Purification and Properties of a High Specific Activity Protein Kinase from Wheat Germ[1]

JEFFREY R. DAVIES AND GIDEON M. POLYA
Department of Biochemistry, La Trobe University, Bundoora, Victoria 3083 Australia

ABSTRACT

A protein kinase was extensively purified to near-homogeneity from wheat germ by a procedure involving affinity chromatography on casein-Sepharose 4B, gel filtration, and repeated chromatography on carboxy-methyl-Sepharose CL-6B. The protein kinase preparations have the highest specific activities (up to 656 nanomoles phosphate incorporated per minute per milligram of protein) yet reported for plant protein kinases. The major polypeptides in purified preparations were revealed as two barely-resolved bands (molecular weight 31,000) on polyacrylamide gel electrophoresis in subunit-dissociating conditions. The molecular size of the protein kinase as determined from gel filtration is 30,000. The protein kinase catalyzes the phosphorylation of casein, phosvitin, and the wheat germ cyclic AMP-binding protein cABPII but not of bovine serum albumin and histones nor of the wheat germ cytokinin-binding protein CBP. The protein kinase has a pH optimum of 7.9 and a K_m value for ATP of 10 micromolar. The protein kinase differs from wheat germ CBP kinase in molecular weight, differential sensitivity to inhibitors, and in substrate specificity.

Protein phosphorylation-dephosphorylation represents an important means of enzyme regulation in eukaryote cells and a key process involved in hormone action in animal cells through the modulation of specific protein kinases by cyclic AMP, cyclic GMP, or Ca^{2+} (7). Proteins known to be phosphorylated in higher plants include chromosomal proteins (13; see 27), thylakoid proteins (1, 26), ribosome-associated proteins (21, 24, 25; see 27), RNA polymerase (10), and pyruvate dehydrogenase (18). Phosphorylation of pyruvate dehydrogenase results in inactivation of the enzyme complex (18), and phosphorylation of wheat embryo protein synthesis initiation factor eIF-2 causes inactivation of the factor and inhibition of translation (19, 24). The phosphorylation of the chloroplast light-harvesting Chl a/b-binding protein appears to be involved in regulation of the distribution of absorbed excitation energy between the two photosystems, and the phosphorylation of this protein is light dependent (1). The mechanism of regulation of other plant protein phosphorylation reactions is unknown.

Protein kinases have been resolved from higher plants (2, 5, 6, 8, 10, 12, 16, 17, 22) but no cyclic nucleotide-dependent or Ca^{2+}-calmodulin-activated plant protein kinases have been reported. Highly purified plant protein kinase preparations have been isolated with specific activities in the range of approximately 10 to 200 nmol phosphate transferred/min·mg of protein (5, 6, 8, 10, 12, 16, 22). Two casein-phosphorylating protein kinases (mol wt 39,000 and 120,000) have been purified to apparent homogeneity from soybean cotyledons (6), and electrophoretic homogeneity has

also been obtained for a wheat germ protein kinase (22). This latter enzyme preparation phosphorylates casein and phosvitin, contains an approximately 20,000 D subunit, and has a specific activity of 215 nmol/min·mg protein (22). This enzyme differs from another wheat germ casein-phosphorylating protein kinase, CBP kinase[2], in not phosphorylating the wheat germ cytokinin-binding protein CBP (16). The present paper describes the purification and properties of a further wheat germ casein-phosphorylating kinase that differs in many properties from the protein kinase purified by Rychlik and Zagórski (22) and is distinct from CBP kinase (16) in not phosphorylating CBP.

MATERIALS AND METHODS

Protein and Protein Kinase Assays and Electrophoresis. Protein kinase was assayed routinely with 100 μg dephosphorylated casein as substrate as described previously (16). Protein was determined by the Folin procedure (9) using crystalline BSA as a standard or by measuring A_{280}. Electrophoresis in 0.1% SDS-10% (or 15%) polyacrylamide slab gels, hydrolysis of ^{32}P-labeled protein, and high-voltage electrophoresis of phosphorylated amino acids were conducted as described previously (16).

Plant Material and Chemicals. Raw wheat germ and chemicals were obtained as described previously (16). CM-Sepharose CL-6B was obtained from Pharmacia Fine Chemicals AB. cABPII was purified to homogeneity as described previously (15). BSA, dephosphorylated casein (partially hydrolyzed and dephosphorylated for protein kinase studies), calf thymus histones (Sigma type II-A), wheat seed α-amylase inhibitor, and wheat germ lectin were obtained from Sigma Chemical Co. CBP and CBP kinase were purified, and casein was coupled to cyanogenbromide-activated Sepharose 4B as described in the accompanying paper (16).

Purification of Casein Kinase. All purification steps were conducted at 0 to 4°C. Wheat germ (500 g) was suspended in 1.8 L of 50 mM Tris (Cl⁻, pH 8.0) (buffer A) containing 0.1 M NaCl and homogenized for 5 min at top speed with an Ultra-Turrax blender (Janke and Kunkel, Staufen, West Germany). The homogenate was filtered through muslin, and the filtered homogenate was centrifuged at 16,000g for 45 min. The resulting supernatant was filtered through Miracloth, and the filtered supernatant was added to about 200 ml bed volume of casein-Sepharose 4B (3.5–4.4 mg casein bound/g wet weight of gel) in 0.1 M NaCl-buffer A. The suspension was stirred intermittently over 15 min and then washed on a sintered glass funnel with about 2 L 0.1 M NaCl-buffer A. The washed gel was resuspended in 0.1 M NaCl-buffer A and packed in a column (16 cm² × 12 cm). The column was eluted stepwise with about 1 L 0.2 M NaCl-buffer A followed by about 200 ml 0.5 M NaCl-buffer A to elute the casein kinase. The protein

[2] Abbreviations: CBP kinase, wheat germ protein kinase phosphorylating CBP; CBP, wheat germ cytokinin-binding protein; cABPII, wheat germ 3′,5′-cyclic AMP-binding protein II.

