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Purification and characterization of the protein kinase eEF-2 isolated from rat liver cells

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The elongation factor 2 (eEF-2) protein kinase was isolated from rat liver cells, purified and partly characterized. It was found that the enzyme exists in an inactive form in the homogenate of rat liver. The active fraction of kinase eEF-2 was obtained after removal of the inhibitory substance by hydroxyapatite column chromatography.

The purified enzyme is an electrophoretically homogeneous protein with relative molecular mass of approximately 90000 and isoelectric point, pI = 5.9. The enzyme specifically phosphorylates the elongation factor eEF-2 in the presence of calmodulin and Ca^{2+}.

The protein kinase eEF-2, calmodulin and Ca^{2+}-dependent (kinase III), which specifically phosphorylates elongation factor eEF-2 is an object of interest of our laboratory, but so far little information on isolation and purification of the enzyme has been published. Protein kinase III, partially purified, was obtained by Nairn *et al.* in 1985 [1] and by Ryazanov *et al.* in 1988 [2]. In 1991 the enzyme was purified from rabbit reticulocytes and its relative molecular mass was determined to be 90000 [3]. Up to the present time satisfactory purification of kinase eEF-2 from rat liver has not been achieved [4]. In this paper the results of the purification and characterization of the protein kinase eEF-2 from rat liver cells are presented.

MATERIALS AND METHODS

Male Wistar rats, 150–200 g in weight, were used. All the introductory procedures: preparation of the cell free system from rat liver (ribosomes, elongation factors, ^{14}C-labelled aminoacyl-tRNA) and assay of eEF-2 activity were described earlier [5–7].

The kinase eEF-2, dependent on calmodulin and Ca^{2+}, was isolated from rat liver as described by Nilsson *et al.* [3] with some modifications.

Preparation of homogenate. Rat livers were homogenized in buffer A containing: 20 mM Tris/HCl, pH 7.6, 1 mM EDTA, 1 mM EGTA, 15 mM 2-mercaptoethanol, 100 mM KCl, 0.02% NaN_3, 0.1 mM PMSF[1], using a Potter homogenizer. Homogenate was centrifuged for 0.5 h at 15000 r.p.m. and the pellet was discarded. Supernatant was centrifuged for 3 h at 105000 $\times g$ and the sediment was discarded.

Ammonium sulphate fractionation. To the postribosomal supernatant ammonium sulphate was added to 0.4–0.6 saturation. The precipitate was centrifuged for 10 min at 10000 $\times g$ and the pellet dissolved in 40–50 ml of buffer B

[1]Abbreviations: PMSF, phenylmethylsulfonylfluoride; DTT, dithiothreitol.

(20 mM Tris/HCl, pH 7.6, 0.1 mM EDTA, 15 mM 2-mercaptoethanol, 100 mM KCl, 10% glycerol, 0.1 mM PMSF) was dialyzed in vacuum against buffer B.

Chromatography on DE-52 cellulose. To the top of the column (2.5 cm × 17 cm), packed with DE-52 cellulose and equilibrated with buffer B, 10 ml of the concentrated dialysate (1.5 g of protein) was applied. Elution was performed with buffer B containing 0.1, 0.225 or 0.4 M KCl. The flow rate was maintained at about 25 ml per hour and the fractions of 5 ml were collected. The fractions eluted with 0.4 M KCl were concentrated to the volume of 3–5 ml with the use of Centriper 10 separator and dialyzed against buffer C containing: 10 mM KH_2PO_4/ /K_2HPO_4, pH 7.0, 10% glycerol, 7 mM 2-mercaptoethanol, 0.1 mM PMSF.

Chromatography on hydroxyapatite. The concentrated, dialyzed preparation obtained after DE-52 cellulose column chromatography eluted with 0.4 M KCl (approximately 15 mg of protein), was placed on the top of the column (1 cm × 22 cm) packed with hydroxyapatite and equilibrated with buffer C. The elution was carried out with the same buffer till no protein was present in eluate, and then with the linear gradient (0.1 to 0.3 M) of KH_2PO_4/K_2HPO_4, pH 7.0 (2 × 50 ml). The flow rate was maintained at 25 ml per hour and fractions of 3 ml were collected. Active fractions were concentrated by vacuum dialysis against the buffer containing: 25 mM Tris/HCl, pH 7.6, 0.1 mM EDTA, 7 mM 2-mercaptoethanol, 100 mM KCl, 15% glycerol.

