Production of Recombinant Human Creatine Kinase (r-hCK) Isozymes by Tandem Repeat Expression of M and B Genes and Characterization of r-hCK-MB

YOSHIKO SUNAHARA, KOHI UCHIDA, TOSHIO TANAKA, HIROKAZU MATSUKAWA, MANABU INAGAKI, and YUHSI MATUO*

**Background:** Serum creatine kinase-MB isoenzyme (CK-MB) is widely used as a marker of myocardial injury. We prepared recombinant human CK (r-hCK) MB isoenzyme and examined its potential for use as a control material for assay of CK-MB in serum.

**Methods:** cDNAs encoding CK-M and CK-B subunits were inserted into the same plasmid vector, followed by transformation of *Escherichia coli*. The resulting three types of CK isoenzymes were purified by conventional chromatography.

**Results:** The ratio of MB to MM to BB was 50:40:10 on the basis of CK activity. Highly purified CK-MB with a specific activity of 533 U/mg was produced in a yield of 5.7 mg/g of packed cells. Purified r-hCK-MB had the isoelectric point (pI 5.3) and molecular size (46 kDa for the subunit) of native CK-MB. Its immunoreactivity in an ELISA using antibody against native heart enzyme was similar to that of cardiac CK-MB. The r-hCK-MB retained >90% activity for at least 4 months at 11 °C in a delipidated serum matrix in a liquid form at a concentration of 118 U/L.

**Conclusions:** r-hCK-MB shows key properties of the native cardiac isoenzyme and may be useful as a control and calibrator for serum assays of CK-MB.

© 2001 American Association for Clinical Chemistry
Biotech UK Ltd.). Expression plasmid pTRP-CK-M was constructed by insertion of the DNA fragment prepared by digesting the pBluescript II SK(−)-CK-M with EcoRI/BamHI, which encodes the human CK M-type subunit, into expression vector pTRP(3) that had been digested with EcoRI/BamHI. For expression of 1146 bp of the human CK B-type isoenzyme (4), plasmid pTRP-CK-B was constructed according to a method similar to the one mentioned above using a human brain cDNA library as a template.

CONSTRUCTION OF CK-MB EXPRESSION VECTOR

HindIII/SalI-digested pTRP-CK-M fragment was inserted into pBluescript II SK(−) to construct pBluescript II SK(−)-trpP+CK-M. This plasmid was digested with XbaI, and the fragment in which the trp promoter and human CK M-type subunit gene are located was purified. This DNA fragment was inserted into XbaI-digested pTRP-CK-B, the 5′ terminus of which was dephosphorylated, to construct pTRP-CK-B/M (Fig. 1).

This expression vector was transformed into Escherichia coli.
coli. r-hCK-MB, -MM, and -BB were expressed according to the method described previously (3).

**Purification of r-hCK-MB**

r-hCK-MB was purified from cell lysate by three chromatographic methods (hydrophobic, ion-exchange, and gel-filtration) after ammonium sulfate fractionation. The precipitate that formed at 80% saturation of ammonium sulfate was dissolved in 50 mmol/L sodium phosphate buffer (pH 8.0) containing 40% saturation of ammonium sulfate, 1 mmol/L MgCl₂, 0.2 mmol/L EGTA, and 2 mmol/L β-mercaptoethanol, and subjected to hydrophobic chromatography on a phenyl-Sepharose column with 40–0% linear gradient elution. Fractions containing CK activity were equilibrated with 50 mmol/L sodium phosphate buffer (pH 7.4) containing 2 mmol/L β-mercaptoethanol, applied to a Q-Sepharose column, and washed with the same buffer. r-hCK-MB was eluted from the column with a linear NaCl gradient of 0–300 mmol/L. r-hCK-MB fractions were subjected to buffer exchange with 20 mmol/L Tris-HCl buffer (pH 8.5) containing 0.15 mol/L NaCl, 10 mmol/L β-mercaptoethanol, 500 g/L glycerol, and 1 g/L NaN₃ by gel filtration and then stored at −80 °C. The total protein concentration was determined by the bicinchoninic acid method (Pierce Chemical Company) (5) with bovine serum albumin as the calibrator.

