Biochemical and Immunochemical Properties of Modified Human Neuron-Specific Enolase

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¹²⁵I-Labeled recombinant human neuron-specific enolase (R-NSE) was inadequate for RIA as a labeled antigen. The binding activity of labeled R-NSE to the antibody was markedly decreased. To supplement this defect and facilitate purification, we constructed two R-NSE derivatives, Y-NSE (one tyrosine residue was added at the N-terminal of R-NSE) and Y-NSE.H6 (six histidine residues were further added at the C-terminal of Y-NSE). The biochemical and immunochemical characteristics of these R-NSE derivatives were essentially the same to those of R-NSE. These derivatives were useful not only as standards for enzyme immunoassay (EIA), but also as labeled antigens for RIA. These results clearly indicate that the reactivity of these modified NSEs to anti-NSE antibody is almost equivalent to that of human brain $\gamma\gamma$ -enolase (B-NSE), and that even if the modified NSEs are labeled, they retain their binding affinities to antibodies in contrast to R-NSE.

Key words neuron-specific enolase; recombinant NSE; modified NSE; tyrosine-tagging; histidine-tagging; immunoassay

Enolase is a glycolytic enzyme catalyzing the reaction pathway between 2-phosphoglycerate and phosphoenolpyruvate. Enolase is present as a dimeric isoenzyme composed of two of three immunologically distinct 46–47 kDa subunits (α , β and γ).^{2–4)} The $\alpha\gamma$ - and $\gamma\gamma$ -enolases are known collectively as neuron-specific enolase (NSE) and are localized mainly in nervous tissue and neuroendocrine cells.⁵⁾ The tissue specificity of enolase isoforms and the mechanisms of its organ-specific expression are of genetic and biochemical interest. NSE is, furthermore, a useful marker for the diagnosis and monitoring of patients with neuroendocrine tumors and renal cell carcinoma, especially for neuroblastoma^{6,7)} and small-cell carcinoma of the lung.^{8,9)} The serum NSE concentrations have been measured by RIA or EIA (enzyme immunoassay). NSE, used as the labeled antigen in the RIA system or as assay standard, is usually purified from human brain tissue. However, it is difficult to obtain human brain. Instead of human brain $\gamma\gamma$ -enolase (B-NSE), we previously used recombinant human $\gamma\gamma$ -enolase (R-NSE) purified from *Escherichia coli* as the standard material for EIA.¹⁰⁾ The biochemical and immunochemical properties of R-NSE were almost identical to those of B-NSE. Here, we report the application of labeled R-NSE to RIA, and the construction and characterization of R-NSE derivatives which are more suitable for labeling.

MATERIALS AND METHODS

Bacterial Strain and Plasmids *E. coli* JM109 was used as the host strain for recombinant plasmids. The expression vector pKK223-3 containing a *tac* promoter was purchased from Pharmacia Biotech, Tokyo, Japan. The *tac* promoter was induced with 0.3 mM isopropyl- β -D-thiogalactopyranoside (IPTG). Plasmid pHTK503,¹⁰⁾ containing a complete cDNA for γ -subunit of enolase, was used as the template for PCR. The γ -subunit of enolase produced in *E. coli* cells exist almost as the active $\gamma\gamma$ -form ($\gamma\gamma$ -enolase), and this $\gamma\gamma$ -enolase was designated R-

NSE.¹⁰⁾ In this study, the cDNA for γ -subunit of enolase was thus used as R-NSE cDNA, and the modified R-NSE cDNAs were designated Y-NSE cDNA or Y-NSE.H6 cDNA. Therefore, Y-NSE and Y-NSE.H6 produced in *E. coli* cells are both the $\gamma\gamma$ -enolase derivatives.