Molecular filtration through Ultrogel AcA-44. The concentrated, dialyzed preparation obtained after hydroxyapatite chromatography (approximately 1 mg of protein) was placed on the Ultrogel column AcA-44 (1.5 cm × 70 cm) equilibrated with the dialyzing buffer. The elution was carried out with the same buffer. The flow rate was maintained at 20 ml per hour and fractions of 1.3 ml were collected. The active fractions were combined, concentrated and stored at –20°C.

Analytical procedures

Protein determination. In all of the soluble fractions from the chromatographic column, protein concentration was measured spectrophotometrically at 260 nm and 280 nm, after correction for the absorption at 320 nm [8]. Protein in combined active fractions was determined according to Bradford [9] using bovine serum albumin as a standard.

Assay of eEF-2 protein kinase activity. The incubation mixture, in 50 µl, contained: 20 mM Tris/HCl, pH 7.6, 1 mM $MgCl_2$, 0.6 mM $CaCl_2$, 5 mM DTT, 20 µM [γ-^{32}P]ATP (about 1000 c.p.m./pmol), 10 µg/ml of calmodulin, 1 to 10 µg of eEF-2 and 5 to 10 µl of the kinase fraction to be assayed. Incubation was performed at 30°C for 10 min. The reaction was stopped by adding buffered solution in a volume adjusted to obtain final concentrations of: 62.5 mM Tris/HCl, pH 6.8, 10% glycerol, 2% SDS, 1.5% DTT, 0.1% bromophenol blue. Samples were heated for 3 min in a boiling water bath and used for electrophoretic separation according to Laemmli [10]. Dried electrophoretograms were autoradiographed [11]. The varying intensity of the bands, corresponding to relative molecular mass of kinase eEF-2, were interpreted as corresponding to the concentrations of kinase eEF-2 in particular fractions.

The activity of the purified preparations of eEF-2 protein kinase was assayed as described previously [12].

The standard proteins used for the assay of relative molecular mass by polyacrylamide-SDS gel electrophoresis were: myosin (205000), β-galactosidase (116000), phosphorylase *b* (97400), bovine serum albumin (66000), chicken egg albumin (45000), carbonic anhydrase (29000).

Chemicals. Tris, ATP, PMSF, bovine serum albumin were from Serva; histone IIA, high molecular weight standard mixture for SDS--polyacrylamide gel electrophoresis, trypsin from bovine pancreas, EGTA from Sigma; calmodulin from Calbiochem; hydroxyapatite for column chromatography from Merck; DEAE-cellulose DE-52 from Pharmacia; Ultrogel AcA-44 from BF Biotechnics (France); [^{14}C]leucine (60 mCi/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were from Amersham. Other reagents were of analytical grade and obtained from commercial sources.

RESULTS AND DISCUSSION

The results presented in Fig. 1 show that the 0.4–0.6 ammonium sulphate saturation fraction was separated by column chromatography on