The purity of r-hCK-MB was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10–20% gradient gels (Daiichi Pure Chemicals). Electrophoresis was performed using Cassette Electrophoresis Unit “DAILICH”, followed by staining with Rapid Stain Coomassie Brilliant Blue (nacalai tesque).

**Characterization of r-hCK-MB**

The pI of r-hCK-MB was determined on the Phast System (Amersham Pharmacia Biotech) in a pH range of 3–9. Gels were stained with Phast Gel Blue R (Coomassie Brilliant Blue R-350).

CK activity was measured at 37 °C according to the method recommended by the Japanese Society of Clinical Chemistry with the reaction mixture shown in Table 1 on a Hitachi-7150 automated analyzer unless otherwise stated. Enzyme samples were diluted up to ~1000 U/L with 20 mmol/L Tris buffer (pH 8.5) containing 1 g/L bovine serum albumin.

To determine optimum pH, enzyme activity was measured using the buffer shown in Table 1 as a basic solution; 100 mmol/L imidazole or bis-Tris buffer was used as the buffer for reactions at pH 6.0–7.2.

**Sandwich ELISA for CK-MB**

For the ELISA, a 96-well plate was coated with anti-CK-MB monoclonal antibody (20 g/L), which was cross-reactive with CK-MB and -BB but not CK-MM, and incubated at 4 °C overnight. The plate was washed five times with phosphate-buffered saline (PBS) containing 0.5 g/L Tween 20 (Tween-PBS). PBS containing 10 g/L bo-

---

**Table 1. Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.60 (30 °C)</td>
<td>100 mmol/L</td>
</tr>
<tr>
<td>Imidazole buffer</td>
<td>2 mmol/L</td>
</tr>
<tr>
<td>EDTA</td>
<td>10 mmol/L</td>
</tr>
<tr>
<td>Magnesium acetate</td>
<td>20 mmol/L</td>
</tr>
<tr>
<td>ADP</td>
<td>5 mmol/L</td>
</tr>
<tr>
<td>d-Glucose</td>
<td>20 mmol/L</td>
</tr>
<tr>
<td>NADP</td>
<td>3.0 kU/L (30 °C)</td>
</tr>
<tr>
<td>CP</td>
<td>1.5 kU/L (30 °C)</td>
</tr>
</tbody>
</table>

---

**Table 2. Summary of purification of r-hCK-MB.**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume, mL</th>
<th>Total protein, mg</th>
<th>Total CK activity, U</th>
<th>Yield, %</th>
<th>Specific activity, U/mg</th>
<th>Purification, fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extractc</td>
<td>1410</td>
<td>35 532</td>
<td>579 200</td>
<td>100</td>
<td>16</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate fractionation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernatant at 40% saturation</td>
<td>1360</td>
<td>20 550</td>
<td>466 793</td>
<td>81</td>
<td>23</td>
<td>1.4</td>
</tr>
<tr>
<td>Precipitate at 80% saturationd</td>
<td>338</td>
<td>2075</td>
<td>46 958</td>
<td>81</td>
<td>23</td>
<td>1.4</td>
</tr>
<tr>
<td>Phenyl-Sepharose CL-4B</td>
<td>207</td>
<td>684</td>
<td>42 290</td>
<td>73</td>
<td>62</td>
<td>3.8</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>98</td>
<td>218</td>
<td>19 221</td>
<td>33</td>
<td>88</td>
<td>5.4</td>
</tr>
<tr>
<td>Sepharacryl S-200</td>
<td>24</td>
<td>48</td>
<td>6894*</td>
<td>12</td>
<td>144*</td>
<td>8.8</td>
</tr>
</tbody>
</table>

