

Molecular characteristics of cytostatic factors in amphibian egg cytosols

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Summary

In amphibians, zygotes microinjected with cytosol of unactivated eggs are arrested at metaphase of mitosis. The factor responsible for this effect has been designated 'cytostatic factor, (CSF)'. CSF is inactivated by Ca^{2+} addition to cytosols. During storage of the Ca^{2+} -containing cytosols, a stable CSF activity develops. Therefore, the first Ca^{2+} -sensitive CSF and the second Ca^{2+} -insensitive CSF have been referred to as primary CSF (CSF-1) and secondary CSF (CSF-2), respectively. We have partially purified CSF-1, which had been stabilized with NaF and ATP, and CSF-2 from cytosols of *Rana pipiens* eggs by ammonium sulphate (AmS) precipitation and sucrose density gradient centrifugation or gel filtration, and investigated their molecular characteristics. CSF-1 was sensitive to protease, but resistant to RNase, and inactivated within 2 h at 25°C. CSF-1 could be sedimented in a sucrose density gradient from a fresh cytosol or its crude fraction precipitated at 20–30 % saturation of AmS, showing the sedimentation coefficient 3S. When analyzed by SDS–polyacrylamide gel electrophoresis (PAGE), all the proteins in partially

purified CSF-1 samples entered the gel and were separated into numerous peptide bands. In contrast, CSF-2 was an extremely large molecule, being eluted from Sepharose columns as molecules larger than 2×10^6 , and failed to enter the gel when analyzed by SDS–PAGE. It could be purified 40 times from cytosols. CSF-2 was a highly stable molecule, being neither inactivated nor dissociated at pH 11.5 or by 4 M-NaCl and LiCl and 8 M-urea. It was also resistant to RNase treatment. However, CSF-2 could be broken down into small peptides of variable sizes by trypsin, α -chymotrypsin, and papain, but not by *S. aureus* V8 protease, although it was less sensitive to proteases than CSF-1. The dose-dependency test showed that the activity of CSF-2 is independent of its concentration and that an amount of CSF-2 could cause cleavage arrest earlier when injected into a blastomere in a larger volume.

Key words: amphibian, oocyte, cytosols, meiosis, proteins, cell cycle.

Introduction

When meiosis is resumed in full-grown prophase-arrested oocytes, the nuclear envelope of the germinal vesicle or oocyte nucleus breaks down and the chromosomes condense to a metaphase state. After completing the first meiotic division, the oocytes of most vertebrates are arrested at the second meiotic metaphase before fertilization. CSF is a cytoplasmic factor responsible for maintaining this arrest at metaphase in unfertilized eggs. When cytoplasm or cytosols of unactivated *Rana* or *Xenopus* eggs are injected into a zygote, the recipient embryo is arrested at metaphase for prolonged periods of time (Masui & Markert, 1971; Meyerhof & Masui, 1977, 1979; Heidemann & Gallas, 1980; Shibuya & Masui, 1982; Newport & Kirschner, 1984; Gerhart *et al.* 1984; Karsenti *et al.* 1984).

The inactivation of CSF during activation of eggs may be explained by the increase in intracellular Ca^{2+} during egg activation observed by Wasserman *et al.*

(1980) and Busa & Nuccitelli (1985), since CSF-1 activity in fresh cytosols of unactivated eggs rapidly disappears after addition of Ca^{2+} (Meyerhof & Masui, 1977; Shibuya & Masui, 1988). However, fresh cytosols, to which Ca^{2+} has been added to inactivate CSF, develop CSF activity again during storage at 2°C. To distinguish these two CSFs from each other, the initial, Ca^{2+} -sensitive, unstable CSF in fresh cytosols has been designated primary CSF (CSF-1), and the latter, Ca^{2+} -insensitive, stable CSF which appears in stored cytosols designated secondary CSF (CSF-2) (Meyerhof & Masui, 1977). Both CSF-1 and CSF-2-arrested blastomeres contain one or sometimes two spindles with metaphase chromosomes devoid of asters (Meyerhof & Masui, 1977; Shibuya & Masui, 1988).

However, CSF-2 is different from active CSF-1. They can be separated from each other by ultracentrifugation and AmS precipitation. When fresh cytosols of unfertilized eggs were subjected to prolonged ultracentrifugation in the absence of ATP and NaF (7.5 h at 170 000 g),

CSF-1 activity was sedimented. CSF-2 activity developed after Ca^{2+} addition to the lighter fractions that did not contain CSF-1, and was also sedimented by ultracentrifugation. CSF-1 could be precipitated with AmS at 20 to 30% saturation, whereas CSF-2 could only develop in fractions of fresh cytosols that could be precipitated with AmS at a concentration higher than 50% saturation. Once it had appeared in these fractions, however, CSF-2 could be precipitated with AmS at the concentration of 20–40% saturation (Shibuya & Masui, 1989). The specific activity of the CSF-2 thus partially purified was increased up to 30 times. These results, taken together, clearly showed that CSF-1 and CSF-2 are different molecular entities and that CSF-2 originates from inactive precursors which are assembled into an active, larger molecule(s). In this paper, we describe further differences in the molecular characteristics of CSF-1 and CSF-2 partially purified from cytosols of unfertilized *R. pipiens* eggs.

Materials and methods

Frogs, eggs and embryos

Rana pipiens were obtained from a supplier in Vermont (Hazen), maintained at 2°C and induced to ovulate as described previously (Shibuya & Masui, 1982). Unfertilized eggs were dissected out of the ovisac and dejellied for the preparation of cytosols. To obtain embryos, eggs were stripped from frogs that had been maintained at 6 to 8°C for up to one week after ovulation and fertilized *in vitro*. Zygotes were incubated at 15°C to the 2-cell stage prior to microinjection, as described previously (Shibuya & Masui, 1988).

Solutions and media

The compositions of the modified Ringer's solution and dejelling solution have been described previously (Wasserman & Masui, 1976; Shibuya & Masui, 1982). The standard extraction medium contained 0.25 M-sucrose, 0.2 M-NaCl, 5.0 mM-MgSO₄, 2.5 mM-ethylene glycol-bis-(β -aminoethyl ether)N,N,N',N'-tetra acetic acid (EGTA, Sigma), 10 mM- β -mercaptoethanol, buffered with 10 mM-Na₂HPO₄-NaH₂PO₄, at pH 6.5, which was sometimes supplemented with 10 mM-NaF and/or 3 mM-ATP or 0.5 mM- γ -S-ATP from stock solution. These ATP stock solutions contained 0.12 M-ATP (or γ -S-ATP), 0.2 M-MgSO₄, and 0.2 M-Tris (pH 7.0) (Shibuya & Masui, 1988).

