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PURIFICATION AND CHARACTERIZATION OF CREATINE KINASE, AN ESTROGEN-INDUCED UTERINE PROTEIN(IP) FROM IMMATURE RATS

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An estrogen-responsive translational product, the induced protein (IP) first described by Notides and Gorski (8), was obtained solely from the target organ, immature rat uterus, and purified to homogeneity in a procedure using two chromatography steps. The purified IP has a molecular weight of 49,000, and the isoelectric point is 5.2. Creatine kinase activity is associated with the homogeneous IP. There are some differences between the uterine enzyme and the creatine kinase BB isoenzyme, including differences in stability, and sensitivity to mercaptans. Estrogen-induced creatine kinase purified by this simple, reproducible method is a useful antigen for further studies on the translation and transcription processes involved in hormone-modulated synthesis.

The prevalent concept of steroid hormone action is that the steroid, after entering the target cell, binds to a specific cytosol receptor protein. The steroid holoreceptor is then translocated to the nucleus, where it binds to an acceptor site, the identity of which is the subject of speculation, experimentation, and controversy. Earlier studies in our laboratory indicated that estrogen receptors possess polydeoxynucleotide binding sites which discriminate between the component bases of DNA (1-3). This recognition underlies the specific binding of glucocorticoid and progesterone receptors with

Abbreviations used: IP, induced protein; MEM, Minimal Eagle Medium; SDS, Sodium dodecyl sulfate; E_2, 17β-estradiol.

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limited, high-affinity sequences of DNA within or proximate to coding regions of mouse mammary tumor virus and chick oviduct egg-white proteins respectively (4-7).

Another protein whose synthesis is tightly regulated by a steroid hormone is the estrogen-induced protein (IP) of immature rat uterus. Induction of IP is estrogen-specific, is earliest in the train of hormone-induced translational events, and is dependent on prior synthesis of RNA (8, 9). A direct correlation exists between the number of estrogen receptors translocated to the nucleus and the amount of IP synthesized (10). IP is thus an obvious candidate for studying the mechanism of estrogen-receptor regulation of gene expression, including identification of the putative holoreceptor binding site within DNA. For such studies purification of IP from a hormone target tissue in reasonable amount is essential; providing antibodies of high fidelity for isolation of IP mRNA and subsequent production of specific probes. The work reported here is a first step towards this goal.

Materials and Methods

Animals and materials. Female Wistar rats, 18-21 days old, were obtained from the Griffin Laboratory of this Center. [4,5-^{3}H]L-leucine (130 Ci/mmol) and [U-^{14}C]leucine (330 mCi/mmol) were purchased from Amersham; estradiol-17β (E_2) from Sigma Chemical Co.; DEAE-Sephacel from Pharmacia; hydroxyapatite Ultrogel from Reactifs IBF (France); and aprotinin (Trasylol) from the Mobay Chemical Corp.

All other reagents were from standard supply houses. Creatine kinase assay kits (CK-NAC) were bought from Biodynamics/Boehringer. Minimal Eagle medium (MEM) minus leucine was prepared from the MEM-Selectamine kit sold by Gibco.

Preparation of rat uterine cytosol containing IP. The experimental protocol for *in vivo* treatment with E_2 and subsequent labeling with radioactive leucine was adapted from the procedure of Katzenellenbogen and Gorski (11). Fifty rats were injected intraperitoneally with E_2 (5 μg in 0.3 ml of 1% ethanol in saline); 50 others received only the vehicle. After 1 to 1.5 h the animals were killed, and the uteri were removed and dissected free of adherent fat. The uteri from E_2-treated rats were placed in a vial with 20 ml of MEM containing 10 μCi of [^{3}H]leucine, 0.3 μg of actinomycin D/ml and 400 units of aprotinin/ml. The uteri from vehicle-injected animals were placed in another vial containing the same incubation mixture except that the labeled amino acid was 2.5 μCi of [^{14}C]leucine. The suspensions were flushed with 5% CO_2 in O_2 and incubated in a Dubnoff shaker at 37°C for 2 h.

