PURIFICATION AND IDENTIFICATION OF INTERMEDIATE CATABOLIC PRODUCTS IN THE *IN VIVO* DEGRADATION OF PIG LIVER PHOSPHOFRUCTOKINASE

by Tosifusa Toda

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CURRICULUM VITAE

氏　名　　戸田年総

生年月日　　昭和２６年７月２３日

本籍地　　福井県福井市順化２丁目１１０２番地

現住所　　東京都東久留米市２丁目５番１４号

略　歴

１９７５年３月　大阪大学理学部化学科卒業

１９７７年３月　大阪大学大学院理学研究科生物化学専攻前期課程修了

１９７７年４月　東京都老人総合研究所・生化学部勤務　現在に至る

研　究　歴

１９７５年４月－１９７７年３月

大阪大学蛋白質研究所・酵素反応学部門・院生

１９７７年４月－

東京都老人総合研究所・生化学部・助手

現在に至る

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SUMMARY

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) was purified to homogeneity from pig liver. The final preparation, which showed a specific activity of 160 units/mg of protein, contained only a single polypeptide of Mr = 80,000 on a silver-stained SDS-polyacrylamide gel. Polyclonal antibody against the purified enzyme was induced in a rabbit, and its IgG fraction was obtained by chromatography on a Protein A-Sepharose CL-4B column. The IgG showed the binding activity to the predominant isozyme of phosphofructokinase in pig liver, rat liver and human erythrocytes, but not to isozyme in muscle. The specific antibody was purified further by immunoaffinity chromatography on a phosphofructokinase-conjugated Affi-Gel 10 column. Intermediate catabolic products in the in vivo degradation of phosphofructokinase were extracted from fresh pig livers under inhibition of proteinases. And they were adsorbed on an anti-phosphofructokinase IgG-conjugated Affi-Gel 10 column. Polypeptides in the eluate from the immunoaffinity column were further separated by preparative two-dimensional polyacrylamide gel electrophoresis, and their cross-reactivity to liver-type phosphofructokinase was substantially

assessed by an immunoelectrotransfer blot method. The polypeptide of Mr = 84,000, which was an intact form of phosphofructokinase in pig liver, was demonstrated in a major spot on the blot. Phosphofructokinase of Mr = 80,000, which was purified by the multi-step chromatographic method, seemed to be an unphysiologically digested form of Mr = 84,000 polypeptide, since no spot corresponding to Mr = 80,000 was detected on the blot. Polypeptides of Mr = 68,000, Mr = 64,000, Mr = 56,000 and Mr = 51,000 were assigned to catabolic products of Mr = 84,000 polypeptide, since they were obviously demonstrated on the blot. However, it was still in doubt that all of these polypeptides were recognized with the clones of IgG which recognized Mr = 84,000 polypeptide in their commom antigenic determinants. Hence IgG subspecies in the polyclonal antibody preparation were separated by two-dimensional cellulose acetate electrophoresis under a native condition, and their immunoreactivities to the radioiodinated polypeptides (Mr = 84,000, Mr = 68,000, Mr = 64,000, Mr =56,000 and Mr = 51,000) were examined by a reversed-phase immunoblot method. Thus obtained autoradiogram indicated that these polypeptides were bound to the common IgG subspecies (pI 5.3, pI 5.7, pI 6.0, pI 6.3). The structural homology among these cross-reactive polypeptides was further

assessed by proteinase-V8 digestion followed by SDS-polyacrylamide gel electrophoresis and autoradiography. The resulted autoradiogram showed that the common sized fragment of Mr = 7,500 was yielded from all of these polypeptides, and Mr ≒ 16,000, Mr = 12,500 and Mr = 6,300 fragments were from Mr = 84,000, Mr = 80,000, Mr = 68,000, Mr = 64,000 and Mr = 56,000 polypeptides. The similarity in the digestion profiles supports our conclusion that these polypeptides are intermediate catabolic products of phosphofructokinase of Mr = 84,000.

The possibility of artifacts in preparation was ruled out as follows. Radioiodinated phosphofructokinase (Mr = 84,000) was added to the buffer for homogenization. The tracer molecule accompanied intermediate catabolic products in the whole procedures including homogenization, centrifugation and immunoaffinity chromatography. Radioactive polypeptides recovered from the immunoaffinity column were separated by SDS-polyacrylamide gel electrophoresis, and detected by autoradiography. It was confirmed that the enzyme was not digested in preparation, because no band corresponding to Mr = 68,000, Mr = 64,000, Mr = 56,000 or Mr = 51,000 was detected even on the extensively exposed X-ray film. On the results, it is concluded that the predominant isozyme of

phosphofructokinase in pig liver (Mr = 84,000) is *in vivo* degraded through intermediate catabolic products of Mr = 68,000, Mr = 64,000, Mr = 56,000 and Mr = 51,000.

INTRODUCTION

Since Spence (1) reported that the impairment of glucose tolerance was likely to progress in aged men, many groups have discussed on the relation between the glucose intolerance and aging. Most of them with a few exeptions (2,3) have reported that the glucose tolerance deteriorates in elderly men. Davidson (4) has carefully analyzed the data in the exeptional reports, and has concluded that the data also indicate the age-related impairment of glucose utilization in tissues.

The cases of abnormal blood glucose pattern after oral glucose load may include pathological (diabetic) and non-pathological (non-diabetic) deterioration in glucose tolerance. Nonaka et al. (5) made efforts to exclude pathological cases from the test subjects, and reported as follows. The 100-g oral glucose tolerance test (OGTT) performed on 34 healthy, non-obese subjects aged 60 to 89 years without family history of diabetes revealed a high incidence of abnormal tolerance: 21% chemically diabetic and 53% borderline type. The incidence of chemical diabetes (diabetes decipiens) increased with advancing age; 10% in the 7th, 24% in the 8th and 29% in the 9th decades. The incidence of borderline type also increased, and it resulted

in the decrease of absolutely normal cases in elderly subjects. To elucidate the causes of the impairment, capacity for insulin secretion in aged subjects was examined. Immunoreactive insulin (IRI) levels during 100-g OGTT in 2 hours were plotted. An area under the curve of IRI during 2 hours of OGTT (IRI area) was divided by an area of blood glucose (glucose area) in the same test. The ratio thus obtained in subjects aged 60 to 89 years was not statistically different from that in subjects aged 20 to 49 years. Therefore, the decreased tolerance to glucose load could not be ascribed to the impaired insulin secretion, which was characteristics of primary diabetes (diabetes mellitus). 'Paradoxical rise' of immunoreactive glucagon (IRG), which was first reported by Buchanan and McCarrol (6) as the characteristics of diabetes mellitus, was not observed in elderly subjects of chemically diabetic and borderline type. Hence Nonaka and his co-workers have concluded that the abnormality of glucose tolerance in elderly subjects appears to be caused by pathogenesis other than insulin deficiency and glucagon overproduction. And, they have speculated that decrease in glucose-metabolizing capacity in the liver and decrease in sensitivity of tissues to the insulin action might be the factor responsible for the glucose intolerance in elderly subjects.

Imura determined the glucose disposal rate in healthy elderly men (N = 13, 73± 3 years old) and young controls (N = 12, 29± 2 years old) under insulin administration. And, he found that there was no appreciable difference in the maximal disposal rate between aged and young groups, but the blood insulin level required to induce a half maximal disposal rate increased with aging. The result means that the insulin sensitivity in tissues is likely to decrease with aging. To assess the contribution of receptor level to the decrease in sensitivity, he further assayed the insulin receptor on erythrocytes from young and aged men, and concluded that there was no statistical change in both receptor level and receptor affinity to insulin binding. On the results, he has concluded that the post-receptor system for glucose utilization deteriorates with aging.

No change in insulin receptor has also been observed by Hollenberg and Schneider (7) in human skin fibroblasts, Fink et al. (8) in human monocytes, and by Helderman (9) in human T-lymphocytes. And yet the reduced responce to insulin stimulation has also been observed by Ito (10) in human skin fibroblasts, Muggeo et al. (11) in human monocytes, and by Pagano et al. (12, 13) in human adipocytes and rat hepatocytes.

On the basis of these findings, we have set up our working hypothesis as follows. 1. The post-receptor system for glucose utilization deteriorates with advancing age. 2. The age-related deterioration may be ascribed to alteration of proteins in the post-receptor signal transmission system and/or key enzymes in glycolytic pathway. And we have arranged two distinct approaches to the age-related post-receptor defect. One is the screening of age-relatedly changing proteins in tissues. For this purpose, we developed a new method of two-dimensional electrophoresis (14,15) and two-dimensional densitometry (16-18). Liver proteins of 1-, 6-, 12-, 24- and 28-months-old rats were separated by the two-dimensional electrophoresis on cellulose acetate membrane (2DCAE). Protein spots on the membrane were stained out with Coomassie Brilliant Blue G-250, and quantified by two-dimensional densitometry using a TV-camera-equipped system (FIMC system). And we found out two unique proteins of pI 4.9 and pI 7.3, which changed in quantity with aging. pI 7.3 protein was observed to decrease in Wistar and Fischer strain male rats with advancing age. However, it could not be speculated that the protein was in the direct relation to aging process, because female rat liver contained only a trace amount of the protein at any age, and the castration of young male rat

resulted in marked decrease of the protein. On the contrary, pI 4.9 protein (Mr = 26,000) could participate the defect of liver in insulin response, since the protein was observed to decrease in both male and female rats of Wistar and Fischer strains, and it was not affected by castration (19). Hence we have continued our studies on pI 4.9 protein to elucidate its physiological meanings in the post-receptor defect.

Another approach to the age-related deterioration of glucose metabolism under insulin stimulation has been arranged on the studies on catabolic pathways of phosphofructokinase. There is a general agreement in the literature that phosphofructokinase plays a major role in the control of glycolysis in nearly all types of cells (20-26). The activity of this enzyme is under the allosteric regulation with several metabolites. Among them, ATP is the most potent inhibitor which is believed to be a feedback mediator in the Pasteur's effect. And the ATP inhibition is relieved by fructose 2,6-bisphosphate which has been suggested to be a final messenger from glucagon receptor (27-30).

In addition to the allosteric regulation, phosphofructokinase activity is known to be regulated in enzyme amount itself. Dunaway and his co-workers (31-34)

have reported that the immuno-reactive protein of PFK-L_2 (the predominant isozyme of phosphofructokinase in the liver) decreases 60-70% in 6-day fasted rats or rats made diabetes with alloxan or streptozotocin. Refeeding induces a return to normal level within 72 h, and insulin treatment for 72 h increases the amount of this isoenzyme to 6- to 8-fold greater than the diabetic levels. They have determined the synthetic and the degradation rates and have concluded that the reduction of PFK-L_2 in fasting and diabetes is the result of an increased degradation rate with little or no change in synthetic rate. In our experiments, liver phosphofructokinase decreased with alloxan diabetes faster in aged rats (N = 3, 2 months old) than in young controls (N = 3, 30 months old). Recovery from the diabetic level by insulin administration was also slower in aged rats than in young controls. And the recovered levels were still lower in aged rats than in young controls. On these observations we have speculated that the age-related alterations in phosphofructokinase responce to alloxan diabetes and insulin administration might be ascribed to decrease in synthetic rate and to increase in degradation rate of the enzyme. And we have continued the studies on degradation pathway of phosphofructokinase, taking the latter possibility for our primary working hypothesis.

In our preliminary experiments, we have observed that the enzyme is inactivated faster in liver extracts from diabetic rats than in those from normal controls. And the enzyme in liver extracts from normally aged (non-diabetic) rats is less stable than that from young controls. The inactivation is markedly prevented by addition of proteinase inhibitors to the extracts. And under the inhibition, no appreciable difference in inactivation rate has not been observed between aged and young rats. The results means that some intracellular proteinase, which inactivates phosphofructokinase, is likely to increase with advancing age. However, such 'phosphofructokinase-degrading proteinase' has still not been assigned. Hence intermediate catabolic products in the in vivo degradation of phosphofructokinase should be identified to assign the specific proteinase.

In this paper, the techniques of immunoaffinity chromatography, preparative two-dimensional electrophoresis of ISOTACHO/DALT system, enzyme-linked immunodetection, cellulose acetate two-dimensional electrophoresis, radioimmunodetection and proteinase-V8 peptide mapping were used to purify and identify the intermediate catabolic products of phosphofructokinase. The possibility of

artifacts in preparation was ruled out by a tracer method using radioiodinated phosphofructokinase.

MATERIALS AND METHODS

Reagents -- Phosphocellulose (P-11) and Glass Fiber Filter GF/B were purchased from Whatman, Ltd. (Maidstone, England); DEAE-Sephacel and Protein A-Sepharose CL-4B were from Pharmacia Fine Chemicals (Uppsala, Sweden); acrylamide, N,N'-methylene bisacrylamide, Affi-Gel Blue and Affi-Gel 10 were from Bio-Rad Laboratories (Richmond, Calif., U.S.A.); Cellulofine GCL-2000-sf was from Seikagakukogyo Co., Ltd. (Tokyo, Japan). Tris, dithiothreitol, ATP, AMP, fructose 6-phosphate, fructose 2,6-bisphosphate, antipain, leupeptin, pepstatin, triosephosphate isomerase, glycerophosphate dehydrogenase and bovine serum albumin were obtained from Sigma; NADH was from Oriental Yeast Co., Ltd. (Tokyo, Japan). Na[^{125}I]iodide was purchased from New England Nuclear (Boston, Mass., U.S.A.). Horseradish peroxidase-linked goat anti-rabbit IgG antibody and proteinase V8 were purchased from Miles Laboratories, Inc. (Naperville, U.S.A.); Pansorbin was from Calbiochem-Behring Corp. (La Jolla, Calif., U.S.A.). Titan III cellulose acetate plates was purchased from kabushiki-kaisha Helena Kenkyujyo (Urawa-shi, Japan). Separax, Separax EF and Sepaline were products of Fuji Photo Film Co., Ltd. (Tokyo, Japan). Visking dialysis tubing was obtained from Union

Carbide (Chicago, U.S.A.); X-ray film (RP Royal X-Omat) was from Eastman Kodak Co. (Rochester, U.S.A.); Cronex intensifying Screen (Lightning Plus) was from E.I. Du Pont Nemours & Co. (Wilmington, U.S.A.); nitrocellulose membrane (Membrane Filter BA 85, 0.45 μm) was from Schleicher & Schull GmbH (Dassel, West Germany); Filterpaper No. 51A was from Toyo Roshi Kaisha (Tokyo, Japan); Coomassie brilliant blue G-250 was from Nakarai Kagaku Yakuhin (Kyoto, Japan); Ampholine carrier ampholytes were from LKB Productor AB (Stockholm, Sweden). Other chemicals of analytical reagent grade were purchased from Wako Pure Chemicals (Osaka, Japan).

Apparatus -- Two types of apparatus were made for two-dimensional cellulose acetate electrophoresis (Fig. 1).

Fig. 1

Isotachophoretic concentration and the following first-dimensional separation were performed on apparatus A. The apparatus was equipped with suspension bridges (c) to supply electric current to the strips of cellulose acetate membrane (f) simultaneously with the contact of the strips with the bridging pads (d and e). If electric current was not supplied to the strips of cellulose acetate membrane,

the sharp boundary of the two different electrolytes could not be maintained on the strips. The apparatus had four holes on the lid for sample application.

Isoelectric focusing was performed on apparatus B (Fig. 1) for the second-dimensional separation. The sharp edges of the platinum electrodes and the minimum dead space in the apparatus made it possible to reproduce isoelectric focusing on the thin membrane (140 μm, 110 x 60 mm). Apparatus A and B were kindly made by Jookoo Co. (Tokyo, Japan) in accordance with our designs.

Regulated DC power supply Model 2000-200 Auto was purchased from Koike Precision Instruments (Kawasaki-shi, Japan). Peristaltic pump Model P-3 was from Pharmacia Fine Chemicals (Uppsala, Sweden). The temperature of circulating coolant was regulated by Coolnics Model CTR-220 (Komatsu-Yamato, Tokyo, Japan). The pH was measured using pH Meter 26 with combined electrode GK 2321C (Radiometer A/S, Copenhagen, Denmark).

The hardware of two-dimensional densitometer (IP-1), shown in Fig. 2, was designed and made by ADS Co. (Nara,

Fig. 2

Japan) on the basis of our specifications. A CCD solid-state video camera BS-703 (Matsushita Electric Co., Osaka, Japan) is equipped for image acquisition. The array size of the CCD image sensor is 404 x 256, and the S/N is 50 dB (300:1). The image data sent from the camera unit are temporarily resistered in a display memory (DM) of 512 x 256 matrix array. Processing of the image data is carried out on the DM. Storage of the original and the processed image data is on four pages of frame memory (FM). Subroutine programs for data acquisition, data transfer, data display, and other primitive tasks were executed with Z-80 CPU in IP-1. And the CPU is controled by SORD microcomputer M223 mark V (SORD Computer Systems, Inc., Tokyo, Japan) via parallel interfaces (PIO). The subprograms (FIMC) for control of IP-1 and the main programs (2DD) for evaluation of two-dimensional electrophoretograms were prepared on the SORD microcomputer in both ASSEMBLY and BASIC languages by ourselves.

