

## Purification and Characterization of $\gamma$ -Enolase from Various Mammals

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The gamma subunit of enolase ( $\gamma$ -enolase) was purified from the brain tissues of cow, dog, goat, pig, rabbit, and rat. The purification was achieved in only three steps: ammonium sulfate-precipitation, DE 53 cellulose ion-exchange chromatography, and polyacrylamide gel electrophoresis (PAGE) in a preparative mode. The purification procedure was comparatively more simple than previously reported methods, and the yield of  $\gamma$ -enolase was sufficient for subsequent structural and immunological analyses. In all mammals, the purified  $\gamma$ -enolase migrated in sodium dodecyl sulfate-PAGE (SDS-PAGE) with a molecular mass of 46 kilodaltons (kDa), and the immunological cross-reactivity between those  $\gamma$ -enolases was very strong. The structural homology of these  $\gamma$ -enolases was examined by peptide mapping using cyanogen bromide cleavage and subsequent two-dimensional electrophoresis. The resulting peptide patterns were highly similar and in cow, dog, and goat, the patterns were almost identical. These results indicate that structural homology, that is, the species non-specificity of  $\gamma$ -enolase, appears to be very high.

**Keywords** neuron-specific enolase; protein purification; peptide mapping; cyanogen bromide cleavage; two-dimensional electrophoresis; species non-specificity

Enolase (2-phospho-D-glycerate hydrolyase, EC 4.2.1.11), which is a glycolytic enzyme, catalyzes the interconversion of 2-phosphoglycerate to phosphoenolpyruvate. Enolase is present in the cells of all organs as a dimeric isoenzyme, composed of two out of three homologous but distinct subunits ( $\alpha$ ,  $\beta$  and  $\gamma$ ).<sup>1,2)</sup> Nucleotide sequence analyses of enolase-encoding complementary deoxyribonucleic acids (cDNAs) isolated from various mammals indicate that the enolase subunits are encoded by three distinct but closely related genes.<sup>3-11)</sup> In the five active forms ( $\alpha\alpha$ ,  $\beta\beta$ ,  $\gamma\gamma$ ,  $\alpha\beta$ , and  $\alpha\gamma$ ), the  $\gamma$  subunit-containing enolases ( $\alpha\gamma$  and  $\gamma\gamma$  forms) are found mainly in nervous tissue and neuroendocrine cells; therefore, these two forms are called neuron-specific enolase (NSE).<sup>12)</sup>

Although the tissue specificity of enolase isoforms is of genetic and biochemical interest, the molecular mechanisms of its organ-specific expression have not yet been clarified. NSE is, furthermore, of medical interest as a tumor marker. In patients with tumors of neuroendocrine cells, especially neuroblastoma<sup>13,14)</sup> and small-cell carcinoma of the lung,<sup>15-17)</sup> serum NSE concentrations are significantly elevated compared with healthy controls, and a high correlation is found between serum NSE level and patient response to therapy or the clinical course of the disease.<sup>15)</sup>

Comparative studies of NSEs isolated from various mammals revealed immunological cross-reactivity between many species.<sup>18)</sup> Moreover, cDNA and direct amino acid sequence analyses of NSE  $\gamma$  subunits of human,<sup>5-7,10)</sup> rat,<sup>3,4,9)</sup> and mouse<sup>8,11)</sup> showed a high degree of homology. For other mammals, however, no detailed information is available. The present report describes a simple purification procedure for the  $\gamma$  subunit of enolase ( $\gamma$ -enolase) from various mammals and the structural properties of this subunit.