After incubation the two groups of uteri were mixed, washed thoroughly with saline, and homogenized at 0-4°C in 10 ml of 1.5 mM EDTA, pH 7.0, with 400 units of aprotinin. A Polytron homogenizer was operated in two bursts of 20 s each at setting 6 with a 1-min interval. The homogenate was centrifuged

for 60 min at 200,000 x g and 0-4°C. The supernatant (cytosol) was analyzed for the presence of IP and used for purification of this protein.

Purification procedure. A simple two-step procedure was developed to isolate estrogen-induced IP from uterine cytosol. After dialysis against 200 volumes of 1.5 mM EDTA, pH 7.6, the cytosol was chromatographed on a DEAE-Sephacel column with a linear NaCl gradient in 10 mM Tris-HCl buffer, pH 7.6. the $^{3}H/^{14}C$ ratios (see below) in fractions eluting between 0.2 and 0.3 M NaCl were twice as high as in other fractions along the entire gradient (Fig. 1A). The fractions containing IP were pooled, dialyzed against 200 volumes of 1.5 mM EDTA, pH 7.6, and chromatographed on hydroxyapatite Ultrogel with a linear gradient of 0 to 0.1 M $NaPO_4$, pH 6.8 (Fig. 1B). A single radioactive band eluted in the early part of the gradient (0.005 to 0.010 M). The $^{3}H/^{14}C$ ratio was doubled over the prior step.

Identification of IP. An increase in the $^{3}H/^{14}C$ ratio was taken as the criterion for purification of IP. Aliquots of cytosol (∿25 µl) and subsequent purification fractions were analyzed by SDS-gel electrophoresis according to the procedure of Laemmli (12). After staining with Coomassie blue and destaining in ethanol/acetic acid/water (25/8/67), the gel was sliced into 2-mm segments, digested with 0.5 ml of 30% H_2O_2 at 80°C, and assayed for ^{3}H and ^{14}C emissions in a scintillation counter (Beckman model LS-350) using Aquasol fluor (New England Nuclear). A plot of the $^{3}H/^{14}C$ ratio (in cpm), showing a band with an increased ratio, indicated the presence of IP (see Fig. 3A).

Creatine kinase assay. Fractions were assayed for creatine kinase by using CK-NAC kits with creatine phosphate and ADP as substrates. The ATP generated in the reaction was measured by coupling the reaction to hexokinase, glucose-6-phosphate dehydrogenase, and NADP. One unit of activity was defined as the amount of enzyme which converts 1 µmole of NADP to NADPH in 1 min.

Results and Discussion

The DEAE-Sephacel step did not significantly reduce the number of polypeptide bands detected in Coomassie blue-stained SDS-gels (Fig. 2), but after hydroxyapatite Ultrogel chromatography the enriched fraction was found in a single stained band (Fig. 3). In the cytosol a discrete peak with a doubled $^{3}H/^{14}C$ ratio was seen. Peak fractions from the subsequent columns had ratios enhanced four- and sixfold respectively. The hydroxyapatite fraction was devoid of other labeled proteins. In all three samples the peaks with increased $^{3}H/^{14}C$ ratios were associated with a polypeptide of molecular weight (M_r) 49,000. The purified IP contained 2% of the total [^{3}H]leucine but only 0.8% of the total [^{14}C]leucine from the cytosol. The purified protein represented 0.13% of the total cytosol protein. In uterine preparations from rats injected with the vehicle alone there was no difference between the rates of [^{3}H]- and [^{14}C]leucine incorporation into cytosol protein(s), and the ratio remained constant in both chromatographic steps (Fig. 4).

