STUDY OF PURIFICATION AND PROPERTIES OF CREATINE KINASE FROM PORCINE SKELETAL MUSCLES

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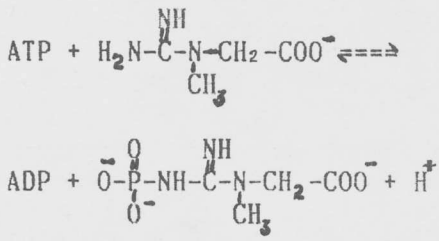
Introduction

Creatine kinase EC 2.7.3.2. is an important enzyme, which participates in the cell energy metabolism and has direct relations with muscle contraction and ATP regeneration. It catalyzes the transphosphorylation reaction between creatine and ATP.



This kinase is found in skeletal muscle, cardiac muscle and brain tissue, presenting three different types, viz.: MM-type (in muscle), BB-type (in brain) and MB-type (in cardiac and other tissues). In recent years, researches reveal that creatine kinase has important role in Na^+-K^+-ATP pump system.(1) Clinically it is also highly evaluated as in the early stage diagnosis of myocardial infarction and in estimating the dimension of infarction.(2) Especially when certain myocardial infarctions can not be judged by electrocardiogram, the analysis of serum creatine kinase is of great use. (2),(3). Therefore, the study of the purification and properties of creatine kinase has significance not only in theory and also in practice.

In 1954 Kuby et al firstly purified and crystallized this enzyme from rabbit liver. (4).Thereafter, a series of isolation and purification works were performed from muscles and brains of various animals e.g. rat(5), monkey(6), carp(7), chicken(8) and human being(9) and horse (10). Among those, the study of creatine kinase from rabbit muscle was the most detailed. The physico-chemical properties, the catalytic mechanisms and the structure of subunits were all studied. (11), (12), (13). Recently, along with the development of molecular biology, even the amino acid sequence of creatine kinase from rabbit, chicken and rat were analysed. (14)(15). But studies of creatine kinase from pig are more less. For pig has a great bulk of muscles, and pig muscle is a good source of many biomolecules, moreover pig is nearer to human beings in biological species, so it is appropriate to isolate and purify creatine kinase from pig muscles instead of from muscles of other minor animals.

Materials and methods

Materials:
Fresh muscles from Hangzhou Meat Factory
Chemical reagents: All A.R. grade or biochemical grade
Apparatus: Centrifuges, visible & ultraviolet spectrophotometers, partition collectors, AA-analyzer (Hitachi 835-50)

Methods:
1) Isolation and purification procedures
a) Fraction I Crude extract
Minced pig muscles (back and leg) 400g. are homogenized with 0.01M.KCL solution (2ml./g.muscle) and then centrifuged (3000 r.p.m.). The supernatant is filtered through 4 layers of cheesecloth. The solution is the crude extract.
b) Fraction II 60% ethyl alcohol-$MgSO_4$ precipitation
The pH of the crude extract is adjusted with 5M.ammonia solution to 9.0. Solid NH_4Cl is added till the concentration is brought to 0.1 M., and adjust pH to 9.0 once more. After stirring for 1hr., 95% ethyl alcohol is added at -10 ℃., to a concentration of 60% (taking the 95% alcohol as 100%). Stirring is continued for half an hour. The solution is warmed to 10℃., centrifuge (3000 r.p.m.)for 15 minutes. To the supernatant, pH 8.5, 2.0 M $MgSO_4$ solution is added to the concentration of 0.03 M.. Restore the concentration of alcohol to 60%, stir for 15 minutes at about 10℃.. Centrifuge for 30 minutes (4000 r.p.m.). The precipitate is extracted twice with pH 9.0, 0.07 M. $Mg(Ac)_2$(The amount for the first time is 8% of that of crude extract, for the

second time is 4%). Centrifuge for 30 minutes (4000 r.p.m.). Combine the supernatants and add 95% ethyl alcihol at -10℃. to the concentration of 50%. Stir for 30 minutes, centrifuge at -5℃.for 30 minutes (4000 r.p.m.). The precipitate is dissolved in pH 8.0, 0.01 M. tris buffer solution.

c) Fraction III $(NH4)_2SO_4$ precipitation (0.45-0.75 saturation)