The saturated AmS solution was prepared in double glass-distilled H₂O at 2°C, adjusted to pH 7.0 with NH₄OH and filtered prior to use. The composition of the dialysis buffer (DB) used for redissolving AmS precipitates was the same as the extraction buffer without the β -mercaptoethanol.

The column buffer (CB) used for gel filtration consisted of 0.2 M-NaCl buffered with 10 mM-Na₂HPO₄-NaH₂PO₄, pH 7.0. Also, a solution of 6.0 or 8.0 M-urea (ultrapure, Schwarz-Mann) buffered with 10 mM-Na₂HPO₄-NaH₂PO₄ at pH 7.0, with or without 0.5 M- β -mercaptoethanol was used.

Preparation of cytosols and CSFs

Cytosols were prepared from dejellied, unactivated eggs by the 1-step extraction method described previously (Shibuya & Masui, 1988). Eggs in a minimum of extraction medium were crushed at 150 000 g for 2 h in a Beckman L3-40 ultracentrifuge using a SW-50.1 rotor. The clear supernatant (cytosol)

was withdrawn and subjected to AmS fractionation or sucrose density gradient centrifugation.

CSF-1 was precipitated from fresh cytosols between 20 and 30% saturation of AmS and immediately dissolved in DB supplemented with NaF and ATP (or γ -S-ATP) as described above.

To obtain partially purified CSF-2, the supernatant obtained after precipitating proteins from fresh cytosols at 50% AmS was dialyzed against DB without EGTA and containing 2.5 mM-CaCl₂ or DB with 5 mM-CaCl₂, at pH 5.5–6.0 (Shibuya & Masui, 1989). After CSF-2 appeared, it was precipitated from the supernatant at 20 to 40% saturation of AmS.

Samples were desalted by either repeated centrifugation in ultrafiltration cones (Amicon, C25 or C50) (CSF-1 and CSF-2) or gel filtration (CSF-2).

Assay of CSFs

One blastomere of each 2-cell embryo was injected in the animal half with 60 nl of preparation, after which recipients (20 to 25 for each preparation) were incubated in 10% modified Ringer's solution at 15°C for 18 to 24 h. To determine the concentration dependence of CSF-2, 20, 30, 40, 50 or 60 nl volumes were injected as above. The injected volumes were verified by measuring the diameter of a drop of DH₂O expelled into silicone oil (specific gravity = 0.99).

To determine the unit and specific activity of CSF in a sample, each preparation was diluted to varying degrees and injected into recipients as described previously (Shibuya & Masui, 1988). One unit of CSF-activity was defined as the activity that arrests 50% of recipients at the 1- to 4-cell stage. The specific activity of a preparation was expressed as units of CSF per μg protein ($\text{U } \mu\text{g}^{-1}$).

Sucrose density gradient centrifugation

Linear sucrose density gradients from 10 to 20% were made by mixing two sucrose solutions containing 0.2 M-NaCl, 5 mM-MgSO₄, 2.5 mM-EGTA, 10 mM-NaF, 3 mM-ATP (or 0.5 mM- γ -S-ATP) and 10 mM-Na₂HPO₄-NaH₂PO₄, pH 6.5. Fresh cytosol, or the 20 to 30% AmS fraction of fresh cytosol (0.5 ml), was layered on a 5.0 ml gradient and spun at 85 000 g for 17 h in a swinging bucket rotor (SW 50.1, Beckman). Following centrifugation, 0.22 ml fractions were collected, kept on ice and assayed for CSF-1 activity within 3 h of fractionation. Reference standard proteins were separated in individual gradients accompanying those with cytosolic preparations.

Gel filtration

CSF-2 preparations (0.3 to 0.5 ml containing approximately 1.7 to 2.5 mg of protein) were applied to columns (0.9 cm \times 15 cm) loaded with Sepharose CL-2B, -4B, -6B or Sephacryl S-200 (Pharmacia), which had relative molecular mass exclusion limits of 40×10^6 , 20×10^6 , 4×10^6 , and 2×10^6 , respectively. The columns were equilibrated with CB or the urea solutions described earlier. Samples (125 μl) were collected, desalted with a Sephadex G-25 (fine, Pharmacia) column and concentrated using ultrafiltration cones (Amicon, C50). Blue dextran (Sigma) of average M_r 2×10^6 was used as a reference.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Cytosolic preparations were analyzed by SDS microslab PAGE as described by Matsudaira & Burgess (1978). Proteins were ethanol-precipitated from preparations at -20°C overnight, dried and then dissolved in 0.125 M-sucrose buffered with 10 mM-Tris-HCl, pH 6.8. These samples were mixed 1:1 with a SDS sample buffer consisting of 3.2% SDS (Sigma),