kinase solution was concentrated to 6 ml by ultrafiltration using an Amicon UM10 filter. The concentrated protein kinase solution was applied to a column (4.9 cm^2 × 70 cm) of Ultrogel AcA 34 equilibrated with 0.25 M NaCl-buffer A, and the protein kinase was eluted with the same buffer. The high specific activity protein kinase fractions from gel-filtration on Ultrogel AcA 34 were pooled. The pooled protein kinase solution was diluted 2.5-fold with buffer A (to give 0.1 M NaCl, final concentration), made 2 μM with respect to ATP, and then was applied to a column (5 cm^2 × 6 cm) of CM-Sepharose CL-6B equilibrated with 0.1 M NaCl-buffer A containing 2 μM ATP. The CM-Sepharose CL-6B column was washed with about 100 ml of 0.1 M NaCl-buffer A-2 μM ATP and then eluted with a linear gradient of increasing NaCl concentration (from 0.1–0.4 M in buffer A-2 μM ATP). High specific activity protein kinase fractions were pooled. In some preparations, a second chromatography on CM-Sepharose CL-6B was carried out in order to achieve specific protein kinase activities of greater than 500 nmol phosphate incorporated/min · mg of protein.

RESULTS

Purification of Wheat Germ Casein Kinase. The wheat germ protein kinase purified in this study catalyzes the phosphorylation of casein but not of CBP. Accordingly, we will refer below to this protein kinase as 'casein kinase' and to the distinct wheat germ protein kinase that catalyzes the phosphorylation of CBP and casein as 'CBP kinase.'

Table I summarizes the purification of casein kinase from wheat germ. The key step in the purification protocol involves chromatography of the initial high speed supernatant through a casein-Sepharose 4B column—of 47,000 mg of protein (from 500 g wheat germ) in the supernatant applied to the column, only 52 mg are retained in the casein kinase fraction (Table I). Because of the presence in the starting supernatant of many potentially interfering agents (and a multiplicity of protein kinases), one cannot determine the yield of the casein kinase from this initial affinity chromatography step. Indeed, there is an apparent 2-fold increase in casein kinase activity after this step (Table I). However, a maximum estimate of the purification achieved (assuming no loss of casein kinase activity at this stage) is about 1,000-fold. The bulk of the casein kinase is eluted from the casein-Sepharose 4B column by 0.5 M NaCl-buffer A; only about 20% of the total retained casein phosphorylating activity is eluted in the prior 0.2 M NaCl-buffer A washing. Extensive washing of the column with 0.2 M NaCl-buffer A was required to achieve high specific activities of the bulk casein kinase and to remove a large proportion (80% of total) of CBP kinase. When a 0.2 M NaCl-buffer A wash of the

Table I. *Purification of Wheat Germ Casein Kinase*

Wheat germ (500 g) was extracted; the yields at each stage have been corrected for losses due to sampling for analysis. Protein kinase activity was determined as described in "Materials and Methods". Protein was determined from A_{280} taking $E_{280}^{0.1\%} = 0.61$ for the purified casein kinase.

Fractionation Stage	Protein	Protein Kinase	Protein Kinase Specific Activity
	mg	*nmol phosphate incorporated/min*	*nmol/min·mg protein*
High-speed supernatant	47,000	743	0.016
Casein-Sepharose 4B	52	1,600	31
Ultrogel AcA 34	10.5	823	78
CM-Sepharose CL-6B (1)	2.3	580	245
CM-Sepharose CL-6B (2)	0.5	220	478

casein-Sepharose 4B column was not employed, the specific activity of the casein kinase eluted in 0.5 M NaCl-buffer A was only about 7 nmol/min·mg protein as opposed to 29 ± 5 nmol/min· mg protein (mean ± SD from five preparations) obtained after extensive washing in 0.2 M NaCl-buffer A. After affinity chromatography on casein-Sepharose 4B, the active bulk casein kinase fractions were concentrated by ultrafiltration prior to gel filtration. Concentration by ultrafiltration resulted in yields of greater than 50%. Subsequent gel filtration on Ultrogel AcA 34 in 0.25 M NaCl-buffer A resolved one major peak of casein kinase activity (Fig. 1). ATP (2 μM) was included in the buffers from this gel filtration step onwards since high specific activity preparations of wheat germ casein kinase resolved by Rychlik and Zagórski [22] were stabilized by inclusion of ATP. The present casein kinase preparation loses all activity in 1 week at 4°C in the absence of ATP. In the final purification step, the casein kinase was eluted from CM-Sepharose CL-6B with a linear gradient of increasing NaCl concentration. At pH 8.0, the peak casein kinase is eluted with a major peak of A_{280} at about 0.2 M NaCl concentration; constant specific protein kinase activity is obtained on the trailing edge of the peak (Fig. 2). In some preparations, a second gradient elution from CM-Sepharose CL-6B was required to achieve specific activities of about 500 nmol/min·mg of protein (Table I). The maximum specific activity observed for a final preparation of casein kinase was 656 nmol/min·mg protein. The $E_{280}^{0.1\%}$ for the purified casein kinase is 0.61 ± 0.02 (mean ± SD from six determinations). The purification protocol consistently yielded final preparations with specific activities of about 500 nmol phosphate incorporated/ min·mg protein as measured in the standard assay with dephosphorylated casein as the protein substrate. Final preparations were devoid of significant CBP kinase activity—this was largely removed at the casein-Sepharose 4B stage with further traces eluting just prior to the peak of casein kinase on both gel filtration on Ultrogel AcA 34 and gradient elution from CM-Sepharose 4B. These preparations lose 29% of activity in 1 week at 4°C.