### Materials and Methods

**Materials, Chemicals and Enzymes** Brain tissues obtained from various mammals (cow, 190 g; dog, 130 g; goat, 170 g; pig, 100 g; rabbit, 93 g; rat, 204 g) were stored at  $-80^{\circ}\text{C}$  until use. DE 53 cellulose was purchased from Whatman Ltd. Adenosine diphosphate (ADP), Coomassie Brilliant Blue (CBB) R-250, and 3,3'-diaminobenzidine tetrahydrochloride (DAB) were obtained from Sigma Chemical Co. Lactate dehydrogenase, nic-

otinamide adenine dinucleotide reduced form (NADH), 2-phosphoglycerate, and pyruvate kinase were obtained from Boehringer Mannheim. All the other chemicals were purchased from Wako Pure Chemical Industries Ltd. and Katayama Chemical Industries Co.

**Purification of the  $\gamma$ -Enolase** Enolase  $\gamma$  subunits were purified from the brain tissue of various mammals by the methods of Suzuki *et al.*,<sup>19)</sup> and Paus and Risberg<sup>20)</sup> with modifications. Brain tissue was homogenized in buffer A: 15 mM Tris-HCl buffer containing 5 mM  $\text{MgSO}_4$  and 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.9, and centrifuged at  $27000 \times g$  for 20 min. The supernatant was then fractionated with solid ammonium sulfate. The materials that precipitated between 40 and 80% saturation were dissolved in buffer A and dialyzed against the same buffer. The insoluble materials were removed by centrifugation and the supernatant was then applied to a DE 53 cellulose column ( $2.5 \times 45$  cm), pre-equilibrated with buffer A. The column was sufficiently washed with the same buffer and eluted with a linear gradient of NaCl from 0 to 0.6 M in buffer A.

The fraction containing the  $\gamma\gamma$ -enolase was concentrated and further purified by the Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system<sup>21)</sup> in a preparative mode. The separated proteins in 11% polyacrylamide gels ( $10 \times 16$  cm, 1.5 mm-thick) were stained in a solution containing 0.5% CBB R-250, 50% methanol, and 10% acetic acid, for 5 min. Gels were destained in a solution containing 10% methanol and 5% acetic acid for 30 min and equilibrated in an electrode buffer: 25 mM Tris-HCl, 0.192 M glycine, 0.1% SDS, pH 8.3. The protein band corresponding to the  $\gamma$ -enolase was cut out from the gels and then cut into small pieces. Electroelution of the dye and protein from the gel pieces was carried out with a disc gel tube ( $1.3 \times 12$  cm). The gel pieces were fixed in the stacking gel (5% T, 4 cm high) and placed on the resolving gel (11% T, 2 cm high). After the line of CBB passed through the gel tube, a protein-trapping cap with a dialysis membrane was connected to the bottom of the tube and the  $\gamma$ -enolase was collected in the cap. This preparation was dialyzed and used for subsequent experiments.

**Enzyme Activity and Protein Concentration** Enolase activity was assayed spectrophotometrically at 340 nm by coupling the reaction with pyruvate kinase and lactate dehydrogenase as described by Rider and Taylor.<sup>1)</sup> The reaction was carried out at  $30^{\circ}\text{C}$  in 1.0 ml of assay buffer containing 67 mM KCl, 7.7 mM  $\text{MgSO}_4$ , 3.2 mM EDTA, 1.8 mM ADP, 0.48 mM NADH, 38 mM triethanolamine buffer (pH 7.5), 1.0 mM 2-phosphoglycerate, 2.25 units/ml pyruvate kinase, and 5.4 units/ml lactate dehydrogenase. One unit of enolase activity is defined as the amount of enzyme converting 1  $\mu\text{mol}$  of substrate per minute under the conditions described.

Protein concentration was measured with a Bio-Rad Protein Assay (Bio-Rad Laboratories), using bovine serum albumin as a standard.

