Similar results are obtained using a consensus substrate peptide.[15] For the assays involving a peptide substrate, the reactions contain 30 n*M* kinase, 500 n*M* consensus peptide, 50 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS), 50 m*M* NaCl, 10 m*M* $MgCl_2$, 1.0 m*M* ATP, 6.25 n*M* [γ-^{32}P]ATP, 2.0 m*M* DTT, and bovine serum albumin (100 μg/ml, pH 7.0) in a 50-μl volume. The reactions are incubated for 30 min at 30°, spotted on P81 cation-exchange filter paper (Whatman, Clifton, NJ), and washed with 30% (v/v) acetic acid to remove free radiolabeled ATP from the phosphorylated peptide, which remains bound to the filter paper. The filters are briefly washed with 100% acetone and allowed to air dry. The radiolabeled phosphate incorporated into the peptide during the phosphorylation reaction is then measured by scintillation counting.

[15] Z. Songyang, S. Blechner, N. Hoagland, M. F. Hoekstra, H. Piwinica-Worms, and L. C. Cantley, *Curr. Biol.* **4,** 973 (1994).

[2] Purification of Recombinant Cyclin B1/cdc2 Kinase from *Xenopus* Egg Extracts

By HEIKE WILHELM, SØREN S. L. ANDERSEN, and ERIC KARSENTI

Introduction

Native mitotic cdc2 kinase (or cdk1 kinase) can be purified from synchronized tissue culture cells, starfish eggs, and *Xenopus* eggs.[1-4] The starfish preparation is probably the easiest to carry out, although one may not have access to starfish eggs. The drawback of all these preparations is twofold. First, it is not easy to obtain large amounts of pure enzyme. Second, it is difficult to ensure that the purified protein is composed only of cdk1 and one type of cyclin, because they are purified by virtue of their kinase activity (usually assayed on histone H1). An alternative is to prepare these enzymes from recombinant proteins expressed or coexpressed in baculovirus,[5] yeast,[6] human cell systems,[7,8] or *Xenopus*.[9] Here, we describe a procedure that makes use of glutathione *S*-transferase (GST)-tagged recombinant cyclin that complexes to and activates the native cdk1 protein present in *Xenopus* egg extracts devoid of mitotic cyclins. This method was first described by Solomon *et al.*[9] Here we describe an improved version of this preparation as well as the characterization of the purified enzyme. The principle of the method is diagrammed in Fig. 1: the cyclin is added to the concentrated interphase extract that contains inactive free cdc2 protein. An inhibitor of type 2 A phosphatase (microcystin) is added to the extract to keep cdc25 active[10,11] and produce a fully active cyclin B1–cdc2 complex. The cdc2–cyclin B1 complex is then retrieved on glutathione beads, eluted, concentrated, and further purified on a Mono S column (Fig. 1).

[1] L. Brizuela, G. Draetta, and D. Beach, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 4362 (1989).
[2] J. C. Labbé, A. Picard, G. Peaucellier, J. C. Cavadore, P. Nurse, and M. Dorée, *Cell* **57,** 253 (1989).
[3] J. C. Labbé, J. P. Capony, D. Caput, J. C. Cavadore, J. Derancourt, M. Kaghdad, J. M. Lelias, A. Picard, and M. Dorée, *EMBO J.* **8,** 3053 (1989).
[4] M. J. Lohka, M. K. Hayes, and J. L. Maller, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 3009 (1988).

METHODS IN ENZYMOLOGY, VOL. 283 0076-6879/97 $25.00

Cyclin Purification

Materials and Solutions

EX: Phosphate-buffered saline (PBS: 137 m*M* NaCl, 2.7 m*M* KCl, 10 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4) containing 1 m*M* EGTA, 1 m*M* EDTA, 0.1% (v/v) Tween 20, lysozyme (0.1 mg/ml), and protease inhibitor mix [1 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml each of aprotinin, pepstatin, and leupeptin]
WB: PBS with 300 m*M* KCl, 1 m*M* DTT, and protease inhibitor mix
ELB: 50 m*M* Tris (pH 8.1), 300 m*M* KCl, protease inhibitor mix, and 5 m*M* reduced glutathione (Sigma, St. Louis, MO)
FB: 50 m*M* HEPES (pH 7.6), 250 m*M* KCl, and 30% (v/v) glycerol