The above solution is dialysed against the same tris buffer solution for 24 hrs. Centrifuge for 30 minutes (4000 r.p.m.). To the supernatant, $(NH4)_2SO_4$ is added to 0.45 saturation, while 1 M. NH4OH is added to maintain the pH to 7.8. Put aside for 2 hrs, centrifuge for 30 minutes (4000 r.p.m.) To the supernatant $(NH4)_2SO_4$ is again added to 0.75 saturation. Stand by for 3 hrs. Centrifuge for 30 minutes (11000xg.). Dissolve the ppt.in the same buffer solution and dialyse to remove $(NH4)_2SO_4$. And then use pH 8, 0.02 M. tris solution containing 0.01 M. mercaptan to equilibrate.

d) Fraction IV DEAE chromatography

Use 0.02 M. pH 8.5 tris buffer to equilibrate the DEAE column (20x1.5 cm.). Add sample in 3 batches. At room temperature (8℃.), use 0.02 M. tris and 0.1 M. tris buffer (pH 8.1) 100 ml. each to elu ate. Collect in tubes, 7 min. one tube (4 ml.). Combine the eluates which show enzyme activity. Add $(NH4)_2SO_4$ to 0.9 saturation, centrifuge, suspend the ppt. in small amount of $(NH4)_2SO_4$ soln. with the same degree of saturation. Keep at -25℃.

2) Determination of enzyme activity

a) Creatine color reaction method (16)

b) pH method (17)(18)

3) Protein determination Biuret method

4) PAGE method for purity determination (19)

5) Sephadex gel chromatography for molecular weight determination (19)

6) SDS-PAGE method for molecular weight determination (19)

7) Amino acid analysis (20)

8) Tryptophane determination (19)

RESULTS

1) Purification and identification of creatine kinase

From 400 g. of pig muscles, 30 ml. of creatine kinase solution (16 mg.protein /ml.) were obtained. Activity recovery was 42.5%, specific activity was 85 units/1mg. protein. (table I)

step / Item	I crude extract	II Alc. $MgSO_4$ ppt.	III $(NH_4)_2SO_4$ ppt.	IV DEAE chromat.
Vol. (ml.)	600	87	68	30
total protein	11080	1183	734	480
total activity	96000	55680	47056	40800
recovery %	100	58.6	49.0	42.5
specific activity units/mg. protein	8.66	47.06	64.10	85.00
purification fold	1.0	5.42	7.40	9.82

Table I Purification of creatine kinase

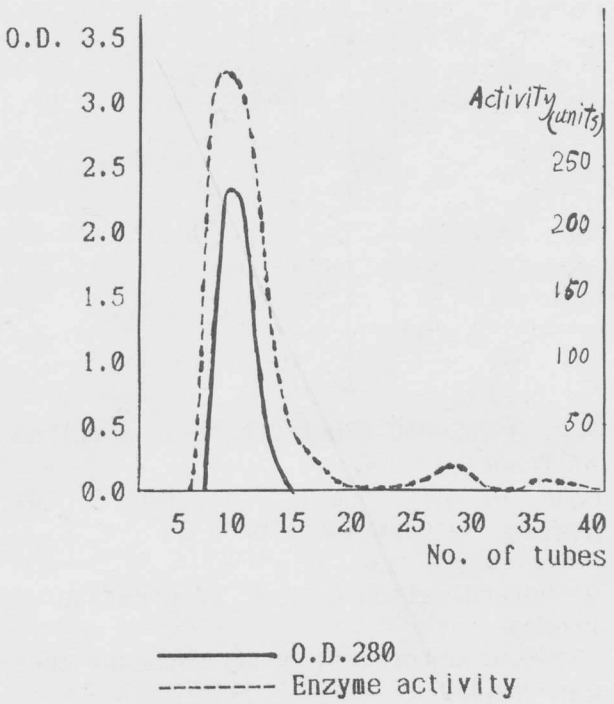


Fig.1 DEAE chromatography

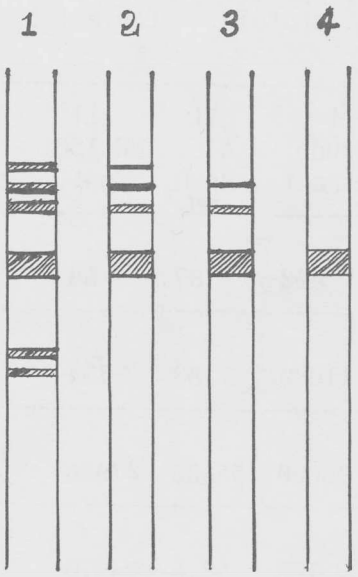


Fig.2 PAG-electrophoretic spectrum
Conc. of gel 7%,
stained by aniline black
Tube 1,2,3,4 correspond the fraction I, II, III, IV respectively
Tube 4 (one band only) shows the enzyme preparation has attained electrophoretic pure