Molecular Size and Subunit Composition of Casein Kinase. The apparent molecular size of casein kinase is 30,000 ± 3,000 (mean ± SD from three determinations) as determined from gel filtration on an Ultrogel AcA 34 column (4.0 cm^2 × 70 cm) in 0.25 M NaCl-buffer A. The Ultrogel AcA 34 column was eluted with buffer A containing 0.25 M NaCl to avoid possible protein aggregation and was calibrated by determining the elution volumes of proteins of known mol wt including pyruvate kinase, fumarase, aldolase, BSA, ovalbumin, myoglobin, and Cyt *c*. Typically, SDS-polyacrylamide gel electrophoresis revealed one major band (mol wt 31,000 ± 1,000) (Fig. 3B) as well as traces of higher mol wt polypeptides (*e.g.* Fig. 3C). In many electrophoretic resolutions, the 31,000 D polypeptide zone was composed of two barely resolved bands (mol wt difference <500) (Fig. 3C). The intensity of the 31,000 D polypeptide band on SDS-polyacrylamide gels correlates with casein kinase activity when samples through the enzyme peak from CM-Sepharose CL-6B are subjected to electrophoresis (Fig. 3, A and B). This is consistent with the 31,000 D polypeptide being the casein kinase subunit. In addition, the 31,000 D polypeptide is the major polypeptide present in purified preparations and has a mol wt the same as the mol wt of the native casein kinase (as determined from gel filtration). The present evidence therefore suggests that the casein kinase has a mol wt of about 30,000 and is composed of one subunit.

Effects of pH, Divalent Cations, and Ionic Strength on Casein Kinase Activity. The casein kinase shows maximal activity at pH 7.9 and 50% of maximum activity at pH values of 4.4 and 9.4 (Fig. 4). The casein kinase activity is largely dependent upon added Mg^{2+}, with maximal activity being obtained at 15 mM MgCl$_2$ concentration; higher concentrations of MgCl$_2$ are inhibitory (Fig. 5). The casein kinase is not activated by CaCl$_2$, MnCl$_2$, nor by zinc acetate when these salts are included in assays at
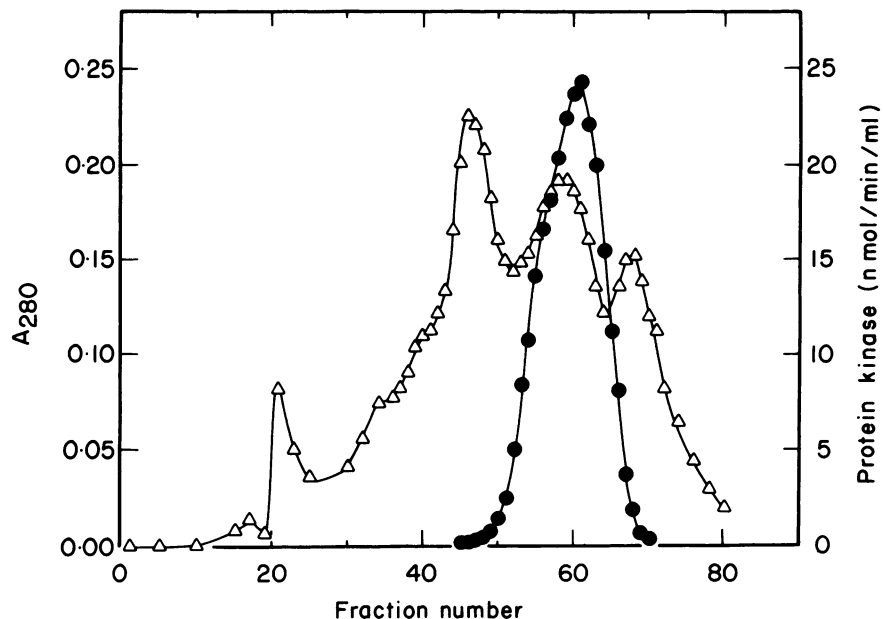
FIG. 1. Gel filtration of casein kinase on an Ultrogel AcA 34 column. Casein kinase eluted from casein-Sepharose 4B was concentrated and chromatographed on Ultrogel AcA 34 as described in "Materials and Methods." ($\triangle$), A_{280}; ($\bullet$), protein kinase activity determined in the standard assay conditions.
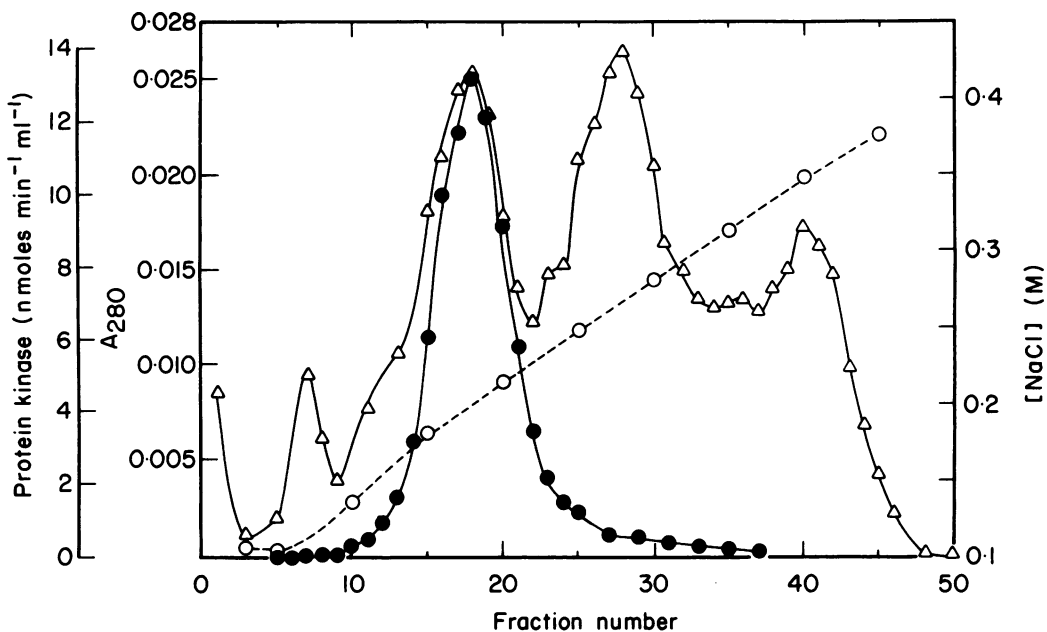