Plasmid Construction Modified NSE cDNAs were obtained by standard PCR. To amplify Y-NSE cDNA (one tyrosine codon was added at the 5'-end of R-NSE cDNA), 5' sense primer "Y1" [5'-TTTGAATTCGATGTATTCCATACAGAAGATCTGGGC-3'] and 3' antisense primer "Y2" [5'-CCCGAATTCCTTATCACAGCACACTGGGATTACGGA-3'] were used (*Eco*RI sites are underlined). To obtain Y-NSE.H6 cDNA (six histidine codons were further added at the 3'-end of Y-NSE cDNA), 5' sense primer "Y1" and 3' antisense primer "H6" [5'-GGGAAGCTTTTATCAATGGTGATGGTGATGGTGC-AGCACACTGGGATTACGGA-3'] were used (*Hind*III site is underlined). These modified NSE cDNAs were digested with *Eco*RI or *Hind*III, and then cloned into pKK223-3. The resulting plasmids containing Y-NSE cDNA or Y-NSE.H6 cDNA were designated as pYEH101 or pYEH310, respectively. Enzymes were purchased from Gibco BRL or Nippon Gene Co. DNA was transformed into *E. coli* JM109 with CaCl_2 .¹¹⁾ Plasmids were purified with a FlexiPrep kit (Pharmacia Biotech) from the transformants.

Expression and Purification of Y-NSE and Y-NSE.H6 A Y-NSE producing strain bearing pYEH101 was cultivated in 4 l of LB broth until exponential phase. After IPTG induction, cells were cultivated for a further 4 h and then harvested by centrifugation. Y-NSE was purified as described¹⁰⁾ with some modifications. The cells were suspended in 20 mM Tris-HCl buffer containing 30 mM NaCl and 10 mM EDTA (pH 7.9), disrupted by sonication, and centrifuged at $27000 \times g$ for 20 min. The supernatant was salted out with ammonium sulfate, and fraction precipitated between 20 and 60% saturation was dissolved in buffer A: 15 mM Tris-HCl buffer (pH 7.9)

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containing 5 mM MgSO₄ and 0.1 mM EDTA. After dialysis against the same buffer, the supernatant was applied to a DEAE-Sephacel column (2.5 × 45 cm), and the bound materials were eluted with a NaCl gradient from 0 to 0.6 M. The fractions containing Y-NSE were pooled, dialyzed against buffer B: 5 mM sodium phosphate buffer (pH 7.4) containing 5 mM MgSO₄, and passed through a Blue Sepharose CL-6B column (2.8 × 9 cm). Fractions containing Y-NSE were chromatographed on a Butyl-Toyopearl 650M column (1.5 × 5 cm) equilibrated with buffer B containing 25% ammonium sulfate. Y-NSE was eluted with a linear gradient of ammonium sulfate (25–0%).

Y-NSE.H6 producing strain, bearing pYEH310, was cultivated and collected at same conditions described above. The cells were suspended in 50 ml of buffer C: 50 mM sodium phosphate (pH 8.0) containing 0.1% Tween 20 and 0.01 mM PMSF (phenylmethylsulfonyl fluoride), and disrupted by sonication. After centrifugation, the supernatant was applied to a Ni-chelate affinity column (3 × 7.5 cm; Qiagen Inc., Chatsworth, CA, U.S.A.). The column was then washed by buffer C of pH 5.8, and Y-NSE.H6 was eluted with buffer C of pH 4.5.

Analytical Techniques Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and EIA were performed as described previously.¹⁰⁾ Enolase activity was assayed by the method of Rider and Taylor.¹²⁾ Protein concentration was measured with a protein assay kit (Nippon Bio-Rad Laboratories, Tokyo, Japan) using bovine serum albumin as the standard. N-terminal sequence analysis was performed with a PPSQ-10 amino acid sequencer (Shimadzu Corp., Kyoto, Japan). DNA was sequenced using an FMBIO-100 fluorescence imaging analyzer (Takara Shuzo Co., Ltd., Otsu, Japan). EIA was performed by the one-step sandwich method as described.¹⁰⁾ RIA was of the typical double-antibody type. Recombinant NSEs were labeled with ¹²⁵I using chloramine T (1.2–2.2 MBq/μg protein). The standard inhibition curves were obtained with an NSE RIA kit (Eiken Chemical Co., Ltd., Tokyo, Japan) using 25–30 kBq of

labeled NSEs. Assays were performed according to the manufacturer's instructions.