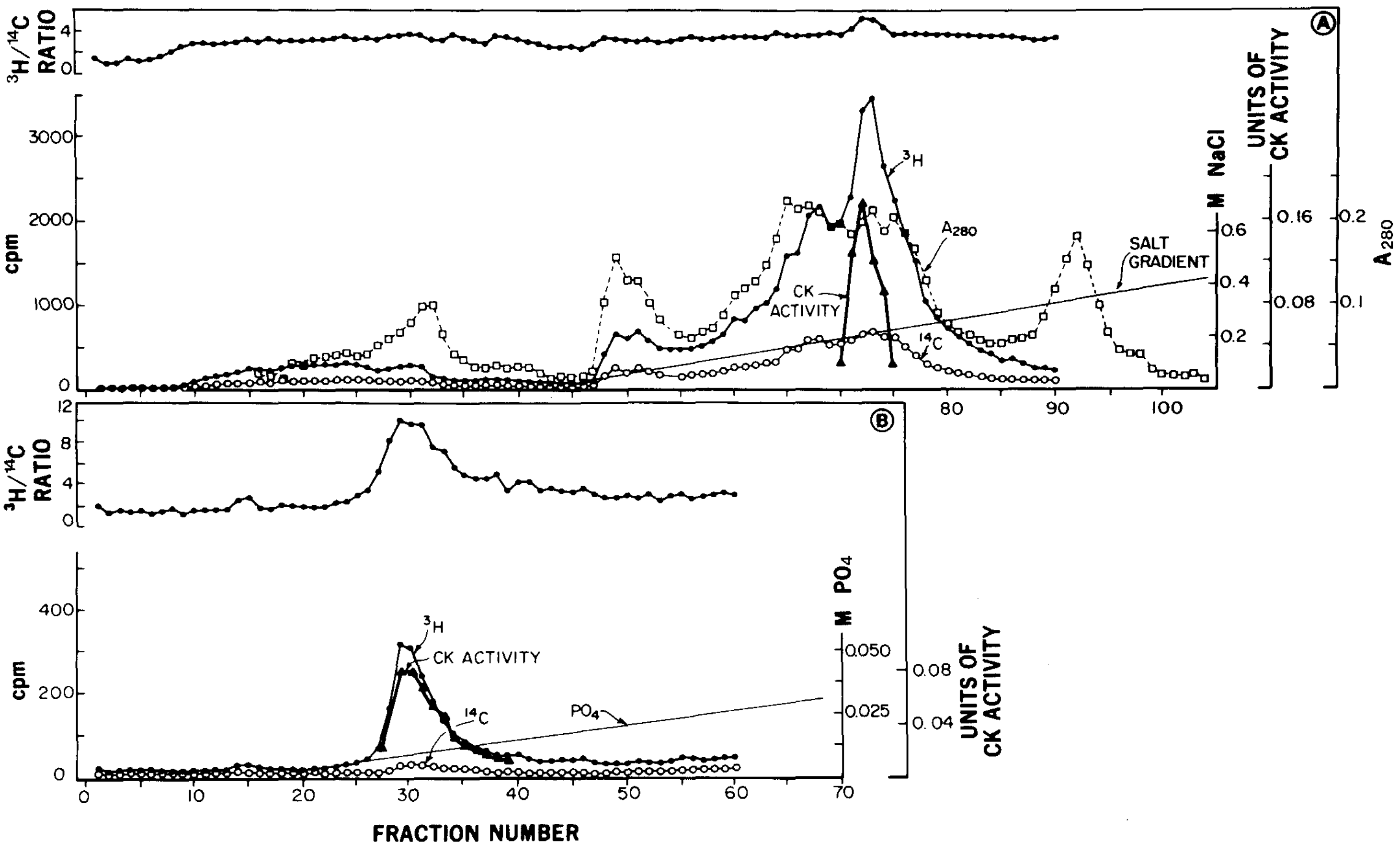

Fig. 1. Purification of IP by (A) DEAE-Sephacel and (B) hydroxyapatite Ultrogel chromatography. (A) Dialyzed cytosol, diluted to a protein concentration of 0.2 mg/ml, was passed through a DEAE-Sephacel (1.5 x 30-cm) column equilibrated with 0.01 M Tris-HCl, pH 7.6, containing 1.5 mM EDTA at a flow rate of 30 ml/h. Elution was carried out with a linear NaCl gradient (0 to 0.1 M, total volume 600 ml), and 5-ml fractions were analyzed as indicated. (B) DEAE-Sephacel fractions 60-70, which had an increased $^3H/^{14}C$ ratio, were pooled, dialyzed against 1.5 mM EDTA, pH 7.0 , and passed through a column of hydroxyapatite Ultrogel (1.5 x 30-cm) equilibrated with 1.5 mM EDTA, pH 7.0. A linear gradient of 0 to 0.1 M sodium phosphate buffer, pH 6.8, was used to elute, and 5-ml fractions were analyzed as indicated.

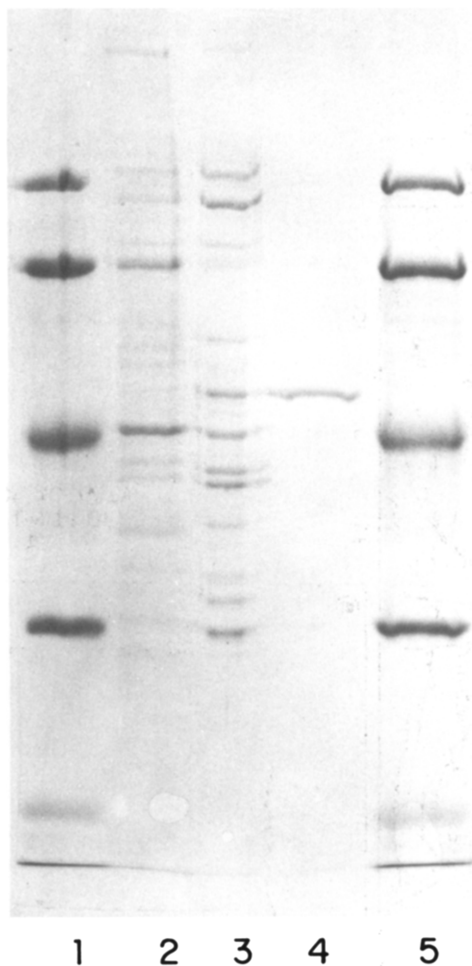

Fig. 2. SDS-gel electrophoretic analysis of fractions containing IP. Lanes 1 and 5 are molecular weight markers (phosphorylase b, 94,000; serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,000). Lane 2, cytosol, Lane 3, pooled DEAE-Sephacel column eluates (fractions 60-70 from Fig. 1A), Lane 4, pooled hydroxy-apatite Ultrogel column eluates (fractions 28-32 from Fig. 1B). All gels were stained with Coomassie brilliant blue.

Iyengar et al. (13) reported that estrogen-stimulated growth of bovine myometrium was correlated with a twofold increase in creatine kinase activity. Reiss and Kaye (14), using mixed preparations of radiolabeled, estrogen-stimulated, immature rat uterine and unlabeled brain cytosols, found after extensive purification that the major IP was indistinguishable from the BB isoenzyme of brain creatine kinase. Although we could not demonstrate a difference in the specific activities of cytosol creatine kinase derived from uteri of E_2-