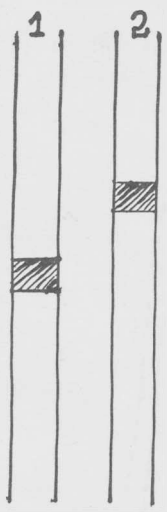


Fig. 3 SDS-PAG electrophoretic spectrum of fraction IV
Conc. of gel: Tube 1, 7.5%, Tube 2, 10%, stained by commaise blue R-25

2) Determination of M.W. of creatine kinase

a) M.W. determination by sephadex chromatography

The M.W. of creatine kinase 83,000 was obtained from standard curve made from the data on table II.

Item / Proteins	M.W.	Vol. of eluate
horse liver dehydrogenase	80,000	27
bovine serum albumin	67,000	32
horse radish peroxidase	40,000	43
trypsin	23,300	52.5
sample	83,000	25

Table II Volume of eluate of standard proteins and sample protein

b) M.W. by SDS-PAGE-method

The M.W. of creatine kinase subunit, 42,000 was obtained from the standard curve made from the data on table III.

The relative motility of sample was 0.525, the corresponding M.W. was about 42,000.

Item	dye frontier	band dist.	relative motility	log M.W.	M.W. x10
phosphorylase B	6.4	1.2	0.187	4.937	9.40
TMV	6.4	5.8	0.906	4.243	1.75
carbonic anhydrase	6.4	4.5	0.703	4.447	3.00
actin	6.4	3.2	0.500	4.633	4.30
bovine serum albumin	6.4	2.2	0.343	4.826	6.70
sample	6.1	3.2	0.525	4.630	4.20

Table III Band distances (cm.) of standards and sample

3) Amino acid analysis

a) Tryptophane content

50mg./g.protein obtained from the standard curve.

b) Total amino acids analysis (except Trp.) by amino acid analyzer

amino acid	content mg.% protein	amino acid	content mg.% protein
Asp.	113.33	Cys.	9.95
Thr.	37.875	Val.	56.34
Ser.	37.837	Met.	28.71
Glu.	118.47	Ile.	26.53
Ala.	26.37	Leu.	86.74
Gly.	46.76	Tyr.	13.22
Phe.	47.43	Lys.	89.18
His.	29.78	Arg.	47.40
Pro.	33.07		

Table IV Amino acid Analysis results

4) Kinetic properties of pig muscle creatine kinase

a) Optimum pH

The optimum pH of this enzyme is between 6.5~7.0, obtained from the figure 4 based upon the data on table V. The enzyme activity was determined by creatine color reaction method. Enzyme conc.0.06 mg. protein /ml..

Item pH	Activity	Activity ratio
5.5	2.975	35
6.0	5.950	70
6.5	7.990	94
7.0	8.500	100
7.5	5.100	60
8.0	2.215	25

Table V The influence of pH on creatine kinase activity

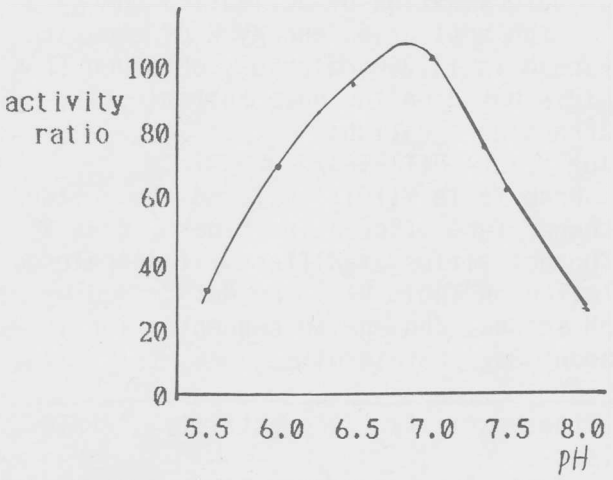


Fig.4 The optimum pH of creatine kinase

b) The influence of temperature on creatine kinase

Keep creatine kinase solution at different temperatures for 15 minutes, the inactivation is significant at 60 ℃. (Table VI, Fig. 5)

activity	before incubation	after incubation	ratio
20° C	60	60	100%
25° C	60	60	100%
30° C	60	60	100%
35° C	60	60	100%
40° C	60	53	88%
45° C	60	50	83%
50° C	60	30	50%
60° C	60	0	0