---

* Protein concentration was determined by the BCA protein assay (Pierce).
* CK activity was assayed by the method described in Materials and Methods with some modifications.
* Cells (350 g wet weight) were disrupted by Dyno Mill (Willy A. Bachofen AG Maschinenfabrik).
* The precipitate was dissolved with a small volume of the buffer described in Materials and Methods.
* Enzyme activity shown was assayed at 25 °C in the presence of 1 mmol/L N-acetyl-cysteine. When the activity was assayed by the Japanese Society of Clinical Chemistry method shown in Table 1 (20 mmol/mL at 37 °C), the fraction obtained from the column had a specific activity of 533 U/mg.
vine serum albumin was added for blocking and incubated for 1 hour at room temperature. After the wells were washed with Tween-PBS, a sample solution containing CK-MB was added and incubated at room temperature for 2 hours, followed by washing with Tween-PBS. Rabbit anti-CK-BB polyclonal antibody (10 g/L) was added and incubated at room temperature for 1 hour. After the wells were washed with Tween-PBS, goat anti-rabbit IgG-horseradish peroxidase conjugate was added and incubated at room temperature for 1 hour, followed by washing with Tween-PBS. Substrate solutions for horseradish peroxidase, o-phenylenediamine (0.4 g/L), and H2O2 were added. After a 10-minute incubation at room temperature, the reaction was stopped by the addition of 12 mol/L H2SO4. The absorbance at 492 nm was measured using a microplate reader (MRP A4; TOSOH). Samples were diluted to final concentrations of 0, 12.5, 25, 50, 100, and 200 mg/L, and the absorbances measured at these concentrations were plotted.

**STABILITY OF r-hCK-MB**

To study the stability of r-hCK-MB in buffer, r-hCK-MB was diluted to 2000 kU/L in 20 mmol/L Tris-HCl buffer (pH 8.5) containing 0.15 mol/L NaCl, 10 mmol/L β-mercaptoethanol, 500 g/L glycerol, and 1 g/L NaN3 and stored at −80 or 4 °C. To study the stability of r-hCK-MB...
in a serum matrix, r-hCK-MB was diluted to 120 U/L in delipidated serum purchased from Strategic BioSolutions and stored at −80 or 11 °C. r-hCK-MB stability was examined by an immunoinhibition assay (6) using the CK-MB E-HA Test (Wako Pure Chemical Industries).

Results and Discussion

Expression and Purification

The insertion of cDNAs that encoded for CK-M and CK-B subunits into the same plasmid vector enabled three isoenzymes, r-hCK-MB, -MM, and -BB, to be expressed simultaneously in E. coli. r-hCK-MB was purified from crude extract by ammonium sulfate fractionation, followed by hydrophobic, anion-exchange, and gel-filtration chromatography (Table 2). Three CK peaks were eluted from the anion-exchange column (Fig. 2). The first peak did not react with the anti-CK-MB antibody. The second and third peaks were identified as CK-MB and CK-BB, respectively, based on their cross-reactivity to the antibody, and were confirmed by pI. r-hCK-MM was eluted in the flow-through fraction, whereas the other two isoenzymes were bound to the column, and r-hCK-MB was eluted at 125 mmol/L NaCl. r-hCK-MB with a specific activity of 533 U/mg was obtained in a yield of 12%. The isoelectric points of these three recombinant CK enzymes were indistinguishable from those of their native counterparts, suggesting that the native proteins are minimally processed. The expression ratio of these three types of isoenzymes was MM:MB:BB = 40:50:10 on the basis of enzyme activity. Thus, the technology allowed production of all three types of CK.

Purity of r-hCK-MB

The estimated purity of r-hCK-MB was ~90% when 2 μg of protein was analyzed by SDS-PAGE. The molecular mass of the subunit was ~46 kDa (Fig. 3), and the isoelectric point was 5.2 (data not shown), consistent with the reports of Kanemitsu and Okigaki (7, 8). No band was detected at pI 6.5, which corresponds to CK-MM, or pI 4.5, which corresponds to CK-BB, even when 10 μg of enzyme

---

**Table 3.** $K_m$ values and optimum pH of hCK-MB.

<table>
<thead>
<tr>
<th>Enzyme source</th>
<th>$K_m$ a mmol/L</th>
<th>Optimum pH</th>
<th>ADP</th>
<th>CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>1.19</td>
<td>0.14</td>
<td>6.2–6.8</td>
<td></td>
</tr>
<tr>
<td>Recombinant in this report</td>
<td>1.23</td>
<td>0.13</td>
<td>6.2–6.8</td>
<td></td>
</tr>
<tr>
<td>Recombinant$^d$ from Genzyme</td>
<td>1.19</td>
<td>0.13</td>
<td>6.2–6.8</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ $K_m$ was calculated using Lineweaver-Burk plot.