**Gel Electrophoresis and Immunoblotting** Proteins were separated on 0.1% SDS-14% polyacrylamide gel according to Laemmli.<sup>21)</sup> Prestained SDS-PAGE standards (phosphorylase B, 110 kDa; bovine serum albumin, 84 kDa; ovalbumin, 47 kDa; carbonic anhydrase, 33 kDa; soybean trypsin inhibitor, 24 kDa; lysozyme, 16 kDa; Bio-Rad Laboratories) were used as

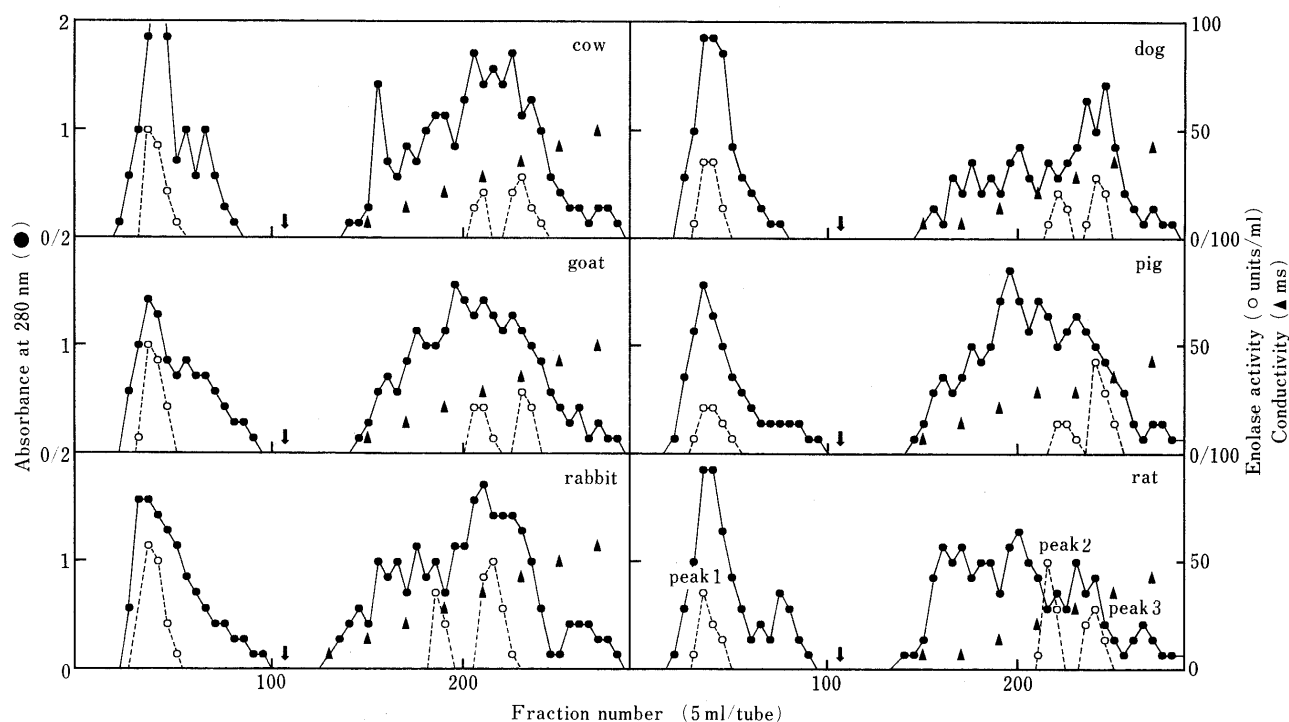


Fig. 1. DE 53 Cellulose Column Chromatographies of the 40–80% Ammonium-Precipitated Fractions

Brain tissues from various mammals were homogenized and fractionated with solid ammonium sulfate. The fractions which precipitated between 40 and 80% were applied to a DE 53 cellulose column (2.5 × 45 cm) which was equilibrated with 15 mM Tris-HCl buffer, pH 7.9, containing 5 mM MgSO<sub>4</sub> and 0.1 mM EDTA. The  $\gamma\gamma$ -enolases were eluted with a NaCl linear gradient from 0 to 0.6 M in the equilibration buffer. The arrows indicate the start of the gradient. Peaks 1, 2 and 3 correspond to the enolase activity of the  $\alpha\alpha$ ,  $\alpha\gamma$  and  $\gamma\gamma$  forms, respectively. ○, enolase activity; ▲, conductivity; ●, protein concentration monitored by absorbance at 280 nm.