FIG. 2. Resolution of the casein kinase by chromatography on CM-Sepharose CL-6B. Casein kinase fractions from gel filtration (see Fig. 1) were applied to a column of CM-Sepharose CL-6B which was eluted with a gradient of increasing NaCl concentration as described in "Materials and Methods." ($\triangle$), A_{280}; ($\bullet$), protein kinase activity determined in the standard assay conditions; (O), NaCl concentration.

concentrations from 0.25 to 50 mM. When included in the standard assay (containing 10 mM $MgCl_2$), 1 mM $CaCl_2$, $CuCl_2$, and $BaCl_2$ cause only 27%, 21%, and 16% inhibition, respectively; inclusion of 1 mM $CoCl_2$ and 1 mM $MnCl_2$ inhibits 62% and 74%, respectively. Zn^{2+} and Hg^{2+} are potent inhibitors of the casein kinase—inclusion of 1 mM zinc acetate or 1 mM $HgCl_2$ in the standard assay causes 98% and 100% inhibition, respectively, of the enzyme. At 1 mM concentration, none of the divalent cations tested cause precipitation with casein as substrate in the assay.

The casein kinase is inhibited by elevated salt concentrations (Fig. 6). The casein kinase is inhibited 50% by 25 mM $(NH_4)_2SO_4$, 70 mM potassium phosphate, and by 190 mM NaCl, NH_4Cl, or KCl. Neither K-acetate nor Na-acetate are inhibitory when included in the assays at concentrations up to 200 mM in the presence

of 5 mM initial NaCl concentration (Fig. 6). In contrast, CBP kinase is inhibited 55% by 100 mM Na- or K-acetate in these conditions in the presence of 75 mM NaCl (see Ref. 16); in identical assay conditions at 75 mM NaCl concentration, 100 mM Na-acetate inhibited casein kinase by only 12%.

Substrate Specificity of Casein Kinase. Table II shows the substrate specificity of the casein kinase. Casein, phosvitin, and cABPII are phosphorylated by the purified casein kinase, which has absolute dependence on added protein substrate for activity (Table II). Neither BSA nor a preparation of calf thymus histones are phosphorylated by the casein kinase (Table II). Neither purified wheat seed α-amylase inhibitor (at 0.1 mg/ml) nor wheat germ lectin (at 0.4 mg/ml) are substrates for the casein kinase. The K_m values of the casein kinase for dephosphorylated casein
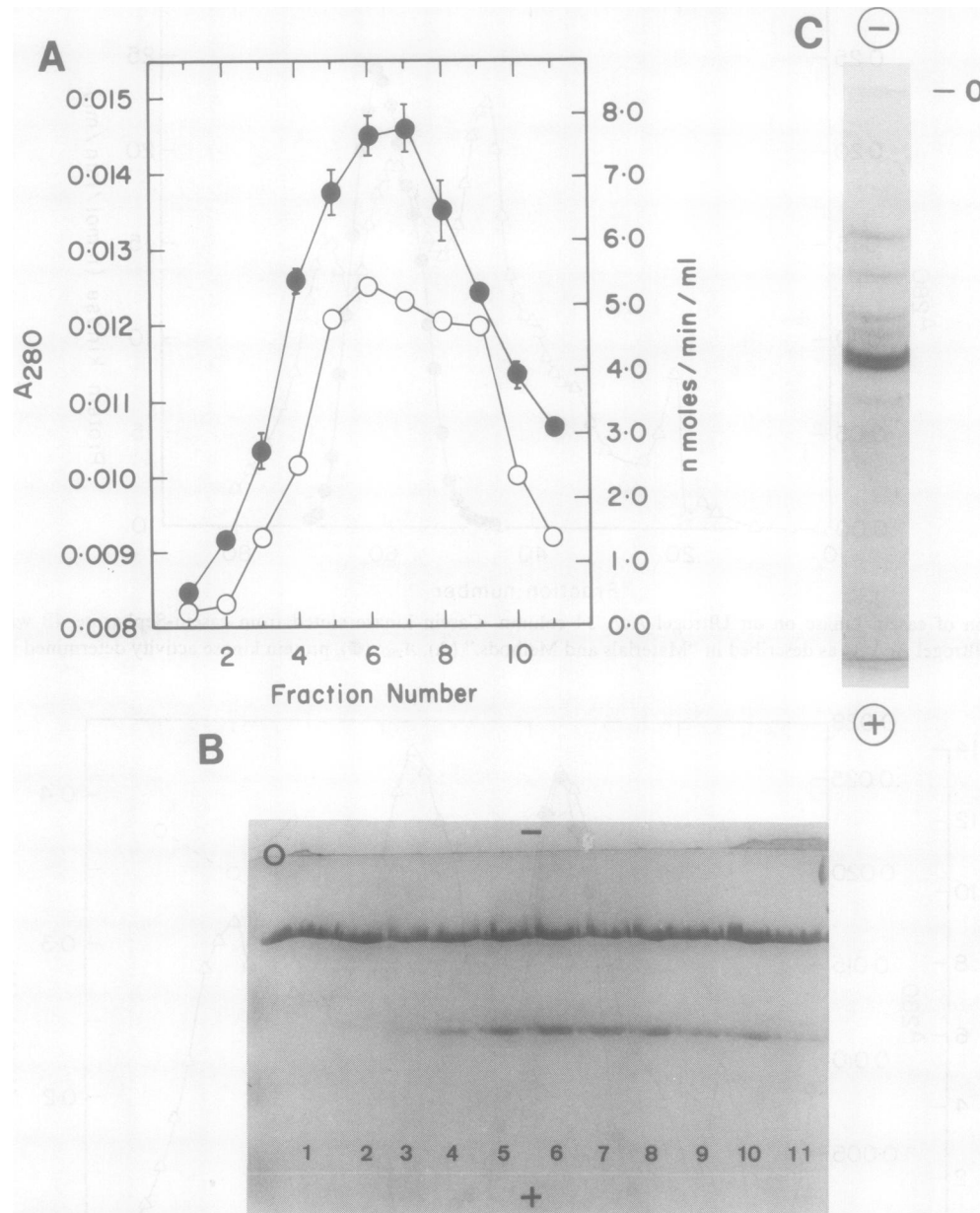
FIG. 3. SDS-polyacrylamide gel electrophoresis of purified casein kinase. A, Casein kinase fractions resolved on CM-Sepharose CL-6B. (○), A_{280}; (●), protein kinase (nmol/min·ml). B, Electrophoresis in 0.1% SDS-12.5% polyacrylamide gel of fractions from (A). Protein in 2-ml samples of each fraction was precipitated by addition of 0.2 ml 66% TCA in the presence of 20 μg BSA (as carrier protein), and the pellets were washed with ethanol-ether (1:1, v/v) before electrophoresis. The numbers refer to the fraction numbers in (A); the major lower mobility band corresponds to BSA; (O), origin. C, Electrophoresis of a highly purified casein kinase preparation in a 0.1% SDS-15% polyacrylamide gel. Ten μg of protein was applied.