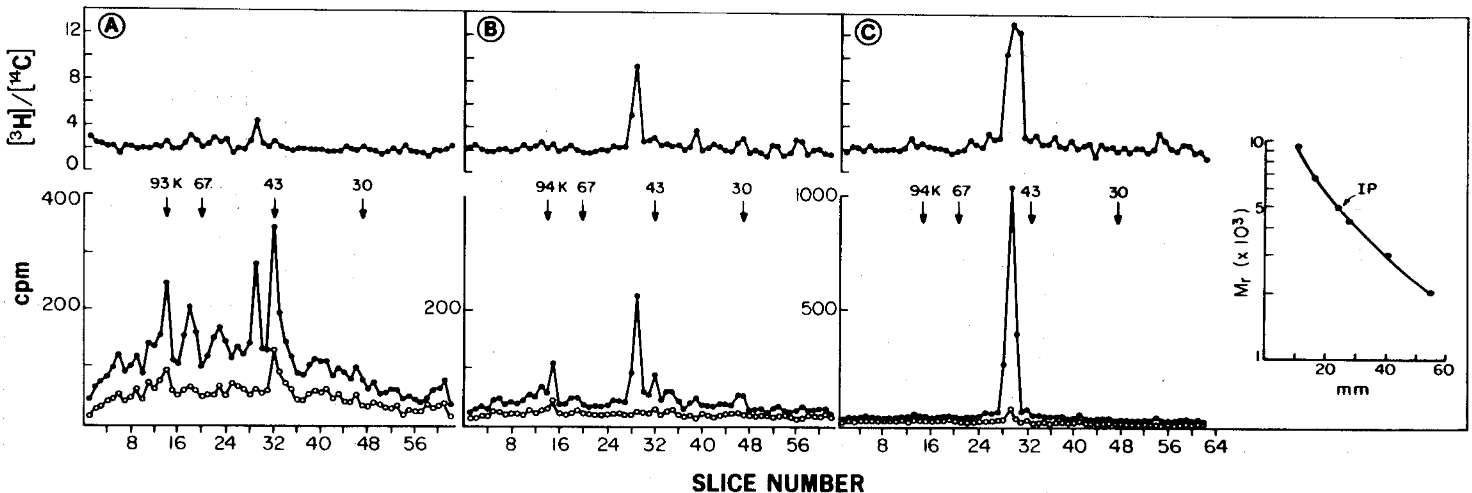

Fig. 3. Distribution of ^3H and ^{14}C on SDS-gel electrophoresis. Segments (2 mm) were sliced from each lane (Fig. 2, lanes 2-4), digested with 0.5 ml of 30% hydrogen peroxide at 80°C, and assayed for radioactivity. (A) cytosol; (B) DEAE-Sephacel pooled fractions (60-70); (C) Hydroxyapatite Ultrogel pooled fractions (28-32) closed circles represent $[^3H]$ and open circles represent $[^{14}C]$. Inset: Semilogarithmic plot of M_r versus distance travelled by marker proteins and IP.

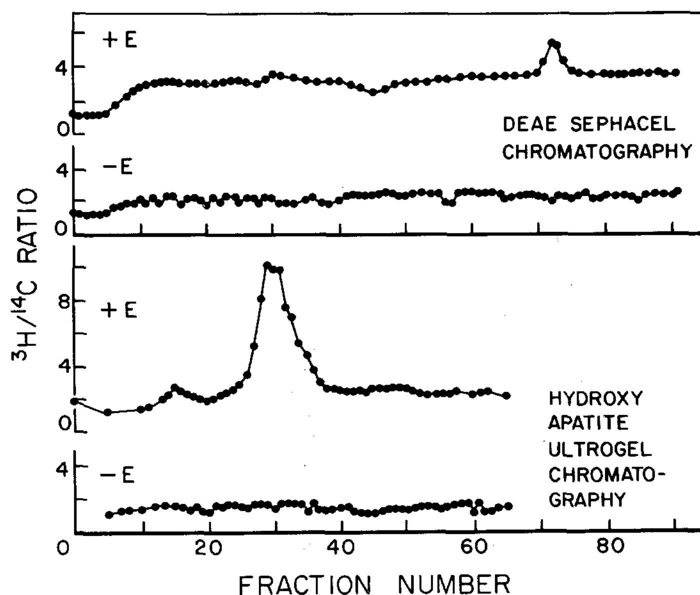

Fig. 4. Elution pattern of estrogen-treated and -untreated rat uterine cytosol from (A) DEAE-Sephacel and (B) hydroxyapatite Ultrogel columns. Double-labeled cytosol was prepared as described in Methods. Rat uteri were incubated with [^{3}H]leucine with (+E) or without (-E) estradiol. Chromatography was carried out as described for Fig. 1.

constants of the BB isoenzymes of kinase from a variety of sources (15). The and vehicle-treated rats, E_2 treatment did increase the incorporation of leucine into IP (Fig. 3A).