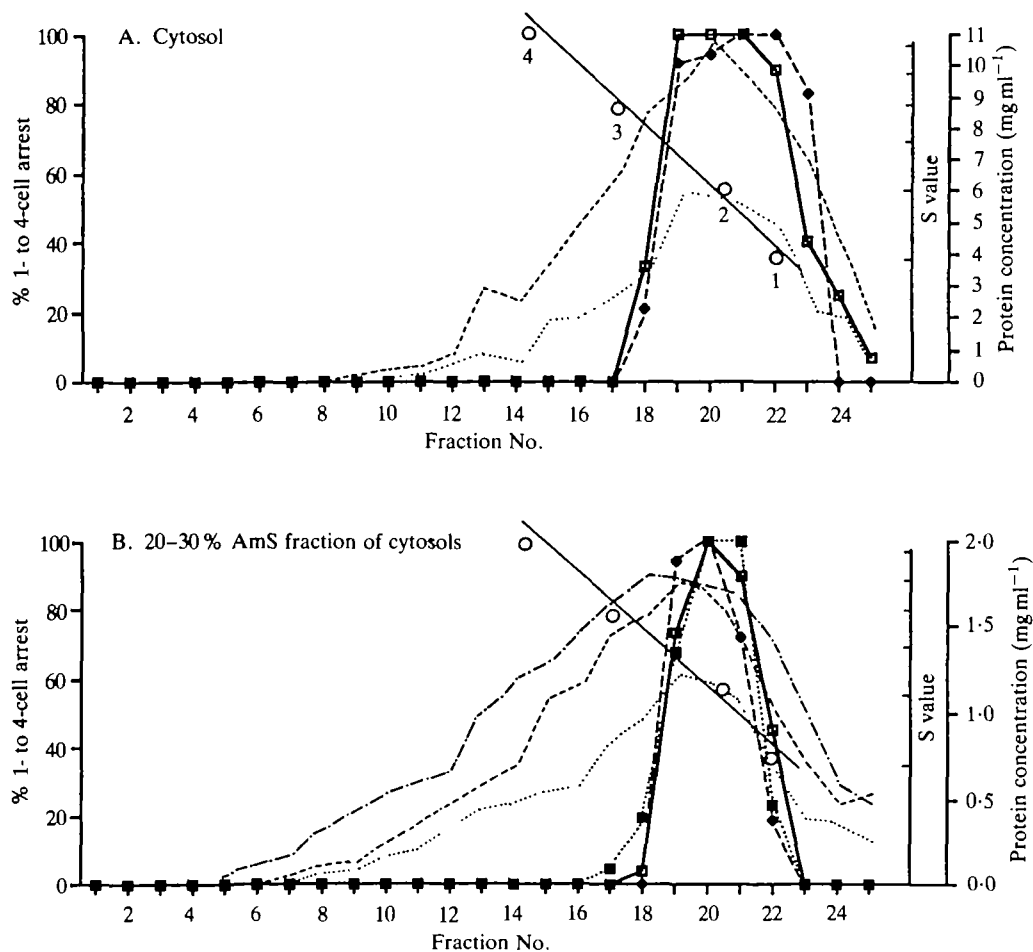


Fig. 1. Sucrose density gradient fractionation of fresh cytosols and fractions of cytosols containing CSF-1. Fresh cytosol or its 20–30% AmS fraction (0.5 ml) was layered on a 5 ml linear gradient of 10 to 20% sucrose (see Materials and Methods) and centrifuged at 85 000 g for 17 h. Fractions (0.22 ml) were kept on ice until assayed for CSF-1. Molecular markers used were as follows: 1, myoglobin, 2.04S; 2, β -lactoglobulin, 2.85S; 3, alcohol dehydrogenase, 4.8S; 4, lactate dehydrogenase, 7.3S. (A) Fresh cytosol (i) Cytosol prepared with standard extraction medium. Before layering on the gradient, 10 mM-NaF and 6 mM-ATP was added to the cytosol. \blacklozenge — \blacklozenge : % 1- to 4-cell arrest; —: protein concentration in each fraction (mg ml^{-1}); amount of protein loaded = 12.0 mg. (ii) Cytosols prepared with standard extraction medium containing 30 mM-NaF. Before layering on the gradient, 6 mM-ATP was added to the cytosol. \square — \square : % 1- to 4-cell arrest; \cdots : protein concentration in each fraction (mg ml^{-1}); amount of protein loaded = 10.4 g. (B) 20–30% AmS fraction of fresh cytosol was redissolved in standard extraction medium containing 10 mM-NaF and 6 mM-ATP and 0.5 ml was loaded on gradients without desalting. (i) Fraction of fresh cytosol prepared with standard extraction medium. \square — \square : % 1- to 4-cell arrest; \cdots : protein concentration in each fraction (mg ml^{-1}); amount of protein loaded = 2.6 mg. (ii) Fraction of fresh cytosol in A.i. above. \blacklozenge — \blacklozenge : % 1- to 4-cell arrest; —: protein concentration in each fraction (mg ml^{-1}); amount of protein loaded = 4.7 mg. (iii) Fraction of cytosol prepared with standard extraction medium. 10 mM-NaF was added to the cytosol before fractionation. \blacksquare — \blacksquare : % 1- to 4-cell arrest; \cdots : protein concentration in each fraction (mg ml^{-1}); amount of protein loaded = 5.8 mg.

16% glycerol (Sigma), 2.8 M- β -mercaptoethanol and 0.0016% bromphenol blue (Sigma). Samples were then boiled for 5 to 15 min, centrifuged, and the supernatants (5 to 10 μl containing 10 to 20 μg protein) were applied to 10, 12 or 17% separating gels (30% (29:1) stock acrylamide-bis solution). Gels were stained with Coomassie blue, and dried between sheets of transparent cellophane membrane backing (Bio-Rad).

Tests for ionic conditions

CSF-2 was dissolved in DB and dialyzed for 18 h at 2°C against solutions of 4 M-NaCl (pH 7.1), 4 M-LiCl (pH 7.0), 8 M-

urea (pH 6.8) (pH adjusted with NaOH), or a solution of 0.25 M-sucrose and 0.1 M-NaCl buffered with 10 mM- Na_2HPO_4 -NaOH (pH 11.25). The dialysates were then dialyzed against DB for 24 h at 2°C before assaying for activity. Also, CSF-2 was treated with these agents for 8 h at 2°C by dissolving directly in the test solutions, before dialyzing against DB as above.

Enzyme tests

For treatment with insoluble enzymes, cytosols or redissolved AmS fractions were exposed to carboxymethyl cellulose-bound protease from *Streptomyces griseus* (Sigma,