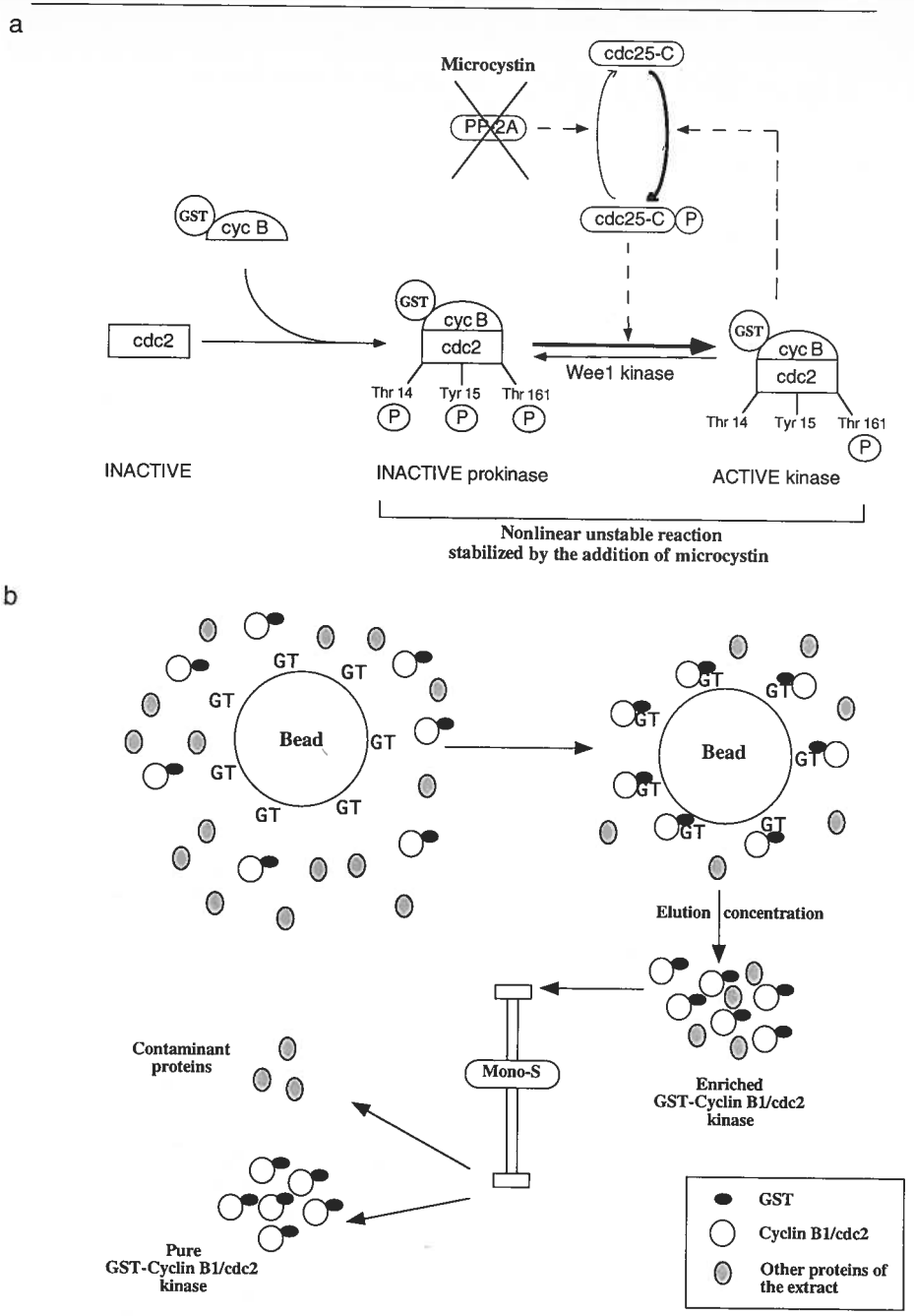
The buffers may be stored without protease inhibitors and reduced glutathione at 4°.

GT–agarose: glutathione–agarose (Sigma)
Reduced glutathione (Sigma)
GST–cyclin B1 plasmid: A kind gift from D. Kellogg [in Bl 21 (DE3) pLys S strain]

Procedure

1. Grow 100 ml of preculture overnight in Luria-Bertani medium (LB) with ampicillin (0.1 mg/ml) at 37°.

[5] D. Desai, Y. Gu, and D. O. Morgan, *Mol. Biol. Cell* **3,** 571 (1992).
[6] D. Leroy, V. Baldin, and B. Ducommun, *Yeast* **10,** 1631 (1994).
[7] Z. Q. Pan, A. Amin, and J. Hurwitz, *J. Biol. Chem.* **268,** 20443 (1993).
[8] Z. Q. Pan and J. Hurwitz, *J. Biol. Chem.* **268,** 20433 (1993).
[9] M. J. Solomon, T. Lee, and M. W. Kirschner, *Mol. Biol. Cell* **3,** 13 (1992).
[10] M. A. Félix, P. Cohen, and E. Karsenti, *EMBO J.* **9,** 675 (1990).
[11] I. Hoffmann, P. R. Clarke, M. J. Marcote, E. Karsenti, and G. Draetta, *EMBO J.* **12,** 53 (1993).



2. Dilute, the next day, 1:50 (v/v) in LB with ampicillin.
3. Grow until an OD_{600} of 0.6 is reached and induce with 0.4 m*M* isopropyl-β-D-thiogalactopyranoside (IPTG) overnight at 18°.
4. Pellet the cells and, after washing once in ice-cold PBS, freeze the pellet in liquid nitrogen and store at −80°.
5. Lyse the cells by adding a 5× pellet volume of EX to the frozen pellet and stir for 30 min on ice.
6. Sonicate five to seven times, for 30 sec each, at setting 7–8 on a Branson sonifier at 30-sec intervals.
7. Bring the suspension to 300 m*M* KCl and 15 m*M* dithiothreitol (DTT).
8. Dialyze in a 20-fold suspension volume against WB without protease inhibitor mix. Change the buffer three times (45 min each).
9. Clarify by ultracentrifugation for 1 hr at 100,000 *g*.
10. Bind the supernatant to GT–agarose equilibrated in WB (use 1 ml of GT–agarose for every 1 liter of cells). Rotate for 1 hr at 4°.
11. Wash the suspension once batchwise, and then pour it into a column (e.g., a Poly-Prep chromatography column; Bio-Rad, Richmond, CA) and wash until no protein can be detected in the flow-through.
12. Elute with 10 column volumes of ELB.
13. Dialyze peak fractions separately overnight in FB (200 times the fraction volume).
14. Freeze aliquots in liquid nitrogen and store at −80°.
15. Analyze preparation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE).