RESULTS

Construction and Purification of Modified NSEs The standard inhibition curves obtained from labeled B- and R-NSE were compared. As shown in Fig. 1, the labeled R-NSE was less reactive to anti-NSE antibody. The reason for this difference is still unclear, but R-NSE is probably unstable upon labeling. We thus modified R-NSE for use in RIA. Two R-NSE derivatives, Y-NSE (one tyrosine residue added at N-terminal) and Y-NSE.H6 (one tyrosine and six histidine residues added at N- and C-termini, respectively), were examined. The constructed plasmids containing modified NSE cDNAs are shown in Fig. 2. The tagged tyrosine residue at the N-terminal was expected to

Fig. 1. Standard Inhibition Curves Obtained from Labeled B- or R-NSE

An RIA system constructed with the labeled R-NSE was compared with that of human brain γ -enolase (B-NSE). Assays were performed using an NSE RIA kit under the conditions recommended by the manufacturer. Briefly, 0.1 ml of standard NSE solutions (2, 4, 8, 16, 32, 64, 128 ng/ml), anti-NSE rabbit serum, and labeled B- or R-NSE (25–30 kBq/0.1 ml) were mixed and incubated for 3 h at 30 °C. After reaction (30 min at 30 °C) of the second antibody against rabbit IgG, the reaction mixtures were centrifuged, then the radioactivities of precipitates were determined (●, B-NSE; ○, R-NSE). B/T, bound/total.

Fig. 2. Construction of Recombinant Plasmids Encoding Modified NSEs

Y-NSE cDNA and Y-NSE.H6 cDNA were obtained by PCR using pHTK503 as a template. These cDNAs were then cloned into the *E. coli* expression vector pKK223-3. Y-NSE and Y-NSE.H6 contained not only the introduced tyrosine and histidine residues but also an initiator methionine residue. See text for details. (A) pHTK503, encoding R-NSE; (B) pYEH101, encoding Y-NSE; (C) pYEH310, encoding Y-NSE.H6.

Fig. 3. SDS-PAGE of Purified Y-NSE and Y-NSE.H6, and Application to Immunoassays

(A) Purified Y-NSE and Y-NSE.H6 (1 μ g each) were loaded in lanes 2 and 3, respectively. As a reference, R-NSE (46 kDa) was loaded in lane 1. The positions of molecular mass markers are shown on the left. (B) Purified Y-NSE (Δ), Y-NSE.H6 ($\square$) and B-NSE ($\bullet$) in the NSE EIA kit (Eiken Chemical Co., Ltd.) were assayed under the same conditions as described.¹⁰⁾ Five known concentrations (1.65, 5, 15, 45 and 135 ng/ml) were used as standards. (C) RIA systems constructed with the labeled Y-NSE (Δ), Y-NSE.H6 ($\square$) or B-NSE ($\bullet$) were examined. Assay conditions are described in the text and in the legend to Fig. 1.

be labeled preferentially to those inside the molecule. Therefore, decreases in antigenicity by labeling of internal tyrosine residues should be prevented. Six histidine residues were tagged to facilitate purification.¹³⁾ The structures of these plasmids were confirmed by DNA sequencing.

Y-NSE, expressed in the strain bearing pYEH101, was analyzed by SDS-PAGE and immunoblotting with anti-NSE antibody. A major protein band which reacted specifically with the antibody was detected with a molecular weight of 46 kDa on SDS-PAGE. Then, Y-NSE.H6, expressed in the strain bearing pYEH310, was examined by the same procedure. A major protein band reactive with the antibody was detected at 47 kDa. Y-NSE was purified from the strain bearing pYEH101 by several chromatographic separations. Finally, 9.1 mg of Y-NSE was obtained from 9.0 g cells with a specific activity of 80 units/mg. As expected, Y-NSE.H6 containing the histidine tag was purified by only Ni-chelate affinity chromatography, and 19.2 mg of Y-NSE.H6 was obtained from 3.7 g of cells. This recovery was clearly superior to those of Y-NSE or R-NSE (6.0 mg from 8.1 g cells).¹⁰⁾ These NSEs were certified as highly purified materials by SDS-PAGE (Fig. 3A).