Table VI The influence of temperature on creatine kinase activity

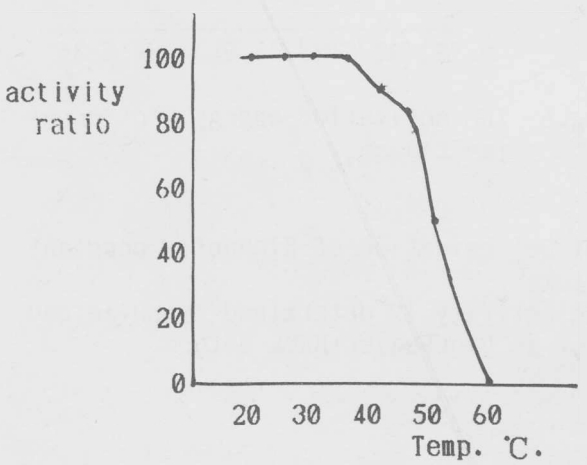


Fig.5 The influence of temperature on creatine kinase

c) Determination of activation energy E

The activation energy E of creatine kinase is 14,280 calories, obtained from Fig.6 based on the data on table VII, by Arrhenius eguation:

$\log a_2/a_1 = E(T_2 - T_1)/2.303\ RT_1T_2$

From Table VII it is found the optimum temperature of creatine kinase is 35 ℃. The activities at different temperatures (cited on Table VII) are determined by pH-method. The enzyme concentration is about 3mg.protein/ml.

Item Temp.	activity	log activity	1/Tx10
15 ℃	48	1.681	34.7
20 ℃	60	1.778	34.2
25 ℃	110	2.041	33.5
30 ℃	170	2.230	33.0
35 ℃	240	2.380	32.5
40 ℃	200	2.300	31.9

Table VII The activity under different temperatures

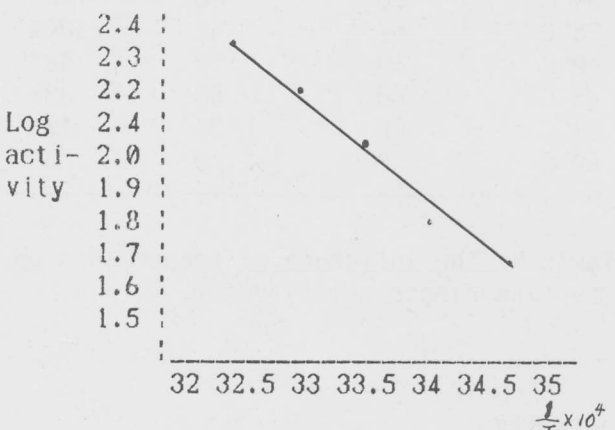


Fig.6 The activation energy E of creatine kinase

d) Determination of Michaelis constant Km

The activity is determined by pH-method, plot as Lineweaver-Burk method.

(1) Km-ATP

Creatine is fixed at 40 mM. 1/Km is obtained from Fig.7 and hence Km=2.04 mM.

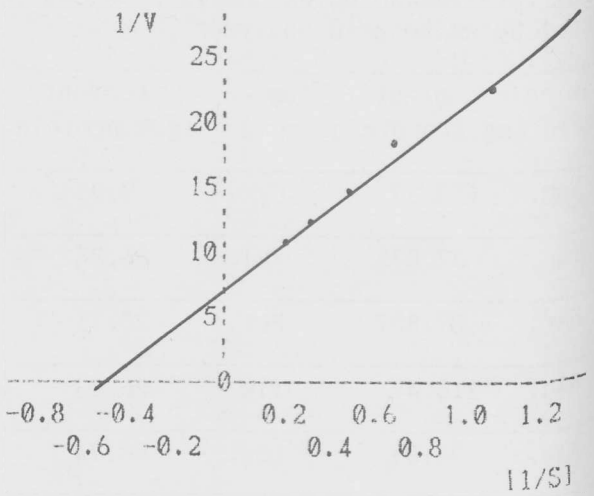


Fig.7 Lineweaver-Burk plot of Km-ATP

(2) Km-creatine

ATP concentration is fixed at 4mM/3ml. 1/km is obtained from Fig.8, hence Km-creatine equals 14.28mM.