Assay of phosphofructokinase -- The following assay system was used throughout the purification procedure. The reaction mixture contained in a final volume of 1 ml: 50 mM Tris-HCl, pH 8.0, 100 mM KCl, 20 mM ammonium sulfate, 2 mM dithiothreitol, 6 mM $MgCl_2$, 1 mM ATP, 1 mM fructose

6-phosphate, 0.5 mM AMP, 1 μM fructose 2,6-bisphosphate, 0.15 mM NADH, aldolase (0.4 unit), triosephosphate isomerase (2.4 units), glycerophosphate dehydrogenase (0.4 unit), and phosphofructokinase (0.001-0.01 unit). After 5-min preincubation without substrate, enzyme reaction was initiated by addition of fructose 6-phosphate. The rate of oxidation of NADH was measured in a spectrophotometer at 340 nm. A unit of phosphofructokinase was defined as the amount of enzyme that catalyzed the formation of 1 μmol of fructose 1,6-bisphosphate per min at 25°C.

Determination of protein -- Protein concentration was determined by Lowry's method (35) modified by Bensadoun and Weinstein (36) with crystalline BSA as a protein standard.

Titration of anti-phosphofructokinase antibody -- Titer of anti-phosphofructokinase antibody preparation was determined by ELISA on multiwell plate as follows. Wells of Falcon 9311 plate were treated with antigen by holding 50-ng each of purified phosphofructokinase in 50 μl of PBS (10 mM KH_2PO_4-KOH, pH 7.4, 0.14 M NaCl) at 4°C overnight. Unadsorbed phosphofructokinase was removed by washing with TPBS (PBS containing 0.05%(v/v) Tween 20). Fifty-microliter aliquots of diluted antibody preparations were held in the

wells at room temperature with a gentle shaking for 2 h. The wells were then washed with TPBS, and treated with 100-µl aliquots of 2,000-fold diluted HRPO-conjugated anti-rabbit IgG antibody (Miles Lab.) at room temperature for 1 h. The wells were washed with TPBS, and filled with 150-µl each of peroxidase reaction medium (5 mM 5-aminosalicylic acid-NaOH, pH 6.0, 0.005% H_2O_2). The reaction was carried out at 37°C with a gentle shaking for 30 min, and terminated by addition of 50 µl of 1.5 N NaOH. Products of the reaction was quantified by photometry at 492 nm reffering to 600 nm. Titer was defined as the dilution of antibody preparation which gave 0.8 O.D. in this assay system. Specific titer was defined as the titer extrapolated to 1 mg/ml in protein concentration.

Purification of phosphofructokinase -- Pig livers were obtained from a local slaughterhouse within 30 min after slaughter, and frozen in a dry-ice box immediately. Two kilograms of the tissue was thawed in a cold room at 4°C overnight, and minced before use.

Step 1: Extraction. The minced tissue was homogenized in a blender with 3,000 ml of buffer A (50 mM Tris/phosphate, pH 8.0, 50 mM NaF, 5 mM EDTA, 2 mM dithiothreitol, 1 mM ATP,

0.5 mM PMSF). The homogenate was centrifuged at 53,000 x g for 90 min.

Step 2: Ammonium sulfate precipitation. The supernatant (3,000 ml) was supplemented with 830 g of ammonium sulfate to give 45% saturation, and stirred overnight in a cold room. Precipitate was spun down at 18,000 x g for 30 min, and dissolved in 1,500 ml of buffer B (50 mM Tris/acetate, pH 8.0, 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 0.1 mM PMSF), and dialyzed against buffer B overnight in a cold room.

Step 3: Heat treatment. The dialyzed preparation was transferred to conical flasks, and heated in a water bath at 80°C. The enzyme solution was kept at 60°C for 1 min, then chilled on ice. The precipitate formed was removed by centrifugation at 5,000 x g for 30 min. The supernatant was dialyzed against buffer B overnight.

Step 4: Affinity chromatography on a phosphocellulose column. The heat extract was diluted with buffer B to 3,000 ml, and applied to a phosphocellulose column (Whatman P-11, 2,000-ml bed) equilibrated with buffer B. The column was washed with 2,000 ml of buffer B. Phosphofructokinase activity was eluted with 2,500 ml of buffer C (75 mM Tris/phosphate, pH 8.0, 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, 3 mM ATP, 1 mM AMP, 25 μM fructose

2,6-bisphosphate, 0.1 mM PMSF). Fractions of high phosphofructokinase activity (1,050 ml) were pooled.

Step 5: Anion-exchange chromatography on a DEAE-Sephacel column. Phosphofructokinase activity from the phosphocellulose column was applied to a DEAE-Sephacel column equilibrated with buffer C. The column was washed with 100 ml of buffer D1 (0.1 M Tris/phosphate, pH 8.0, 50 mM NaF, 10%(w/v) sucrose, 1 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 1 mM AMP, 2 μM fructose 2,6-bisphosphate, 0.1 mM PMSF). Phosphofructokinase was eluted in a linear gradient from buffer D1 to buffer D2 (1 M Tris/phosphate, pH 8.0, 0.1 M ammonium sulfate, 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 1 mM AMP, 2 μM fructose 2,6-bisphosphate, 0.1 mM PMSF). Major peak fractions of high phosphofructokinase activity were combined and dialyzed against buffer E (25 mM Tris/phosphate, pH 8.0, 40 mM ammonium sulfate, 10 mM NaF, 0.1 mM EDTA, 4 mM dithiothreitol, 0.05 mM ATP, 0.1 mM AMP, 50 μM fructose 2,6-bisphosphate).

Step 6: Affinity chromatography on an Affi-Gel Blue column. Phosphofructokinase activity from the DEAE-Sephacel column was applied to an Affi-Gel Blue column (60-ml bed) equilibrated with buffer E. The column was washed with 500 ml of buffer E. Phosphofructokinase activity was eluated

with 2,000 ml of buffer F (25 mM Tris/phosphate, pH 8.0, 40 mM ammonium sulfate, 10 mM NaF, 0.1 mM EDTA, 4 mM dithiothreitol, 3 mM ATP, 1 mM AMP, 50 μM fructose 2,6-bisphosphate).

Step 7: Gel permeation chromatography. Fractions of high phosphofructokinase activity from the Affi-Gel Blue column was concentrated to 4 ml, and applied to 190-ml bed of Cellulofine GCL-2000-sf column (exclusion limit: Mr = 2,000,000) equilibrated with buffer C supplemented with 0.1 M ammonium sulfate. Phosphofructokinase activity recovered just behind the void fractions was stored at -70°C until use.

SDS-polyacrylamide gel electrophoresis and silver staining -- For the test of purity of phosphofructokinase preparations, SDS-polyacrylamide gel electrophoresis was performed according to the method of Laemmli (37) on 1-mm thick gel slab (70 x 90 mm). Protein bands on the gel were visualized by silver staining of Poehling and Neuhoff (38) with our modifications. In brief, the gel was dipped in 50%(v/v) methanol, 10%(v/v) acetic acid for 30 min, and in 10%(v/v) methanol, 15%(v/v) acetic acid, 2%(v/v) glutalaldehyde for 15 min. The gel was rinsed with distilled water for 15 min with refreshing the water every 5

min, and incubated with 0.1 %(w/v) dithiothreitol for 10 min. After rinsing with distilled water for 5 min, the gel was incubated with 0.5%(w/v) silver nitrate, 0.5%(w/v) ammonia, 0.01 M NaOH for 15 min, and then rinsed with distilled water for 20 min with refreshing the water every 5 min. Protein bands were developed in 0.01%(w/v) formaldehyde, 0.005%(w/v) citric acid.

Preparation of polyclonal antibody -- Anti-phospho-fructokinase antibody was induced in a rabbit according to the method of Vaitukaitis (39) with slight modifications. For the primary immunization, 0.1 mg of purified pig liver phosphofructokinase (160 units/mg) in 1.5 ml of phosphate-bufferred saline was mixed with an equal volume of Freund's incomplete adjuvant (Difuco 0639-60) supplemented with 7.5 mg of non-viable descicated Myobacterium tuberculosis H37 RA (Difuco 3144-33-8), and emulsified by a syringe-transfer method. A male rabbit of 2.85-kg body weight was immunized with the emulsion at 40 sites on the back intradermally and on every toe pad. The rabbit was boosted with 0.2 mg and 0.35 mg of phosphofructokinase emulsified with Freund's complete adjuvant (Difco 0638-59) on day 49 and 63 respectively. Anti-serum was harvested from day 70 after the primary immunization. The rabbit was

bled 10 ml a day from the central ear artery over 2 weeks. On day 84, the whole blood was extruded from the heart. Titer of the anti-serum was evaluated by an ELISA. The IgG fraction was purified by chromatography on a Protein A-Sepharose CL-4B column. The 10-ml aliquot of the anti-serum was applied to 4-ml bed of Protein A-Sepharose CL-4B column equilibrated with buffer G (10 mM Tris-HCl, pH 7.5, 0.14 M NaCl). Nonspecifically adsorbed proteins were washed out with 70 ml of buffer H (10 mM Tris-HCl, pH 7.5, 1.5 M NaCl, 0.05%(v/v) Tween 20). IgG was eluted in buffer I (0.1 M glycine-HCl, pH 3.0). The eluate was neutralized to pH 7.5 by addition of 1 M Tris. A large portion of the purified IgG was covalently fixed on Affi-Gel 10. A small portion of the IgG preparation was further purified by affinity chromatography on a phosphofructokinase-conjugated Affi-Gel 10 column. The immunoaffinity-purified IgG was used as a first antibody for enzyme-linked immunostaining of phosphofructokinase and its intermediate catabolic products on nitrocellulose membrane.

Conjugation of phosphofructokinase and IgG to Affi-Gel 10 -- One-ml bed of Affi-Gel 10 was suspended in 10 ml of distilled water, and sedimented by centrifugation at 2,000 x g for 30 sec. The washing cycle was repeated 3 times within

10 min. The washed Affi-Gel 10 was then suspended in 10 ml of 50 mM HEPES-NaOH, pH 7.5 containing purified phosphofructokinase or IgG. The suspension was shaken gently overnight at 4°C, and centrifuged at 2,000 x g for 30 sec. The phosphofructokinase- and IgG-conjugated Affi-Gel 10 preparations were treated with 1 M ethanolamine-HCl, pH 7.5 for 1 h at room temperature to mask the remained sites for conjugation. The gels were then washed with buffer G containing 0.05%(v/v) Tween 20, and stored in a refrigerator at 4°C until use.

Immunoaffinity chromatography on anti-phosphofructokinase IgG-conjugated Affi-Gel 10 column -- A hunfred grams of fresh pig liver was homogenized with 900 ml of buffer J (0.1 M Tris-HCl, pH 8.0, 50 mM NaF, 5 mM EDTA, 0.5 mM PMSF, 5 μg/ml antipain, 5 μg/ml leupeptin, 5 μg/ml pepstatin). The homogenate was centrifuged at 175,000 x g for 90 min. The supernatant was filtered through Millipore membrane (0.45 μm), and applied to an anti-phosphofructokinase IgG-conjugated Affi-Gel 10 column (i.d. 10 mm x 100 mm). Nonspecifically adsorbed proteins were washed out with 300 ml of buffer J, and 20 ml of buffer H. Intact phospho-fructokinase and its catabolic products were eluted with 30 ml of buffer I.

Preparative two-dimensional polyacrylamide gel electorphoresis -- Two-dimensional electrophoresis in O'Farrell's system (40, 41) has been a powerful technique to isolate polypeptides from complex protein mixtures. However, it has been pointed out that the proteins, which are less soluble in a buffer of low ionic strength, likely to remain at the top of isoelectric focusing gel in the first dimension. And phosphofructokinase has been suggested to precipitate under low ionic strength by Massey and Deal (42). In fact, we observed that the purified phosphofructokinase and its catabolic products hardly migrated into isoelectric focusing gel in our preliminary experiments. Hence the method of two-dimensional electrophoresis in ISOTACHO/DALT system should have been developed. In this method, polyacrylamide gel isotachophoresis is performed in the first dimensional direction, and SDS-polyacrylamide gel electrophoresis is in the second dimensional direction.

Because of its large capacity for sample loading, isotachophoresis is suitable for the first dimension of two-dimensional electrophoresis in preparative use.

Isotachophoretic gel for the first dimensional separation was prepared as follows. One milliliter of 27%(w/v) acrylamide was mixed with 1.5 ml of 2%(w/v) BIS, 1 ml of 0.5

M Tris/tartarate, pH 4.5, and 3 ml of 6.5%(w/v) NP-40. Five grams of urea was dissolved in the mixed solution. After a brief deaeration in vacuo, 20 µl of 10%(w/v) ammonium persulfate and 7 µl of TEMED were added. The solution was immediately poured into 2.5-mm-i.d. x 13-cm gass tubes, and the surfaces were overlayered with a small amount of water. The acrylamide was polymerized overnight at room temperature. The overlayered water was removed before use.

Isotachophoresis was performed as follows. Polypeptides in the eluate from the immunoaffinity column were precipitated with 15%(w/v) trichloroacetic acid, and washed 3 times with acetone/HCl (100:1). The precipitate was dried in vacuo and dissolved in 200 µl of sample-application medium (8.5 M urea, 2%(w/v) NP-40, 5%(v/v) 2-mercaptoethanol, 0.1 M Tris). Insoluble residue was removed by centrifugation. About 1 mg of protein in a 100-µl aliquot of the supernatant was mixed with 30 µl of Ampholine (pH 3.5-9.5), and applied to the gel. The sample solution was overlayered with terminating electrolyte (0.5 M 6-amino-n-caproic acid, 50 mM Tris), and the upper electrode vessel was filled with the same electrolyte. Lower electrode vessel was filled with leading electrolyte (50 mM tartaric acid, 50 mM Tris). Isotachophoresis was carried out at 1 mA for 3 h. The gel was extruded from the glass

tube, and incubated with 100 ml of SDS-treatment solution (50 mM Tris-HCl, pH 6.8, 1%(w/v) SDS, 2%(v/v) 2-mercaptoethanol). The SDS-treated gel was stuck on the top of a gel slab (150 x 130 x 1 mm) for SDS-polyacrylamide gel electrophoresis in Laemmli's system (37). Electrophoresis was carried out at 15 mA for 1 h and then at 30 mA for 4 h.

After electrophoresis, the gel was stained overnight with 0.06%(w/v) Coomassie Brilliant Blue G-250 in 5%(w/v) trichloroacetic acid, 3.2%(w/v) sulfosalicylic acid, 50%(v/v) ethanol. The exess dye was removed by rinsing with 10%(v/v) acetic acid, 20%(v/v) ethanol. Coomassie-stained spots on the gel slab were analyzed by image processing on our FIMC system of two-dimensional densitometer as described blow. And the polypeptides in the spots were subjected to the following immunochemical detection and to radio-iodination.

Detection of intact phosphofructokinase and its catobolic products in polyacrylamide gel -- Spot areas of Coomassie-stained polypeptides on the gel slab were punched out. And from each of the spots, small and large gel disks were obtained. Small disks were thoroughly washed with 40%(v/v) ethanol to remove Commassie dye, and dried in

vacuo. The dried gel disks were swollen in a solubilization buffer (25 mM Tris, 0.192 M glycine, 1%(w/v) SDS), and polypeptides in the gel were electrophoretically transferred to a sheet of nitrocellulose membrane. The membrane was then treated with 3%(w/v) liquid gelatin in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl), and incubated with the affinity-purified anti-phosphofructokinase IgG in TBS. After washing with TTBS (TBS containing 0.05%(v/v) Tween 20), the membrane was incubated with HRPO-linked goat anti-rabbit IgG antibody in TBS. Peroxidase activity was visualized by incubation with 2 mg/ml 3,3'-diaminobenzidine, 0.06%(w/v) H_2O_2 in TBS.

Radioiodination and V8-digestion of intact phosphofructokinase and its catabolic products -- Polypeptides in the large disks were radioiodinated according to the method of Elder (43). Radioiodinated polypeptides were purified again by SDS-polyacrylamide gel electrophoresis, and electrophoretically eluted in TGS buffer (25 mM Tris, 0.192 M glycine, 0.1%(w/w) SDS). The apparatus for electrophoretic elution was made as follows. The bottoms of a micro test tubes (Sarstedt 72-699) were cut off, and jointed to a dialysis tubings (Union Carbide Corp. 8/32). The ends of the tubings were closed with plastic

clips (Spectram Med. Ind., Inc. 132734). The gel pieces containing radioiodinated polypeptides were put into the micro test tubes, and the tubes were mounted on an apparatus for disc electrophoresis. The tube was filled with TGS buffer containing 10% glycerol, and both electrode vessels were with TGS buffer. Electrophoretic elution was performed at 100 V for 30 min (cathodic side up). After removing the electrolytes from the upper vessel, eluates in the tubes were obtained. Radioactivities in the eluates were measured with a gamma counter. The eluates were supplemented with BSA (0.2 mg/ml) in accordance with the recommendation of Takeda and Cone (44), and diluted with TGS buffer containing 0.2 mg/ml BSA to adjust the radioactivity to 0.4 μCi/ml. Five micrograms of proteinase V8 in 10 μl of TGS buffer was added to 100-μl each of the diluted eluates. Digestion was carried out at 25°C for 20 h. Enzyme reaction was terminated by heating at 100°C for 3 min. The digestion products were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Two-dimensional electrophoresis of anti-phosphofructokinase antibody on cellulose actate membrane -- Cellulose acetate strips (Titan III, 76 x 10 mm)

were wetted with a leading electrolyte (described in Table I) and laid on a glass plate (2 x 80 x 75 mm). The plate

Table I

was mounted on the cooling bed of apparatus A (Fig. 1). The anode and the cathode vessels were filled with the leading and the terminating electrolytes (Table I), respectively. The bridging pads of filter paper, which were moistened with respective electrolytes, were mounted on the suspension bridges on the lid (see Fig. 1). Excess electrolytes on the strips were removed by blotting with a filterpaper. The lid was then loosely closed to keep the bridges suspended. In this step, the strip of cellulose acetate did not contact with either the anodic or the cathodic bridging pads. A power supply was connected to the platinum electrodes, and 200 V was applied. The lid was then tightly closed to make them in contact. The flow of electric current was initiated by the contact. After a short prerun (for 5 min at 200 V), the strips were loaded with 10 µl aliquots of anti-phosphofructokinase antiserum near the cathodic end. Proteins in the applied specimens were concentrated in narrow bands at the boundary between leading and terminating

electrolytes according to the theory of 'steady state stacking' in isotachophoresis.