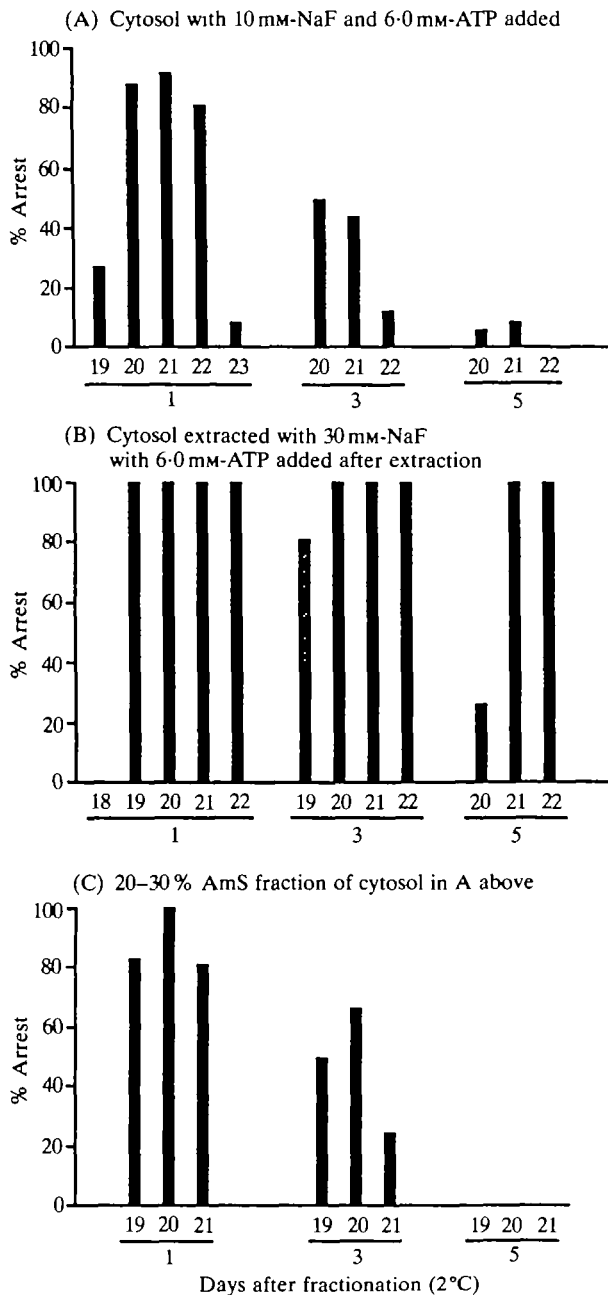


Fig. 2. Stability of CSF-1 after sucrose density gradient fractionation. Following sucrose density gradient centrifugation, fractions were stored at 2°C and assayed for CSF-1 activity 1, 3 and 5 days later. Ordinates: % 1- to 4-cell arrest; Abscissae: Number under each bar indicates fraction no. (see Fig. 1). Number under groups of bars indicates days of storage at 2°C; Solid bar: 1- to 2-cell arrest; Hatched bar: 4-cell arrest; 20 to 25 recipients were injected for each sample. (A) Fractions of cytosol prepared with standard extraction medium. 10 mM-NaF and 6 mM-ATP were added to the cytosol before layering on the gradient (see Fig. 1Ai). (B) Fractions of cytosol prepared with 30 mM NaF in the standard extraction medium. ATP (6 mM) was added to the cytosol before layering on the gradient (see Fig. 1Aii). (C) Fractions of 20-30% AmS fraction of cytosol in A (see Fig. 1Bii).

1 unit ml⁻¹), agarose-bound trypsin (Sigma, 2 units ml⁻¹), or polyacrylamide-bound ribonuclease (RNase, Sigma, 1 unit ml⁻¹) with constant agitation at 25°C. Periodically, the enzymes were sedimented (8000 g for 10 min), and 25 µl of the supernatant was removed and kept on ice until assaying for CSF activity. The enzyme was resuspended, and treatment was continued until the next assay time.

For treatment with soluble enzymes, partially purified CSF-2 was dissolved in DB or 0.2 M-NaCl buffered with 10 mM-Na₂HPO₄-NaH₂PO₄ (pH 6.5) to which *Streptomyces griseus* protease (Sigma, 10 units ml⁻¹), α-chymotrypsin (Sigma, 10 units ml⁻¹) or RNase A (Sigma, 10 units ml⁻¹) was added. After 6 h at 25°C, the digested samples were precipitated 3 times with AmS at 20-40% saturation. The final redissolved precipitate was dialyzed overnight before assaying for CSF activity.

Fingerprint analysis followed the method of Cleveland *et al.* (1977). Partially purified CSF-2 was ethanol-precipitated, dissolved in buffer (0.5% SDS, 10% glycerol, 0.001% bromophenol blue, buffered with 0.125 M-Tris-HCl, pH 6.8) to a concentration of 0.5 mg ml⁻¹, boiled for 2 min, and subjected to proteolysis at 37°C for 30 to 60 min. The proteases used were α-chymotrypsin (Sigma, 0.1 mg ml⁻¹), papain (Sigma, 0.05 mg ml⁻¹), and *Staphylococcus aureus* V8 protease (Sigma, 0.5 mg ml⁻¹). After addition of β-mercaptoethanol and SDS to final concentrations of 10% and 2%, respectively, the reaction was terminated by boiling samples for 2 min before analysis by SDS-PAGE.

Protein determinations

Using bovine serum albumin (BSA) (Bio-Rad) as a standard, protein concentrations of cytosolic samples were determined with Bio-Rad protein assay kit, based on the procedure by Bradford (1976).

Cytology

After fixation in Smith's solution (Smith, 1912), embryos were dehydrated and embedded in Paraplast Plus (Monoject Scientific) and serially sectioned at 10 µm. Sections were stained by the Feulgen procedure, and counterstained with light green (Moore & Ryan, 1940).

Results

CSF-1

Fresh cytosol was treated with insoluble protease or RNase for 1 and 2 h at 25°C. After 1 h of treatment, the protease-treated cytosol lost most of its activity, resulting in only 10% of recipients arrested at the 1- to 4-cell stage. In contrast, the activities of both the RNase-treated and untreated cytosols remained high (89% and 100% of recipients arrested). However, by 2 h all samples had low activity. These results suggest that CSF-1 activity is associated with a labile protein, which remains active for at least 24 h at 2°C (Shibuya & Masui, 1988), but is quickly inactivated at 25°C.

When fresh cytosol containing CSF-1 was fractionated on sucrose density gradients without NaF and ATP, activity was not recovered in any fractions. However, if 10 mM-NaF and 3 mM-ATP were included in the gradient, activity was recovered from either fresh cytosol, or its 20-30% AmS fraction (Fig. 1). There was a broad peak of CSF-1 activity ranging from sedimentation coefficient 1.5S to 4S for fresh cytosols,

Table 1. Summary of CSF-1 recovery after sucrose density gradient fractionation. The samples obtained as described in Fig. 1Biii were assayed for specific activity of CSF-1

CSF-1 preparation	Total protein (mg)	Specific activity ($\text{U } \mu\text{g}^{-1}$)	Total activity ($\text{U} \times 10^{-3}$)	Recovery (%)	Relative increase in specific activity (\times)
Cytosol	73.0	1.7	123.5	100.0	—
20–30 % AmS fraction	5.8	4.0	23.3	19.0	2.4
Sucrose density gradient:					
Fraction 19	0.38	11.0	4.1	3.3	6.3
Fraction 20	0.39	14.0	5.3	4.3	8.1
Fraction 21	0.36	12.0	4.3	3.5	7.0
Total	1.13	—	13.7	11.1	—

and a steep peak at approximately 3S for the 20–30 % AmS fraction of fresh cytosols (Fig. 1). AmS fractionation followed by sucrose density gradient centrifugation could achieve about 8 times purification of CSF-1 (Table 1).

Fractions recovered from sucrose density gradients were stored at 2°C to determine the stability of the partially purified CSF-1. After centrifugal fractionation of cytosols prepared with NaF, CSF-1 persisted for at least 5 days. However, the CSF-1 in the centrifugal fractions of the 20 to 30 % AmS precipitate was less stable than that in fractions obtained from fresh cytosol. In only 1 experiment, CSF-1 activity lasted for 3 days after fractionation (Fig. 2C), whereas in all other experiments, activity had disappeared by 1 day after fractionation. The addition of 0.5 mM- γ -S-ATP to both the gradient and the sample failed to prolong CSF-1 activity.

To examine the peptide compositions of the AmS fractions that contain CSF-1 activity, individual fractions obtained by sucrose density gradient centrifugation (Fig. 1B.iii.) were electrophoresed on a 10 % gel. All fractions tested completely entered the gel,

showing numerous stained bands. However, it is rather difficult to correlate the activity of CSF-1 with the presence of particular peptide bands stained on the gel (Fig. 3).

