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Molecular Cloning and Expression of Human Dihydrolipoamide Dehydrogenase-Binding Protein in *Escherichia coli*

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Abstract The pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of pyruvate with the formation of CO_2, acetyl-CoA, NADH, and H^+. This complex contains multiple copies of three catalytic components including pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3). Two regulatory components (E1-kinase and phospho-E1 phosphatase) and functionally less-understood protein (protein X, E3BP) are also involved in the formation of the complex. In this study, cloning and characterization of a gene for human E3BP have been carried out. A cDNA encoding the human E3BP was isolated by database search and cDNA library screening. The primary structure of E3BP has some similar characteristics with that of E2 in the lipoyl domain and the carboxyl-terminal domain, based on the nucleotide sequence and the deduced amino acid sequence. However, the conserved amino acid moiety including the histidine residue for acetyltransferase activity in E2 is not conserved in the case of human E3BP. The human E3BP was expressed and purified in *E. coli*. The molecular weight of the protein, excluding the mitochondrial target sequence, was about 50 kDa as determined by SDS-PAGE. Cloning of human E3BP and expression of the recombinant E3BP will facilitate the understanding of the role(s) of E3BP in mammalian PDC.

Key words: Pyruvate dehydrogenase complex, dihydrolipoamide dehydrogenase-binding protein, molecular cloning

The pyruvate dehydrogenase complex (PDC) is a large enzyme complex that serves essential roles in biological energy metabolism. The PDC provides the link between glycolysis and the citric acid cycle, and produces acetyl-CoA for the citric acid cycle and acetyl groups for acetylcholine synthesis; in omnivores, 50-80% of metabolism goes through the PDC [3]. The PDC is composed of 30 copies of a tetrameric ($\alpha2\beta2$) pyruvate dehydrogenase (E1; EC 1. 2. 4. 1), 60 meric dihydrolipoamide acetyltransferase (E2; EC 2. 3. 1. 61), 12 copies of homodimeric dihydrolipoamide dehydrogenase (E3; EC 1. 8. 1. 4), and 12 copies of monomeric E3-binding protein (protein X, E3BP) [12, 13]. With an exception of E3BP, these three catalytic components catalyze a series of coordinated reactions involving five coenzymes, which include thiamin pyrophosphate (TPP), lipoic acid, coenzyme A, FAD, and NAD^+. E1 catalyzes a decarboxylation of pyruvate with production of CO_2 and hydroxyethyl-TPP. The hydroxyethyl-TPP is then oxidized to acetyl-TPP with the reduction of lipoic acid. The acetyl group is then transferred to lipoic acid yielding the 8-S-acyl compound. E2 catalyzes transfer of the acetyl group to coenzyme A yielding acetyl-CoA, leaving a reduced E2 lipoyl group that E3 uses as an electron source for FAD-dependent reduction of NAD^+ to NADH [12].

The specific feature of mammalian PDC is that PDC contains protein X, recently called E3BP for its function, 12 copies of which are very tightly associated with the E2 core assembly. Its major role appears to be in binding and positioning the E3 component at the correct sites on the surface of the E2 core [10, 20]. It was difficult to establish the function(s) of E3BP in early studies because E3BP was tightly associated with the E2 core of the complex. The studies using limited proteolysis revealed that protein X contributes to the binding and function of E3 [16].

The fact that E3BP has a sequence similarity with E2 and a covalently bound lipoyl moiety has received much interest [4, 11]. However, E3BP seems not to be a tissue-specific isozyme of E2, since it is found in the liver, kidney, heart, adipose tissue, spleen, skeletal muscle, testes, uterus, red blood cells, and brain of the rat [5]. Maeng and colleagues [10] found that E3BP anchors E3 homodimers inside each of the 12 pentagonal faces of the 60-mer E2.

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Steric hindrance by the lipoyl and E3 binding domains limits the binding of E2 [4]. A novel E3BP that lacks the amino terminal lipoyl domain has been found in *Ascaris suum* [7]. The stoichiometry of the E3BP interaction is 12 moles per mol of PDC. The lipoyl domains of E3BP may substitute for the lipoyl domains of E2 in an overall complex catalytic activity [21]. However, the unique catalytic function(s) and role(s) in the complex are still unknown.