and phosvitin in the standard assay conditions at pH 8.0 are 0.9 and 0.8 mg/ml, respectively. The K_m for ATP in the standard assay conditions with 2 mg/ml casein as substrate is 10 μM as determined from Lineweaver-Burk analysis. While inclusion of CTP, UTP, or ITP at 0.1 or 1.0 mM causes less than 15% inhibition of ^{32}P incorporation from [γ-^{32}P]ATP (25 μM) in the standard assay conditions, inclusion of 0.1 or 1.0 mM GTP causes 20% and 60% inhibition, respectively. In the same conditions, inclusion of 0.1 or 1.0 mM unlabeled ATP inhibits by 87% and over 99%, respectively.

^{32}P-labeled casein from reactions catalyzed by casein kinase was hydrolyzed in 6 N HCl at 100°C for 1 to 4 h, and the hydrolysates were subjected to high-voltage electrophoresis as described previously (15). Peaks of radioactivity corresponding to phosphothreonine (6% of total) and to phosphoserine (17%) were found, in addition to lower mobility material near the point of application

(37%) and a higher mobility anodic peak corresponding to Pi (24% of total).

Effects of Various Compounds on Casein Kinase Activity. No activation of the casein kinase by 3',5'-cyclic AMP (100 μM), N^6BA (100 μM), nor by kinetin (50 μM) was observed. Included at 0.1 mM in the standard assay, 5'-AMP, 2',3'-cyclic AMP, 3',5'-cyclic AMP, 3',5'-cyclic GMP, and 2',3'-cyclic GMP have no effect on casein kinase. At 1 mM concentration, 3',5'-cyclic GMP and 2',3'-cyclic GMP have no effect, while at 1 mM 5'-AMP, 2',3'-cyclic AMP, and 3',5'-cyclic AMP are slightly inhibitory, causing 14%, 20%, and 26% inhibition, respectively. Ten mM 3',5'-cyclic AMP causes 84% inhibition. At 0.1 mM concentration, ADP, adenosine, and adenine cause 64%, 45%, and 20% inhibition of the casein kinase, respectively; at 1 mM, 5'-ADP and adenosine cause 94% and 89% inhibition, respectively.
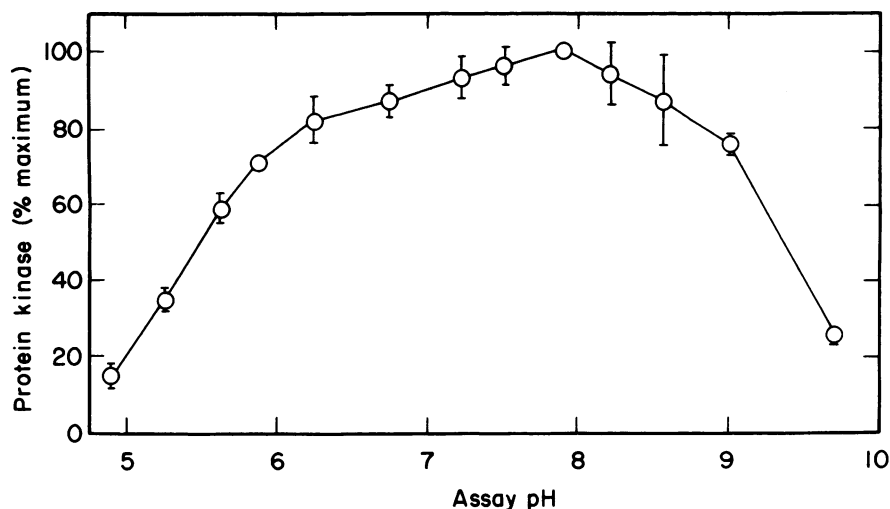
FIG. 4. Casein kinase activity as a function of assay pH. Standard reaction mixtures were buffered employing final concentrations of 50 mM acetate (Na$^+$, pH 4.9–5.6), 50 mM bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane (Cl$^-$, pH 5.9–7.2), 50 mM N-tris(hydroxymethyl)methylglycine (K$^+$, pH 7.5–8.6), and 50 mM glycine (K$^+$, pH 9.0–9.7). Values given represent the means of triplicate determinations, and the vertical bars indicate the SD.
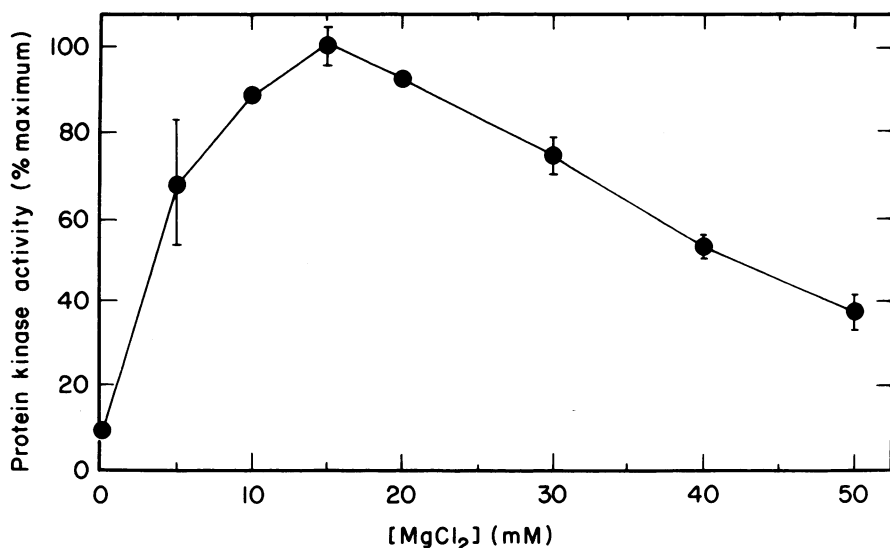