molecular mass standards. The proteins separated in the polyacrylamide gel were stained with 0.25% CBB R-250 or electroblotted onto nitrocellulose membrane according to Towbin *et al.*<sup>22)</sup> The membrane was blocked with a 5% skim milk solution and incubated with rabbit antiserum to human  $\gamma\gamma$ -enolase diluted 1:1000 with TBS (50 mM Tris-HCl, 200 mM NaCl, pH 7.4) containing 3% bovine serum albumin. The bound antibody was visualized with horseradish peroxidase-labeled sheep antibody against rabbit immunoglobulin G and DAB as described by Hawkes *et al.*<sup>23)</sup>

**Cyanogen Bromide Cleavage** Purified  $\gamma$ -enolase was cleaved with cyanogen bromide by the method of Gross and Witkop.<sup>24)</sup> Thirty micrograms of the sample were dissolved in 100  $\mu$ l of 70% formic acid containing 25 mM cyanogen bromide. The reaction was continued for 24 h at room temperature, and then evaporated to dryness in a Speed Vac Concentrator (Savant Instruments). Distilled water was added to the residue and the solution was evaporated to dryness again. This procedure was repeated once more to remove the residual reagents completely.

**Two-Dimensional Electrophoresis** The peptide fragments, obtained by cyanogen bromide cleavage, were analyzed by two-dimensional electrophoresis as described by O'Farrell.<sup>25)</sup> Samples were dissolved in 10  $\mu$ l SDS-lysis buffer; 0.5% SDS, 9.5 M urea, 5% 2-mercaptoethanol, 2% Ampholine (pH 3.5–10, LKB Biotechnology). The first dimension (isoelectric focusing) gels were poured to a height of 6 cm in glass tubes (8 cm × 1.5 mm i.d.) and electrophoresis was performed at 400 V for 4 h. For the second dimension (SDS-PAGE) separation, 16% polyacrylamide gels were used.

**Antisera and Immunodiffusion Technique** Purified rat  $\gamma$ -enolase (50  $\mu$ g/ml), emulsified with an equal volume of Freund's complete adjuvant, was injected subcutaneously into young male rabbits. Five injections were given at 2-week intervals. Two weeks after the last injection, antisera were collected. Rabbit antiserum to human  $\gamma\gamma$ -enolase was provided by Eiken Chemical Co. Double immunodiffusion in an agar gel was performed according to the method of Ouchterlony.<sup>26)</sup>

## Results and Discussion

**Purification of the Rat  $\gamma$ -Enolase** The neuron-specific  $\gamma$ -enolase was first purified from rat brain tissue using a DE 53 cellulose ion-exchange column chromatography and preparative SDS-PAGE. The elution profile on a DE 53

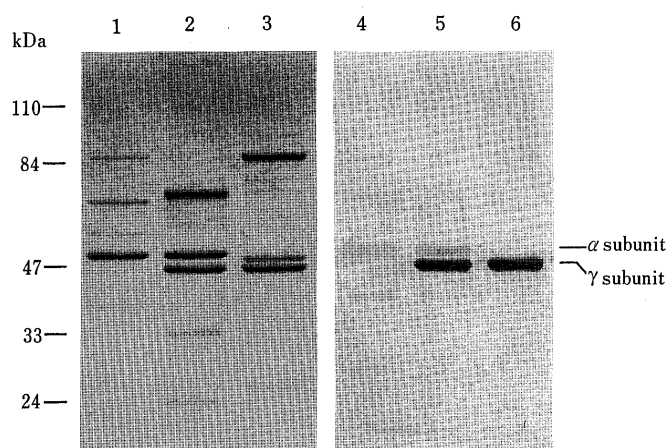


Fig. 2. Analysis of the Rat Enolase Isoforms by SDS-PAGE and Immunoblotting

The three fractions exhibiting the enolase activity, the peaks 1–3 in Fig. 1, were analyzed on a 0.1% SDS-14% polyacrylamide gel. The separated proteins were stained with CBB (lanes 1–3) or immunostained with antibody to the  $\gamma\gamma$ -enolase (lanes 4–6). Lanes 1 and 4, peak 1; 2 and 5, peak 2; 3 and 6, peak 3. Molecular mass markers are shown on the left.

