Expression and Properties of Human Liver \( \beta \)-Ureidopropionase

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Summary A cDNA encoding \( \beta \)-ureidopropionase (BUP) was isolated from a human liver
cDNA library, expressed in \( E. \) coli, and purified from the culture extract. The 2,006 bp cDNA
contained a 1,152 by open reading frame encoding a protein of 384 amino acids with a mo-
lecular weight of 43,165 Da. The subunit molecular weight of the enzyme expressed was
about 43,000 Da. The enzyme was inhibited by 1 mM propionate, but not by 10 mM \( \beta \)-ala-
nine. Chemical analysis of the purified human BUP showed 0.54 zinc atoms per subunit,
and the sequence of BUP cDNA contained one putative zinc-binding site motif. The purified
enzyme had a pI of 5.65, and exhibited positive cooperativity with N-carbamoyl-\( \beta \)-alanine
as the substrate with a Hill coefficient 2.0. These properties of human BUP, except the inhi-
bition by \( \beta \)-alanine, were similar to the rat liver purified enzyme. \( \beta \)-Alanine inhibits rats
BUP activity. The complex regulatory function and the negative cooperative mechanism of
BUP by \( \beta \)-alanine have been observed in rats. This kind of mechanism may not exist in hu-
mans, because \( \beta \)-alanine did not inhibit human BUP.

Key Words \( \beta \)-ureidopropionase, dihydropyrimidinase, dihydropyrimidine dehydro-
genase, tissue distribution, propionate

\( \beta \)-Ureidopropionase (N-carbamoyl-\( \beta \)-alanine amidohydrolase, EC 3.5.1.6; BUP) is the third enzyme in
pyrimidine catabolism. The enzyme catalyzes the irre-
versible hydrolysis of N-carbamoyl-\( \beta \)-alanine to \( \beta \)-ala-
nine. Dihydropyrimidinase dehydrogenase (DHP) is a rate-
limiting enzyme in this pathway (1). BUP is also rate-
limiting in mutant mice which have low BUP activity
(1). BUP activity is decreased in rats fed a low-protein
diet (5% protein), and is increased by a high-protein
diet (60% protein) (2); therefore BUP can be rate-limiting
in rats which are fed a low-protein diet. When the
enzyme activities of the pyrimidine-degradation path-
way are decreased, the accumulation of metabolites
may cause a severe disease, because rats fed a high-ur-
dine diet showed severe hematuria and accumulated
uracil in the liver (3). A recent study reported that a pa-

tient with BUP deficiency excreted N-carbamoyl-\( \beta \)-ala-
nine in urine, and showed a severe developmental delay
and a dystonic movement disorder (4). The antitumor
drug 5-fluorouracil (5-FU) is also degraded to \( \alpha \)-fluoro-
\( \beta \)-alanine by the pyrimidine-degradation pathway (5).
Severe neurotoxicity due to 5-FU has been described in

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Abbreviations: BUP, \( \beta \)-ureidopropionase; DHP, dihydropy-
rimidinase; DPD, dihydroxypridinidime dehydrogenase; PCR,
polymerase chain reaction; EDTA, ethylenediaminetetraacetic
acid

The nucleotide sequence in this paper has been submitted
to the DDBJ/EMBL/GenBank DNA databases under the acces-
sion number AB013885.

MATERIALS AND METHODS

Chemicals All chemicals used were of analytical
grade, and were purchased from Nacalai Tesque Ltd.
(Kyoto, Japan) unless otherwise stated. Human
leukemia cell line, U-937 DE-4, was obtained from the
Riken Cell Bank, Japan.

Cloning of human liver cDNA To obtain a human
cloned BUP cDNA, rat liver BUP cDNA was prepared by PCR as a probe for screening. The cDNA

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encoding human BUP was screened from a cDNA library in TriplEx (Clontech, USA) by the method described previously (10). DNA sequence analysis was performed using an ALF red autosequencer (Amersham Pharmacia Biotech, USA).