CSF-2

Partially purified CSF-2 was prepared from the supernatant obtained after precipitating proteins from fresh cytosol by 50 % AmS (Materials and methods). This CSF-2 has a specific activity at least 20 times higher than crude stored cytosols (Shibuya & Masui, 1989) and remained stable for at least 24 h at 25°C. When this preparation was treated with insoluble and soluble enzymes, a significant reduction in the activity occurred in the samples incubated with proteolytic enzymes, whereas those treated with RNase did not lose activity (Table 2). Also, there was no precipitate recovered from preparations digested for 6 h with proteases when the digests were precipitated with AmS between 20 and 40 % saturation, whereas CSF-2 was precipitated from both the control preparations as well as the preparation treated with RNase (Table 2). These results strongly

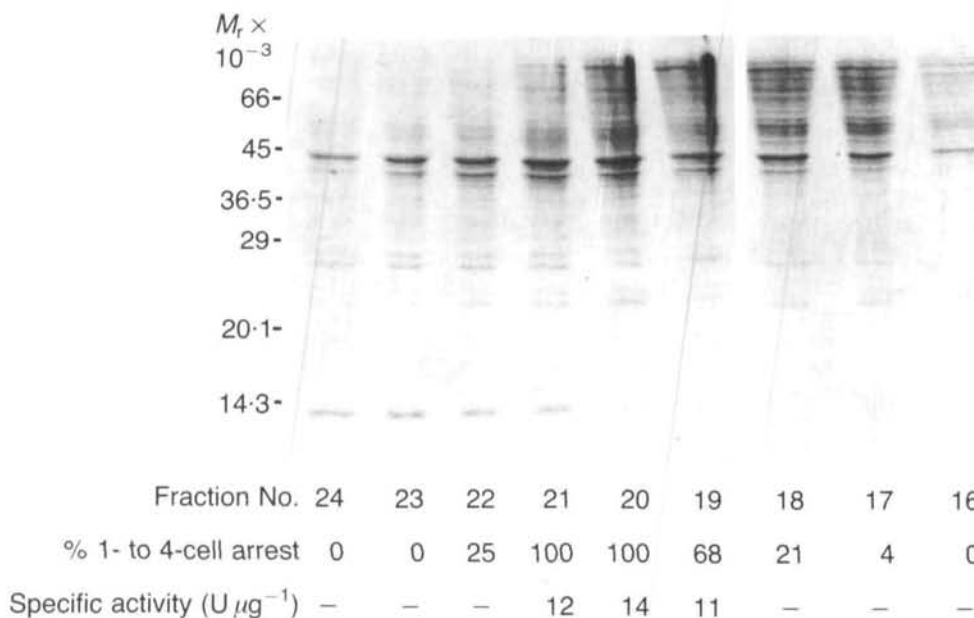
**Fig. 3.** Polyacrylamide gel analysis of fractions obtained by sucrose density gradient centrifugation of the AmS precipitate at 20–30 % saturation. Fractions were obtained by sucrose density gradient centrifugation as described in Fig. 1B and electrophoresed through a 10 % SDS-polyacrylamide gel. The % of recipients arrested by injection and specific activity of each fraction are indicated below each lane.

Table 2. Sensitivity of CSF-2 to enzyme digestion

CSF-2 preparation tested	Enzyme	% 1- to 4-cell arrest (No. of recipients)			
		3 h	4 h	6 h	24 h
(A) Crude CSF-2 (Stored cytosol)	0	92 (24)	—	—	—
	protease	100 (24)	—	—	—
	RNAse	96 (24)*	—	—	—
(B) Partially purified CSF-2 (20–40 % AmS fraction)					
(i) Insoluble enzymes	0 (pH 6.5)	100 (21)	—	100 (21)	—
	0 (pH 7.0)	100 (41)	—	100 (22)	—
	0 (pH 8.0)	—	91 (23)	91 (23)	91 (22)
				40 (23)§	—
	protease (pH 7.0)	100 (26)	—	100 (20)	—
	RNAse (pH 6.5)	91 (21)	—	95 (20)	—
	trypsin (pH 8.0)	—	86 (18)	52 (23)	0 (23)
				4 (25)§	—
					—
					—
(ii) Soluble enzymes	0 (DB)	—	—	100 (20)	—
	0 (0.2 M NaCl)	—	—	100 (20)	—
	protease	—	—	†	—
	α -chymotrypsin	—	—	†	—
	RNAse	—	—	76 (21)	—

* all recipients showed signs of degeneration

§ preparation diluted to 50 % of original concentration

† no precipitation between 20–40 % AmS saturation

— not tested

Stored cytosol or partially purified CSF-2 was digested with insoluble enzymes and assayed for activity as described in Materials and Methods. Digestion of partially purified CSF-2 with insoluble protease, trypsin or RNAse used DB (pH 7.0), a solution of 0.25 M-sucrose buffered with 10 mM-NaH₂PO₄-Na₂HPO₄ (pH 8.0), or DB (pH 6.5), respectively.

Digestion with soluble enzymes was carried out using protease and α -chymotrypsin dissolved in DB (pH 7.0) and RNAse in a buffer of 0.2 M-NaCl and 10 mM-NaH₂PO₄-Na₂HPO₄ (pH 6.5). After treatment, proteins were precipitated 3 times from the digested samples at 20–40 % saturation of AmS to remove the enzymes, and redissolved samples were dialyzed overnight before assaying for activity.

indicate that CSF-2 activity is associated with a stable protein.

However, when the partially purified CSF-2 was electrophoresed on 10 % gels, almost no protein entered into the spacer gel (Fig. 4, lane C). This was never observed in the original supernatant before CSF-2 had developed. The partially purified CSF-2 was also excluded from 2 % polyacrylamide gels as well as 2 % agarose gels. Dissolving CSF-2 in sample buffer with 4×SDS, followed by excessive boiling (up to 15 min) did not render the protein capable of penetrating the gel. To test further the sensitivity of CSF-2 to protein-denaturing conditions, preparations were treated with 4 M-NaCl, 4 M-LiCl, 8 M-urea or a buffer of high pH (11.25). In all cases, not only was high CSF-2 activity recovered after treatment, but the specific activities of the CSF-2 preparations remained approximately the same as prior to the treatment. Therefore, CSF-2 activity is resistant to extreme ionic conditions and pH that denature most proteins.

These observations suggest that CSF-2 is a highly stable, very large molecule. To determine the approximate molecular size of CSF-2, partially purified preparations were applied to gel filtration columns loaded with Sepharose CL-2B, 4B, 6B or Sephacryl S-200. However, in all cases except for the CL-2B column, CSF-2 activity and almost all of the protein eluted as a single narrow peak in the same fractions in which blue

dextran was eluted, and little protein remained in the column (Fig. 5). In the case of the CL-2B column, CSF-2 activity was eluted slightly ahead of blue dextran. The best recovery of CSF-2 activity was achieved when samples treated overnight with 6 or 8 M-urea were applied to Sepharose CL-4B or Sephacryl S-200 columns and then desalted with a Sephadex G-25 column (Table 3). If the columns were equilibrated with urea, CSF-2 activity was still eluted in the same fractions as those containing molecules of similar size to blue dextran. Therefore, the relative molecular mass of CSF-2 must be at least 2×10^6 .

