1.5m gel filtration at high ionic strength (Fig. 1a). The high ionic strength used during gel filtration is necessary to prevent aggregation of both casein kinase and RNA polymerase with contaminating nucleic acids and proteins. Elution of the agarose column at low ionic strengths results in both enzyme activities eluting as an aggregate fraction in the void volume. Casein kinase may be further purified by DEAE-cellulose ion exchange chromatography. Although cauliflower casein kinase does not bind to DEAE-cellulose at 25 mM ammonium sulfate, passage over this anion exchange resin does give significant purification by removal of contaminating proteins. Further purification of the casein kinase activity is achieved by phosphocellulose ion exchange chromatography (Fig. 1b). Sucrose density gradient sedimentation gives a further 3-fold purification (Fig. 1c). Casein kinase activity sediments as a single symmetrical peak of 3.1 ± 0.9S corresponding to a mol wt of 39,000 ± 6,000 daltons (average and standard deviation of eight determinations).

The purification scheme for casein kinase is summarized in Table 1. The over-all purification based on the enzyme applied to the agarose column was approximately 80-fold with a recovery of 24%. Although the final specific activity is higher than generally reported for comparable casein kinases from animal systems, the preparation was not homogeneous. Electrophoresis of the preparation on polyacrylamide in the presence of SDS revealed the presence of three major polypeptides of approximately 32,000, 43,000, and 73,000 daltons. The casein kinase is stable indefinitely if frozen at -20 C in the presence of 30% glycerol and 0.5 mM phenylmethylsulfonylfluoride.

Casein Kinase Characterization. Casein kinase activity shows a broad pH curve reaching a plateau at about pH 7.5 (Fig. 2). Broad pH curves have been observed in a number of other systems and are thought to be the result of increased casein solubility at higher pH (19). Phosphorylation by casein kinase requires a

![Fig. 1](https://example.com/f1.png)

**Fig. 1.** (a): agarose A-1.5m gel exclusion chromatography. Solubilized casein kinase was loaded onto an agarose column (90 x 2.5 cm) and eluted as described under "Materials and Methods." Dashed line shows relative elution profile of cauliflower RNA polymerase activity (6). Fractions 55 through 80 were pooled for further purification. (b): phosphocellulose ion exchange chromatography. After passage over DEAE-cellulose, casein kinase was bound to a phosphocellulose column (30 x 1.5 cm) and eluted with a 25 to 400 mM ammonium sulfate gradient. Fractions 30 through 45 were pooled for further purification. (c): sucrose gradient sedimentation. Casein kinase was centrifuged on 5 to 20% isokinetic sucrose gradients as described under "Materials and Methods."

**Table 1. Summary of cauliflower casein kinase purification.** Details of purification are described in Methods. A unit of casein kinase activity is defined as the transfer of 1 pmol of 32P from [γ-32P]ATP to casein per min at 28 C.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total units</th>
<th>% recovered</th>
<th>Units/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Applied Sample</td>
<td>6.5 x 10^6</td>
<td>100</td>
<td>320</td>
</tr>
<tr>
<td>Agarose</td>
<td>4.0 x 10^6</td>
<td>62</td>
<td>970</td>
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<tr>
<td>DEAE-cellulose</td>
<td>3.7 x 10^6</td>
<td>57</td>
<td>1770</td>
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<tr>
<td>Phosphocellulose</td>
<td>3.46 x 10^6</td>
<td>53</td>
<td>8260</td>
</tr>
<tr>
<td>Sucrose gradient</td>
<td>1.6 x 10^6</td>
<td>24</td>
<td>23,670</td>
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</table>
divalent cation. When tested over the range of 0 to 50 mM Mg\(^{2+}\), casein kinase showed optimal activity with 5 mM Mg\(^{2+}\); Mn\(^{2+}\) at 1 mM gave about 60% of the maximal activity observed with Mg\(^{2+}\), but Ca\(^{2+}\) would not support phosphorylation at any concentration tested (data not shown).

Phosphorylation donor specificities were investigated by attempting to compete out incorporation from labeled ATP with excess unlabeled CTP, GTP, or UTP (Table II). Addition of 6-fold excess unlabeled ATP competes out label incorporation proportionally, but CTP, GTP, or UTP will not compete incorporation from labeled ATP. The apparent $K_m$ for ATP with the optimal Mg\(^{2+}\) concentration (5 mM) is about $2 \times 10^{-6}$ M (data not shown), and activity is inhibited by ATP concentrations greater than 200 $\mu$M. While the $K_m$ for ATP is higher than that reported for either of the soybean kinases (12, 15), it is substantially lower than is typically observed in animal systems (4, 8). Neither cAMP nor cGMP has any effect on casein kinase activity.

Phosphorylation acceptor specificities are shown in Table III. Casein kinase will phosphorylate casein and phosvitin, but not histones, to high levels. The low levels of incorporation in the absence of any additional substrate indicate that the enzyme preparation is not contaminated with casein kinase substrates. SDS-polyacrylamide gel electrophoresis followed by autoradiography failed to show any autophosphorylation of casein kinase after incubation in the presence of labeled ATP (data not shown).
from control and auxin-treated soybean hypocotyl. Plant Physiol 56: 78-82
9. GUILFOYLE TJ, CY LIN, YM CHEN, JL KEY 1976 Purification and characterization of RNA polymerase from a higher plant. Biochim Biophys Acta 418: 344-357