Analysis of Modified NSEs and Their Application to Immunoassays The biochemical properties of R-NSE and its derivatives are shown in Table 1. The specific activities of R-NSE and Y-NSE were almost the same. Although the activity of Y-NSE.H6 was fairly low, other parameters (optimal pH and the Michaelis constant for 2-phosphoglycerate) were similar to those of R- and Y-NSE. Y-NSE and Y-NSE.H6 had not only an additional

Table 1. Biochemical Properties of R-NSE and Its Derivatives

	R-NSE ^{a)}	Y-NSE	Y-NSE.H6
Molecular size of subunits (kDa)	46	46	47
Specific activity (units/mg)	86	80	64
Optimal pH	7.4–7.6	7.4–7.6	7.4–7.6
K_m for 2-phosphoglycerate (m)	2.4×10^{-4}	2.5×10^{-4}	2.2×10^{-4}
N-Terminal sequence	Ser-Ile-Gln-	Met-Tyr-Ser-Ile-Gln-	Met-Tyr-Ser-Ile-Gln-
N-Terminal blocking	no	no	no

^{a)} Data taken from Ref. 10.

tyrosine residue, but also an initiator methionine residue as follows: Met-Tyr-Ser-Ile-Gln-. Y-NSE and Y-NSE.H6 were then tested as EIA standards. In the tested range, the calibration curves of modified NSEs were almost identical to that of B-NSE (Fig. 3B). This result indicates clearly that the immunoreactivities of these modified and native NSEs to the antibody are similar, and that Y-NSE and Y-NSE.H6 can be used as standard materials in this system. Moreover, as shown in Fig. 3C, these modified NSEs were, in contrast to R-NSE, applicable to the RIA system as labeled antigens. We expected that the tyrosine residue at the N-terminal was labeled preferentially and that the decreases in binding affinities by labeling internal tyrosine residues was prevented. This result confirmed that the additional tyrosine residue at the N-terminal works as we predicted.

DISCUSSION

Previously, we expressed and purified R-NSE from *E. coli*.¹⁰⁾ The biochemical properties of R-NSE were almost identical to those of human brain NSE (B-NSE), and R-NSE could be used as the standard material for EIA. However, the ¹²⁵I-labeled R-NSE was inadequate for use as the labeled antigen in the double-antibody RIA system (Fig. 1). Although the reason(s) remains unclear, this result indicated that the application of R-NSE to RIA is difficult. However, B-NSE is also relatively unstable for isotope-labeling. Sometimes, the B-NSE reactivity to the antibody decreases after labeling (data not shown). The NSE molecule may be intrinsically unstable for labeling. For application of R-NSE to RIA, we constructed two modified NSEs, Y-NSE and Y-NSE.H6. Although recombinant protein overproduced in *E. coli* often forms enzymatically inactive inclusion bodies,¹⁴⁾ the modified NSEs were expressed in the soluble fraction in enzymatically active forms similarly to R-NSE. These NSEs comprised approximately 10 to 15% of total proteins in cell extracts. This relatively low level of expression may be one of the reasons why they did not form inclusion bodies. Recombinant proteins produced in *E. coli* often possess an initiator methionine residue at the N-terminal,¹⁵⁾ and Y-NSE and Y-NSE.H6 had such a methionine residue (Table 1). Although non-modified NSEs (R- and B-NSE) lack not only the tyrosine and histidine residues at their termini but also this initiator methionine residue, the biochemical and immunochemical properties of these four NSEs were almost identical. This indicates that these additional amino acids at the termini of NSE molecules do not influence these properties.

The specific radioactivities (MBq/ μ g protein) of labeled Y-NSE or Y-NSE.H6 were about 2-fold higher than that of R-NSE (data not shown), and Y-NSE and Y-NSE.H6 could be used as labeled antigens in the RIA system. These results suggest that (i) the additional tyrosine residue is probably present on the surface of the

molecule, (ii) this tyrosine residue is labeled preferentially to those within the molecule, (iii) thus, decreases in antigen activity by labeling internal tyrosine residues is prevented, and (iv) the additional histidine residues have no effect on the antigen-antibody interaction. Tyrosine-tagging may be an effective method for modification of antigens such as R-NSE which are unstable for labeling. Moreover, in many cases histidine-tagged antigens may also be applicable for RIA without compromising the specificity or sensitivity.

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