Product Obtained

The average recovery from a 5-liter preparation is 4–5 ml of relatively pure 3–7 μ*M* GST–cyclin B1 solution (Fig. 2a).

FIG. 1. Principle of preparation and purification of active GST–cyclin B1/cdc2 complex. (a) Mechanism of activation of the cdc2 kinase in an interphase extract by the addition of GST–cyclin B1. The extract contains unphosphorylated, inactive cdc2 subunits and no mitotic cyclin. The added GST–cyclin binds to the cdc2 subunit and targets the kinase for phosphorylation of Thr-161 by CAK kinase (activatory) and phosphorylation of Thr-14 and Tyr-15 (inhibitory). The latter phosphates can be removed by cdc25-C, which is activated by phosphorylation under the control of active cdc2. This positive feedback loop is a nonlinear reaction step that causes irreproducible activation of the kinase in the extract. To eliminate irreproducibility, we added microcystin, which inactivates the type 2A phosphatase that dephosphorylates cdc25. (b) Two-step purification of the active GST–cyclin B1, first on glutathione (GT) beads and then on a Mono S column.

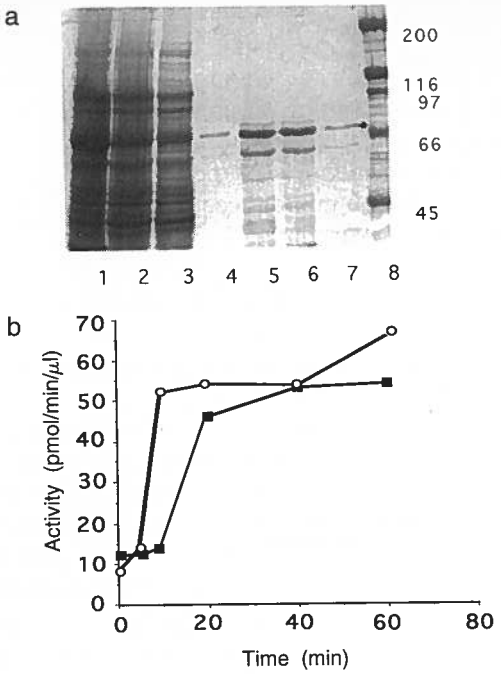


FIG. 2. (a) Purification of GST–cyclin B1 from bacterial extract. Coomassie blue staining of an SDS–polyacrylamide gel. Lane 1, total extract after sonication; lane 2, 100,000 *g* supernatant; lane 3, flow-through of the GT–agarose column; lanes 4–7, eluted fractions 1–4; lane 8, molecular mass marker proteins (kDa). (b) cdc2 kinase activation by addition of GST–cyclin B1 to an interphase extract. (■) No microcystin; (○) 0.2 μM microcystin added (final concentration).

cdc2 Kinase Activation in Interphase Extract

Materials and Solutions

Pregnant mare serum gonadotropin (PMSG; Intergonan, Vermie Veterinar, Tönisvorst, Germany), 200 units/ml
Human chorionic gonadotropin (HCG, Sigma), 2000 units/ml
MMR: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EGTA, 5 mM HEPES (pH 7.8) (2 liters for a typical preparation)
Cysteine hydrochloride (2%, w/v; Sigma), adjusted to pH 7.8 with NaOH (2 liters for a typical preparation)
Calcium ionophore A23187 (Sigma), 20 mg/ml in dimethyl sulfoxide (DMSO)
Cycloheximide (Sigma), 10 mg/ml in water

Cytochalasin D (Sigma), 10 mg/ml in DMSO
Microcystin LR (GIBCO-BRL, Gaithersburg, MD), 1 mM in DMSO
XB[12]: 100 mM KCl, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM potassium HEPES (pH 7.7), 50 mM sucrose, cytochalasin D diluted 1:1000 (v/v), and protease inhibitor mix (100 ml of XB for a typical preparation)
Energy mix (20×): 150 mM creatine phosphate, 20 mM ATP, creatine kinase (0.2 mg/ml), 20 mM $MgCl_2$

Procedure

Preparation of Extracts

1. Inject frogs with 100 units of PMSG per frog (we usually use 10 frogs).
2. After at least 4 days and a maximum of 10 days, inject with 1000 units of HCG and keep the frogs for 18 hr at 16° in MMR (in plastic boxes, 26 × 12 cm, with 500 ml of buffer).
3. Collect the eggs and wash in MMR two or three times.
4. Remove the jelly coat by washing the eggs in several changes of 2% (w/v) cysteine hydrochloride followed by three or four washes with MMR.
5. Activate the eggs in MMR with calcium ionophore diluted 1:10,000 (v/v) for 2 min (first rinse once to change the buffer; total volume used is 200 ml).
6. Wash in MMR and incubate for 20 min in MMR with cycloheximide diluted 1:50 (v/v) (first rinse once to change the buffer; total volume used is 200 ml).
7. Wash again in MMR and finally in XB.
8. Transfer into SW50.1 tubes (Beckman, Palo Alto, CA) containing XB and pack them by spinning at 16° in a Heraeus Megafuge 1.0 R (Osterocle, Germany) for 60 sec at 180 *g*.
9. Remove the excess buffer and place tubes (plus contents) in a 12-ml polypropylene tube (Sarstedt, Nümbrecht, Germany).
10. Crush the eggs at 4° in a Sorvall (Newton, CT) centrifuge, using the HB4 rotor (with rubber adaptor) for 15 min at 16,000 *g*.
11. Collect, on ice, the cytoplasmic layer between the bottom yolk pellet and the top lipid layer; use a 2-ml syringe and 18-gauge needle to puncture the side of the tube.
12. Add energy mix (1:20, v/v) and cytochalasin D (1:1000, v/v), then freeze in 200-μl aliquots in liquid nitrogen.