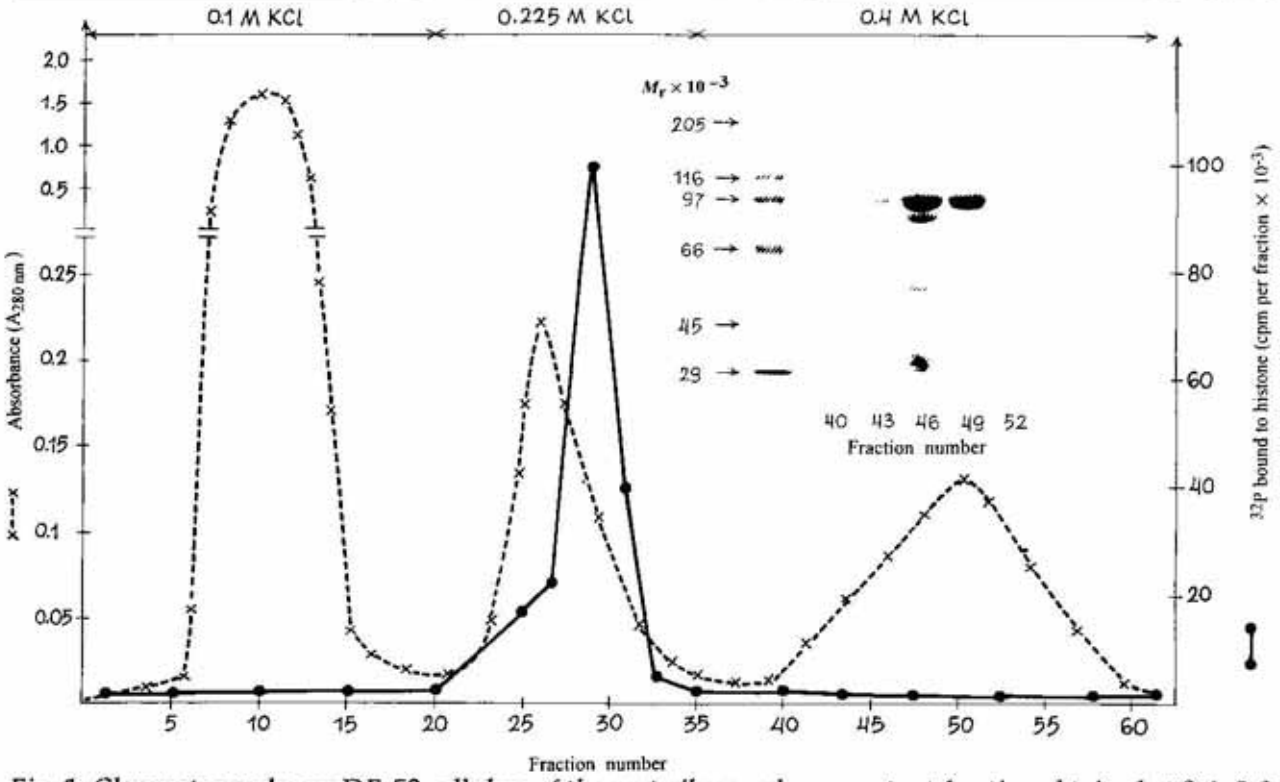

Fig. 1. Chromatography on DE-52 cellulose of the post-ribosomal supernatant fraction obtained at 0.4–0.6 ammonium sulphate saturation.

The protein corresponding to kinase eEF-2 was identified electrophoretically [10]; cAMP-dependent kinase activity was localized with the use of histone IIA as a substrate [13]. Absorbance was measured in the 20-fold diluted fractions. For details see Methods.

DE-52 cellulose into three protein fractions. Unfortunately, the fractions containing a protein with relative molecular mass corresponding to that of eEF-2 kinase (about 90000) were inactive (Fig. 1, electrophoretogram). According to Nygard *et al.* [4] this kinase can be activated by cAMP-dependent protein kinase. The latter enzyme was identified in fractions eluted with 0.225 M KCl (Fig. 1) with the use of histone IIA as an exogenous substrate of cAMP-dependent kinase. The enzyme did not phosphorylate protein fractions eluted from the column with 0.4 M KCl or exogenous eEF-2.

The fractions eluted with 0.4 M KCl were combined and separated again into three fractions on hydroxyapatite column (Fig. 2).

The fractions eluted with 0.1 to 0.14 M phosphate buffer intensively phosphorylated eEF-2 (Fig. 2). The fraction not adsorbed on hydroxyapatite distinctly inhibited the activity of kinase eEF-2 (Fig. 3, B,b). After trypsinolysis of this fraction no inhibitory effect was observed (Fig. 3, B,c), thus pointing to the protein character of the inhibitor. The above results show that protein kinase eEF-2 exists in the postribosomal supernatant together with its inhibitor, and this fact makes clear why protein kinase eEF-2 was inactive in the crude preparation.

Final purification of the protein kinase eEF-2 was achieved by molecular filtration through Ultrogel AcA-44 of the combined active fractions obtained after chromatography on hydroxyapatite (Fig. 4). The peak fractions (76–80) migrated during PAGE-SDS electrophoresis [10] as a single protein band with relative molecular mass of about 90000, in agreement with the results obtained by the group of Nilsson & Nygard [3, 4]. The results show that the purification procedure described above yields a pure, homogeneous eEF-2 protein kinase.

Active fractions obtained after molecular filtration through Ultrogel AcA-44 were concentrated and characterized. The enzyme was free of eEF-2 and it did not undergo autophosphorylation. Its activity was Ca^{2+}-dependent and increased by about 70% after addition of exogenous calmodulin. Specificity of protein kinase for eEF-2 as a substrate is presented in Fig. 5, c. Low kinase activity, observed in the system assayed with no calmodulin added (Fig. 5, b), was caused probably by the presence of slight amounts of endogenous calmodulin.