**BUP expression in E. coli.** The Nco I/Nae I fragment from BUP cDNA in TriplEx was cloned into the expression vector pSE420 (Invitrogen Corp., USA). A single colony from a freshly made transformation of DH-5α cells was inoculated into LB broth and grown to a stationary phase. A 1/100 aliquot from culture was used to inoculate 500 mL of terrific broth containing 100 μg/mL of ampicillin. Following a 90-min incubation at 29°C, the trp-lac promoter in the vector was induced with 1 mm isopropyl-β-D-thiogalactopyranoside, then the culture was incubated for an additional 48 h. The cells were centrifuged, washed twice with phosphate-buffered saline, resuspended in 50 mL of 10 mM phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 10% glycerol, and lysed at 4°C by sonication.

**Measurement of BUP activity.** BUP activity was determined at pH 7.0 by measuring the rate of formation of ammonia by glutamate dehydrogenase and NADH (11).

**Purification of human BUP.** Purification of human recombinant BUP began with the sonicated extract. The lysed extract was centrifuged for 20 min at 27,000×g. The supernatant was heated at 50°C for 5 min, cooled to 4°C, and then centrifuged at 27,000×g. Next, purification was performed as it was for rat BUP (11). The purified enzyme was stored at 4°C in 10 mM phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol and 50% glycerol. The isoelectric value of the enzyme (pI value) was determined by Chromatofocusing (Amersham Pharmacia Biotech, USA) as described previously (11).

**Molecular weight determination and western blot analysis.** The subunit molecular weight was determined by SDS polyacrylamide gel electrophoresis. The molecular weight of the native enzyme was determined by gel filtration with a Superose-6 column (1.5×57 cm) equilibrated with 20 mM Tris, and 50 mM NaCl (pH 7.5). The following proteins were used as standards: thyroglobulin (670,000 Da), ferritin (445,000 Da), catalase (240,000 Da), aldolase (158,000 Da), and albumin (65,000 Da). Western blot analysis was performed using the method described previously (12) with anti-Rat BUP rabbit serum.

**Zinc content.** The content of zinc was measured by atomic absorption spectroscopy with a Hitachi Z-53000 Polarized Zeeman Atomic Absorption Spectrophotometer (Hitachi Ltd., Tokyo, Japan).

**Northern blot analysis.** Northern blot analysis was performed with the human adult multiple tissue blot (Clontech, USA) or mRNAs prepared by the method described previously (10). For human BUP, a 32P-labeled Nco I/Nae I fragment was used as a probe. For dihydropyrimidinase (DHP) and DPD, rat probes were reported previously (10, 13) were used for the northern blot analysis.