Here, the cDNA of human E3BP was cloned and sequenced. The deduced amino acid sequence has a similarity to E2 in amino-terminal, lipoyl-bearing domains. The histidine residue of transacetylase activity in E2 is not conserved in the human E3BP. Overexpression in bacteria was carried out and the molecular weight of the matured E3BP was determined.

MATERIALS AND METHODS

Strain, Plasmid, and Culture Conditions

Escherichia coli DH5α and XL1-Blue (Stratagene, La Jolla, CA, U.S.A.) was used for transformation and propagation of appropriate plasmids. *E. coli* BL21 (DE3) (Novagen, Madison, WI, U.S.A.) was used for expression of human E3BP cDNA cloned to pET32a expression vector (Novagen, Madison, WI, U.S.A.). *E. coli* strains were grown in LB medium (1% Bactotryptone, 0.5% yeast extract, and 1% NaCl, pH 7.3) at 37°C by vigorous shaking. For the selection of plasmid-containing transformants, ampicillin (50 μg/ml) was added into the media. Isopropyl-β-D-thiogalactopyranoside (IPTG, 40 μl of 20 mg/ml solution) and 5-bromo-4-chloro-3-indoyl-β-galactopyranoside (X-gal, 40 μl of a 20 mg/ml solution) were used for selection of transformants harboring recombinant plasmids [9].

cDNA Sequence Analysis

Several clones found by searching of the expressed sequence tag (EST) database of the National Center for Biotechnology Information (NCBI, National Library of Medicine, Bethesda, MD, U.S.A.) were analyzed in this study [9]. A cDNA clone derived from human E3BP mRNA was screened from human placenta cDNA library. Approximately 1.2×10^6 phages of a human placenta cDNA library constructed in the λZAPII (Stratagene, La Jolla, CA, U.S.A.) were screened according to the standard procedures [19, 23]. A 0.5 kb cDNA fragment of human E3BP generated from reverse transcriptase-polymerase chain reaction (RT-PCR) based on previously reported information of human ESTs was used as a probe [2, 6]. Positive clones were isolated and sequenced. Nucleotide sequence was determined using an ABI 377 genetic analyzer (ABI, Foster City, CA, U.S.A.). Preparation of the reaction mixture and the nucleotide sequence analysis using computer was carried out according to the manufacturer's guidance.

Construction of a Bacterial Expression Vector for Human E3BP

*Nco*I and *Bam*HI restriction sites flanking the coding region of E3BP cDNA were constructed by PCR with *Taq* DNA polymerase (Promega, Madison, WI, U.S.A.). The forward primer (E3BPF) containing an *Nco*I site (underlined sequence) was 5'-G CGC GCC ATG GGT GAT CCC ATT AAG ATA-3' (28-mers), corresponding to bases 160-177 of the E3BP cDNA. The reverse primer (E3BPR) containing a *Bam*HI site (underlined sequence) was: 5'-GCG CGC GGA TCC CTA GGC AAG TCG GAT AGG-3' (30-mers), corresponding to bases 1,489-1,506 of the E3BP cDNA. PCR was carried out in the following conditions: hot start at 94°C for 5 min, 35 cycles of denaturation (94°C, 1 min), annealing (56°C, 1 min), extension (72°C, 1 min 30 s), and a final extension at 72°C for 5 min. Resulting PCR product was digested with *Nco*I and *Bam*HI, and then ligated to a bacterial expression vector pET32a (Novagen, Madison, WI, U.S.A.). The recombinant vector, pE3BP32a, was transformed into *E. coli* DH5α for plasmid propagation. The transformant was selected on LB agar plates containing ampicillin (50 μg/ml). The construct was confirmed by the process of restriction enzyme digestion and nucleotide sequencing after the plasmid DNA had been isolated from transformants [19].