[12] A. Murray, "Cell Cycle Extracts," pp. 581–605. Academic Press, New York, 1991.

Activation of cdc2 by Cyclin B1

1. Thaw (at 37° until most but not all ice has thawed) the low-speed interphase extract and centrifuge in a TL 100 centrifuge (Beckman) at 200,000 *g* for 30 min at 4° (we usually use 10 ml of low-speed extract per preparation).
2. Carefully take the clear middle phase of the extract, using an Eppendorf pipette.
3. Activate the cdc2 kinase in the extract by adding GST–cyclin B1 to a final concentration of 200 n*M* in the presence of 0.2 μM microcystin-LR at 20° for 45 min. Proceed to the next section.

Result

Normally, the cyclin B1 activates cdc2 kinase in the interphase extracts after a lag phase that can vary from a few minutes to half an hour (Fig. 2b). Sometimes, it does not activate at all. We have noticed that this variability occurs even in aliquots of the same extract preparation. It is not yet clear why this happens, but we believe this is a consequence of the mechanism of activation of the kinase, which involves a nonlinear autoamplification loop.[13–15] To avoid irreproducibility, we routinely add microcystin to the extracts. By inhibiting a type-2A phosphatase this keeps cdc25 active. This procedure allows an immediate activation of cdc2 kinase by the GST–cyclin B1 in all extracts.

Purification of Active Kinase

Materials and Solutions

KDB: 80 m*M* β-glycerophosphate (pH 7.3), 20 m*M* EGTA, 15 m*M* $MgCl_2$, 300 m*M* KCl, 1 m*M* DTT, and protease inhibitor mix
GT–agarose
Reduced glutathione
KWB: KDB containing 0.5% (v/v) Nonidet P-40 (NP-40), bovine serum albumin (BSA, 10 μg/ml; Sigma) (approximately 99% pure)
KEB: KWB with 50 m*M* reduced glutathione
Centricon-30 cells (Amicon, Beverly, MA)
MDB: 40 m*M* Tris (pH 6.8), 1 m*M* EGTA, 0.5% (v/v) NP-40, 5% (v/v) glycerol (Sigma), BSA (10 mg/ml), 0.5 μM microcystin-LR, 1 m*M* DTT, and protease inhibitor mix
MEB: 40 m*M* Tris (pH 6.8), 20 m*M* KCl, 1 m*M* EGTA, 0.5% (v/v) NP-40, 1 m*M* DTT, and protease inhibitor mix
MA: 40 m*M* Tris (pH 6.8), 20 m*M* KCl, 1 m*M* EGTA, 0.04% (v/v) NP-40, 1 m*M* DTT, and protease inhibitor mix
MB: 40 m*M* Tris (pH 6.8), 1 *M* KCl, 1 m*M* EGTA, 0.04% (v/v) NP-40, 1 m*M* DTT, and protease inhibitor mix
Mono S PC 1.6/5 column (100 μl; Pharmacia-LKB Biotechnology, Uppsala, Sweden)
SMART System, version 1.50 (Pharmacia-LKB Biotechnology)

[13] B. Novak and J. J. Tyson, *J. Cell Sci.* **106,** 1153 (1993).
[14] I. Hoffmann and E. Karsenti, *J. Cell Sci.* **18,** 75 (1994).
[15] I. Hoffmann, G. Draetta, and E. Karsenti, *EMBO J.* **13,** 4302 (1994).

Procedure

1. Dilute the GST cyclin B1-activated extract (see cdc2 Kinase Activation), 1:1 (v/v) with KDB.
2. Mix at a 1:1 (v/v) ratio (diluted extract:packed beads) with GT–agarose equilibrated in KDB.
3. Incubate the extract–bead suspension for 30 min, rotating, at 4°.
4. Pellet and wash the beads extensively in KWB.
5. Elute in KEB (volume twice that of packed beads), for 30 min at 4°, under rotation.
6. Pellet the beads again and concentrate the eluate in Centricon-30 cells to a volume less than 3 ml.
7. At this point the concentrate can be frozen as 200-μl aliquots in liquid N_2 and stored at −70°.
8. Dilute the concentrate in MDB four times and load onto the Mono S column equilibrated in MEB, at 100 μl/min.
9. Wash in MEB until the OD_{280} reaches baseline again.
10. Elute by washing first with MA (four column volumes), followed by a step gradient from 0 to 22% MB (eight column volumes), then a step gradient from 22 to 60% MB (eight column volumes), then a step gradient from 60 to 100% MB (eight column volumes).
11. Collect one-column volume fractions.
12. Assay fractions for histone H1 kinase activity.
13. Analyze the peak fraction by gel and Western blot.