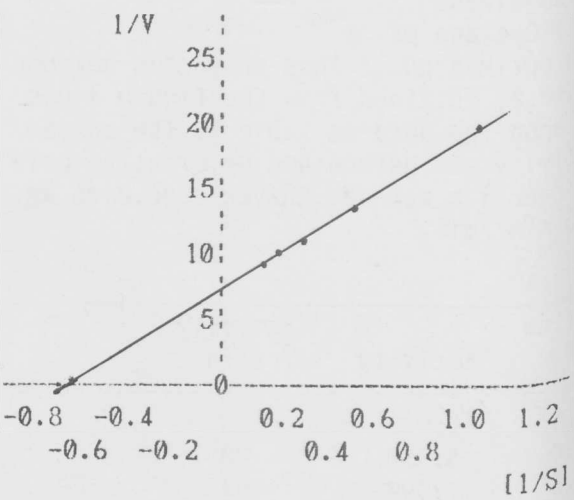


Fig. 8 Lineweaver-Burk plot of Km-creatine

DISCUSSION

In 1954, Kuby et al (4) firstly isolated and purified creatine kinase from rabbit muscle. Owing to the crystallization process was more time-comsuming, many modifications were performed. In 1981 Toshihide and Takasawa (21) purified without crystallization, creatine kinase from pig muscles with DEAE ion exchange chromatography and CM-cellulose chromatography. The activity recovery was 41%, purification fold was 8. We used fractional precipitation method with alcohol & ammonium salts, and DEAE ion exchange chromatography method, in condition that no cold room was available, got a satisfactory result--activity recovery 42.5%, purification fold 10. The M.W. of porcine creatine kinase 83,000, we obtained by sephadex chromatography, is nearer to that of rabbit creatine kinase,82,000. (23) The M.W. of subunit by SDS-PAGE method, is 42,000. The reproducibility of these two methods was high. The amino acid analysis results of porcine creatine kinase were nearer to that of rabbit creatine kinase. (24) The optimum pH, heat stability were nearer to those of chicken (25) and human (26) creatine kinase. Activation energy, Michaelis constant are all nearer to those of rabbit creatine kinase. (21) Therefore it is highly feasible to use porcine creatine kinase instead of rabbit creatine kinase in scientific and clinical practice.

REFERENCES

1) Saks,V.A.et al (1977) Biophs. Acta. 463 (3), 550
2) Inst. of Cardio-vascular Diseases, Chinese Medical Acad. (1974) J. of Card. Vasc. Dis. 2(4), 278
3) Sobel, B.E. et al (1972), Circulation, 46, 640
4) Kuby, S.A., Noda,L. & Lordy, H.A. (1954), J.Biol.Chem. 209, 191-201
5) Hooton, B.T. & Watts, D.C. (1966) Biochem. J. 100, 637-646
6) Kumudavalli, I., Moroland, B.H. & Watts, D.C. (1970) Biochem. J. 117,513-525
7) Gosselin-Rey, Cetal (1970) Biochim. Biophys. Acta 221, 241-254
8) Roy, B.P., Laws, J.F. & Thomson, A.R. (1970)
9) Keutel, H.J., Okabe, K., Jacobs, H.K., et al (1972) Arch. Biochem. Biophys. 150, 648-678
10) Takasawa, T., Fukushi, K. et al (1981) J. Biochem. 89, 1619-1631
11) Watts, D.C. The Enzymes, 1962,8,384
12) Kuby, S.A. The Enzymes, 1962,6,515
13) Bicherstaff, G.F. et al J. Biochem. 1978, 91
14) Pamela, A.B., Robert, A.Z. et al (1984) J.Biol. chem. 259 (23), 14979-14984
15) Arther E.J. and Pavid, P. (1984) J. Biol.Chem. 259, (24), 14985-14992
16) Clinical Biochemical Tests and Determinations Part 1, P.338, 1984, Edited by Shanghai Municipal Medical Labortory
17) Zeng Shi-yuan et al (1986) J.of Hangzhou Institute of Commerce, Natural Science Edition, Vol.I, P.28-30
18) Yao Qi-zhi and Zou Chen-lu (1981) An assay method for creatine phospho kinase activity (pH colorimetry) J.of Biochemistry and Biophysics (China) 13,1
19) Zhang Long-xiang et al Biochemical Methods and Technology, Higher Education Edition (1985) P.94, 112, 124
20) Hua Jia-sheng et al Practical Techniques of Protein Chemistry, Science Edition (1980)
21) Takasawa, T. and Fukushi, K. (1981) J.Biochem. 90, 195-204
23) Yue, R.H., Palmieri, R.H., Olson, O.E. & Kuby, S.A. (1967) Biochemistry, 6, 3204-3227
24) Ouchterlony, C. (1949) Acta. Pathol. Microbiol. Scand. 26, 507
25) Hans, M. Eppenberger et al (1967) J.Biol.Chem. 242,(2),204-209
26) Cheng Lan-ying et al (1981) J. of Biochemistry and Biophysics (China) Vol.I, 17-27