**RESULTS AND DISCUSSION**

Two cDNA clones were isolated from a human cDNA library, and the longer insert was 2,006 bp. As shown in Fig. 1, the deduced amino acid sequence containing 384 residues was shorter than that of rat BUP by 9 residues in the C terminal, and had an 84% homology with rat BUP. The predicted molecular mass and isoelectric point were 43,165 and 6.08, respectively. Rat BUP has two zinc-binding sites in the sequence (6). The first of the putative zinc-binding sites in rat BUP was compatible with the one in carboxypeptidase from rats, and the second proposed zinc-binding site was compatible with the motif found in phospholipase C (14). In human BUP, one of the zinc-binding sites had the E for K substitution at E-101. To show that this clone encoded the BUP activity, the 1,156 bp Nco I/Nae I fragment, which contained the coding region of BUP, was excised from human cDNA, and inserted into a pSE420 expression vector as pSE420BUP. pSE420BUP was expressed in E. coli, and the supernatant from centrifugation of the lysed extract was used for western blot analysis, measurement of BUP activity, and determination of the molecular weight by gel filtration. By western blot, about 43 kDa of BUP protein was found in the cells transformed by pSE420BUP; however, it was not found in the cells transformed only by the pSE420 vector (Fig. 2A). When BUP activity was assayed with 2 mM N-carbamoyl-β-alanine, the activities in the cells with pSE420BUP and pSE420 were 0.0665 and 0 μmol·min⁻¹·mg protein⁻¹, respectively. The elution pattern of human BUP activity by gel filtration had two peaks in the buffer without adding N-carbamoyl-β-alanine (Fig. 2B), while rat BUP increased in size with the ligand (7). The molecular weight of the larger peak was about 720,000 Da, which was more than that of thyroglobulin (670,000 Da), and the smaller peak was about 380,000 Da, which was between ferritin (445,000 Da) and catalase (230,000 Da). The smaller protein was comprised of 8.8 subunits, and the larger protein was comprised of 16.7 subunits, because the subunit molecular weight was 43,000 Da (Fig. 2A). In a previous study, we reported rat BUP to be a hexamer protein (11). We must amend the rat BUP size, because we calculated the subunit molecular weight as higher. Rat liver BUP is an octamer protein. Similarly, human BUP was organized as an octamer or its polymer. Figure 3 shows the results of purification of human BUP. The same protocol for rat BUP purification was used for human BUP. At the stage of gel filtration by Sephacryl S-300, BUP was separated into two fractions (Fr. A had a higher molecular weight than Fr. B. The specific activities of BUP from Fr. A and Fr. B were higher than that of the purified rat liver BUP: 1.59 μmol·min⁻¹·mg protein⁻¹). Fr. B was applied to Chromatofocusing, and purified to homogeneity. The zinc content of the purified enzyme was 0.54 atoms per subunit. As a result, the human enzyme contains about 1 zinc atom per subunit, while rat enzyme contains 1.8 zinc atoms per sub-
Fig. 1. Alignment of the amino acid sequences of human and rat BUP. Identical amino acid residues are shown in boxes. Zinc-binding sites are underlined.
Property of Human $\beta$-Ureidopropionase

Fig. 2. Molecular weight of the human recombinant BUP. (A) Western blot analysis. Western blot analysis was performed with anti-rat BUP rabbit serum. (B) Gel filtration analysis. Samples were applied to a Superose 6-gel filtration column (1.5×57 cm) equilibrated with buffer containing 20 mM Tris, and 50 mM NaCl (pH 7.5). $\downarrow$ shows the elution position of T, thyroglobulin; F, ferritin; C, catalase.

unit (6). The differences in the zinc content between human recombinant BUP and rat purified BUP may be derived from the different protein foldings in the bacterial cultures. However, the human recombinant BUP had higher specific activity than rat purified BUP. For this reason, the difference may support the findings that there are two zinc-binding site sequences in rat BUP cDNA (6), and one of the sites of human BUP cDNA has a one-amino-acid substitution. The $pI$ value of the purified enzyme was 5.65, which is lower than the $pI$ (6.4) of the rat enzyme. As shown in Fig. 4, the purified enzyme activity did not follow Michaelis-Menten kinetics. The Hill plot showed cooperativity with N-carbamoyl-$\beta$-alanine with a Hill coefficient of 2.0, and the $S_{1/2}$ value was 0.10 mM. Next, we evaluated the inhibitory effects of various compounds using a crude extract of BUP. The inhibitory effects of various compounds with 0.2 mM N-carbamoyl-$\beta$-alanine are shown in Table 1. $\beta$-Alanine, the product of BUP, inhibits rat purified BUP (7), however, $\beta$-alanine (10 mM) did not inhibit human recombinant BUP. $\beta$-Alanine may have neurological functions and cause mental retardation (15). $\beta$-Alanine is a component of carnosine ($\beta$-alanylhistidine), which is present in the muscles of many animal species. Carnosine in muscle supplies histidine, when rats are fed a histidine-free diet (16). It degrades to histidine and $\beta$-alanine by kidney carnosinase for supplying histidine to the body. Carnosine in rat muscle also decreases and degrades to

Zinc contents of the purified enzyme were 0.54 atoms per subunit. $pI$ value of the purified enzyme was 5.65.