Product

Up to the step requiring use of a GT–agarose column, the protocol is mostly based on that of Solomon *et al.*[9] After elution from the GT–agarose

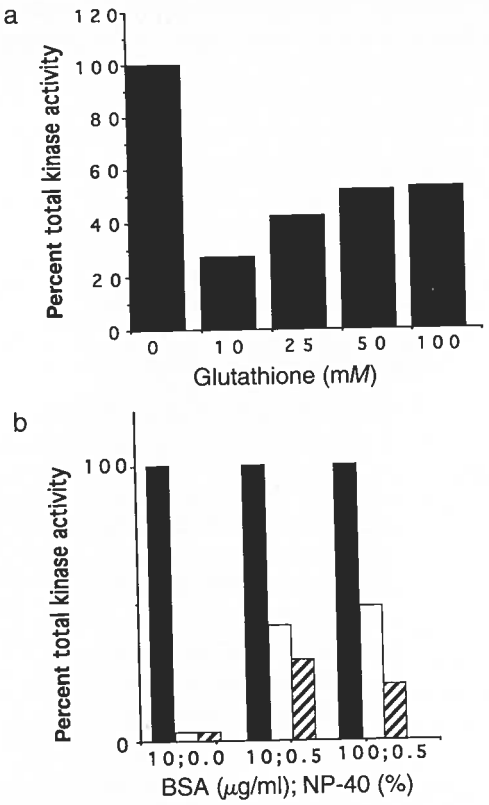


FIG. 3. (a) Elution conditions for the GST–cyclin B1/cdc2 complex from GT–agarose. The cyclin is optimally eluted by 50–100 m*M* glutathione. 100%, Total histone H1 kinase activity bound to GT–agarose (measured on bead aliquots). (b) BSA and NP-40 are required to stabilize and elute cdc2 kinase from the GST matrix. Using KDB containing 50 m*M* reduced glutathione, we find that the optimal elution conditions are obtained by adding BSA (10 μg/ml) and 0.5% (v/v) NP-40. Black bars: Total H1 kinase activity bound to GT–agarose. White bars: Histone H1 kinase activity eluted from GT–agarose. Hatched bars: Histone H1 kinase activity remaining on GT–agarose. Both are expressed as a percentage of the total activity bound to GT–agarose.

column (Fig. 3), the kinase is dilute and must be concentrated. In the concentration step we usually recover about 50% of the initial kinase activity, with an activity of 15 pmol/min/μl. Further attempts to concentrate this preparation resulted in a large increase in viscosity, which stopped the concentration process. This is probably due to the high concentration of contaminating proteins at this step (Fig. 4a). We therefore decided to concentrate further and purify the kinase on an ion-exchange column. Mono

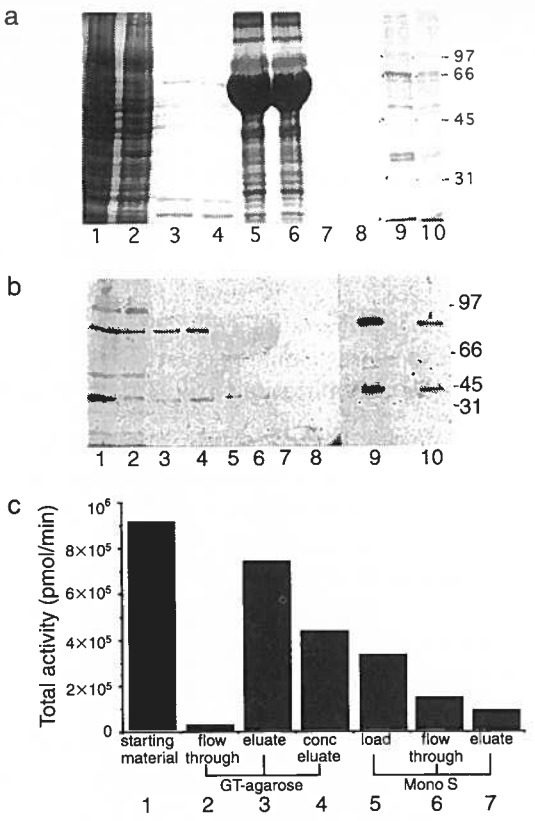


FIG. 4. Purification of GST–cyclin B1/cdc2 complex from an interphase extract. (a) Coomassie blue staining of an SDS–PAGE analysis. The gel was loaded according to volume. Lane 1, interphase extract activated by the addition of 200 n*M* GST–cyclin B1; lanes 2–4, flow-through, eluted fraction, and concentrated fraction eluted from the GT–agarose column, respectively; lanes 5 and 6, load and flow-through from the Mono S column, respectively; lane 7, fraction 3 (peak fraction); lane 8, fraction 4; lane 9, fraction 3, 100-fold increased load; lane 10, fraction 4, 100-fold increased load. Molecular mass marker proteins (kDa) are indicated on the right. (b) Western blot analysis of the fractions described above, using antibodies directed against cyclin B1 and the PSTAIR region of $p34^{cdc2}$. (c) Flow chart of the histone H1 kinase activity purification shown in (a) and (b) (Mono S eluate; histone H1 kinase activity in fraction 3).

Q did not prove helpful because we usually lost a great deal of the starting activity on the column, but a Mono S column worked well. The kinase eluted from the Mono S column is relatively pure, with only few contaminating proteins (Fig. 4a, lanes 7–10). The yield could be easily improved by using a larger column, which would avoid the loss of the kinase that flows

TABLE I
YIELD OF THREE DIFFERENT KINASE PREPARATIONS

Activity	Preparation 1	2	3
Total starting (pmol/min)	92,822	140,929	?
Total final (pmol/min)	8,560	6,516	13,629
Final (pmol/min/μl)	86	33	136

through the Mono S in our preparation (Fig. 4c). We now routinely obtain pure preparations with an average kinase activity of 100 pmol/min/μl, which is largely sufficient for most applications (see Table I).