FIG. 5. Activation of casein kinase by MgCl$_2$. Casein kinase was measured as described in "Materials and Methods" in assays buffered with 20 mM Hepes (K$^+$, pH 8.1). Each point represents the mean of triplicate determinations, and the vertical bars indicate SD. Results are presented as percentage of maximum activity (obtained with 15 mM MgCl$_2$ in the assay).

The protein inhibitor of animal cyclic AMP-dependent protein kinase (4) does not inhibit the casein kinase when included in the standard assay at 1 mg/ml. In contrast, hemin, which inhibits cyclic AMP-dependent protein kinase and the phosphorylation of eukaryote initiation factor 2 (14, 23), completely inhibits the casein kinase when present at 1 mg/ml. In experiments involving 5 min preincubation before assay, 10 mM DTT, 10 mM 2-mercaptoethanol, and 1 mM iodoacetamide have no significant effect on the casein kinase activity, but 0.1 and 1.0 mM concentrations of N-ethylmaleimide inhibit by 31% and 79%, respectively.

A search was made for compounds that might differentially inhibit the casein kinase and CBP kinase from wheat germ. Table III shows that CBP kinase activity with ATP as phosphoryl donor and casein as substrate is more sensitive to inclusion of other nucleoside 5'-triphosphates, guanosine, and of ferrocyanide or ferricyanide than is the casein kinase in the same conditions. The concentrations for 50% inhibition of CBP kinase by ferricyanide and ferrocyanide are 0.4 and 0.17 mM, respectively. Conversely, casein kinase is more sensitive to inhibition by hemin (50% inhibition at 0.12 mM) than is CBP kinase (50% inhibition at 0.25

mM hemin). Casein kinase is more sensitive to inclusion in the assay of 5'-ADP or adenosine (Table III). The concentration for 50% inhibition of casein kinase by adenosine is 100 μM at which concentration there is no inhibition of CBP kinase (Table III).

DISCUSSION

The affinity chromatography-based purification procedure for the casein kinase described here yields the highest specific activity plant protein kinase preparations yet reported. The specific activities obtained approach the value of approximately 1 μmol/min· mg protein obtained with homogeneous preparations of the catalytic subunit of mammalian cyclic AMP-dependent protein kinase (20). The best specific activity of the casein kinase obtained here (656 nmol/min·mg protein) is less than the specific activities of homogeneous calf thymus casein kinases I and II (847 and 1220 units, respectively) (3) but greater than the specific activities of apparently pure soybean casein kinases I and II (332 and 347 units, respectively) (6) or of the wheat germ protein kinase purified by Rychlik and Zagórski (215 units) (22). The yield of purified kinase from the present affinity-based purification procedure (44
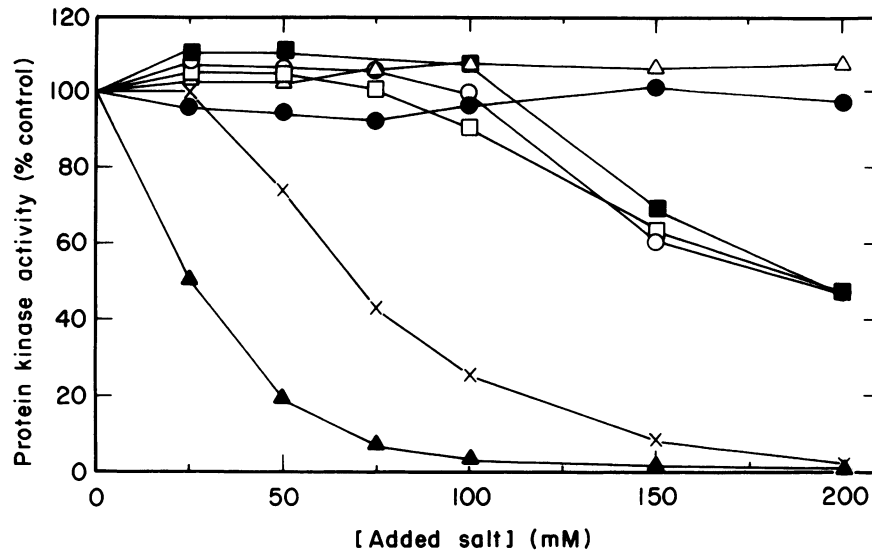
FIG. 6. Inhibition of casein kinase by salts. Casein kinase was assayed as described in "Materials and Methods" with 20 mM Hepes (K^+, pH 8.1), 5 mM NaCl, and 20 mM $MgCl_2$ in all assays. Activities in the presence of added salts were determined in triplicate and are presented as percentage of control activity (with no added salt): (▲), $(NH_4)_2SO_4$ added; (×), K-phosphate; (□), NaCl; (■), KCl; (O), NH_4CL; (△), sodium acetate; (●), potassium acetate.