Creatine kinase activity was associated with the enriched fractions of both purification steps (Table 1). The DEAE-Sephacel fraction was increased

Table 1

Purification of Estrogen-Induced Rat Uterine IP

	Total Radioactivity (cpm) ^{3}H	Total Radioactivity (cpm) ^{14}C	^{3}H/^{14}C	Total Protein (mg)	Creatine Kinase Total Activity (units)[a]	Creatine Kinase Specific Activity (units/mg protein)	Purification (-fold)
Cytosol	4,618,440	1,204,440	3.83	38	84	2.21	1
DEAE-Sephacel	1,395,100	305,200	4.50	9	132	14.6	6.6
Hydroxy-apatite Ultrogel	102,300	10,700	9.50	0.05	124	2480.0	1127.0

[a] One unit is the amount of enzyme which converts 1 μmole of substrate in 1 min at standard assay conditions.

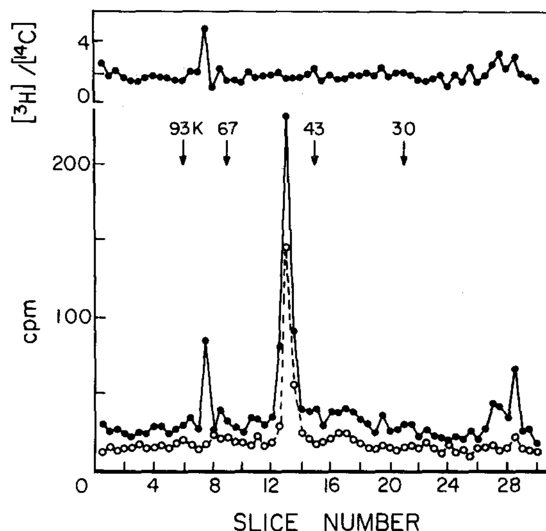

Fig. 5. SDS-gel electrophoretic analysis of hydroxyapatite Ultrogel eluate fraction containing creatine kinase activity purified from double-labeled cytosol from control (-E) immature rats. Fractions containing creatine kinase activity and eluting at the phosphate concentration where IP elutes were dialyzed against water and concentrated to 1/10 the original volume. A 100-μl portion, representing a protein concentration of 5 μg, was applied for electrophoretic analysis; 2-mm segments were sliced and analyzed as described for Fig. 3.

sixfold in specific activity over the cytosol, and the hydroxyapatite fraction showed a further 170-fold increase. The total creatine kinase activity recovered in the DEAE-Sephacel eluate was increased 57%, indicating that the enzyme had been partially inhibited in the cytosol. Nearly 95% of the activity in the enriched fraction from the first column was recovered in the hydroxyapatite-purified fraction.

In uterine preparations from vehicle-treated animals the hydroxyapatite Ultrogel eluate containing creatine kinase activity was concentrated by lyophilization because the total radioactivity was low. When this concentrate was analyzed by SDS-gel electrophoresis, a major labeled band corresponding to M_r 49,000 was seen, but there was no increase in the $^3H/^{14}C$ ratio (Fig. 5). These findings suggested that little synthesis of creatine kinase occurred in the absence of E_2.

In characterizing the creatine kinase activity of purified IP, the K_m for creatine phosphate was found to be 0.4 mM, a value close to the Michaelis-Menten

Table 2

Amino Acid Analysis (Molar Percent) of Estrogen-Induced Proteins

Amino Acid	This IP[a]	Rat Creatine Kinase BB[b]
Aspartic acid + asparagine	11	12.30
Threonine	3.6	4.60
Serine	6.2	5.83
Glutamic acid + glutamine	13	11.80
Proline	5.6	5.86
Cysteine (1/2 Cys)	1.7	1.33
Glycine	14	8.45
Alanine	7.9	5.47
Valine	5.8	5.56
Methionine	2.3	2.50
Isoleucine	4.0	3.40
Leucine	9.0	10.82
Tyrosine	2.4	2.36
Phenylalanine	3.6	4.44
Histidine	2.1	3.52
Lysine	5.2	5.38
Tryptophan	ND[c]	0.71
Arginine	5.1	5.67

[a] Determined by the fluorescamine technique by Dr. Stanley Stein of Hoffman La Roche Inc., Nutley, New Jersey.