Stability of Active Kinase

Materials and Solutions

Concentrated active kinase from GT–agarose (see Purification of Active Kinase)
Glycerol
DMSO
EB: 80 m*M* β-glycerophosphate (pH 7.3), 20 m*M* EGTA, 15 m*M* $MgCl_2$, 1 m*M* DTT, protease inhibitor mix

Procedure

Temperature Stability

1. Assay the histone H1 activity of the concentrated active kinase from the GT–agarose column.
2. Freeze aliquots under different conditions:
   Flash freeze in liquid nitrogen
   Add 10% (v/v) glycerol (final concentration) and flash freeze in liquid nitrogen
   Add 10% (v/v) DMSO (final concentration) and flash freeze in liquid nitrogen
   Add 10% (v/v) glycerol (final concentration) and freeze at −80°
   Freeze at −80°
   Add 10% (v/v) glycerol (final concentration) and freeze at −20°
   Freeze at −20°
   Add 50% (v/v) glycerol (final concentration) and freeze at −20°
3. Thaw out aliquots the next day and assay again.

Results and Comments

We decided to check systematically the freezing conditions, after having had some problems. As shown in Fig. 5, the kinase is best preserved by being frozen at −80° or in liquid nitrogen. It is not necessary to add glycerol to keep the kinase active. A temperature of −20° inactivates the kinase, unless it is in a 50% (v/v) glycerol solution. We always flash freeze it in liquid nitrogen and store the kinase at −80°. We have stored the final preparation of kinase in 10-μl aliquots at −80° for 12 months without any loss of activity.

Native Molecular Weight of Kinase

Materials Solutions and Constants

EB: 80 m*M* β-glycerophosphate (pH 7.3), 20 m*M* EGTA, 15 m*M* $MgCl_2$, 1 m*M* DTT, protease inhibitor mix
BA: 5% (w/v) sucrose in EB, 300 m*M* KCl, 0.25% (v/v) NP-40
BB: 25% (w/v) sucrose in EB, 300 m*M* KCl, 0.25% (v/v) NP-40
BC: 150 m*M* KCl in EB
Standard markers used: Bio-Rad gel-filtration standard (content consists of thyroglobulin, bovine gammaglobulin, chicken ovalbumin, equine myoglobulin, vitamin B_{12}; Bio-Rad Laboratories), catalase (Calbiochem, La Jolla, CA), aldolase (Calbiochem), the last two made up as a 25-mg/ml solution in EB

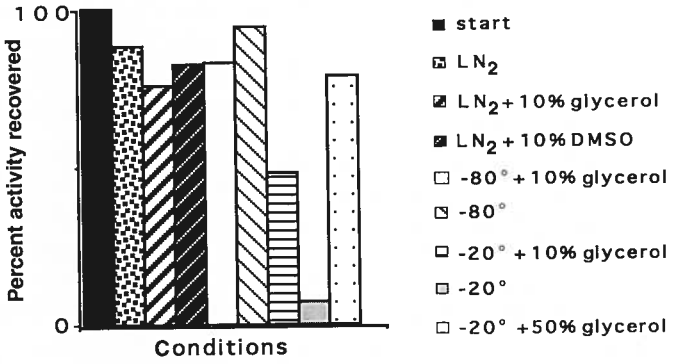


FIG. 5. Storage conditions of the purified kinase. The freezing conditions were tested on the concentrated fraction eluted from the GT–agarose column. Histone H1 kinase activity recovered after thawing samples is shown. The activity is expressed as a percentage of total activity before freezing (black bar). LN_2, Liquid nitrogen.

Superdex 200 PC 3.2/30 Smart column (Pharmacia-LKB Biotechnology)
Smart System, version 1.50 (Pharmacia-LKB Biotechnology)

Constants Used for Calculations

$S_{20,w}$ values for the standard proteins are obtained from Refs. 16 and 17.

$S_{20,w}$ (10^{-13} sec^{-1}) values for the sucrose gradient standard proteins are as follows: thyroglobulin tetramer 19 (M_r 669,000), thyroglobulin dimer 12 (M_r 330,000), catalase 11.3 (M_r 240,000), aldolase 7.35 (M_r 158,000), gammaglobulin 7 (M_r 158,000), ovalbumin 3.54 (M_r 44,000), myoglobulin (M_r 17,500).

The Stokes radius (R_S) values of the gel-filtration standard proteins are determined from their molecular weight by means of Eq. (1):

$$R_S = [(1 - \nu\rho_{20,w})M_r]/(6\pi n_{20,w} N S_{20,w}) \quad (1)$$

R_S values (in nanometers) are as follows: thyroglobulin, 10.64; gammaglobulin, 5.39; ovalbumin, 2.97; myoglobulin, 2.09.