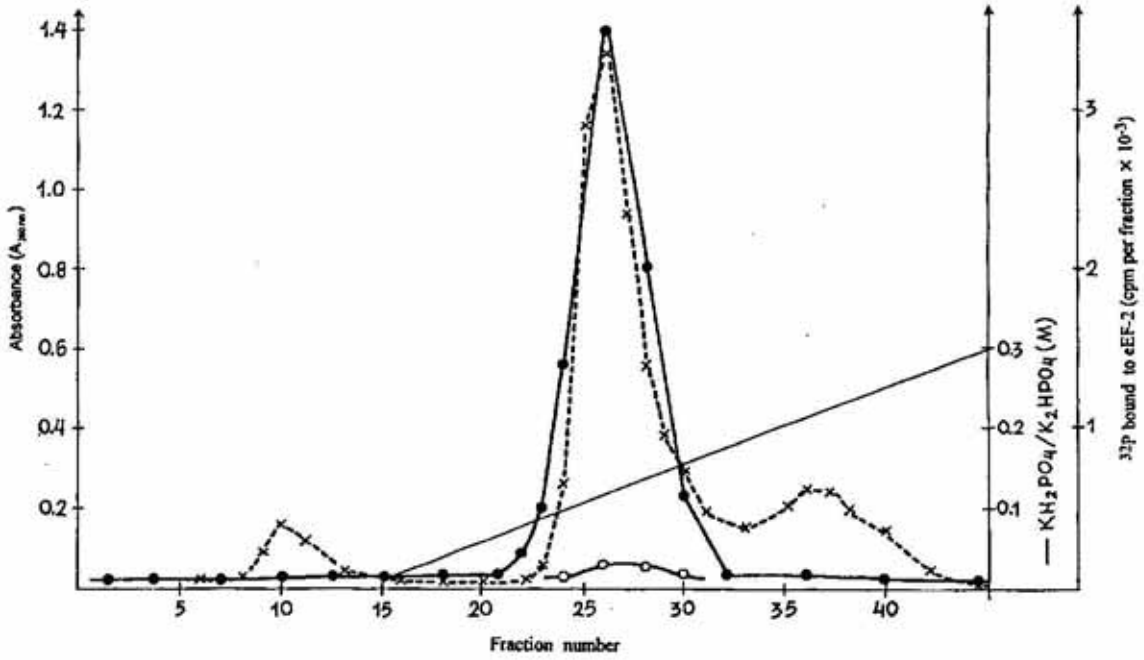

Fig. 2. Chromatography on hydroxyapatite column of the combined fractions (40–52) eluted from DE-52 cellulose.
(×) Absorbance; (●) amount of ^{32}P bound to eEF-2; (○) amount of ^{32}P bound to eEF-2 in the presence of combined fractions 8–12 (about 10 µg of protein). For the assay of protein kinase activity 20 µl of each fraction and 5 µg of the substrate (eEF-2) were used. For details see Methods.

EGTA inhibited phosphorylation of eEF-2 by binding of Ca^{2+} (Fig. 5, e).

The homogeneity and relative molecular mass (90000) of the purified protein kinase eEF-2 was confirmed by two-dimensional electrophoresis (PAGE-IEF) according to O'Farrell [14]; pI of the enzyme was 5.9.

The optimum calmodulin and Ca^{2+} concentrations for kinase eEF-2 activity were 16 µg/ml and 0.6 mM, respectively. The increase in the amount of the phosphorylated form of eEF-2 (100000) was time-dependent. Michaelis constant (K_m) of kinase eEF-2 calculated for eEF-2 as a substrate in the reaction of phosphorylation was 1.1 µM and for ATP it was 16.7 µM.

The results on substrate specificity of protein kinase eEF-2 are collected in Table 1. The enzyme showed the highest activity with eEF-2 (100000) from rat liver; the activity with eEF-2 (100000) from Guerin epithelioma cells being only about one fifth of the former. Casein, total histone and histone IIA were phosphorylated