Immediately after attaining the isotachophoretic concentration, the cathode vessel was replaced with another one filled with the passing electrolyte (Table I). The cathodic bridging pad was also replaced with a fresh pad moistened with the passing electrolyte. With this electrolyte, 'steady state stacking' was broken. Electrophoretic separation was carried out at a constant current (0.4 mA for each strip) for about 45 min. The front of migration was monitored with the band of BPB (bromophenol blue).

In the second-dimensional direction, isoelectric focusing was carried out on two layers of cellulose acetate membrane (Separax EF, 110 x 60 mm) with carrier-ampholytes (Table I). After the first-dimensional electrophoresis, the cathodic end of the Titan III strip was cut off, leaving a 60-mm long piece. The strip was then put on the membrane perpendicularly to the direction of electric current for isoelectric focusing (as Fig. 1). A pad of glass fiber filter (GF/B, 59 x 5 mm) moistened with anode electrolyte (Table I) was put on the anodic end of the piled membrane, and another pad with cathode electrolyte (Table I) was put on the cathodic end. Before the pads were put on the

membrane, excess electrolytes on the pads were removed by blotting with a filterpaper to avoid irregular spread of the electrolytes over the membrane. When the lid is closed, the platinum electrodes were got in touch with the electrode pads. A constant current (2 mA per two layers of membrane) was applied, and the increase in voltage was monitored. When the voltage reached 800 V, the Titan III strip was removed and isoelectric focusing was continued at 800 V for additional 1.5 h.

Staining of protein on cellulose acetate membrane -- After the second-dimensional isoelectric focusing, the upper layer of cellulose acetate membrane was dipped in 20%(v/v) sulfosalicylic acid for 5 min for protein fixation. The membrane was then dipped in 0.06%(w/v) Coomassie Brilliant Blue G-250, 5%(w/v) trichloroacetic acid, 3.2%(w/v) sulfosalicylic acid, 50%(v/v) ethanol for 7 min. Exess dye was removed by rinsing with 10%(v/v) acetic acid, 20%(v/v) ethanol. The membrane was then dipped in 0.5%(w/v) polyvinyl alcohol, 5%(v/v) acetic acid for 5 min, and stuck on a glass plate. The membrane was dried at room temperature overnight.

Double detection of anti-phosphofructokinase antibody and whole IgG on nitrocellulose membrane -- The lower layer of cellulose acetate membrane was sandwiched in between two sheets of nitrocellulose membrane, and they were kept in a moistened box at 4°C overnight. Protein spots were transferred to nitrocellulose membrane by the 'sandwich blot' method. After the blotting, the nitrocellulose membranes were treated with 3%(w/v) liquid gelatin in TBS to block nonspecific binding sites. One sheet of each pair was then incubated with radioiodinated Mr = 84,000 polypeptide, and the other sheet was incubated with radioiodinated Mr = 68,000, Mr = 64,000, Mr = 56,000 or Mr = 51,000 polypeptide overnight. After washing with TTBS, the sheets were dried and applied to autoradiography. Kodak X-Omat AR film (20.3 x 25.4 cm) was held between the nitrocellulose membrane and an intensifying screen (DU PONT Lightning Plus) at -70°C for 15 days. After the use for autoradiography, the nitrocellulose membrane was wetted again with TBS, and incubated with HRPO-conjugated anti-rabbit-IgG antibody for 2 h at room temperature. Unbound antibodies were rinsed out with TTBS, and the IgG pattern was visualized by peroxidase reaction with 3,3'-diaminobenzidine and H_2O_2.

Two-dimensional densitometry -- (i) Image acquisition. Three types of optical readers have been available for us to acquire two-dimensional patterns. A drum scanner is suitable for transparent films such as autoradiograms, but not applicable to opaque materials such as cellulose acetate membrane. Scanning-microscope photometers (45,46) provide the most accurate image acquisition as yet, but they are still time-consuming. Television (TV) cameras have been proposed as the most rapid optical reader for two-dimensional densitometry, and a few groups (47, 48) have already introduced TV cameras into their methods. However, conventional TV cameras with image tubes still have some problems. The virtual signal-to-noise ratios (S/N) of Vidicon and Plumbicon tubes are around 46 dB (200:1), and that of Image Orthicon tube is around 35 dB (60:1). Under these low S/Ns, 8-bit digitization of the video signals is meaningless. In practice, 8-bit digitization into 256 gray levels is required to evaluate 0.01 O.D. in the full range of 0-2 O.D. The 'blooming', 'burning', gain and offset problems of these tube cameras interfere with reliability in quantitative analysis of two-dimensional electrophoretograms. Especially the burning causes gross modification of optical data. These problems have not been solved in any other type of image tube. Recently, a solid-state TV camera

with a CCD image sensor has been available, which may offer a wider dynamic range than tube cameras. The CCD-TV camera shows no burning, less blooming and a high S/N (50 dB, 300:1) matching to 8-bit digitization. Hence, we introduced the CCD-TV camera into our FIMC system of two-dimensional densitometer (17).

(ii) Blank subtraction. It is practically impossible to realize a completely even illumination in the camera's field of view. And the nonuniformity in illumination would cause position dependency of optical measurement in the camera's field of view. We overcame the problem with a method of 'blank subtraction'. Theoretical basis of the method is as follows: I_0^S and I_0^B in Fig. 3 are intensities of the light

Fig. 3

source for a sample and a blank membrane, respectively. Intensities $I_1^S(\underline{x},\underline{y})$ and $I_1^B(\underline{x},\underline{y})$, illuminating a unit area of each membrane at position $(\underline{x},\underline{y})$, are given by Eqs. [1] and [2].

$$I_1^S(\underline{x},\underline{y}) = k_1(\underline{x},\underline{y}) \cdot I_0^S \qquad [1]$$

$$I_1^B(\underline{x},\underline{y}) = k_1(\underline{x},\underline{y}) \cdot I_0^B$$

$$= k_1(\underline{x},\underline{y}) \cdot k_0 \cdot I_0^{S}$$

$$= k_0 \cdot I_1^{S}(\underline{x},\underline{y}) \qquad [2]$$

Here, k_0 means the $I_0^{'B}/I_0^{S}$ ratio, and $k_1(\underline{x},\underline{y})$ is the efficiency of the lighting system at position $(\underline{x},\underline{y})$. $OD^{S}(\underline{x},\underline{y})$ and OD^{B}, which are optical densities at position $(\underline{x},\underline{y})$ on the sample and the blank membrane respectively, are defined by Eqs.[3] and [4].

$$OD^{S}(\underline{x},\underline{y}) = -\log\frac{I_2^{S}(\underline{x},\underline{y})}{I_1^{S}(\underline{x},\underline{y})} \qquad [3]$$

$$OD^{B} = -\log\frac{I_2^{B}(\underline{x},\underline{y})}{I_1^{B}(\underline{x},\underline{y})} \qquad [4]$$

Here, $I_2^{S}(\underline{x},\underline{y})$, and $I_2^{B}(\underline{x},\underline{y})$ are intensities of the light scattered from the sample and the blank membranes at position $(\underline{x},\underline{y})$. OD^{B} is independent from $\underline{x}$ and $\underline{y}$, provided that the blank membrane is optically uniform. Each part of the scattered light is gathered with the lens system, and focused on the surface of the CCD image sensor at each corresponding pixel point. The light intensities $I_3^{S}(\underline{x},\underline{y})$ and $I_3^{B}(\underline{x},\underline{y})$ on the sensor are defined by Eqs.[5] and [6].

$$I_3^S(\underline{x},\underline{y}) = (1-k_2^S)\cdot k_3(\underline{x},\underline{y})\cdot I_2^S(\underline{x},\underline{y}) \quad [5]$$

$$I_3^B(\underline{x},\underline{y}) = (1-k_2^B)\cdot k_3(\underline{x},\underline{y}) \quad [6]$$

Here, k_2^S and k_2^B are scattering coefficients of the sample and the blank, respectively. The light scattering must be even on homogeneous membrane with no crack. $k_3(\underline{x},\underline{y})$ refers to the beam-gathering efficiency of the lens system, and it is same for both the sample and the blank.

Electric signals on the CCD image sensor corresponding to the light intensities $I_3^S(\underline{x},\underline{y})$ and $I_3^B(\underline{x},\underline{y})$ are converted to pixel data $PD^S(\underline{i},\underline{j})$ and $PD^B(\underline{i},\underline{j})$ through the video amplifier (AMP), the analog-to-digital converter (A/D) and the logarithmic transformer (LOG). Then the pixel data are resistered in the display memory (DM). The trapped data corresponding to the sample and the blank membranes are given by Eqs.[7] and [8].

$$PD^S(\underline{i},\underline{j}) = k_4\cdot\log(k_5\cdot I_3^S(\underline{x},\underline{y})) \quad [7]$$

$$PD^B(\underline{i},\underline{j}) = k_4\cdot\log(k_5\cdot I_3^B(\underline{x},\underline{y})) \quad [8]$$

Here, $(\underline{i},\underline{j})$ means an address of the matrix array on the CCD image sensor, which captures photons from the corresponding position on the membrane. The constant k_4 is the

multiplying strength for fitting the pixel data to 8-bit binary integers, and k_5 refers to the gain of AMP.

Subtraction of Eq.[8] from [7] gives:

$$PD^S(\underline{i},\underline{j})-PD^B(\underline{i},\underline{j}) = k_4 \cdot \log\frac{I_3{}^S(\underline{x},\underline{y})}{I_3{}^B(\underline{x},\underline{y})} \qquad [9]$$

Inserting Eqs.[2]-[6] into [9], we obtain:

$$PD^S(\underline{i},\underline{j})-PD^B(\underline{i},\underline{j}) = k_4 \cdot \log\frac{I_3{}^S(\underline{x},\underline{y}) \cdot I_1{}^B(\underline{x},\underline{y})}{I_3{}^B(\underline{x},\underline{y}) \cdot k_0 \cdot I_1{}^S(\underline{x},\underline{y})}$$

$$= k_4 \cdot (\log\frac{I_2{}^S(\underline{x},\underline{y})}{I_1{}^S(\underline{x},\underline{y})} - \log\frac{I_2{}^B(\underline{x},\underline{y})}{I_1{}^B(\underline{x},\underline{y})} + \log\frac{1-k_2{}^S}{(1-k_2{}^B)\cdot k_0})$$

$$= - k_4 \cdot OD^S(\underline{x},\underline{y}) + k_4 \cdot (OD^B + \log\frac{1-k_2{}^S}{(1-k_2{}^B)\cdot k_0}) \qquad [10]$$

The net OD of the sample is ultimately described in the form shown blow:

$$OD_N(\underline{x},\underline{y}) = OD^S(\underline{x},\underline{y}) - OD^B$$

$$= - \frac{PD^S(\underline{i},\underline{j})-PD^B(\underline{i},\underline{j})}{k_4} + \log\frac{1-k_2{}^S}{(1-k_2{}^B)\cdot k_0} \qquad [11]$$

This equation means that $OD_N(\underline{x},\underline{y})$ is obtained from $PD^S(\underline{i},\underline{j})-PD^B(\underline{i},\underline{j})$ by a linear function. Unevenness of illumination in the camera's field of view may not affect

the $OD_N(\underline{x},\underline{y})$, even if it affects the S/N of the optical data. And when the scattering properties of the sample and the blank membranes are the same ($k_2^S = k_2^B$) and the intensities of the light source are the same for the sample and for the blank ($k_0 = 1$), we obtain:

$$OD_N(\underline{x},\underline{y}) = - \frac{PD^S(\underline{i},\underline{j}) - PD^B(\underline{i},\underline{j})}{k_4} \qquad [12]$$

The actual procedure to realize the densitometry according to the above equations is as follows. First, a sheet of unstained membrane is put on the camera stage, and the 'blank image' is acquired with a CCD image sensor. Pixel data $PD^B(\underline{i},\underline{j})$ on the blank image are transferred from the camera unit to DM through AMP, A/D and LOG. Here, DM is a page of display memory comprising 512 x 256 pixels in matrix array, and the image in it is displayed on a cathode-ray tube (CRT) in 256 steps of gray levels. The blank image data are held on page 1 and 3 of frame memory (FM0 and FM3). Then, a sample image is acquired, and the pixel data $PD^S(\underline{i},\underline{j})$ are held on DM and FM1. Pixel data on FM0 are subtracted from those on DM, and the remainders are stored in FM0 and FM2 as blank subtracted data.

(iii) Local background subtraction. The still remained background, which modifies optical density of protein spot,

would be given by the sum of the following three major elements; term $\log((1-k_2^S)/(1-k_2^B)\ k_0)$ of Eq. [11], overlapping of the protein spot with trails of the neighboring peaks, remaining of the dye on the membrane used for protein staining. The latter two elements are included in $OD_N(\underline{x},\underline{y})$. In the program, the overall background is simply determined as the local lowest level of $-(PD^S(\underline{i},\underline{j}) - PD^B(\underline{i},\underline{j}))$ within a window (58 x 80 pixels) as follows. The frame of window appears on the video-monitor CRT, and an operator is allowed to move it to designate a site for the local area. Pixel data in the window is subjected to smoothing for noise reduction, and the lowest level of $-(PD^S(\underline{i},\underline{j})-PD^B(\underline{i},\underline{j}))$ is determined. The gray levelis subtracted from pixel data in the window.

(iv) Integration of pixel data. The local view in the window is, then, extended three times in dimension, and displayed on the left half of the video-monitor CRT. Its perspective view is exhibited on the right half, if required. An operator is allowed to draw a circle on the expanded local image using a digitizer. The pixel data in the circle are recalled from FM0, and summed up to get k_4 $OD_N(\underline{x},\underline{y})$.

(v) Integrated optical density standard. Thus obtained sum is just a relative value of integrated optical density

(IOD), since the pixel data are multiplied by k_4 to be converted into 8-bit-binary integers, and the scale of the length is arbitray in the camera's field of view. Hence the obtained value should be calibrated with an internal standard, of which size (mm^2) and IOD (O.D. mm^2) are previously determined using Joyce Loebl Microdensitometer 6.

Microcomputer programming -- Both ASSEMBLY language and BASIC language were utilized in our programming for the following reasons. BASIC has been the most popular programming language for microcomputer users, and there have been many potential programmers even in our medical institute. Programs in BASIC language are easy to revise. However, execution of a large program written in BASIC language is quite slow. Subroutine programs written in ASSEMBLY language improve the execution speed. Hence, we prepared a package of subroutine modules (FIMC) in ASSEMBLY language, and then wrote the utility program (2DD) for two-dimensional densitometry in BASIC language on the subroutines. FIMC consists of four modules, a common module and three overlay modules. The common module is incorporated in the main frame of BASIC interpreter, and loaded on the random access memory (RAM) of SORD M223 throughout the processing. The overlay modules are held on

the floppy disk. Each of the overlay modules is fetched from the disk and appended to the main frame of BASIC interpreter, when a subroutine in the module is recalled in the BASIC program.

RESULTS

Purification of phosphofructokinase -- The results of purification are summerized in Table II. More than 90% of

Table II

phosphofructokinase activity in pig liver homogenate was extracted in the supernatant fraction. And 92% of phosphofructokinase activity in the extract was precipitated with 45% saturation of ammonium sulfate. The precipitate was dissolved in buffer B, and dialyzed against the same buffer for the following heat treatment. Major contaminants were precipitated by the incubation at 60°C for 1 min. Phosphofructokinase activity was maintained in the treatment. The activity recovered in the supernatant was adsorbed on phosphocellulose. Phosphofructokinase activity was eluted with the buffer of high ATP concentration (Fig. 4).

Fig. 4

Phosphofructokinase in the eluate was then adsorbed on DEAE-Sephacel. The major activity was eluted in a main peak at 0.3 M Tris/phosphate in the buffer-gradient (Fig. 5).

Fig. 5

The peak fractions were dialyzed against buffer E, and adsorbed on Affi-Gel Blue. From the dye-affinity column, the activity was eluted with 3 mM ATP and 1 mM AMP in buffer E (Fig. 6). Phosphofructokinase in the eluates was trapped

Fig. 6

on a small bed of DEAE-Sephacel for concentration.

The enzyme was finally purified by gel permeation chromatography. Phosphofructokinase activity eluted in a symmetric peak just behind the void fractions was pooled (Fig. 7). Purities of the enzyme preparations were examined

Fig. 7

by SDS-polyacrylamide gel electrophoresis followed by silver staining. The final preparation after the gel permeation

chromatography comprised Mr = 80,000 polypeptide only (Fig. 8). The apparent molecular weight (Mr = 1,300,000)

Fig. 8

determined by the gel permeation chromatography corresponds to hexadecamer of Mr = 80,000. The highly purified phosphofructokinase (specific activity = 160 units/mg) was used as an antigen for induction of antibody on a rabbit, as an antigen for titration of the antibody by ELISA, and as a ligand for preparation of an immunoaffinity column.