The relative increases in specific activity after gel filtration ranged from 1.4 to 1.8 times that of the original samples, or up to approximately 40 times the specific activity of the whole cytosols. The specific activity of purified samples reached $60 \text{ U } \mu\text{g}^{-1}$ protein after filtration with Sepharose CL-4B. However, further repeated gel filtration failed to increase the specific activity beyond this level (data not shown). Samples eluted from Sepharose CL-4B columns had higher specific activities (44 and $53 \text{ U } \mu\text{g}^{-1}$), and contained much less protein that could penetrate the gels, when electrophoresed through 10 % gels, than samples eluted from Sephadex G25 column after desalting (32 and $33 \text{ U } \mu\text{g}^{-1}$) as seen in Table 3. Therefore, clearly the gel filtration had removed smaller proteins from the CSF-2 preparations, increasing specific activity.

When partially purified CSF-2 samples were electrophoresed after trypsin digestion, the components that had failed to enter the gel disappeared, and bands of small peptides appeared in the separating gel (Fig. 4). The change in the electrophoretic behaviour of the sample after trypsin digestion was correlated with the reduction of CSF activity. When column-purified CSF-2 was treated with α -chymotrypsin, papain, or *S. aureus* V8 proteases and electrophoresed in 17% SDS-PAGE according to the peptide-mapping method of Cleveland *et al.* (1977), the proteins in the sample were extensively digested by α -chymotrypsin and papain, but not by V8, into very small peptide fragments, which could not be resolved by the gel (Fig. 6C,F,I). BSA treated with these enzymes in the same way as CSF-2 samples showed its characteristic patterns of peptide fragment distribution (Fig. 6B,E,H). Taken together, these results suggest that partially purified CSF-2 activity is associated with an extremely large molecule that is formed from the assembly of protein components by covalent-bonding.

Since CSF-2 appears to be a large molecule, it was of interest to determine its dose-effect relationship. To this end, different volumes of a partially purified CSF-2 preparation, which had been diluted to 80, 60, 40, 20, or 10% of its original concentration, were injected into recipients, and the specific activities were calculated.

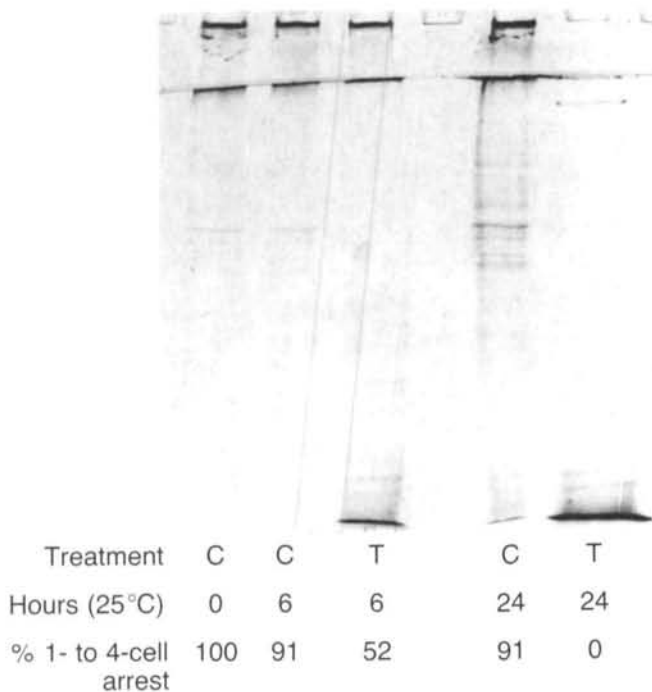


Fig. 4. Polyacrylamide gel electrophoresis of CSF-2 treated with insoluble trypsin. Samples of partially purified CSF-2, which had been digested with insoluble trypsin for 6 or 24 h as described in Table 2, were electrophoresed through a 10% SDS-polyacrylamide gel. Conditions of the treatments applied to the samples (C: control, no enzymes; T: trypsin treatment), and their CSF activities (% of arrested recipients) are indicated below each lane.

The dose-effect curves in the graph did not show a dependence on CSF-2 concentrations in injected volumes (Fig. 7A). In other words, different injected samples containing the same amount of protein brought about the same percentages of arrested recipients regardless of their volumes or concentrations.

However, the stage at which recipients could be arrested appeared to be varied by the volume even if a constant amount of protein was injected. Thus, as seen in Fig. 7B, the same amount of protein injected in a smaller volume causes recipients to be arrested more frequently at the 4-cell stage, whereas a larger injected volume arrests more recipients at the 2-cell stage. This may imply that the effect of CSF-2 is exerted by diffusion through the cytoplasm. Therefore, the delayed arrest at 4-cell stage caused by injection of a small volume of CSF-2 may be a consequence of delay in expansion of the sphere of CSF-2 influence due to its slow-diffusion through the cytoplasm.

Cytological examination

Hemiblastulae that had been injected with sucrose density gradient fractions containing CSF-1 (4 embryos), or urea-treated samples of partially purified CSF-2 (12 embryos) were examined cytologically. In all cases, the nucleus was arrested at metaphase indicative of authentic CSF arrest.

Discussion

Our experiments treating CSF-1 with enzymes and increased temperature strongly indicated that CSF-1 was a labile protein. We previously had suggested that the stability of CSF-1 in fresh cytosols depended on phosphorylation of proteins, since this activity was not only prolonged, but also enhanced, by additions of NaF and ATP or γ -S-ATP. Also, addition of ATP to NaF-containing cytosols could recover and extend CSF-1 activity for days as long as a residual activity was still detected (Shibuya & Masui, 1988).

In the present study, we found that CSF-1 activity could be recovered after sucrose density gradient centrifugation only if NaF and ATP or γ -S-ATP were present in the system. When whole cytosol was fractionated on sucrose density gradients, fractions retained high CSF-1 activity for several days during storage at 2°C. In contrast, AmS precipitates containing CSF-1 had high activity immediately after sucrose density gradient fractionation, but the fractions soon lost activity despite the presence of 10 mM-NaF and 3 mM-ATP or 0.5 mM- γ -S-ATP. Therefore, it appears that the components that stabilize CSF-1 in the presence of NaF and ATP could be present in the whole cytosols and sedimented together with CSF-1 activity through sucrose density gradients, whereas they may become inactivated in, or separated from, the fraction containing CSF-1 during AmS precipitation. Some of these components may be protein kinases, which stabilize CSF-1 activity by protein phosphorylation utilizing ATP.