column of the ammonium sulfate-precipitated fraction (40–80% saturation) from the homogenate is presented in Fig. 1. As described in the previous paper,<sup>20)</sup> the enolase activity was separated into three peaks (peaks 1 to 3 in Fig. 1) which were thought to correspond to the  $\alpha\alpha$ ,  $\alpha\gamma$  and  $\gamma\gamma$  forms of enolase, respectively.

To prove peak 3 to be the  $\gamma\gamma$  form, these three fractions exhibiting enolase activity were collected separately and analyzed by SDS-PAGE and subsequently by immunoblotting using the antibody to human  $\gamma\gamma$ -enolase. The fractions corresponding to peaks 1 and 2 each contained a 49 kDa

protein (Fig. 2, lanes 1 and 2), and a 46 kDa protein was found in the fractions corresponding to peaks 2 and 3 (lanes 2 and 3). Furthermore, the 46 kDa protein reacted strongly with the antibody against the  $\gamma\gamma$ -enolase (lanes 5 and 6), and a minor band was detected at the 49 kDa position (lanes 4 and 5). These results suggested that the 46 kDa protein is the  $\gamma$  subunit and the 49 kDa protein is probably the  $\alpha$  subunit.

Peak 3, containing the  $\gamma\gamma$  form of enolase, was further purified by SDS-PAGE in a preparative mode. The proteins contained in peak 3 were separated on an 11% polyacrylamide gel and the 46 kDa protein band ( $\gamma$ -enolase) was cut out from the gel. The purified  $\gamma$ -enolase (approximately 1.1 mg) was obtained by the electrophoretic elution described. The purity of this preparation was proven on a 14% polyacrylamide gel, and a single protein band with a molecular mass of 46 kDa was detected (Fig. 3, lane 1).

#### Purification of the $\gamma$ -Enolase from Various Mammals

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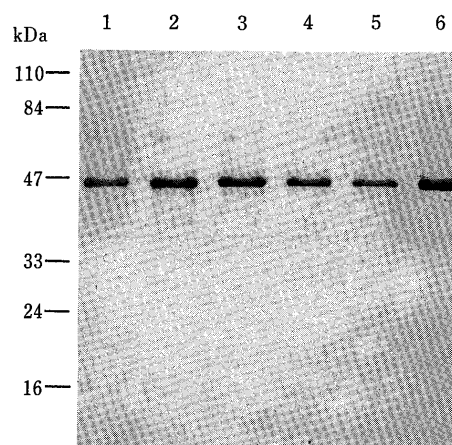


Fig. 3. Enolase  $\gamma$  Subunits ( $\gamma$ -Enolases) Purified from Various Mammals

The  $\gamma$ -enolases purified from rat (lane 1), cow (lane 2), dog (lane 3), goat (lane 4), pig (lane 5), and rabbit (lane 6) were analyzed on a 0.1% SDS-14% polyacrylamide gel. In all samples, a single band with a molecular mass of 46 kDa was detected. Molecular mass markers are shown on the left.