Induction of anti-phosphofructokinase antibody -- A male rabbit of 2.85-kg body weight was immunized with total 0.65 mg of phosphofructokinase as described in Methods. Seven days after the second boosting, titer of the anti-serum rose to 5,500 in ELISA (Fig. 9). The rabbit was bled 10 ml a

Fig.9

day, and total 200 ml of anti-serum was obtained. Immunoreactivity of the anti-serum was examined by a solid-phase immunoadsorption test using Protein A-conjugated particles. And the antibody in the serum was observed to

have the binding activity to liver-type isozyme, but not to muscle-type isozyme.

Purification of the antibody and preparation of an anti-phosphofructokinase IgG-conjugated Affi-Gel 10 column -- Anti-phosphofructokinase antibody was purified by chromatography on a Protein A-Sepharose CL-4B column and on a phosphofructokinase-conjugated Affi-Gel 10 column. The specific titer rose 6 fold by Protein A-affinity chromatography, and 150 fold by antigen-affinity chromatography (total 900-fold purification). About 170 mg of IgG was eluted in buffer I from Protein A-Sepharose CL-4B. The IgG fraction was then filtered through a Sephadex G-25 column equilibrated with 50 mM HEPES-NaOH, pH 7.5, to replace glycine buffer. About 150 mg of IgG was covalently fixed on 8-ml bed of Affi-Gel 10 at 4°C. Remaining sites for conjugation on Affi-Gel 10 were blocked by incubation with 1 M ethanolamine-HCl, pH 7.8. Binding capacity of the IgG conjugated with Affi-Gel 10 was determined as follows. Various volumes of pig liver extract from 10%(w/v) homogenate were incubated with 0.1-ml aliquots of the IgG-conjugated gel. After 10-min incubation, the gel was spun down and phosphofructokinase activity in the

supernatant was assayed. One-milliliter bed of the gel precipitated 7 units of phosphofructokinase.

Immunoaffinity chromatography of intact phosphofructokinase and its catabolic products on the anti-phosphofructokinase IgG-conjugated Affi-Gel 10 column -- Catabolic intermediates were extracted from 100 g of fresh pig liver in a buffer containing proteinase inhibitors as described in Methods. Cross-reactive polypeptides in the extract were adsorbed on the immunoaffinity column (8-ml bed of anti-phosphofructokinase IgG-conjugated Affi-Gel 10). Nonspecific proteins adsorbed on the gel were extensively washed out with buffer J and buffer H. About 2 mg of protein including intermediate catabolic products of phosphofructokinase were eluted in buffer I (Fig. 10).

Fig. 10

Purification of intact phosphofructokinase and its catabolic products by preparative two-dimensional electrophoresis -- The immunoaffinity-purified polypeptides were further separated by a preparative two-dimensional polyacrylamide gel electrophoresis, and stained with

Coomassie Brilliant Blue G-250 (Fig. 11). Distribution of

Fig. 11

polypeptides was analyzed by two-dimensional densitometry using our TV-camera-equipped system. The binarized image (Fig. 12) showed 10 isles of polypeptides numbered 1 to 10.

Fig. 12

Isle 1 with a small tail seemed to contain two components. Isle 9 seemed to consist only of a single component. However, the contour lines (iso-density lines) made it clear that isle 9 consisted of 3 components (Fig. 13). Relative

Fig. 13

amounts of these components were also determined by the two-dimensional densitometer. The results are shown in Table III.

Table III

Peak areas of these components (i.e. polypeptides), which

were circled with the most inner lines of iso-density, was punched out from the gel slab. And small and large disks were obtained from each peak area. Cross-reactivity of polypeptides to phosphofructokonase were assessed by an enzyme-linked immunoelectrotransfer blot (Western blot) method. Polypeptide of Mr = 84,000, which was an intact form of phosphofructokinase in pig liver, was most intensely demonstrated on the blot. Polypeptides of Mr = 68,000, Mr = 64,000, Mr = 56,000 and Mr = 51,000 were also noticeably demonstrated by the immunochemical staining (Fig. 14).

Fig. 14

Those polypeptides in the gel pieces were subjected to radioiodination according to the method of Elder (43). And about 50 μCi of [^{125}I]iodide was incorporated into 1-μg each of polypeptides. The radioiodinated polypeptides were purified again by SDS-polyacrylamide gel electrophoresis (Fig. 15), and eluted electrophoretically from the gel

Fig. 15

slices at the radioactive peaks.

Two-dimensional electrophoresis and radioimmunodetection of anti-phosphofructokinase antibody on cellulose acetate membrane -- Subspecies of IgG in the anti-phosphofructokinase anti-serum were separated by two-dimensional electrophoresis on two layers of cellulose acetate membrane under a native (denaturant-free) condition. Protein patterns were visualized by Coomassie staining on the original cellulose acetate membrane (I-A, II-A, III-A and IV-A in Fig. 16). Distributions of IgG were demonstrated by

Fig. 16

HRPO-linked immunostaining on nitrocellulose membrane after 'sandwich' blotting (I-B, II-B, III-B and IV-B in Fig. 16). Spots of specific antibodies were detected by autoradiography on nitrocellulose membrane after incubation with radioiodinated antigens (I-C, II-C, III-C and IV-C in Fig. 16). The autoradiograms indicate that the antiserum contains at least 6 species of anti-phosphofructokinase antibody (pI 5.3, pI 5.7, pI 6.0, pI 6.3, pI 6.6 and pI 7.3). All six show the binding activity to Mr = 68,000 polypeptide, five (pI 5.3, pI 5.7, pI 6.0, pI 6.3 and pI 6.6) to Mr = 64,000 and Mr = 56,000 polypeptides, and four (pI 5.3, pI 5.7, pI 6.0 and pI 6.3) to Mr = 51,000

polypeptide. The results indicate that the candidate polypeptides for intermediate catabolic products of phosphofructokinase shares the common antigenic determinants (epitopes).

Electrophoretic profiles of proteinase-V8 digests of radioiodinated phosphofructokinase and its catabolic products -- The radioiodinated phosphofructokinase and its catabolic products were digested with proteinase V8. And the digests were separated by SDS-polyacrylamide gel electrophoresis, and detected by autoradiography. The autoradiograms showed that these polypeptides yielded common sizes of V8-digests (Fig. 17). The results support our

Fig. 17

conclution that these cross-reactive polypeptides (Mr = 68,000, Mr = 64,000, Mr = 56,000 and Mr = 51,000) are all catabolic products of phosphofructokinase of Mr = 84,000.

Assessment of the possibility of unphysiological degradation of phosphofructokinase in preparation -- Fresh pig livers were homogenized with buffer J containing radioiodinated phosphofructokinase (Mr = 84,000) or with

radioiodinated catabolic products (Mr = 68,000, Mr =64,000). Centrifugation and immunoaffinity chromatography were carried out as described above. The polypeptides recovered after the immunoaffinity chromatography were separated by SDS-polyacrylamide gel electrophoresis. Bands of radioactive polypeptides were detected by autoradiography (B in Fig. 18). Degradation of the radioiodinated

Fig. 18

phosphofructokinase in preparation was not observed on the autoradiogram. Thus the possibility of artifacts in preparation was ruled out.

DISCUSSION

In 1970, Gershon and Gershon (49) reported the accumulation of inactive isocitrate lyase in aged nematodes in their experiments as follows. They purified the enzyme from young nematodes, and prepared a specific antibody against the purified enzyme. Isocitrate lyase activity in the homogenates of young and aged nematodes were titrated with the antibody. And they observed that more antibody was required to precipitate a unit of the enzyme from aged nematodes than that from young controls. They concluded that cross-reacive materials without enzyme activity accumulated in the aged nematodes. After the report, several groups followed their study, and Reznick et al. (50) observed 50% decrease in specific activity of aldolase in 31-months-old mouse liver, Reis and Gershon (51) observed 60% decrease in that of superoxide dismutase in 27-months-old rat liver, Gafni (52) observed 37% decrease in that of glyceraldehyde-3-phosphate dehydrogenase in 28-months-old rat muscle, and Sharma et al. (53) observed 50% decrease in that of enolase in 26-days-old nematodes. Although the cause of the enzyme defects has not been cleared, intracellular proteinases have been suggested to be responsible for the inactivation process of the enzymes.

Enzyme alterations other than the defects in specific acivity have been also observed in aged animals. Holliday and Tarrant (54) have found that the heat-labile glucose-6-phosphate dehydrogenase appears in cultured human fibroblasts at the late passages in population doubling. And thay have speculated that the heat lability of the enzyme is the result of post-translational alterations. The appearence of such heat-labile enzyme in aged animals has been reported by several other groups; glucose-6-phosphatase in rat liver by Grinna and Barber (55), glucose-6-phosphate dehydrogenase in mouse liver by Schofield and Hadfield (56), and DNA polymerase in rat liver by Taguchi and Ohashi (57). However, it has still been in doubt that the instability is due to the enzyme molecules themselves, since they were examined in crude extracts. Hence intracellular proteinase could be responsible for the appearence of heat-labile enzymes in tissues of aged animals.

Since Schimke (58) reported that the change in arginase activity in rat liver under a dietary restriction was due to the change in degradation rate, the physiological importance of enzyme catabolism have been discussed by many groups. The most advanced studies have been accomplished on prosthetic group-requiring enzymes. Kominami et. al. (59) have reported that the formation of apoenzyme of ornithine

aminotransferase is a rate-limiting step in the degradation pathway of the enzyme. Inactive apoenzyme is more susceptible than the holoenzyme to B6 group-specific proteinase. And they have speculated that the partially cleaved apoenzyme is further digested in lysosomes with most of nonspecific proteinases. However, catabolic pathways of other allosteric enzymes under hormonal regulation are still unclear.

Phosphofructokinase is one of the rate-limiting enzymes on glycolytic pathway in mammalian hepatocytes. The enzyme is under the hormonal and dietary regulation through concentrations of many allosteric modulators. In addition to the allosteric regulation, phosphofructokinase activity is known to be regulated in the balance of synthesis and degradation of the enzyme. Dunaway et al. (31-34) have reported that the reduction in phosphofructokinase in fasted and diabetic liver is the result of an increased degradation rate with little change in synthetic rate. In this paper, we have reported the purification and identification of the intermediate catabolic products in the in vivo degradation of pig liver phosphofructokinase in order to establish the basis for assigning the phosphofructokinase-cleaving proteinases. Intermediate catabolic products were extracted from normal pig livers under the inhibition of proteinases,

and they were chromatographed on an anti-phosphofructokinase IgG-conjugated Affi-Gel 10 column. The immunoaffinity-purified polypeptides were further purified by two-dimensional electrophoresis in ISOTACHO/DALT system. Cross-reactivities of the polypeptides isolated on the gel slab were assessed by an immunoelectrotransfer blot method. The cross-reactive polypeptides were radioiodinated by the chloramine-T method, and digested with proteinase V8. The digestion profiles were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography, and the common-sized peptides were demonstrated on the autoradiogram. Hence we concluded that the cross-reactive polypeptides purified by immunoaffinity chromatography and by two-dimensional electrophoresis are the intant phosphofructokinase and its catabolic products. The possibility of artifacts in preparation was ruled out by an internal tracer method. Radioiodinated phosphofructokinase (Mr = 84,000) accompanied the in vivo catabolic products in the whole procedures of preparation. After immunoaffinity-purification, recovered polypeptides were separated by SDS-polyacrylamide gel electrophoresis, and radioactive bands were demonstrated by autoradiography. Only Mr = 84,000 band was detected even on the extensively exposed X-ray films. On the results, it has been concluded that phosphofructokinase in pig liver is

degraded through the intermediate catabolic products of Mr = 68,000, Mr = 64,000, Mr = 56,000 and Mr = 51,000 physiologically.

Thus we have established the way to assign phosphofructokinase-degrading proteinases in pig liver. Physiological proteinases which degrade phosphofructokinase *in vivo* should digest radioiodinated phosphofructokinase (Mr = 84,000) *in vitro*. The *in vivo* digestion products of radioiodinated phosphofructokinase could be identified by two-dimensional electrophoresis with the use of the radioiodinated *in vivo* catabolic products as markers.

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ABBREVIATIONS

Abbreviations used are: PFK, phosphofructokinase; BSA, bovine serum albumin; STI, soybean trypsin inhibitor; IgG, immunoglobulin G; IRI, immunoreactive insulin; IRG, immunoreactive glucagon; Tris, tris-(hydroxymethyl)-ammino-methane; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid; ATP, adenosine triphosphate; AMP, adenosine monophosphate; NADH, nicotinamide-adenine dinucleotide reduced form; Tween 20, polyoxyethylene sorbitan monolaurate; PBS, phosphate-buffered saline; TPBS, Tween 20-supplemented phosphate-buffered saline; TBS, Tris-buffered saline; TTBS, Tween 20-supplemented Tris-buffered saline; BIS, N,N'-methylenebisacrylamide; SDS, sodium dodecyl sulfate; TEMED, N,N,N',N'-tetramethylethylene diamine; PMSF, phenylmethylsulfonyl fluoride; HRPO, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; OGTT, oral glucose tolerance test; Mr, relative molecular weight; pI, isoelectric point; PAGE, polyacrylamide gel electrophoresis; CAE, cellulose acetate electrophoresis; CE, concentrating electrophoresis; IEF, isoelectric focusing; CA, cellulose acetate; NC, nitrocellulose; AR, autoradiogram; OD, optical density; O.D., unit of optical density; IOD, integrated optical density; FIMC, frame-image-memory controller; CCD,

charge-coupled device; CPU, central processing unit; RAM, random access memory.

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Table I

Electrolytes for two-dimensional electrophoresis on cellulose acetate membrane

First dimension	
Leading electrolyte	62 mM Tris-HCl, pH 6.7
Terminating electrolyte	50 mM arginine-$Ba(OH)_2$, pH 11.7
Passing electrolyte	100 mM Tris-H_3PO_4, pH 7.5
Second dimension	
Carrier-ampholyte	5% (w/v) Ampholine (pH 3.5-10)/10% (w/v) sucrose
Anode electrolyte	1% (v/v) H_3PO_4/30% (w/v) sucrose
Cathode electrolyte	1% (v/v) Ethylenediamine

Table II

Purification of pig liver phosphofructokinase

Step	Volume[a]	Activity[b]	Protein[c]	Specific Activity	Purification	Yield
	ml	*units*	*mg*	*units/mg*		*%*
Crude extract	3,000	3,700	360,000	0.01	1	100
Ammonium sulfate fractionation	2,750	3,400	170,000	0.02	2	92
Heat treatment	2,150	3,500	36,000	0.10	10	95
Phosphocellulose	1,050	2,900	920	3.2	320	78
DEAE-Sephacel	115	1,900	92	21	2,100	52
Affi-Gel Blue	1,000	670	7	96	9,600	18
Cellulofine	10	207	1.3	160	16,000	5.6

[a]With 2 kg of pig liver as starting material.

[b]Activity was measured by a rate assay coupled with aldolase, triosephosphate isomerase and glycerophosphate dehydrogenase. A unit of PFK is defined as the amount of the enzyme that catalyzes the formation of 1 µmol of fructose 1,6-bisphosphate per min at 25°C.

[c]Protein was determined by the method of Lowry et al (35) modified by Bensadoun and Weinstein (36) with crystalline BSA as a protein standard.

Table III

Size, IOD and relative IOD determined by two-dimensional densitometer of FIMC system

isle/peak	size(mm^2)	IOD(O.D. mm^2)	relative IOD
1-a	26.8	10.35	100
1-b	1.9	0.05	0.5
2	2.1	0.05	0.5
3	2.5	0.05	0.5
4	5.5	0.45	4.3
5	5.1	0.21	2.0
6	2.9	0.18	1.7
7	3.6	0.08	0.8
8-a	14.7	6.24	60.3
8-b	12.9	4.53	43.8
8-c	8.3	1.45	14.0
9	8.2	0.30	2.9
10	5.3	0.26	2.5

Fig. 1. Diagram of the apparatus for two-dimensional cellulose acetate membrane electrophoresis. Apparatus A, equipped with suspension bridges, is designed to make a discontinuous electrolytes system on four strips of cellulose acetate membranes. (a) Leaf springs, (b) holes for sample application, (c) suspension bridges, (d) bridging pad of filterpaper moistened with leading electrolyte, and (e) bridging pad moistened with terminating electrolyte in the isotachophoretic concentration step. Pad (e) is replaced with another one moistened with passing electrolyte in the following separation step. (f) Strips of cellulose acetate (76 x 10 mm), (g) glass plate (2 x 80 x 75 mm), (h) anode vessel containing leading electrolyte, and (i) cathode vessel containing terminating electrolyte in the concentration step. Vessel (i) is replaced with another one containing passing electrolyte in the separation step. (j) Electrode wickes of filterpaper, (k) platinum electrodes, (l) terminals, (m) cooling bed, and (n) water circulation (4°C). The lid is designed to close in two states (i.e., suspended and contacted states). In the suspended state, the strips of cellulose acetate membrane (f) are not in contact with either the anodic bridging pad (d) or the cathodic bridging pad (e). Apparatus B, equipped with knife-edge-shaped electrodes, has minimized dead space to

prevent evaporation and condensation of water during isoelectric focusing. (o) Leaf springs, (p) terminals, (q) knife-edge-shaped platinum electrodes, (r) glass plate (3 x 120 x 70 mm), (s) several layers of cellulose acetate membrane (110 x 60 mm), (t) strip of cellulose acetate on which first-dimensional electrophoresis was carried out, (u) pad of glass fiber (59 x 5 mm) moistened with anode electrolyte, (v) pad of glass fiber moistened with cathode electrolyte, (w) cooling bed, and (x) water circulation (4°C). Four sets of apparatus B matches to one set of apparatus A.