In our sucrose density gradients, CSF-1 activity was

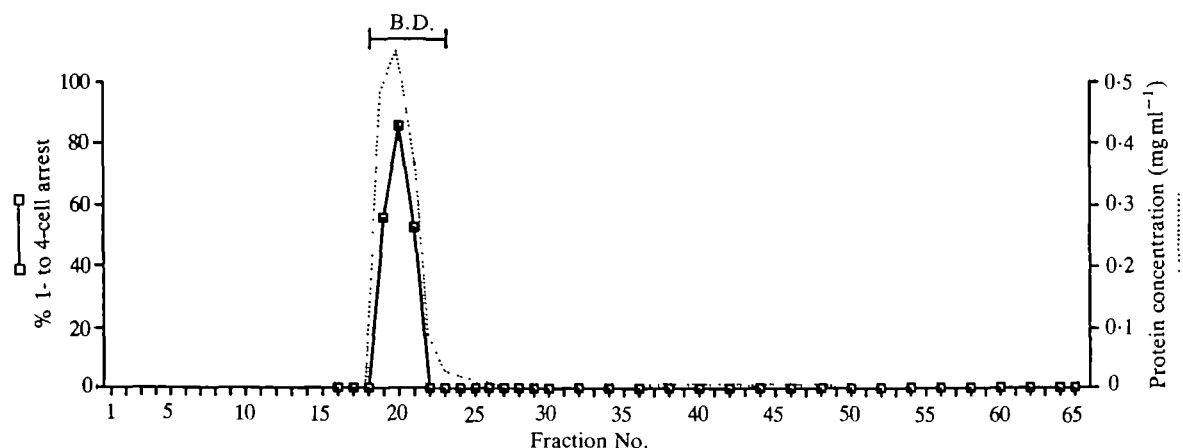


Fig. 5. Gel filtration of CSF-2 on Sepharose CL-4B. Partially purified CSF-2 after AmS precipitation was redissolved in CB for 18 h at 18°C and applied to a Sepharose CL-4B column equilibrated with CB. Almost all the protein and CSF-2 activity was eluted in the same fractions as those in which blue dextran was eluted. B.D.: range of fractions containing blue dextran ($M_r = 2 \times 10^6$).

sedimented with its peak at approximately 3S. This result is not consistent with our previous observation that CSF-1 activity in fresh cytosols extracted without NaF and ATP could be sedimented rather quickly by ultracentrifugation (Shibuya & Masui, 1989). A possible explanation to reconcile the discrepancy between these results may be that CSF-1, which exists in whole cytosols as a large complex of molecules or in association with large molecules, becomes smaller forms in the presence of NaF and ATP during sucrose density gradient centrifugation. This situation is comparable to maturation promoting factor (MPF), a cytoplasmic factor that is responsible for transition from G₂ to M-phase of both the meiotic and mitotic cell cycles (for review, see: Masui & Clarke, 1979; Masui and Shibuya, 1987). MPF has also been shown to change sizes when it is fractionated under different conditions. MPF in cytosols of *R. pipiens* eggs when fractionated by sucrose density gradient centrifugation, showed activity peaks of 32S, 15S and 4S (Wasserman & Masui, 1976). In starfish, MPF was fractionated on a Sephacryl S-300 column as $300 \times 10^3 M_r$ molecules, while the activity was

recovered in the 5S fraction by sucrose density gradient centrifugation (Kishimoto & Kondo, 1986). More recently, highly purified MPF of *Xenopus laevis* (Lohka *et al.* 1988) showed an apparent relative molecular mass of 145×10^3 or higher on column chromatography, but SDS-PAGE showed that it consisted of 45 and 32×10^3 peptides. Therefore, it appears that both MPF and CSF-1 could exist either as large or small molecular forms.

In the present study, it was remarkable that CSF-2 neither lost activity nor reduced its molecular size after treatment at high ionic strength, high pH or with high concentrations of urea. Therefore, in all probability, the molecular assembly of CSF-2 does not involve electrostatic linkages and hydrogen bonds. Although CSF-2 activity was more resistant to proteolytic digestion than CSF-1, when CSF-2 was treated long enough with proteolytic enzymes, it lost activity and, at the same time, was digested into small peptides. Results of peptide mapping suggest the presence of basic and aromatic amino acids and free sulphhydryl groups, but the absence of acidic amino acids in the peptide chains.

Table 3. Summary of gel filtration of CSF-2. Partially purified CSF-2 was dissolved in the sample buffer indicated and desalted with Sephadex G-25, or applied to Sephacryl S-200 or Sepharose CL-4B columns before desalting. The eluted samples containing CSF-2 activity were pooled and their specific activities were determined

Gel filtration	Elution buffer	Sample buffer	Hours at 18°	Specific activity ($U \mu g^{-1}$)	Relative increase in specific activity (×)
Sephacryl S-200	CB	CB	18–24	46–51	1.4–1.5
	urea (6.0 M)	urea (6.0 M)	2	58	1.6
			18–24	46–55	1.4–1.5
Sepharose CL-4B	CB	CB	18–24	63	1.8
	CB	urea (8.0 M)	18–24	64	1.8
	CB	urea (8.0 M)	18–24	60	1.7
		+β-merc. (0.5 M)			
	urea (8.0 M)	urea (8.0 M)	2	62	1.7
Sephadex G-25	urea (6.0 M)	urea (6.0 M)	18–24	44–53	1.5–1.7
	CB	urea (6.0 M)	18–24	33–36	—