Procedure

Sucrose Density Gradient Centrifugation

1. Create linear 4-ml gradients with BA and BB.
2. Gradient a consists of 10 μl of Bio-Rad markers in 90 μl of EB. Gradient b consists of 30 μl of catalase solution, 30 μl of aldolase solution, and 40 μl of EB. Gradient c consists of 50 μl of concentrated kinase from the GT–agarose column and 50 μl of EB.
3. Spin the gradients in an SW 60 rotor at 27,000 rpm for 16 hr at 4°.
4. Collect 250-μl fractions. Measure the sucrose concentration in the fractions, using a densitometer.
5. Analyze fractions on a gel and, for gradient c, also determine the histone H1 kinase activity and analyze the fractions by immunoblotting using anti-cyclin and anti-cdc2 antibodies.

Gel Filtration

1. Equilibrate the Superdex 200 column in BC.
2. Run 10 μl of Bio-Rad markers plus 40 μl of EB before running 50 μl of concentrated kinase from the GT–agarose column (elution speed, 50 μl/min; fraction size, 50 μl).

[16] G. D. Fasman, "Handbook of Biochemistry and Molecular Biology," Vol. II, pp. 258 and 317. CRC Press, Cleveland, Ohio, 1976.
[17] A. B. Schneider, H. Bornet, and H. Edelhoch, *J. Biol. Chem.* **246,** 2835 (1971).

3. Analyze the fractions from the second run by Western blot and determine the histone H1 kinase activity.

Results and Comments

The molecular weight of the GST–cyclin B1/cdc2 complex is determined by gel filtration in combination with sucrose gradients as described in the literature.[18–20] Using this method, three parameters are needed to determine the molecular mass: the apparent Stokes radius (R_S, in nanometers), which is derived from gel-filtration data; the sedimentation coefficient [S, (10^{-13} sec^{-1})], which is derived from the sucrose gradient data; and the partial specific volume [ν, (cm^3/g)] of the protein, which can be calculated from the amino acid composition.[21] From these three values and using Svedberg's equation [Eq. (2)], one can obtain an estimate of the molecular weight (M_r).

$$M_r = 6\pi n_{20,w} N R_S S_{20,w}/(1 - \nu\rho_{20,w}) \quad (2)$$

where M_r (g/mol) is the molecular weight, R_S is the Stokes radius (nm), $S_{20,w}$ ($\times 10^{-13}$ sec) is the sedimentation coefficient at 20°, ν (cm^3/g) is the partial specific volume, $n_{20,w}$ ($g \times m/sec^2$) is the viscosity of water at 20°, $\rho_{20,w}$ (g/cm^3) is the density of water at 20°, and N (1/mol) is Avogadro's number.

Partial specific volume: The partial specific volume for the GST cyclin B1–cdc2 complex (ν) is estimated as 0.75 cm^3/g (according to the amino acid composition[21]) and for the standard proteins we use an average value for ν of 0.73 (cm^3/g).

Determination of R_S from gel-filtration data: Plot $[-\log(K_{av})]^{1/2}$ (y axis) versus the Stokes radius (R_S, x axis) of the standard proteins. K_{av} is the normalized elution volume; $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e is the elution volume for each protein, V_0 is the void volume, and V_t is the total volume of the column. From this plot, the R_S value for the GST–cyclin B1/cdc2 complex is determined to be 8.35 nm.

Determination of $S_{20,w}$ from sucrose gradient data: For the standard proteins, plot $S_{20,w}$ (y axis) against the percentage of sucrose at which the protein elutes (x axis). However, the sedimentation coefficients for the standard proteins are measured at 4°. To calculate $S_{20,w}$ we use Eq. (3):

$$S_{20,w} = s_{4,w}[(1 - \nu\rho_{20,w})/(1 - \nu\rho_{4,w})](n_{4,w}/n_{20,w}) \quad (3)$$

[18] M. L. Siegel and J. K. Monty, *Biochim. Biophys. Acta* **112,** 346 (1966).
[19] C. R. Cantor and P. R. Schimmel, "Biophysical Chemistry: Techniques for the Study of Biological Structure and Function," Vol. II, pp. 539–642. W. H. Freeman, San Francisco, 1980.
[20] G. S. Bloom, M. C. Wagner, K. K. Pfister, and S. T. Brady, *Biochemistry* **27,** 3409 (1988).
[21] A. A. Zamyatin, *Annu. Rev. Biophys. Bioeng.* **13,** 145 (1984).

where $n_{4,w}$ is the viscosity of water at 4° and $S_{4,w}$ is the sedimentation coefficient at 4°. Values are as follows: $n_{4,w} = 1.5138$ mN sec m^{-2}; $n_{20,w} = 1.0019$ mN sec m^{-2}; $\rho_{4,w} = 1$ g cm^{-3}; $\rho_{20,w} = 0.99823$ g cm^{-3}.

The GST–cyclin B1/cdc2 complex sediments in 9.5% (w/v) sucrose, which corresponds to an $S_{20,w}$ value of 3.28×10^{-13} sec^{-1}.

With the R_S value of 8.35 nm and the $S_{20,w}$ value of 3.28×10^{-13} sec^{-1}, the molecular weight (M_r) of the GST–cyclin B1/cdc2 complex is calculated to be 122,302, using Eq. (2). This fits well with the calculated molecular mass of the GST–cyclin B1/cdc2 complex of 103 kDa (41 kDa for cyclin, 28 kDa for GST, and 34 kDa for cdc2), indicating a monomeric complex. The divergence of the shape of the protein from a globular form is expressed by the frictional ratio: $f/f_0 = R_S/(3\nu M_r/4\pi N)^{1/3}$. Using the values obtained here, f/f_0 is calculated to 2.52, which indicates that the GST–cyclin B1/cdc2 complex is asymmetrical. When the purified kinase is cleaved with thrombin (Sigma; we use 3 units of thrombin for kinase from 1 ml of interphase extract and incubate overnight at 4° under rotation) to remove the GST tag, the native molecular weight is calculated to 107,380. The high molecular weight is most likely due to uncleaved GST–cyclin B1/cdc2 complex. The cleaved kinase had an R_S value of 2.95 nm, which gives an f/f_0 value of 1.07, indicating that the cyclin B1/cdc2 complex is globular.