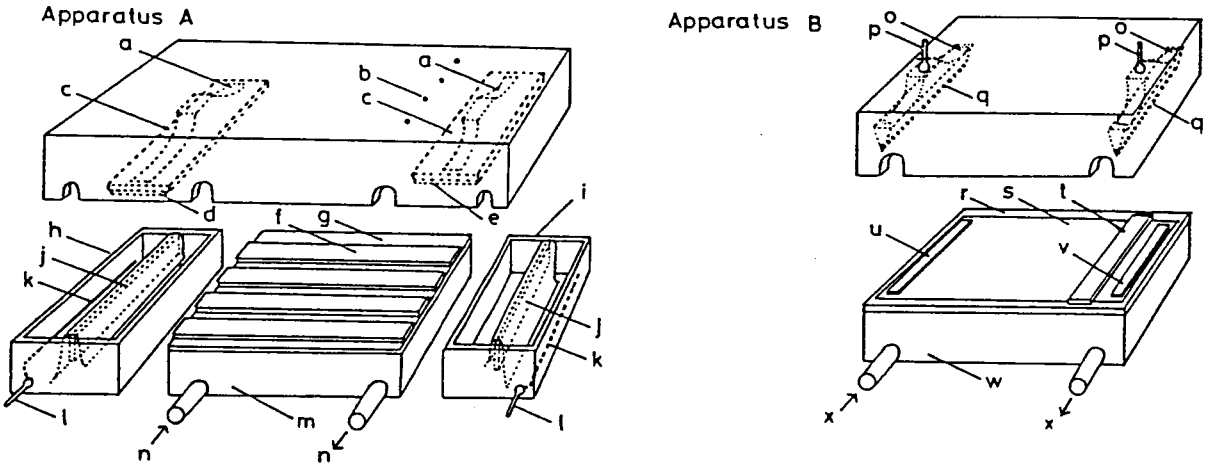


Fig. 2. Block diagram of the hardware system of two-dimensional densitometer. CPU, central processing unit; PIO, parallel interface; DIG, digitizer; FM, frame memory; DM, display memory; D/A, digital-to-analog converter; LOG, logarithmic transformer; A/D, analog-to-digital converter; AMP, video amplifier; CRT, cathode-ray tube; CCD, charge-coupled device.

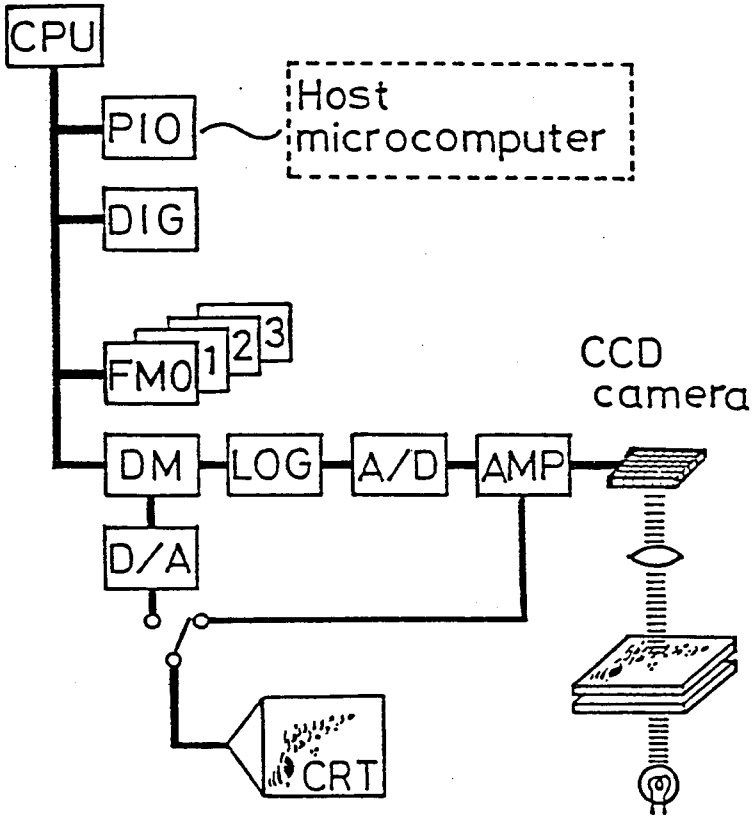


Fig. 3. Optical system of the CCD camera unit for data acquisition. I_0^S and I_0^B, intensities of the light source; $I_1^S(\underline{x},\underline{y})$ and $I_1^B(\underline{x},\underline{y})$, intensities of the incident light upon a sample and a blank membrane at position $(\underline{x},\underline{y})$; $I_2^S(\underline{x},\underline{y})$ and $I_2^B(\underline{x},\underline{y})$, intensities of the light scattered from the sample and the blank membrane at position $(\underline{x},\underline{y})$; $I_3^S(\underline{x},\underline{y})$ and $I_3^B(\underline{x},\underline{y})$, intensities of the incident light upon a surface of the CCD image sensor.

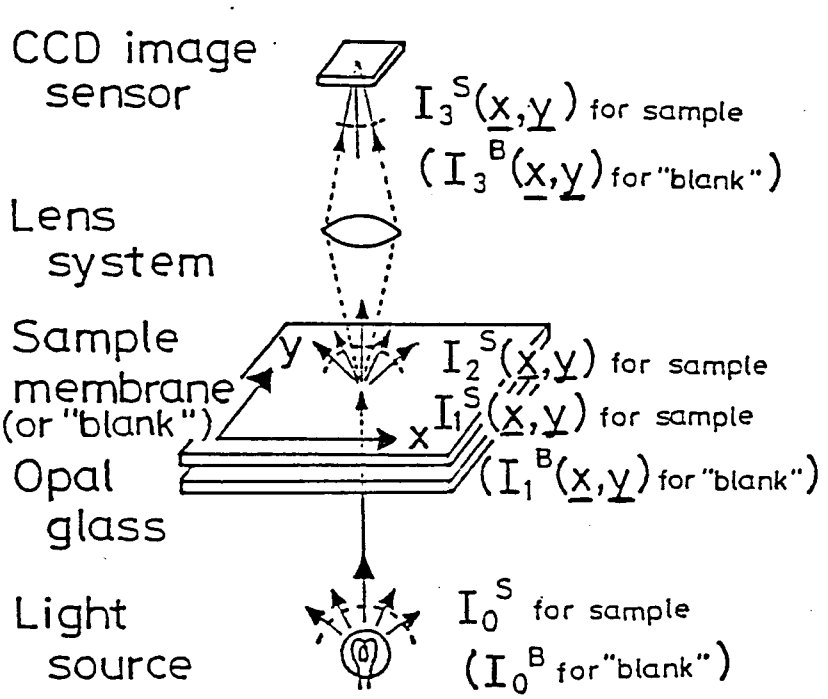


Fig. 4. Phosphocellulose (P-11) column chromatography. The phosphofructokinase activity in the heat extract (step 3) was applied to the phosphocellulose column (Whatman P-11, i.d. 100 mm x 255 mm) equilibrated with buffer B (50 mM Tris/acetate, pH 8.0, 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 0.1 mM PMSF). More than 90% of applied protein was passed through the column. About 80 per cent of applied phosphofructokinase activity was eluted with buffer C (75 mM Tris/phosphate, pH 8.0, 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, 3 mM ATP, 1 mM AMP, 25 μM fructose 2,6-bisphosphate, 0.1 mM PMSF). The peak fractions indicated with a bar were pooled.

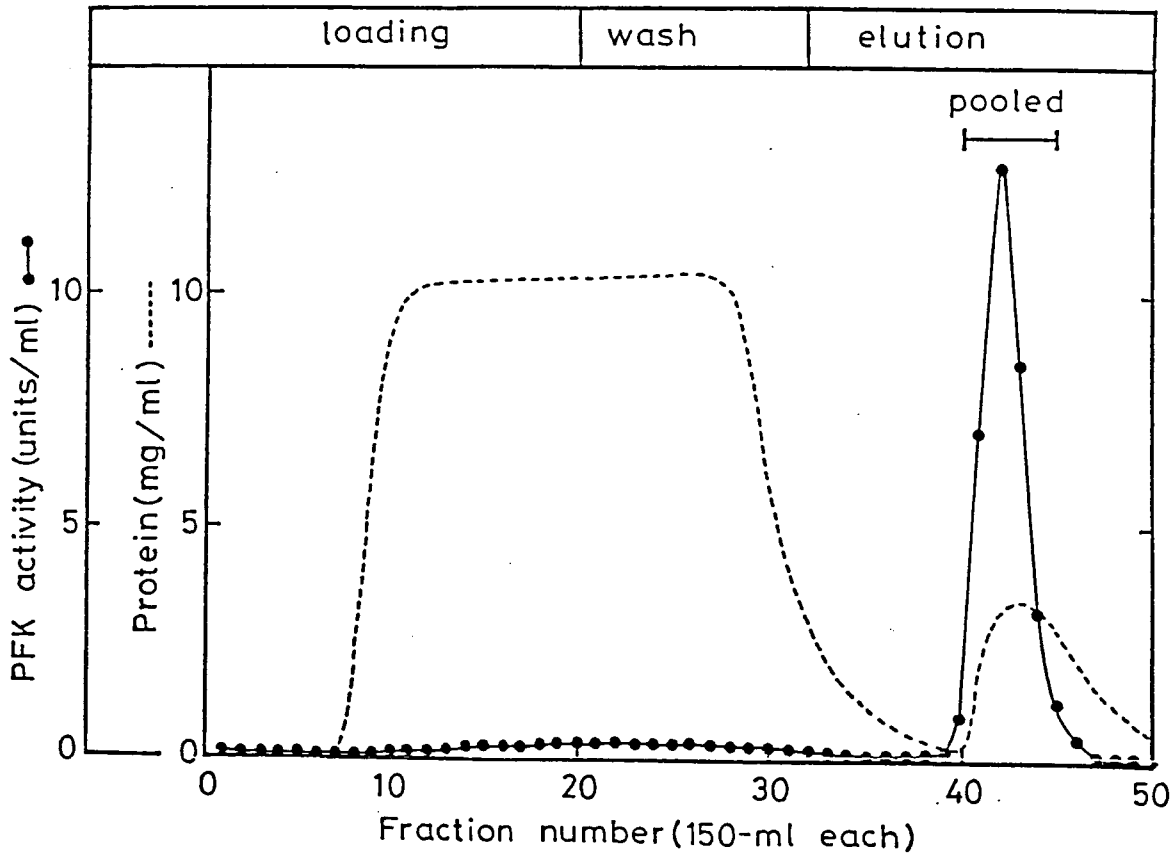


Fig. 5. DEAE-Sephacel column chromatography. The phosphofructokinase activity eluted from phosphocellulose column was adsorbed on DEAE-Sephacel (i.d. 25 mm x 200 mm) equilibrated with buffer C. About 90% of protein passed through the column. After the column was washed with buffer D1 (0.1 M Tris/phosphate, pH 8.0, 50 mM NaF, 10%(w/v) sucrose, 1mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 1 mM AMP, 2 μM fructose 2,6-bisphosphate, 0.1 mM PMSF), phosphofructokinase was eluted with the buffer of a linear salt gradient from buffer D1 to buffer D2 (1M Tris/phosphate, pH 8.0, 0.1 M ammonium sulfate, 50 mM NaF, 1 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, 1 mM AMP, 2 μM fructose 2,6-bisphosphate, 0.1 mM PMSF). About 65% of the activity was recovered in a major peak at 0.3 M of Tris/phophate. The peak fractions indicated with a bar were pooled.

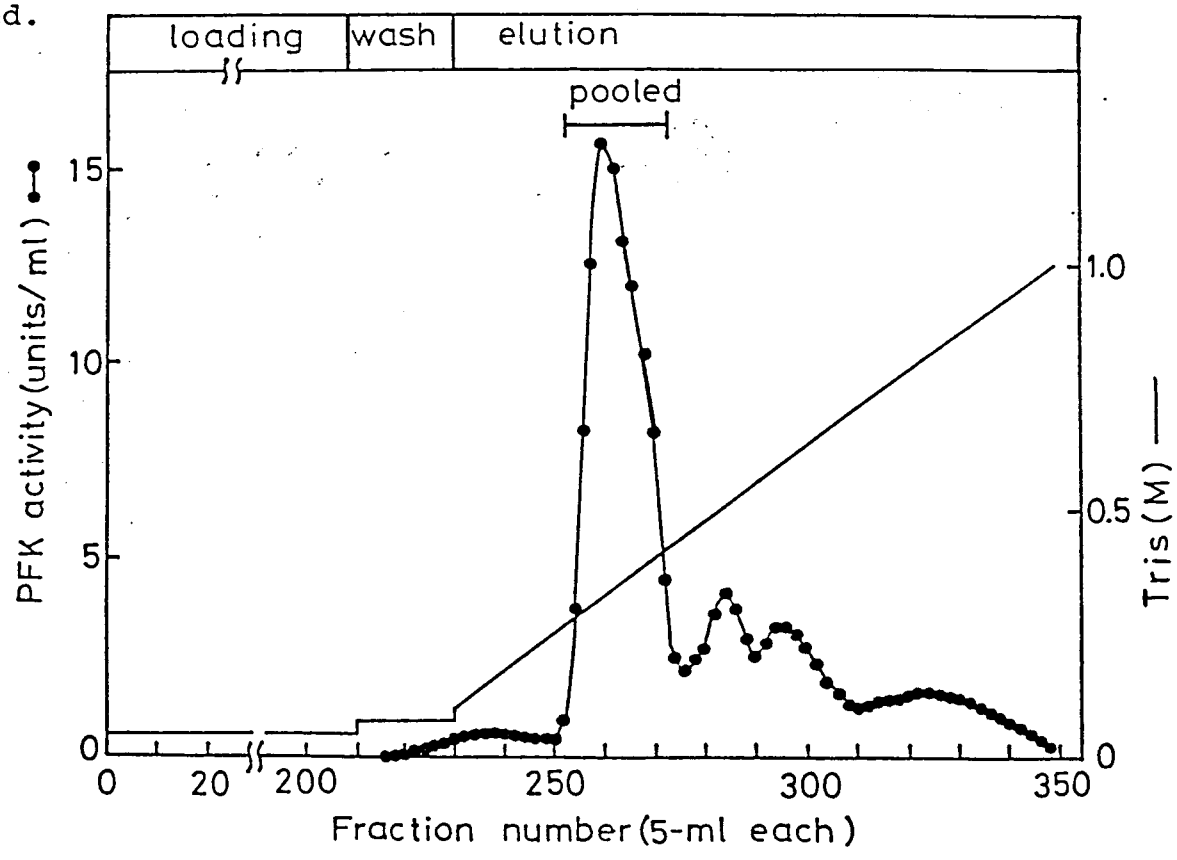


Fig. 6. Affi-Gel Blue column chromatography. The pooled Phosphofructokinase activity from DEAE-Sephacel column was dialyzed against buffer E (25 mM Tris/phosphate, pH 8.0, 40 mM ammonium sulfate, 10 mM NaF, 0.1 mM EDTA, 4 mM dithiothreitol, 0.05 mM ATP, 0.1 mM AMP, 50 μM fructose 2,6-bisphosphate), and applied to an Affi-Gel Blue column equilibrated with the same buffer. After washing the column with buffer E, phosphofructokinase activity was eluted with buffer F (25 mM Tris/phosphate, pH 8.0, 40 mM ammonium sulfate, 10 mM NaF, 0.1 mM EDTA, 4 mM dithiothreitol, 3 mM ATP, 1mM AMP, 50 μM fructose 2,6-bisphosphate). The peak fractions indicated with a ber were pooled.