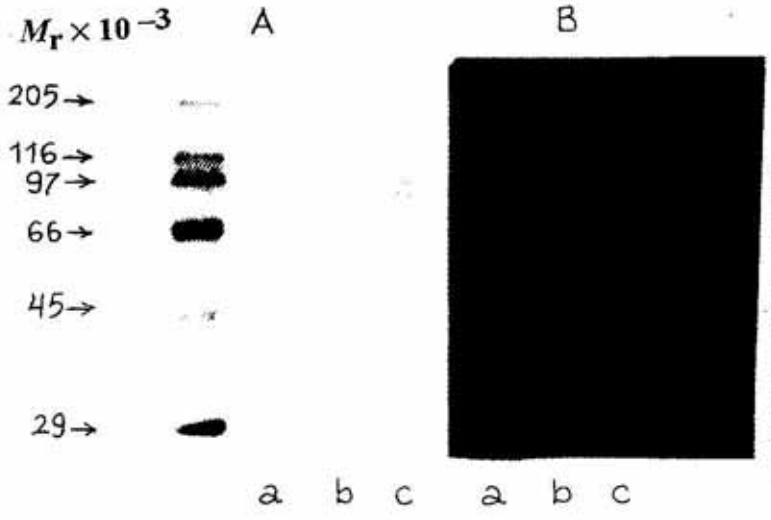

Fig. 3. Electrophoretogram (A) and autoradiogram (B) obtained after phosphorylation of eEF-2: (a) with no kinase inhibitor added; (b) with inhibitor added; (c) in the presence of the inhibitor fraction previously digested with trypsin.
Electrophoresis was performed according to Laemmli [10]; autoradiography [11] was carried out with the use of X-Ray Film (Foton, Poland).