[b] Reiss and Kaye (14).

[c] ND = Not determined.

creatine kinase activity of our purified IP remained very stable at 4°C for several weeks. The enzyme was not activated by mercaptans but was inhibited by p-hydroxymercuribenzoate, iodoacetic acid, and N-ethylmaleimide. Unlike the BB isoenzyme of rat brain, purification of the kinase activity associated with IP of rat uterus did not require addition of mercaptans.

The amino acid composition of the purified IP, as well as the identity of he N-terminal amino acid, was investigated. No N-terminal amino acid could be detected by nine cycles of Edman degradation, suggesting a blocked N-terminal amino group. The amino acid composition (Table 2) indicated that the protein is rich in acidic residues (glutamic, aspartic, and their amides). This was consistent with the low isoelectric point of 5.2, determined by isoelectric focusing. The amino acid composition is quite similar to that of the BB isoenzyme of rat creatine kinase (14).

Other workers have used mixtures of double-labeled cytosols from immature rat uteri with unlabeled extracts of mature rat uteri (16) or brain (14). This is the first report of purification to homogeneity of IP entirely from an

estrogen target organ, immature rat uteri. Using the increase in the $^{3}H/^{14}C$ ratio as the indicator of IP, we have confirmed that IP has creatine kinase activity.

Acknowledgement

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References

1. Thanki, K.H., Beach, T.A., and Dickerman, H.W. (1978) J. Biol. Chem. 253, 7744-7750.
2. Kumar, S.A., Beach, T.A., and Dickerman, H.W. (1980) Proc. Natl. Acad. Sci. 77, 3341-3345.
3. Dickerman, H.W., and Kumar, S.A. (1982) in Hormones and Cancer, Ed. W.W. Leavitt. Plenum Publ. Corp., NY. 1-18.
4. Payvar, F., Wrange, O., Carlstedt-Duke, J., Okvet, S., Gustafsson, J.-A., and Yamamoto, K.R. (1981) Proc. Natl. Acad. Sci. 78, 6628-6632.
5. Mulvihill, E.R., LePennec, J.-P., and Chambon, P. (1982) Cell. 28, 621-632.
6. Govindan, M.V., Spiess, E., and Majors, J. (1982) Proc. Natl. Acad. Sci. 79, 5157-5161.
7. Compton, J.G., Schrader, W.T., and O'Malley, B.W. (1982) Biochem. Biophys. Res. Comm. 105, 96-104.
8. Notides, A., and Gorski, J. (1966) Proc. Natl. Acad. Sci. 56, 230-235.
9. Mueller, G.C., Gorski, J., and Aizawa, Y. (1961) Proc. Natl. Acad. Sci. 47, 164-169.
10. Katzenellenbogen, B.S. (1975) Endocrinology 96, 289-297.
11. Katzenellenbogen, B., and Gorski, J. (1972) J. Biol. Chem. 247, 1299-1305.
12. Laemmli, U.K. (1971) Nature 227, 680-685.
13. Iyengar, M.R., Fluellen, C.E., and Iyengar, C.W.L. (1980) Biochem. Biophys. Res. Comm. 94, 948-954.
14. Reiss, N.A., and Kaye, A.M. (1981) J. Biol. Chem. 256, 5741-5749.
15. Iyengar, M.R., Fluellen, C.E., and Iyengar, C.W. (1982) J. Muscle Res. and Cell Mobility 3, 231-246.
16. Manak, R.C., Wertz, N., Slabaugh, M., Denari, H., Wang, J.-T., and Gorski, J. (1980) in Steroid Induced Uterine Proteins. M. Beato. Ed. Elsevier, NY pp. 21-34.