K_m and V_{max} of Kinase Using Histone H1 as Substrate

Materials and Solutions

EB: 80 m*M* β-glycerophosphate (pH 7.3), 20 m*M* EGTA, 15 m*M* $MgCl_2$, 1 m*M* DTT, protease inhibitor mix
ATP (Boehringer Mannheim), 4 m*M* solution in water; dilute from a 100 m*M* stock kept frozen at −20°, adjust to pH 7.0 using NaOH
Histone H1 (Sigma), 20 mg/ml solution in water
[γ–^{32}P]ATP, 10 mCi/ml (Amersham, Arlington Heights, IL)
RM: 3 μl of [γ–^{32}P]ATP, 45 μl of 4 m*M* ATP, 222 μl of EB, 30 μl of histone H1 (20 mg/ml), and H_3PO_4, 150 m*M*
Phosphocellulose P81 paper (Whatman, Clifton, NJ)

Procedures

To determine K_m and V_{max} values, velocity measurements of phosphorylation as a function of substrate (histone H1) concentration should be carried out. It is important to choose a substrate and a kinase concentration that result in a constant transfer of phosphate to the substrate over time. This means that the substrate concentration should be in large excess

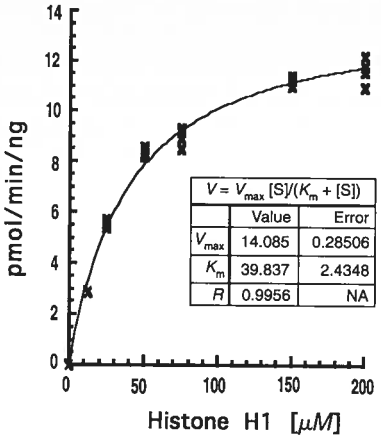


FIG. 6. V_{max} and K_m of the purified kinase. Each point corresponds to the average of four measurements.

relative to the phosphorylation capacity of the kinase. It should also be verified that the V value is proportional to the enzyme concentration. In the experiment described here, we have determined the adequate enzyme concentration for a histone H1 concentration to be 2 mg/ml. For the histone H1 assays, we use the protocol described by Félix *et al.*[22]

Checking Initial Rate at Constant Substrate and Kinase Concentration

1. Dilute the concentrated kinase of the GT–agarose column 1:5 (v/v) in EB and mix with the same volume of RM [3 μl of [γ–^{32}P]ATP, 45 μl of 4 m*M* ATP, 222 μl of EB, 30 μl of histone H1 (20 mg/ml)].
2. Mix well.
3. At various time points after mixing, spot 6 μl on pieces of cellulose and subsequently wash them in H_3PO_4 solution three times.
4. Rinse briefly in ethanol, transfer to scintillation vials, add scintillation fluid, and count.
5. Plot pmol/min/μl of phosphate transferred to histone H1 over time.

Checking That V Is Proportional to Enzyme Concentration

1. Make various dilutions of the kinase in EB.
2. Mix 5 μl of diluted kinase with 5 μl of RM.

[22] M. A. Félix, P. Clarke, J. Coleman, F. Verde, and E. Karsenti, "Frog Egg Extracts as a System to Study Mitosis," pp. 253–283. IRL Press, Oxford, 1993.

3. Take time points (determined in step 1) by spotting 6 μl on a piece of cellulose and wash in H_3PO_4 solution three times.
4. Proceed as in step 4 of the preceding section.
5. Plot pmol/min/μl of phosphate transferred to histone H1 over amount of kinase.

Determining V_{max} and K_m

1. Dilute the kinase in EB (dilution factor determined in previous step; here we use a kinase solution having an activity of 94 pmol/min/μl and dilute it 10-fold).
2. Mix 5 μl of diluted kinase with 5 μl of RM, varying the final concentration of histone H1 in RM.
3. Let the reaction go for the length of time determined by checking the initial rate conditions (here we use 4 min) and stop the reaction by spotting 6 μl on a piece of cellulose. Wash in H_3PO_4 solution three times.
4. Rinse briefly in ethanol, transfer to scintillation vials, add scintillation fluid, and count.
5. Plot V over the concentration of histone H1.
6. Determine V_{max} and K_m values. We calculated V_{max} and K_m using the hyperbola-fitting function in Kaleidagraph, and the classic formula $v = V_{max}[S]/(K_m + [S])$ (Fig. 6).

Results

Figure 6 shows V_{max} and K_m determinations for one preparation of kinase (we use the concentrated fraction of the GT–agarose column). The average V_{max} value is 6.5 pmol/min/ng ($\pm$6.7, $n = 3$) and the K_m is 28.4 μM ($\pm$10.2, $n = 3$). These data can be compared with those in Refs. 23 and 24.

[23] E. Erikson and J. L. Maller, *J. Biol. Chem.* **264,** 19577 (1989).
[24] J.-C. Labbé, J.-C. Cavadore, and M. Dorée, *Methods Enzymol.* **200,** 291 (1991).