Fig. 3. Purification of human recombinant BUP from culture extracts of E. coli. Picture shows SDS polyacrylamide gel electrophoresis of the samples (2 μg protein) from each purification step. The gel was stained with coomassie brilliant blue G-250.

Table 1. Effects of various compounds on human BUP activity.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Concentration (mM)</th>
<th>$\text{BUP activity} (\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control without EDTA</td>
<td>0</td>
<td>0.0182</td>
</tr>
<tr>
<td>+ EDTA</td>
<td>1</td>
<td>0.0180</td>
</tr>
<tr>
<td>Control with EDTA</td>
<td>0</td>
<td>0.0177</td>
</tr>
<tr>
<td>+ $\beta$-Alanine</td>
<td>10</td>
<td>0.0184</td>
</tr>
<tr>
<td>+ Propionate</td>
<td>1</td>
<td>0.0122</td>
</tr>
<tr>
<td>+ Acetate</td>
<td>1</td>
<td>0.0168</td>
</tr>
<tr>
<td>+ DL-3-Hydroxybutyrate</td>
<td>1</td>
<td>0.0185</td>
</tr>
</tbody>
</table>

Various compounds were added to each assay mixture containing 0.2 mM N-carbamoyl-$\beta$-alanine as substrate.
histidine and \( \beta \)-alanine when rats are fed a low-protein diet (17). BUP activity also decreases in rats fed a low-protein diet (2). These data indicate that \( \beta \)-alanine, which is increased by carnosine degradation, regulates rat BUP activity. In rats, the \( K_i \) value of BUP for \( \beta \)-alanine is 1.08 mM, and the concentration of \( \beta \)-alanine in the liver is assumed to be about 0.2 mM (18). Since \( K_i \) is 5-fold the steady state concentration of \( \beta \)-alanine, rat BUP is not greatly inhibited under normal conditions. However, if the \( \beta \)-alanine concentration increases, \( \beta \)-alanine will inhibit BUP in rats (18). In contrast, \( \beta \)-alanine did not regulate human recombinant BUP activity. This finding suggests the reverse regulation by \( \beta \)-alanine does not exist in humans; human BUP activity
may not decrease with a low-protein diet. Propionate (1 mM) inhibited BUP activity by 31%. This value supports the finding that a patient (19) with propionic acidemia due to primary deficiency of propionyl CoA carboxylase showed an elevated excretion of N-carbamoyl-β-alanine. Acetate (1 mM) showed less effective inhibition for the enzyme than propionate. EDTA (1 mM) did not inhibit human BUP activity. When the rats were fed a zinc-deficient diet for 4 wk, liver BUP activity did not change and was similar to that of control rats (20). These findings show that zinc may be strongly bound to BUP, or that zinc-binding is not needed for BUP activity in rats and humans.

There are many studies on the tissue distribution of enzymes of the pyrimidine degradation pathway in rats (11, 12, 18, 21). The first enzyme of the pathway, DPD, is present in many normal tissues in rats (18). The second enzyme, DHP, and BUP activity are detected mainly in the liver and kidneys (18). There is very little information on the tissue distribution of enzymes in human blood cells and the liver (22, 23). As shown in Fig. 5, from the findings of northern blot analysis with human tissues, DPD mRNA was found in all tissues except the brain. DHP and BUP mRNA were found in the liver and kidneys alone. BUP was not expressed in the human leukemia cell line, U-937 DE-4. The enzymes of the pyrimidine-degradation pathway degrade 5-FU. One study reported that a patient with DPD deficiency excreted 89.7% of the dose in the urine as unchanged 5-FU (24). When DPD activity is normal in humans, about 50% of 5-FU is excreted as β-fluoro-α-alanine (25, 26). From these findings and the tissue distributions of human enzymes, it is suggested that the liver and kidneys are the tissues responsible for pyrimidine and 5-FU degradation in humans. As described in the introduction, the accumulation of metabolites of pyrimidine may cause severe diseases, and the regulation of human BUP may have an important role in the occurrence of human diseases. The findings of the present study increased information about the regulation and tissue distribution of BUP in humans.

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REFERENCES