Table II. Protein Substrate Specificity of Casein Kinase

Casein kinase was assayed in triplicate as described in "Materials and Methods" with 20 mM Hepes (K^+, pH 8.1), 20 mM magnesium acetate, 50 mM NaCl, and the indicated amounts of test substrate proteins in the assays. Means ± SD from triplicate determinations are shown. Assays with the various substrates were corrected by subtraction of control assays conducted in the absence of added casein kinase.

Added Protein	Protein Kinase	Activity with Casein
μg	pmol incorporated/10 min	%
None	0	0
Dephosphorylated casein (100)	519 ± 22	100
CBP (80)	0	0
cABP II (25)	23 ± 6	4
Phosvitin (100)	230 ± 10	44
Calf thymus histones (100)	0	0
BSA (100)	0	0

nmol/min·100 g fresh weight of starting material) (Table I) is 3 times better than yields obtained for the protein kinase preparations purified from soybean (6) or purified from wheat germ by Rychlik and Zagórski (22). Our casein kinase preparations are near-homogeneous on the basis of high specific activities (lying between those of apparently pure preparations of casein-phosphorylating animal and plant enzymes), the near-identity of subunit and native mol wt estimates of approximately 30,000, and the correlation of the major 31,000 subunit resolved on SDS-polyacrylamide gel electrophoresis with protein kinase activity (Fig. 3, A and B). The 31,000 D doublet band observed in many preparations (Fig. 3C) could derive from covalent modification of the protein, e.g. by specific terminal proteolysis or phosphorylation (3).

The purification procedure resolves the casein kinase from CBP kinase. The casein kinase is clearly distinguished from CBP kinase by its inability to bind to CBP Sepharose or to phosphorylate CBP* (cf. Ref. 16). The casein kinase differs further from CBP kinase (see Ref. 16) in lack of activation by Mn^{2+} (at concentrations from 0.25-50 mM), in apparent mol wt and in differential sensitivity to

Table III. Differential Inhibition of Casein Kinase and CBP Kinase

CBP kinase and casein kinase were assayed in the standard assay conditions with dephosphorylated casein as substrate in the presence or absence of the indicated compounds. Assays were conducted in triplicate, and assay values (±SD) were expressed as per cent of control activities (with no inhibitor added).

Addition	Casein Kinase	CBP Kinase
mM	% control	
None	100	100
GTP (0.1)	80 ± 5	27 ± 1
GTP (1.0)	40 ± 2	2 ± 1
CTP (0.1)	103 ± 2	100 ± 5
CTP (1.0)	104 ± 4	92 ± 5
UTP (0.1)	98 ± 3	96 ± 4
UTP (1.0)	85 ± 2	50 ± 2
ITP (0.1)	106 ± 6	56 ± 1
ITP (1.0)	97 ± 5	12 ± 1
ADP (0.1)	36 ± 3	76 ± 4
ADP (1.0)	6 ± 1	26 ± 1
Adenosine (0.1)	50 ± 8	103 ± 4
Adenosine (1.0)	11 ± 1	73 ± 7
$K_4Fe(CN)_6$ (1.0)	75 ± 6	9 ± 3
$K_3Fe(CN)_6$ (1.0)	105 ± 6	25 ± 3

a variety of inhibitors (Table III; Fig. 6). The casein kinase, like CBP kinase (16), is inhibited by elevated salt concentrations, but differs from CBP kinase in insensitivity to high sodium or potassium acetate concentrations (Fig. 6; see Ref. 16). Thus, the inhibition of the casein kinase by high salt concentrations is anion specific. The differential inhibition of the casein kinase and CBP kinase by ferrocyanide, ferricyanide, adenosine, and other compounds (Table III) may be useful in distinguishing between these (and related) protein kinases in future studies.

The casein kinase described here differs in subunit mol wt, inhibition by high NaCl and KCl concentrations, and lack of activation by Mn^{2+} or Ca^{2+} from another cyclic nucleotide-independent, casein-phosphorylating protein kinase isolated from wheat germ by Rychlik and Zagórski (22). Apparently, homogeneous preparations of the latter enzyme contain a 20,000 D polypeptide (22); preparations of the casein kinase described in the present paper contain a predominant 31,000 D polypeptide

(Fig. 3). The present casein kinase is inhibited by very high (nonphysiological) concentrations of cyclic AMP as is CBP kinase (16) and a protein kinase activity in wheat germ that catalyzes the phosphorylation of protein synthesis initiation factor eIF-2 (19). However, the latter enzyme, unlike CBP kinase (16) and the casein kinase, is insensitive to N-ethylmaleimide (19). We have previously reported the phosphorylation of casein and cABPII by three wheat germ protein kinase fractions (15). However, these fractions were very impure (specific activities, 0.2–3.0 nmol/min·mg) and were isolated in low yield (2 nmol/min·100 g as compared to 44 nmol/min·100 g for the present purified casein kinase) i.e. the present casein kinase would have been largely eliminated in the isolation procedure. Further, these protein kinase fractions, unlike the present casein kinase, catalyze the phosphorylation of CBP (16).

The wheat germ casein kinase described here has an apparent native mol wt of 30,000 as compared to a mol wt of 55,000 for a chromatin-associated casein kinase from soybean (11) and 39,000 for a casein kinase from cauliflower nuclei (12). The latter enzymes, unlike the wheat germ casein kinase, are activated by low concentrations of Mn^{2+} (11, 12). Hemin gives 50% inhibition of the casein kinase at 120 μM and 50% inhibition of the catalytic subunit of beef heart cyclic AMP-dependent protein kinase at 35μM (23). However, the protein inhibitor of the latter enzyme (4) does not inhibit the wheat germ kinase. The wheat germ casein kinase, like all protein kinases isolated from plants to date, is not activated by cyclic AMP. The wheat germ casein kinase is distinct in native mol wt (30,000) from casein kinases NI (23,000) and NII (85,000) from tobacco leaves (5) and from casein kinase I (mol wt 39,000) and casein kinase II (mol wt 120,000) from soybean cotyledons (6).