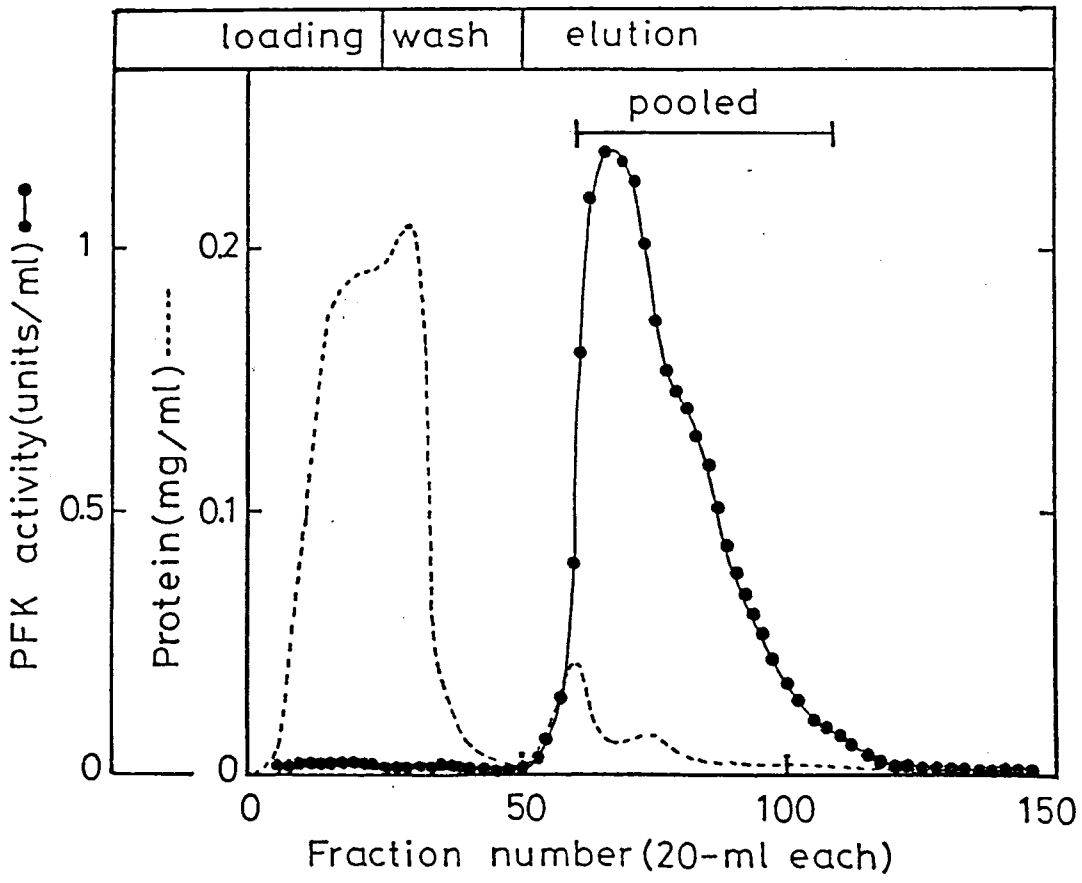


Fig. 7. Gel permeation chromatography on Cellulofine column. The phosphofructokinase preparation purified by dye-affinity chromatography on an Affi-Gel Blue column was applied to Cellulofine GCL-2000-sf column equilibrated with buffer C supplemented with 100 mM ammonium sulfate. Phosphofructokinase activity was recovered in a single peak just behind the void volume. Peak positions of marker proteins, which were chromatographed separately, are indicated with allows. The peak fractions indicated with a bar were pooled as the final preparation of purified phosphofructokinase. The apparent molecular weight (Mr = 1,300,000) corresponds to the hexadecamer of the subunit which shows Mr = 80,000 on SDS-polyacrylamide gel.

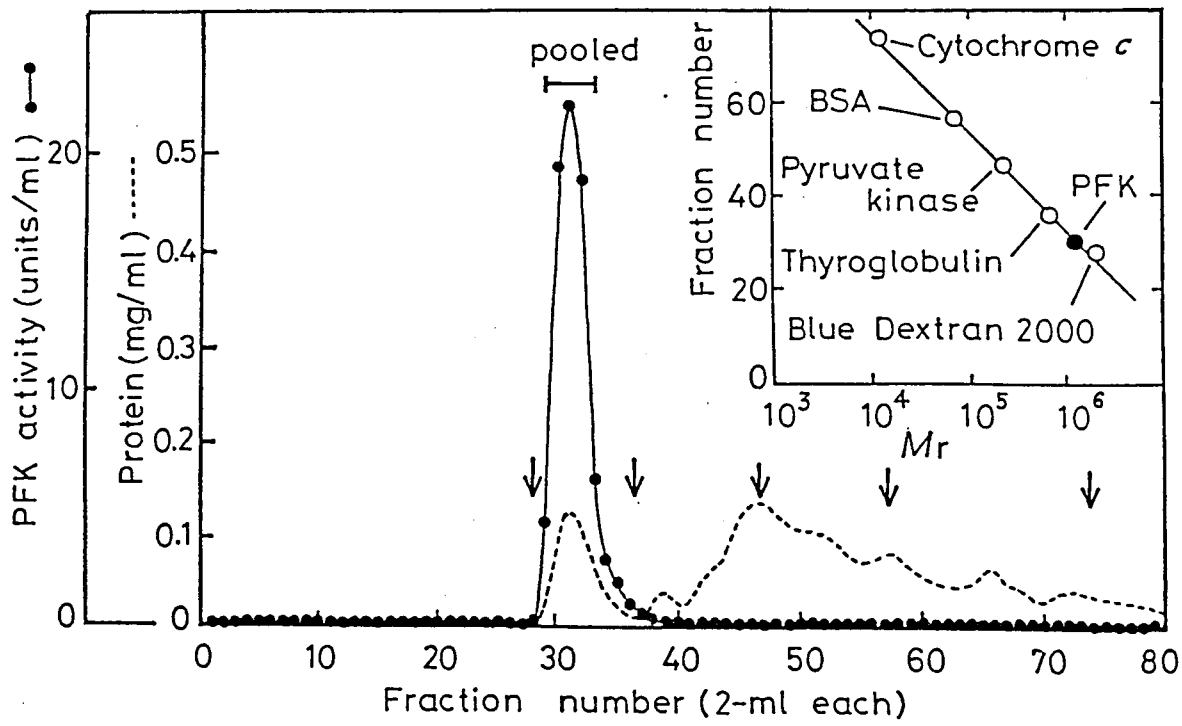


Fig. 8. SDS-polyacrylamide gel electrophorsis. The phosphofructokinase preparations at the heat treatment step (lane b), at the DEAE-Sephacel step (lane c) and at the final step (lane d) were subjected to electrophoresis on SDS-polyacrylamide gel. Protein bands were visualyzed by the silver staining method of Poehling and Neuhoff (38). Monomer, dimer and trimer of BSA were on lane a as molecular weight markers.

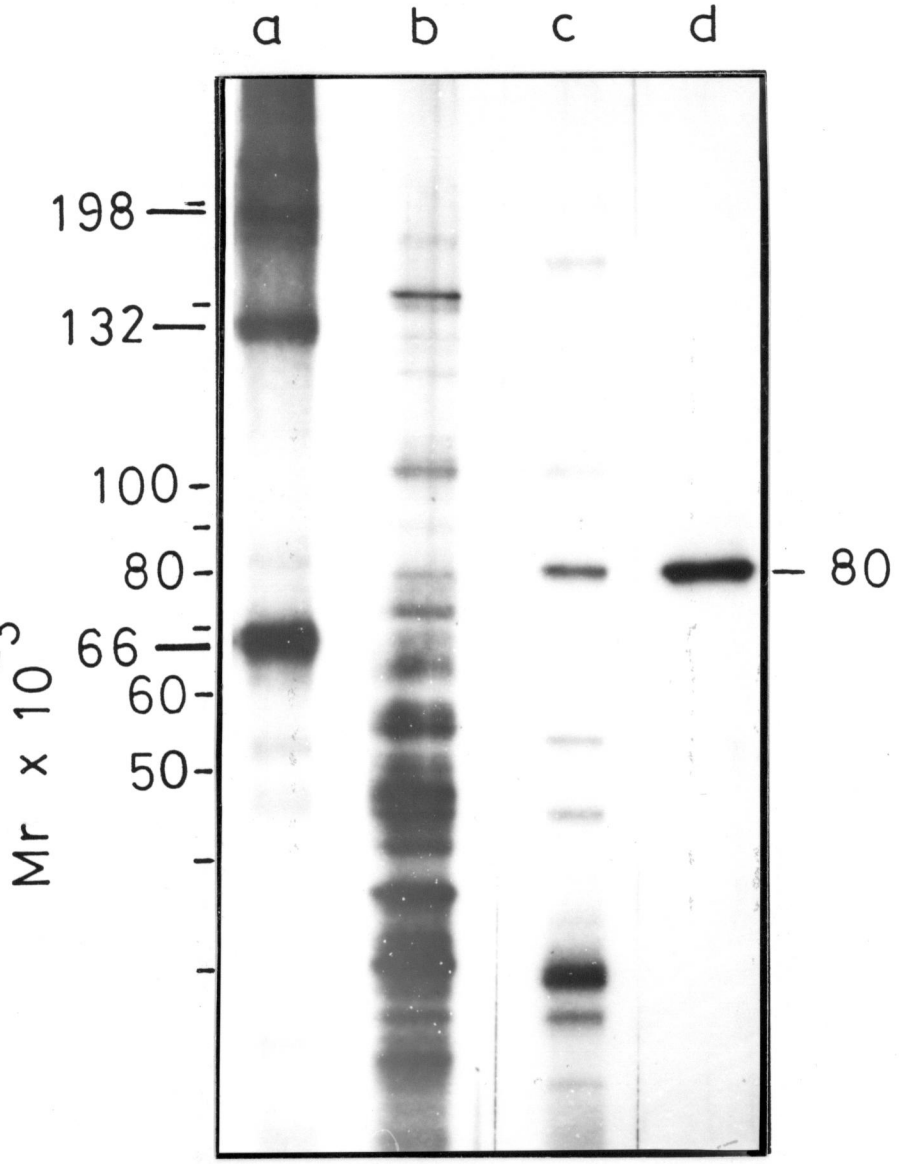


Fig. 9. Induction of anti-phosphofructokinase antibody on a rabbit. The rabbit was immunized primarily with 0.1 mg of phosphofructokinase, and boosted twice with 0.2 mg and 0.35 mg of the enzyme as described in MATERIALS AND METHODS. Titer of the anti-serum was determined by ELISA. From day 70 to day 84 after the primary immunization, anti-serum was harvested and pooled. The inserted figure shows the specificity of the pooled anti-serum. Indicated volumes of the anti-serum were mixed with 1-unit each of liver-type and muscle-type isozymes. IgG-bound enzyme was precipitated with Pasorbin, and the remained activity in the supernatant was assayed.

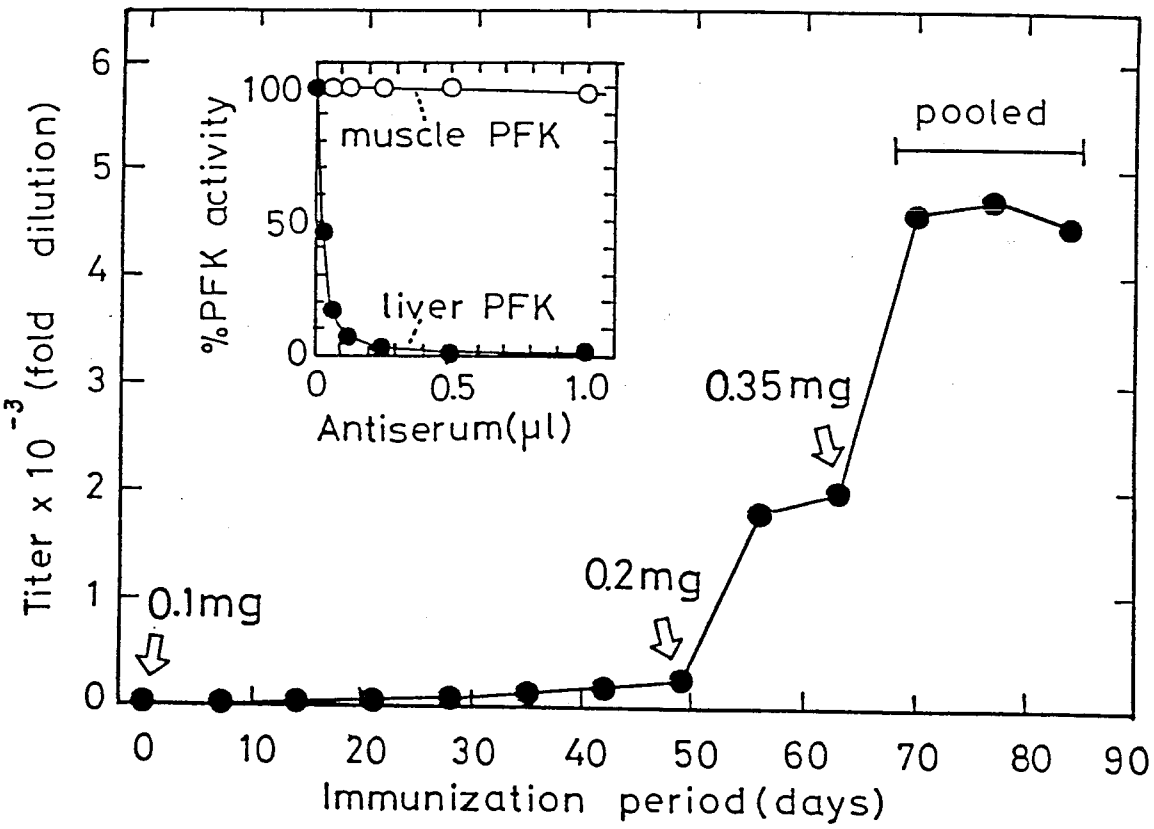


Fig. 10. Immounoaffinity chromatography of intermediate catabolic products of phosphofructokinase on an anti-phosphofructokinase IgG-conjugated Affi-Gel 10 column. Extract from 100 g of pig liver was directly applied to an anti-phosphofructokinase IgG-conjugated Affi-Gel 10 column (i.d. 10 mm x 100 mm). Nonspecific proteins of low affinity to the ligand were washed out with buffer J (0.1 M Tris-HCl, pH 8.0, 50 mM NaF, 5 mM EDTA, 0.5 mM PMSF, 5 μg/ml antipain, 5 μg/ml leupeptin, 5 μg/ml pepstatin) and buffer H (10 mM Tris-HCl, pH 7.5, 1.5 M NaCl, 0.05%(v/v) Tween 20). Specific proteins of high affinity to the anti-phosphofructokinase IgG were eluted with buffer I (0.1 M glycine-HCl, pH 3.0). Protein concentration was estimated from the absorbance at 280 nm, assuming that 1 O.D. corresponded to 1 mg/ml.

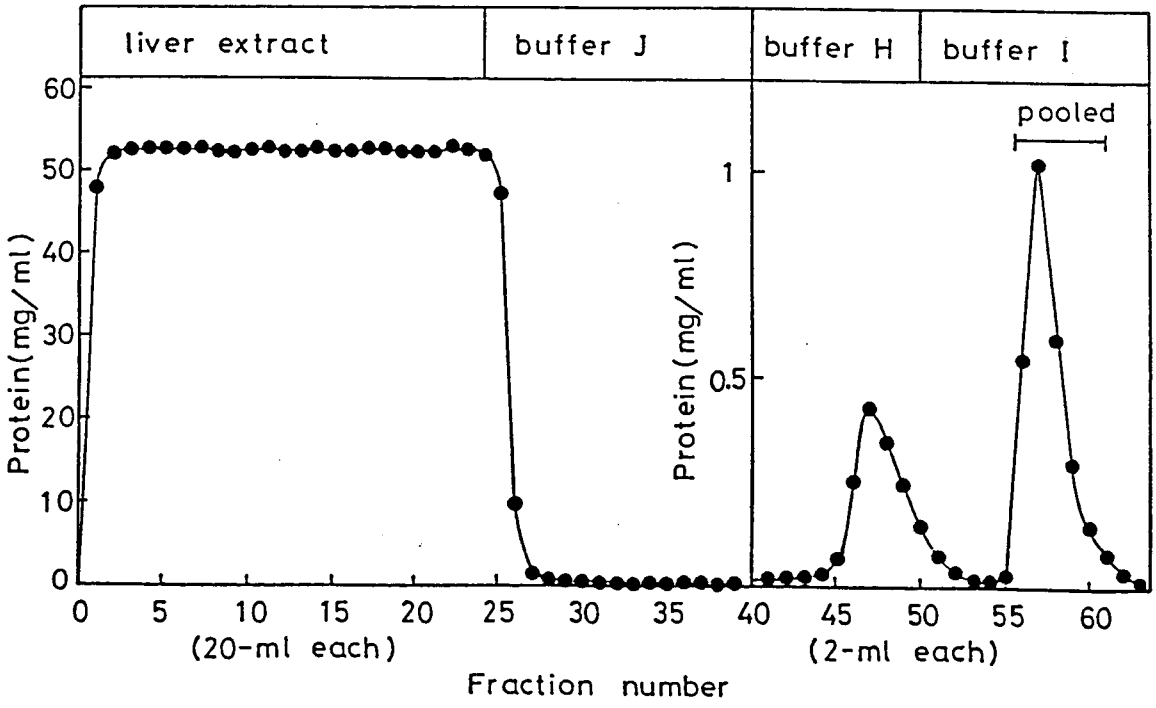


Fig. 11. ISOTACHO/DALT two-dimensional polyacrylamide gel electrophoresis. Thirty microliters of 40% (w/v) Ampholine, pH 3.5-9.5, and 100-µl aliquot of sample-application medium containing 1 mg of immunoaffinity-purified polypeptides were mixed, and applied to the preparative two-dimensional electrophoresis. In the first dimensional direction, isotachophoresis was carried out on polyacrylamide gel containing urea and NP-40. In the second dimensional direction, SDS-polyacrylamide gel electrophoresis was performed. Protein spots on the gel slab were visualized by staining with Coomassie Brilliant Blue G-250.