$^b$ pH range with activity >95% of the maximum is shown.

$^c$ Native hCK-MB from Aalto Scientific Ltd.

$^d$ Recombinant hCK-MB from Genzyme Diagnostics.

---

Fig. 5. Immunoinhibition of r-hCK-MB.

r-hCK-MB, -MM, and -BB samples were incubated for 10 min at room temperature with five 1:2 serial dilutions of goat anti-CK-MM antibody, which inhibits CK-M subunit activity. The remaining CK activity was measured using Merckliquid CK (Kanto), the reagent set for measuring CK activity, and a Hitachi-7150 automated analyzer. The remaining activity of each sample was calculated by defining the value of sample containing no anti-CK-MM antibody as 100%. ○, native hCK-MM (Aalto); ●, r-hCK-MM from this study; □, native hCK-MB (Aalto); ◇, r-hCK-MB (Genzyme); ■, r-hCK-MB from this study; ▲, r-hCK-BB from this study.

Fig. 6. Stability of r-hCK-MB in buffer matrix.

r-hCK-MB was dissolved with 20 mmol/L Tris-HCl (pH 8.5) containing 0.15 mol/L NaCl, 10 mmol/L β-mercaptoethanol, 500 g/L glycerol, and 1 g/L NaN₃ and stored at −80 or 4 °C. At the respective temperatures, the day 0 value was defined as 100%.
protein was applied, suggesting that the contamination of r-hCK-MB with r-hCK-MM or -BB was <1%

PROPERTIES
The enzymologic properties of purified r-hCK-MB were examined by comparing with native hCK-MB or the r-hCK-MB from Genzyme, which has been characterized as a reference material. The $K_m$ values were $1.2 \times 10^{-3}$ mol/L for CP and $1.3 \times 10^{-4}$ mol/L for ADP. The activity was maximum at pH 6.2–6.8 in imidazole buffer as well as in bis-Tris buffer (Table 3). The immunologic reactivity of r-hCK-MB was evaluated by comparing it to native hCK-MB and the r-hCK-MB from Genzyme, as assayed by sandwich ELISA (Fig. 4). The ELISA results, using anti-CK-MB monoclonal antibody as capture antibody and anti-CK-BB antibody as the detection antibody, indicated that the response curve for r-hCK-MB was similar to that of native CK-MB. The immunologic reactivities of r-hCK-MB, -MM, -BB; native hCK-MB; and the r-hCK-MB from Genzyme were analyzed by immunoinhibition assay using an anti-CK-MM antibody that specifically inhibits M-type subunit activity (Fig. 5). The r-hCK-MB activity remaining at a concentration of anti-CK-MM antibody at which M-type activity was entirely inhibited was 42.4%, which was similar to the reactivity of native hCK-MB (42.8%) and the Genzyme r-hCK-MB (43.1%), whereas the remaining activity for r-hCK-BB was 99.4%. These results indicate that r-hCK-MB possesses immunoreactivity similar to that of the native enzyme.

STABILITY
In a buffer matrix, r-hCK-MB activity was stable for 20 days at −80 °C, whereas at 4 °C, a 15% loss of activity was observed after 15 days (Fig. 6). To study stability in a serum matrix, buffer matrix containing r-hCK-MB was diluted 1:100 with 9 g/L NaCl, and further diluted with delipidated serum to an activity of −120 U/L. It was stable at least for 9 months at −80 °C. At 11 °C, 93.2% of the original activity remained after 4 months, and 75% remained after 9 months (Fig. 7).

In conclusion, the results indicate that r-hCK-MB has the potential to be useful as a control/calibrator.

References