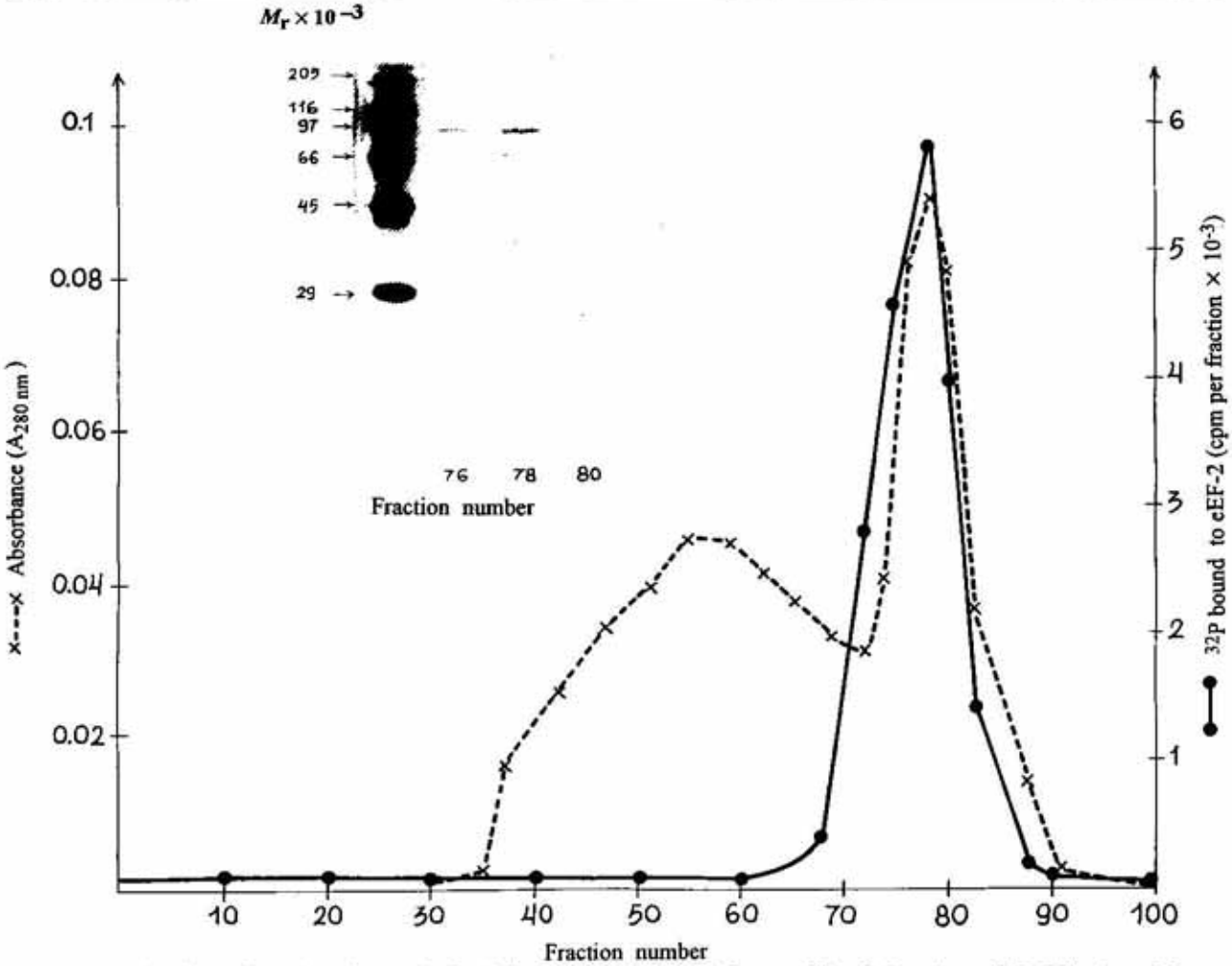

Fig. 4. Molecular filtration through the Ultrogel AcA-44 of the combined fractions (23–30) eluted from hydroxyapatite column.
The activity of kinase eEF-2 was localized using about 2 μg of eEF-2 as a substrate and 10 μl of each fraction eluted from the column. The electrophoretic analysis of the active fractions (about 30 μl) was carried out in 10% polyacrylamide-0.1% SDS [10]. For details see Methods.

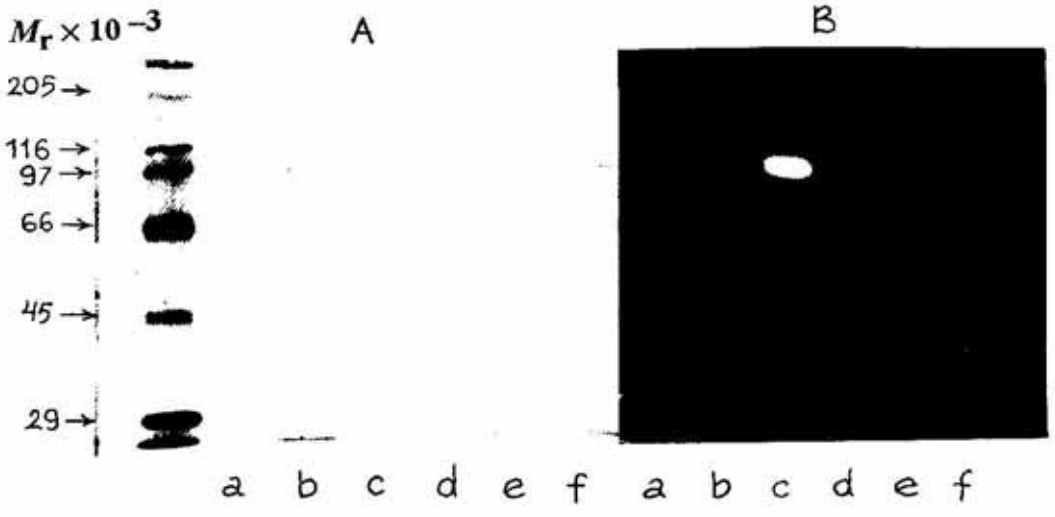

Fig. 5. Characterization of kinase eEF-2 specificity: electrophoretogram (A) and autoradiogram (B).
Autoradiography of the electrophoretogram obtained after phosphorylation of the eEF-2 preparation in the systems: (a) in the absence of eEF-2, (b) in the absence of calmodulin, (c) in complete system (eEF-2, kinase, calmodulin, Ca^{2+}), (d) in the absence of Ca^{2+}, (e) in complete system with EGTA added, (f) in the absence of kinase.

Table 1

The substrate specificity of calmodulin- and Ca^{2+}-dependent eEF-2 kinase.

The phosphorylation was carried out in the incubation mixture (for details see Methods) containing appropriate substrates (2–100 μg).

Substrate	Amount of bound ^{32}P (pmol × min^{-1} per mg of protein)
eEF-2 (100 kDa)	
from rat liver	2400
from Guerin epithelioma	480
eEF-2 (65 kDa)	
from rat liver	0
from Guerin epithelioma	0
eEF-1	
from rat liver ribosomes	0
Casein	0.8
Histone IIA	1.2
Total histone	0.32

slightly, whereas eEF-2 (65000), eEF-1 and rat liver ribosomes were not phosphorylated. Evidently lower specificity of liver protein kinase eEF-2 for eEF-2 (100000) isolated from Guerin epithelioma cells could be caused by different features of the structure of eEF-2 isolated from these tissues, as described previously [15].

In conclusion we can say that the protein kinase eEF-2 has been purified to homogeneity and its relative molecular mass is about 90000; the enzyme phosphorylates specifically eEF-2 (100000) in the presence of calmodulin and Ca^{2+}. The crude preparation of kinase eEF-2 was inactive because of the simultaneous presence of its inhibitor in the postribosomal supernatant. The inhibitor was separated during the purification procedure and its protein character has been demonstrated. Investigations on the inhibitor of protein kinase eEF-2 and on its regulatory role in the phosphorylation process in cells will be continued.

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