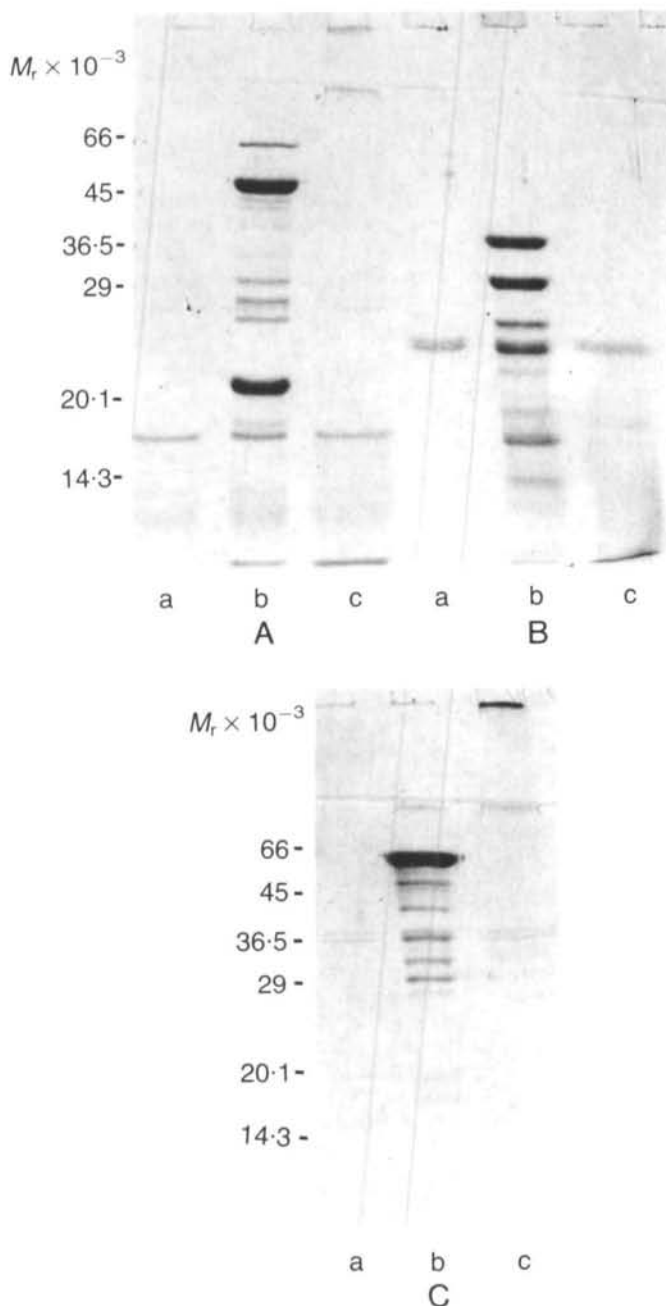


Fig. 6. Proteolytic digestion of CSF-2. Bovine serum albumin (BSA) and column-purified CSF-2 were digested with α -chymotrypsin, papain, or *Staphylococcus aureus* V8 protease at 37°C for 30, 30 or 60 min, respectively, and then electrophoresed through 17% SDS-polyacrylamide gels. a: enzyme alone; b: BSA; c: CSF-2. A: α -chymotrypsin digests; B: papain digests; C: *Staphylococcus aureus* V8 protease digests.

Taken together, these results indicate that CSF-2 activity is associated with an extremely large molecule consisting of covalently linked peptides.

We obtained highly purified samples of CSF-2 with specific activities ranging from 60 to 64 $\text{U } \mu\text{g}^{-1}$ protein after gel-filtration using Sepharose CL-4B. These samples were found to be effective in arresting 50% of

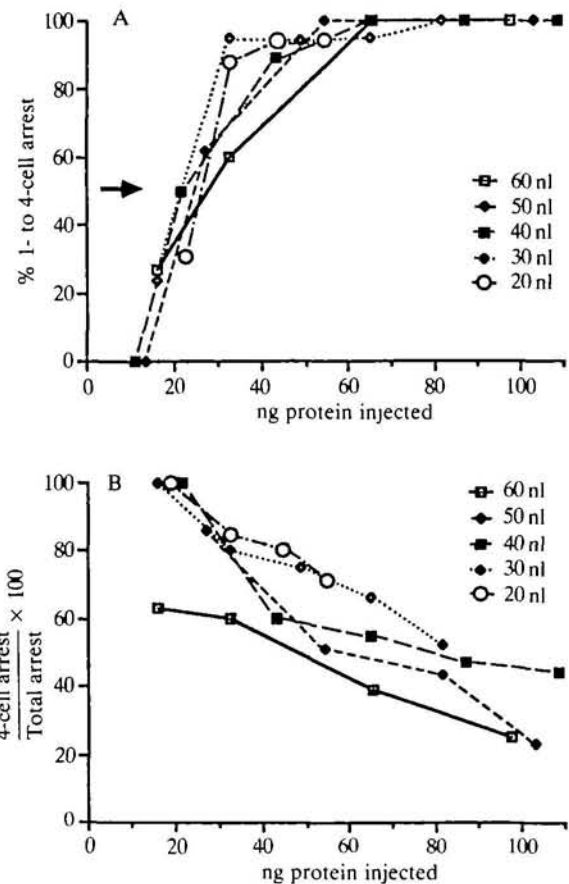


Fig. 7. Effect of different injected volumes and concentrations on the activity of CSF-2 and stage of arrest. A preparation of refractionated CSF-2 was diluted to varying degrees and 20, 30, 40, 50 and 60 nl of each dilution was injected into 2-cell recipients. (A) Determination of 1 unit of CSF activity with respect to the volume of preparation injected. From this graph, the specific activities of the CSF-2 contained in 20, 30, 40, 50 and 60 nl were calculated. They were 42, 45, 44, 39 and 35 $\text{U } \mu\text{g}^{-1}$, respectively. (B) Relationship between CSF-2 concentration and the stage of recipient arrest. For each volume, the percentage of 4-cell arrested embryos ($2 \times 1/4$ and $1/4$ embryo) of the total embryos arrested was calculated and plotted against the amount of protein injected.

recipient blastomeres at doses of about 16 ng per recipient cell. Since CSF-2 was very stable, the fact that repeated gel filtration did not increase its specific activity may suggest that the sample we obtained has approached purity, rather than a loss of the activity during further gel filtration.

We have previously shown that CSF-2 development was accelerated by increasing temperature and optimized near pH 5.5. CSF-2 which developed from the >50% AmS supernatant could be reprecipitated between 20 and 40% saturation of AmS, as well as sedimented by ultracentrifugation. These observations clearly indicated that CSF-2 is the product of a chemical reaction that brings about the assembly of inactive small precursor molecules into a less soluble large CSF-2

molecule(s). This chemical reaction does not require ATP and cannot be prevented by addition of the protease inhibitor, PMSF, or increasing concentrations of β -mercaptoethanol up to 0.1 M (Shibuya & Masui, 1989). Therefore, this molecular assembly reaction does not seem to involve either serine-protease digestion or the formation of disulphide bonds. It is possible that CSF-2 is a product of protein cross-linking reactions similar to the hardening of the fertilization membrane in sea urchins (Lallier, 1971a,b; Foerder & Shapiro, 1977; Veron *et al.* 1977; Hall, 1978; Kay *et al.* 1982; Kay & Shapiro, 1987), or the transglutaminase-catalyzed formation of covalent bridges between glutamine and lysine residues of cytoplasmic proteins (for review, see: Folk, 1980; Lorand & Stenberg, 1982; Cariello *et al.* 1984; Maccioni & Arechaga, 1986). Although the functional significance of these transglutaminases in the control of the cell cycle is as yet unknown, the fact that both CSF-2 development and transglutaminase-catalyzed reactions require Ca^{2+} suggests that CSF-2 could be a product of transglutaminase-mediated cross-linkage of proteins.

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