The only endogenous substrate for the wheat germ casein kinase that we have purified to date is the cyclic AMP-binding protein cABPII (15). The relatively low rates of phosphorylation of cABPII catalyzed by the purified casein kinase cannot be due to contaminating CBP kinase—for which enzyme cABPII is a good substrate (16)—since casein kinase preparations phosphorylating cABPII had no detectable activity with CBP as substrate (Table II). Although it is not known whether cABPII or CBP are phosphoproteins in nature, both these proteins are phosphorylated in vitro by CBP kinase (16), and cABPII is phosphorylated by both the casein kinase and CBP kinase. We are currently employing purified casein kinase in attempts to resolve further endogenous substrates and to search for possible endogenous regulators of this enzyme.

LITERATURE CITED

1. ALLEN JF, J BENNETT, KE STEINBACK, CJ ARNTZEN 1981 Chloroplast protein phosphorylation couples plastoquinone redox state to distribution of excitation energy between photosystems. Nature 291: 25–29

2. CARRATU G, LA MANZOCCHI, GA LANZANI, M GIANNATTASIO 1974 Soluble and ribosome-associated protein kinases from wheat embryos. Plant Sci Lett 3: 313–321

3. DAHMUS ME 1981 Purification and properties of calf thymus casein kinases I and II. J Biol Chem 256: 3319–3325

4. DEMAILLE JG, KA PETERS, EH FISCHER 1977 Isolation and properties of the rabbit skeletal muscle protein inhibitor of adenosine 3',5'-monophosphate dependent protein kinases. Biochemistry 16: 3080–3086

5. ERDMANN H, M BÖCHER, KG WAGNER 1982 Two protein kinases from nuclei of cultured tobacco cells with properties similar to the cyclic nucleotide-independent enzymes (NI and NII) from animal tissue. FEBS Lett 137: 245–248

6. GOWDA S, DTN PILLAY 1982 Cyclic AMP independent protein kinases from soybean cotyledons (*Glycine max* L.). Plant Sci Lett 25: 49–59

7. KREBBS EG, JA BEAVO 1979 Phosphorylation-dephosphorylation of enzymes. Annu Rev Biochem 48: 923–959

8. LIN PPC, JL KEY 1980 Histone kinase from soybean hypocotyls. Purification, properties, and substrate specificities. Plant Physiol 66: 360–367

9. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275

10. MAZUS B, B SZURMAK, J BUCHOWICZ 1980 Phosphorylation *in vitro* and *in vivo* of the wheat embryo RNA polymerase II. Acta Biochim Polonica 27: 9–19

11. MURRAY MG, TJ GUILFOYLE, JL KEY 1978 Isolation and characterization of a chromatin-associated protein kinase from soybean. Plant Physiol 61: 1023–1030

12. MURRAY MG, TJ GUILFOYLE, JL KEY 1978 Isolation and preliminary characterization of a casein kinase from cauliflower nuclei. Plant Physiol 62: 434–437

13. MURRAY MG, JL KEY 1978 2,4-Dichlorophenoxyacetic acid-enhanced phosphorylation of soybean nuclear proteins. Plant Physiol 61:190–198

14. OCHOA S, C DE HARO 1979 Regulation of protein synthesis in eukaryotes. Annu Rev Biochem 48: 549–580

15. POLYA GM, JA BOWMAN 1981 Resolution and properties of two high affinity cyclic adenosine 3':5'-monophosphate-binding proteins from wheat germ. Plant Physiol 68: 577–584

16. POLYA GM, JR DAVIES 1983 Resolution and properties of a protein kinase catalyzing the phosphorylation of a wheat germ cytokinin-binding protein. Plant Physiol 71: 482–488

17. RALPH RK, SJ WOJCIK, P AIREY 1980 In vitro plant protein synthesis and cytokinins. Plant Sci Lett 18: 237–247

18. RANDALL DD, PM RUBIN 1977 Plant pyruvate dehydrogenase complex II. ATP-dependent inactivation and phosphorylation. Plant Physiol 59: 1–3

19. RANU RS 1980 Isolation of a translation inhibitor from wheat germ with protein kinase activity that phosphorylates initiation factor eIF-2. Biochem Biophys Res Commun 97: 1124–1132

20. ROSEN OM, R RANGEL-ALDAO, J ERLICHMAN 1977 Soluble cyclic AMP-dependent protein kinases: review of the enzyme isolated from bovine cardiac muscle. Curr Top Cell Regul 12: 39–74

21. RYCHLIK W, E KUPIDLOWSKI, E NOWAK, W ZAGÓRSKI 1980 Wheat germ protein kinase effects the translation of brome mosaic virus ribonucleic acid in vitro. Biochemistry 19: 5249–5255

22. RYCHLIK W, W ZAGÓRSKI 1980 Purification and characterisation of adenosine-3',5'-phosphate-independent protein kinase from wheat germ. Eur J Biochem 106: 653–659

23. SCOTT CD, BE KEMP, AM EDWARDS 1979 Heme inhibits liver cyclic AMP-dependent protein kinases in cell extracts and intact hepatocytes. Proc Aust Biochem Soc 12: 44.

24. SIERRA JM, C DE HARO, A DATTA, S OCHOA 1977 Translational control by protein kinases in *Artemia salina* and wheat germ. Proc Natl Acad Sci USA 74: 4356–4359

25. SIKORSKI MM, D PRZYBL, AB LEGOCKI, W KUDLICKI, E GASIOR, J ZALAC, T BROKOWSKI 1979 The ribosomal proteins of wheat germ. Distribution and phosphorylation in vitro. Plant Sci Lett 15: 387–397

26. SUSS K-H 1981 Identification of chloroplast thylakoid phosphoproteins: evidence for the absence of phosphoryl-polypeptide intermediates in the ATPase complex. Biochem Biophys Res Commun 102: 724–729

27. TREWAVAS A 1976 Post-translational modification of proteins by phosphorylation. Annu Rev Plant Physiol 27: 349–374