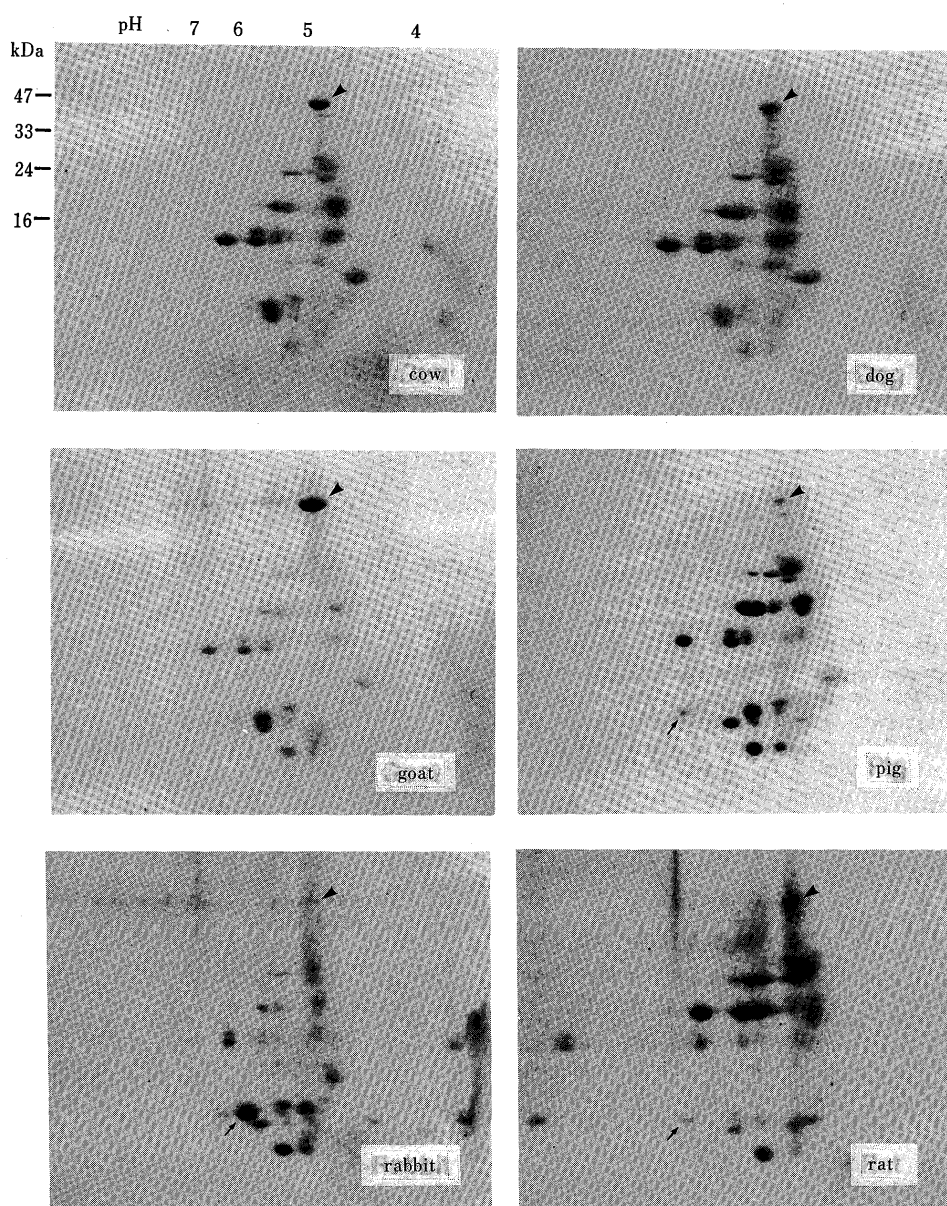


Fig. 4. Two-Dimensional Electrophoresis of Various  $\gamma$ -Enolases Cleaved by Cyanogen Bromide

The  $\gamma$ -enolases were cleaved by cyanogen bromide and analyzed by two-dimensional electrophoresis. For the separation of the first and second dimension, isoelectric focusing and SDS-PAGE were used respectively. The peptide spots indicated by arrows and arrowheads are discussed in the text.

same purification procedure was applied for the purification of the  $\gamma$ -enolase from various mammals (cow, dog, goat, pig, and rabbit). As shown in Fig. 1, the elution patterns of ion-exchange chromatography of extracts from rat and other mammals were essentially identical. In all animals examined, ion-exchange chromatography separated the enolase activity into three peaks and the third peak corresponded to the  $\gamma\gamma$  form. The final preparations of these  $\gamma$ -enolases were analyzed on a 14% polyacrylamide gel. A single band, migrating with a molecular mass of 46 kDa as found in the rat to correspond to  $\gamma$ -enolase, was detected in all other samples (Fig. 3, lanes 2 to 6).