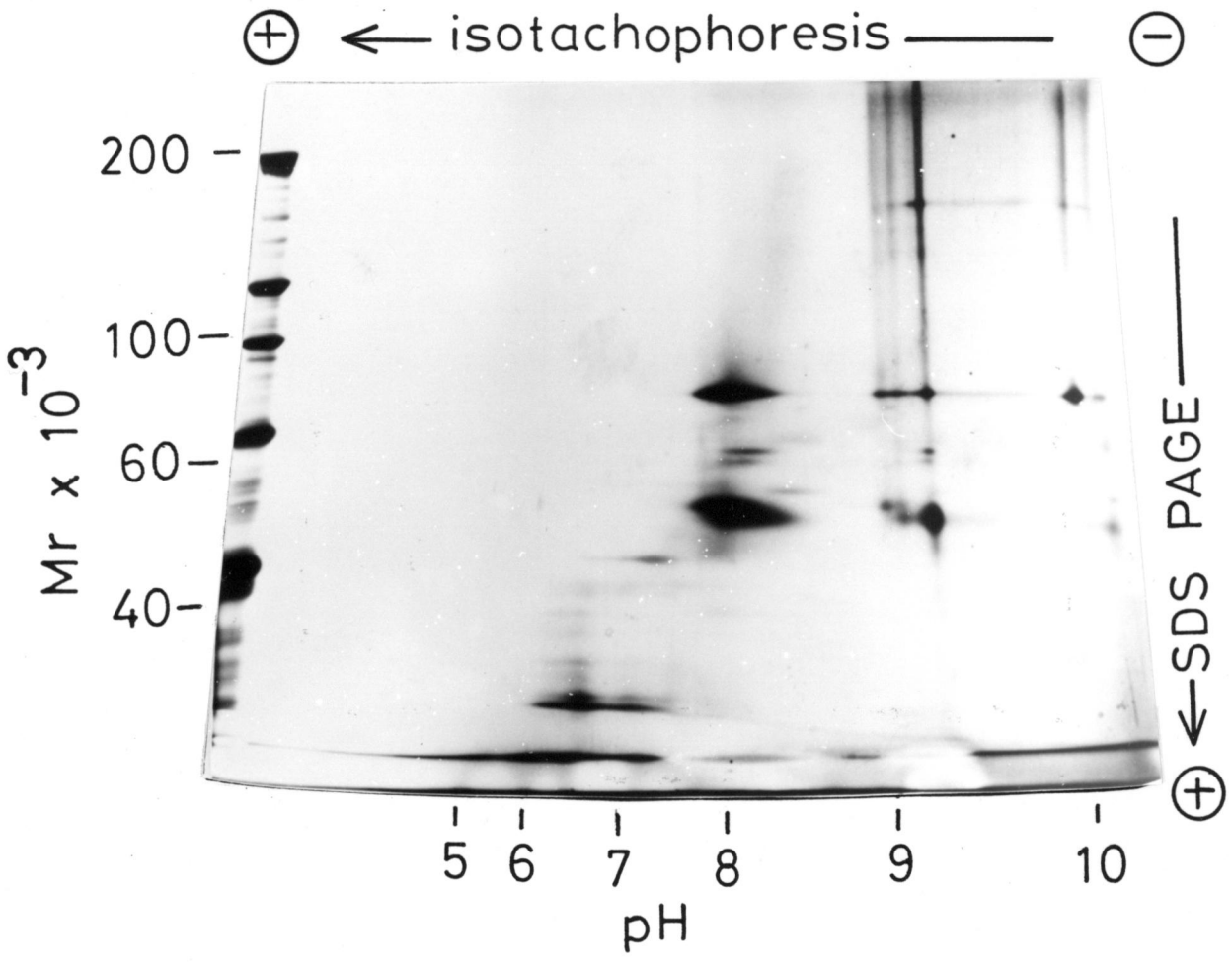


Fig. 12. Image processing of two-dimensional electrophoretogram. I. Silhouetting (binarization). Optical intensities at pixels (picture elements) were acquired with a CCD-TV camera, and stored in a display memory (DM). Unevenness of the light source was cancelled by 'blank subtraction' method. Pixel data within a 'window' area (ca. Mr = 100,000-45,000, pH 6.5-8.8) were transferred to the main memory of the host microcomputer (SORD M223 mark V). Pixels of higher optical levels than a given thresold value were brighten on the CRT screen. The silhouetted image shows the distribution of major isles in the 'window' area.

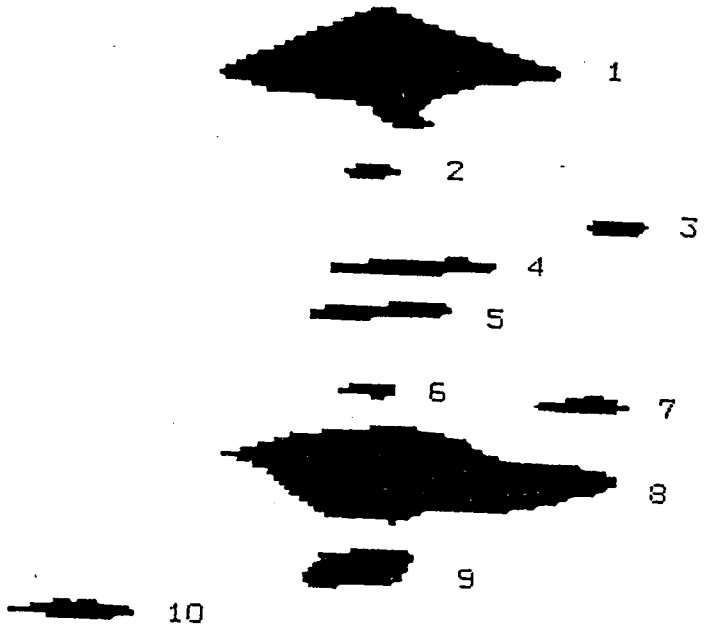


Fig. 13. Image processing of two-dimensional electrophoretogram. II. Contour mapping. Pixel data in the main memory of SORD M223 microcomputer were processed through the subroutine program "CONTOUR" written in the machine language of Z-80 CPU. In brief, the contour lines were obtained as follows; 1. The isles were silhouetted at various contour levels. 2. Boundaries of the silhouettes were drawn as controur lines. Thus obtained contour lines show the distribution of peaks on the isles.

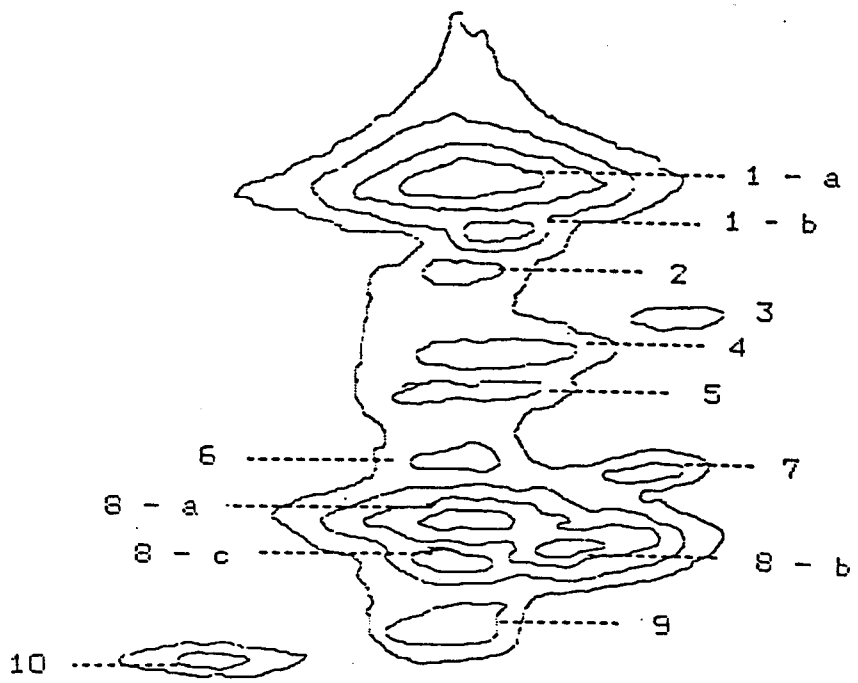


Fig. 14. Immunochemical detection of phosphofructokinase and its catabolic products in polyacrylamide gel disks. The small gel disks were punched out from the peak areas of the Coomassie-stained gel. Polypeptides were transferred from the gel disks onto nitrocellulose membrane electrophoretically. The membrane was treated with liquid gelatin, incubated with rabbit anti-phosphofructokinase IgG (first antibody), and then with HRPO-linked goat anti-rabbit-IgG antibody (second antibody). Peroxidase activity was detected by the reaction with 3,3'-diaminobenzidine.

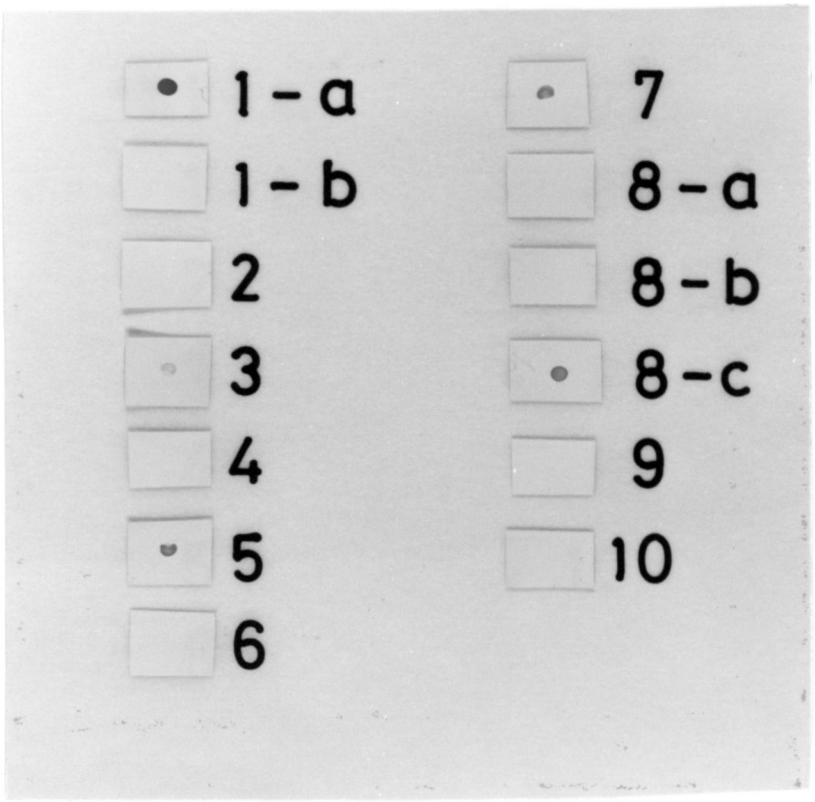


Fig. 15. SDS-polyacrylamide gel electrophoresis of radioiodinated polypeptides. Phosphofructokinase purified by multi-step column-chromatographic method, intact phosphofructokinase and its catabolic products purified by immunoaffinity chromatography, and two-dimensional electrophoresis were radioiodinated in the polyacrylamide gel disks by a chloramine-T method modified by Elder et al. (43). The radioiodinated polypeptides were solubilized with SDS-treating solution and subjected to SDS-polyacrylamide gel electrophoresis. After the electrophoresis, each lane was sliced into 2-mm fractions, and radioactivity was measured with a gamma counter. Polypeptides loaded were; peak 1-a (Mr = 84,000) in A, purified phosphofructokinase (Mr = 80,000) in B, isle 3 (Mr = 68,000) in C, isle 5 (Mr = 64,000) in D, isle 7 (Mr = 56,000) in E and peak 8-c (Mr = 51,000) in F.

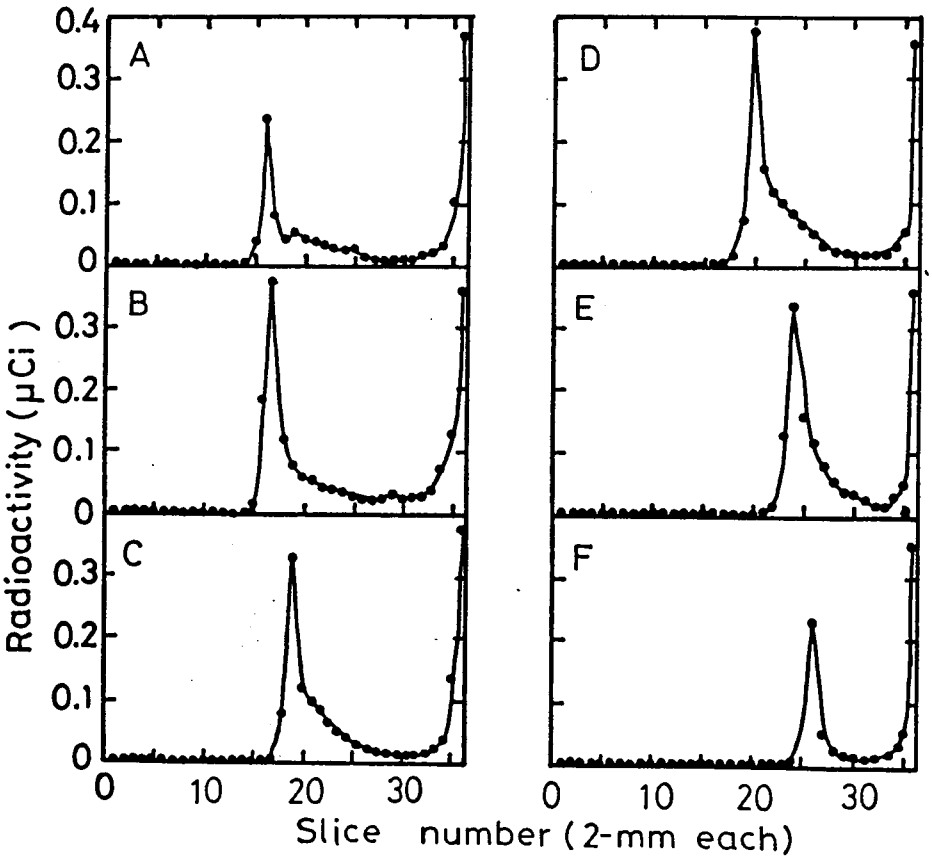
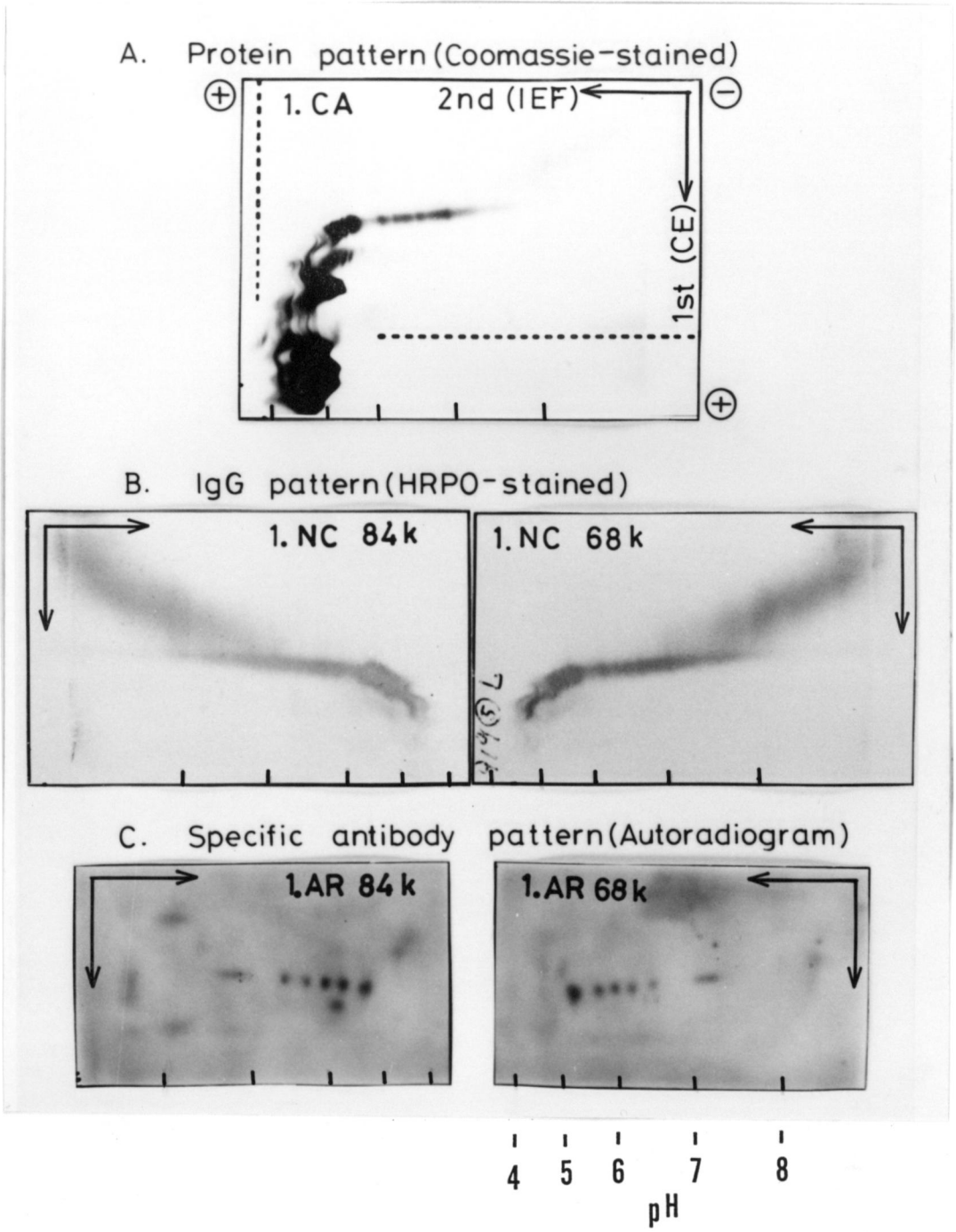
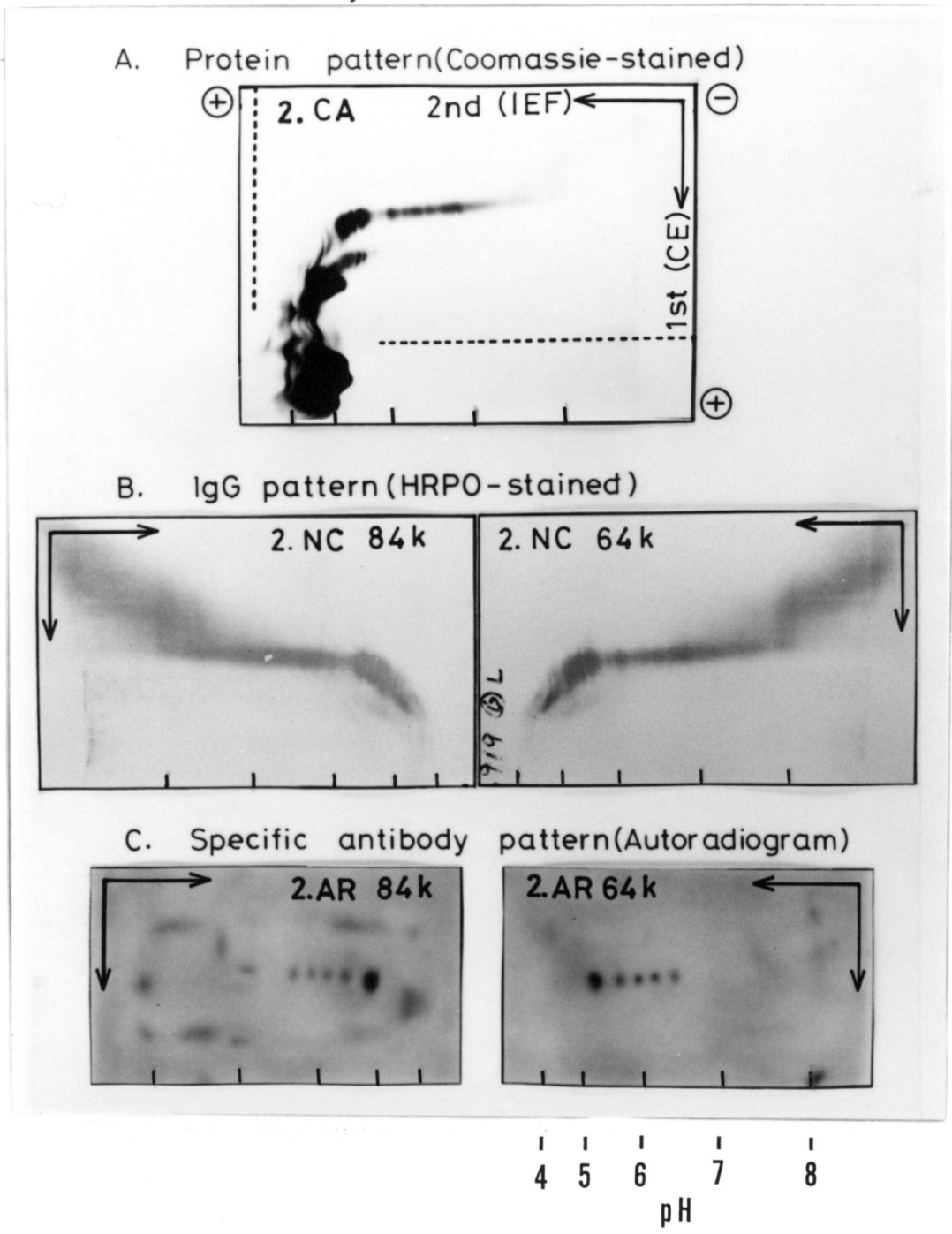