In previous papers, several purification procedures of NSE from rat,<sup>19,27</sup> mouse,<sup>28</sup> cow,<sup>29</sup> and human<sup>20,30</sup> brain tissues have been reported. The  $\gamma\gamma$ -enolase was essentially separated by ion-exchange chromatography in these methods, and further purification was achieved by a combination of chromatographies (e.g., Sephadex G-150, CM-Sephadex, hydroxyapatite, and Blue Sepharose). The main objective of this work was the structural analysis of the various  $\gamma$ -enolases isolated from mammals of many species, so we designed experiments to obtain not the  $\gamma\gamma$  form having the enzyme activity, but only the  $\gamma$  subunit as a purified protein. Therefore, the purification procedure was simpler than the previously reported methods, and it was possible to purify the  $\gamma$ -enolase sufficiently for use in the following structural and immunological analyses.

**Characterization of the Various  $\gamma$ -Enolases** The structural and immunological properties of these  $\gamma$ -enolases were examined by peptide mapping and by a double immunodiffusion test, respectively. The purified  $\gamma$ -enolases were cleaved by cyanogen bromide, and the resulting fragments were separated by two-dimensional electrophoresis combined with isoelectric focusing and SDS-PAGE (Fig. 4). Isoelectric points of the intact (non-cleaved)  $\gamma$ -enolases were approximately 4.7–4.9 in all samples (indicated by arrowheads in Fig. 4). In the cow, dog, and goat, the resulting peptide patterns were almost identical. The patterns of pig, rabbit, and rat differed slightly from the cow–dog–goat pattern. In these patterns, some peptide spots, which were not detected in the cow–dog–goat pattern, were found (indicated by arrows in Fig. 4). However, these six patterns were essentially similar to each other; and therefore, the structural homology, that is, the species non-specificity of  $\gamma$ -enolase, appeared to be very high.

Immunological homology of these  $\gamma$ -enolases was verified by precipitin reaction in agar gel. The rabbit antisera against rat or human  $\gamma$ -enolases reacted strongly with all preparations (except for rabbit), and completely fused precipitation-lines were formed (data not shown).

The NSEs, isolated from human, rat and mouse, have been characterized in detail, and these polypeptide sequences predicted from the  $\gamma$ -enolase-coding cDNAs showed a high degree of homology. Therefore, it had been expected that the  $\gamma$ -enolases of various mammals were also closely related to one another. In this paper, that structural homology was clarified, and an especially strong similarity among cow, dog, and goat, was demonstrated. These results indicate a high degree of conservation of the  $\gamma$ -enolase gene in the process of the evolution of mammals, and it seems that the  $\gamma$ -enolase gene is one of the most conserved genes in various mammals.

Recently, several radioimmunoassay and enzymeimmunoassay systems for NSE measurement in serum were established, and it has been reported that serum NSE level is a potentially useful marker for small cell carcinoma of the lung<sup>15–17</sup> and neuroblastoma.<sup>13,14</sup> However, since it is difficult to obtain a sufficient quantity of NSE from human brain for assays, the application of the bovine or rat NSE to experimental systems has been attempted. Though further experimental work regarding the biological and immunological properties is needed, it is likely that the NSEs of various mammals, not just the bovine or rat NSEs, can be used experimentally without compromising the specificity or sensitivity of the assay system because of structural and immunological similarities.

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