Fig. 16. Two-dimensional cellulose acetate electrophoresis of anti-phosphofructokinase IgG. Proteins in 10 ul aliquots of anti-serum were two-dimensionally separated on two layers of cellulose acetate membrane. One layer of the pair was stained with Coomassie Brilliant Blue G-250 to visualize protein patterns. Proteins on the other layer were transferred onto two sheets of nitrocellulose membrane by 'sandwich' blot method. After nonspecific binding sites were blocked with liquid gelatin, one sheet of the nitrocellulose membrane was incubated with radioiodinated Mr = 84,000 polypeptide, and the other sheet was with radioiodinated polypeptide of Mr = 68,000 in I, Mr = 64,000 in II, Mr = 56,000 in III and Mr = 51,000 in IV, respectively. Distribution of specific antibodies with binding activity to the radioiodinated polypeptides was visualized by autoradiography. Kodak X-Omat film with Cronex Intensifying Screen was exposed with the nitrocellulose membrane for 15 days at -70°C. After the use for autoradiography, the nitrocellulose membrane was incubated with HRPO-linked goat anti-rabbit IgG antibody, and the distribution of whole IgG was detected by peroxidase reaction with 3,3'-diaminobenzidine.

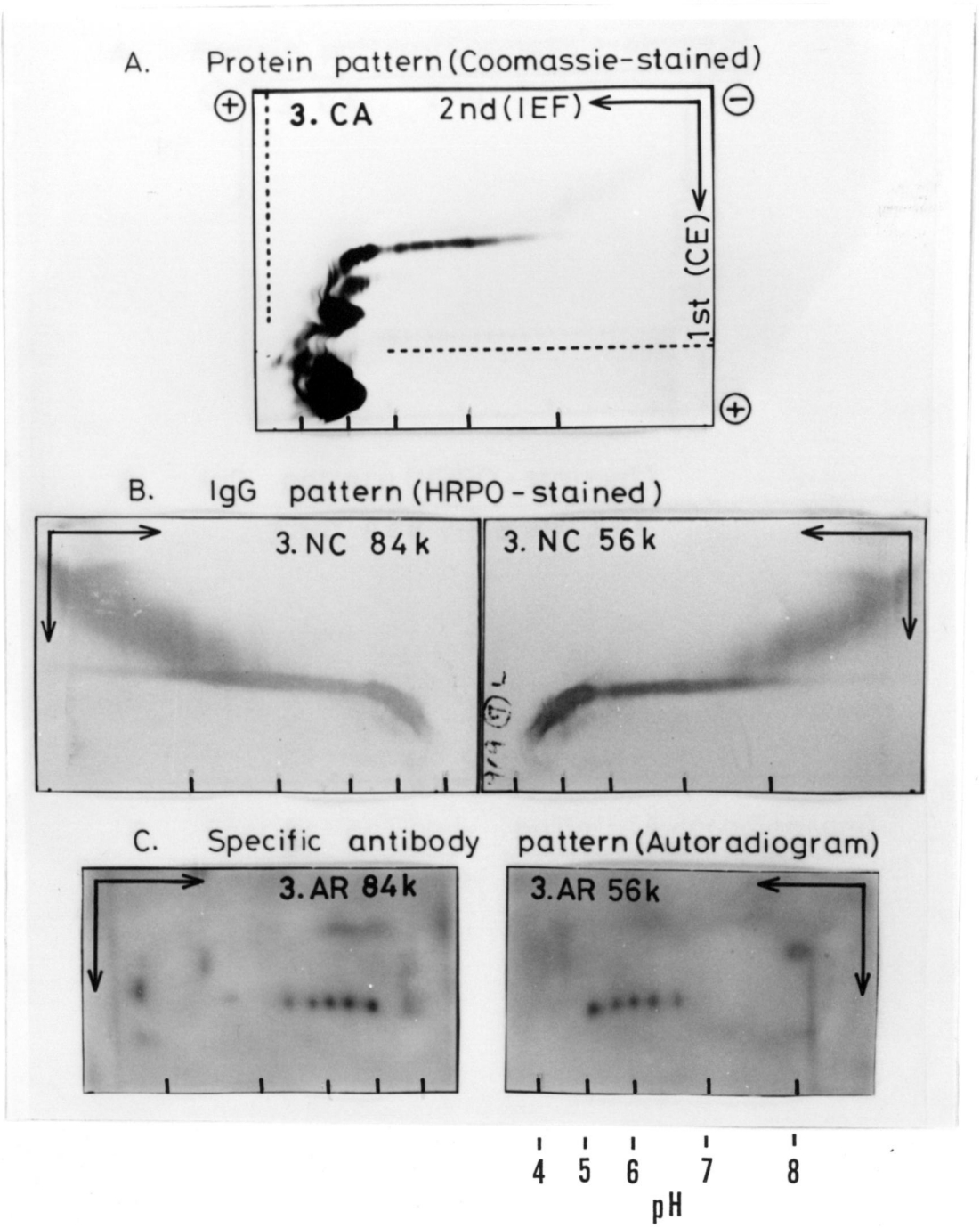
I


A. Protein pattern (Coomassie-stained)
1. CA
2nd (IEF)
1st (CE)
B. IgG pattern (HRPO-stained)
1. NC 84k
1. NC 68k
C. Specific antibody pattern (Autoradiogram)
1. AR 84k
1. AR 68k
4
5
6
7
8
pH

II


A. Protein pattern(Coomassie-stained)
2. CA
2nd (IEF)
1st (CE)
B. IgG pattern (HRPO-stained)
2. NC 84k
2. NC 64k
C. Specific antibody pattern(Autoradiogram)
2.AR 84k
2.AR 64k
4 5 6 7 8
pH

III


A. Protein pattern (Coomassie-stained)
3. CA
2nd (IEF)
1st (CE)
B. IgG pattern (HRPO-stained)
3. NC 84k
3. NC 56k
C. Specific antibody pattern (Autoradiogram)
3. AR 84k
3. AR 56k
4 5 6 7 8
pH

IV

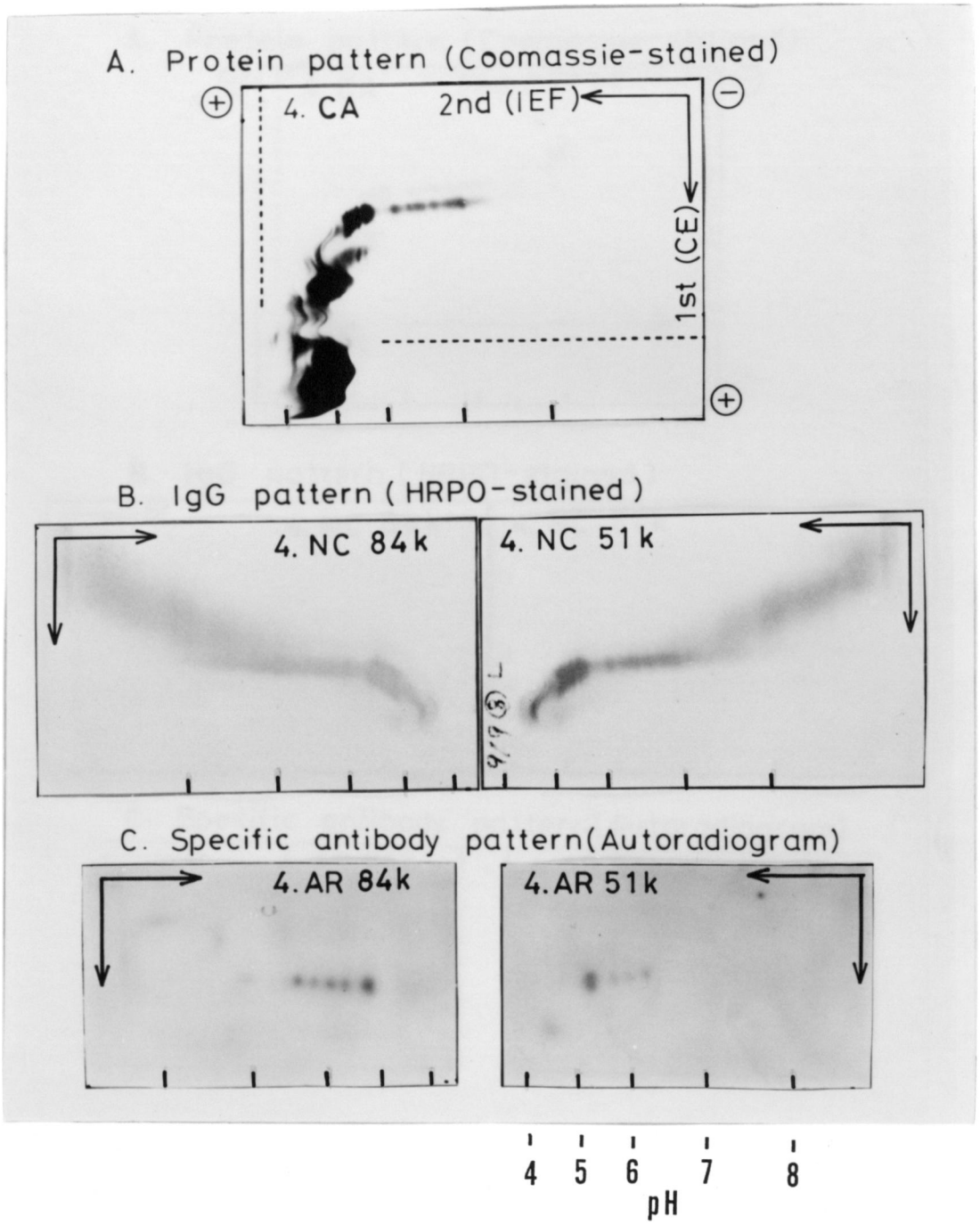

A. Protein pattern (Coomassie-stained)
4. CA
2nd (IEF)
1st (CE)
B. IgG pattern (HRPO-stained)
4. NC 84k
4. NC 51k
C. Specific antibody pattern(Autoradiogram)
4. AR 84k
4. AR 51k
4
5
6
7
8
pH

Fig. 17. SDS-polyacrylamide gel electrophoresis of proteinase-V8 digests of radioiodinated phosphofructokinase and its catabolic products. Purifed phosphofructokinase, intact phosphofructokinase and its cross-reactive polypeptides were radioiodinated and digested with Proteinase V8. The digests were separated by SDS-polyacrylamide gel electrophoresis, and detected by autoradiography. Lane a, Mr = 84,000; lane b, Mr = 80,000; lane c, Mr = 68,000; lane d, Mr = 64,000; lane e, Mr = 56,000; lane f, Mr = 51,000.

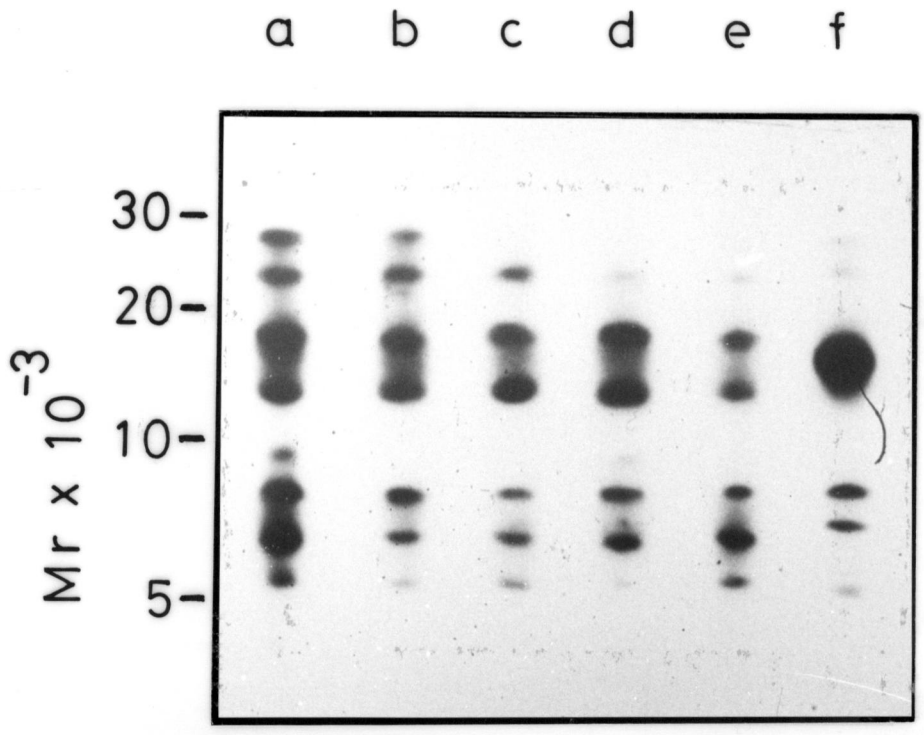


Fig. 18. SDS-polyacrylamide gel electrophoresis of intact phosphofructokinase and its *in vivo* catabolic products prepared under the coexistence of internal tracers. Radioiodinated polypeptides of Mr = 84,000, Mr = 68,000 and Mr = 64,000 were respectively accompanied with intact phosphofructokinase and its catabolic products in the whole procedure from homogenization to immunoaffinity chromatography. polypeptides recovered after the immunoaffinity chromatography were separated by SDS-polyacrylamide gel electrophoresis (5 µg protein per lane in A, 0.1 µg protein per lane in B). A. Cross-reactive polypeptides were detected by HRPO-linked immunoelectrotransfer blot method. B. Radioactive polypeptides were detected by autoradiography. Accompanied tracers were Mr = 84,000 in lane a, Mr = 68,000 in lane b and Mr = 64,000 in lane c respectively.