Expression of Recombinant E3BP in *E. coli*

Competent *E. coli* BL21 (DE3) cells were transformed with pE3BP32a plasmid. Transformants were selected on LB agar plates containing ampicillin (50 μg/ml). Several colonies displaying antibiotic resistance were tested for their ability to express recombinant polypeptide. For carrying out the expression experiment, 10 μl of glycerol stock was inoculated into 5 ml of LB broth supplemented with 50 μg/ml ampicillin. The cells were grown overnight at 37°C with shaking. The cultured cells were inoculated into 1-l of LB medium with ampicillin (50 μg/ml). The cells were allowed to grow at 37°C until A_{600} reached 0.6–0.7. At this point, IPTG (0.7 mM, final concentration) was added to induce the expression of recombinant polypeptide after 1 ml of culture was taken as the control. Cells were cultured for hours at 32°C with vigorous shaking and then harvested.

Purification of Recombinant E3BP

Harvested cells were resuspended in lysis buffer (50 mM of sodium phosphate buffer, pH 8.0, 300 mM of NaCl) supplemented with a mixture of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml of pepstatin A, 20 μg/ml of leupeptin, 0.2 mM of benzamidine, 1% aprotinin) in 2 ml per gram wet weight of cells. Lysozyme (1 mg/ml) was added into the suspension, and the soup was incubated on ice for 30 min. Cells were sonicated six times for 10 seconds on ice with 10-second intervals between each

burst. Extracts were incubated with RNase A (10 μg/ml) and DNase I (5 μg/ml) for 20 min on ice and then clarified by centrifugation at 10,000 ×g for 30 min at 4°C. Clear extracts containing recombinant E3BP were applied on a Ni-NTA Superflow™ affinity column (Qiagen, Valencia, CA, U.S.A.) equilibrated with 50 mM of sodium phosphate buffer, pH 8.0, and 300 mM of NaCl. The column was washed twice with 4 ml of the wash buffer (50 mM of sodium phosphate buffer, pH 8.0, 300 mM of NaCl, 10 mM of imidazole) to remove unbound portions. The bound proteins were eluted three times with 1 ml of elution buffer (50 mM of sodium phosphate buffer, pH 8.0, 300 mM of NaCl, 100 mM of imidazole). Fractions containing human E3BP were collected and treated with enterokinase for eliminating the amino-terminal residues which were added for affinity purification. The expressed recombinant protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [18].

RESULTS AND DISCUSSION

Nucleotide Sequences and Deduced Amino Acid Sequences
Database search of EST in NCBI (National Library of Medicine, Bethesda, MD, U.S.A.) showed several clones that encoded protein sequences similar to those reported previously [11, 15, 21] for the amino terminus and an internal region of bovine along with human E3BP. According to these results of the sequences of E3BP cDNA, we performed cloning and nucleotide sequence analysis of the human E3BP cDNA. Human E3BP cDNA has an open reading frame of 1,503 base pairs that encodes a protein of 501 amino acid residues with a calculated molecular weight of 54,085 (Fig. 1). There was evidence that this cDNA encodes E3BP, that was provided by a comparison of the deduced amino acid sequences with the previously published amino-terminal sequences [11, 15, 21] (Table 1). Based on the amino-terminal sequence of 22 amino acids for bovine heart E3BP reported previously, and the result of database search on sequences specific to the mitochondrial target sequence, the mature form of human E3BP was thought to start at the 54th amino acid. The first 53-amino acid residues were very similar to other mitochondrial targeting sequences [24].

Human E2 has two lipoyl-bearing domains corresponding to amino acids 7 to 87 and 135 through 213. Residues 6-83 of human E3BP shows 44% of sequence identity with the first lipoyl-bearing domain, and 46% identity with the second lipoyl-bearing domain of E2 [2]. That E3BP should have a lipoyllysine residue in its amino-terminal region, and is expected from the previous studies that demonstrate acetylation of this protein upon incubation of PDC with either radioactive pyruvate or acetyl-CoA [4, 11]. Lipoyllysine residues were typically found to occur

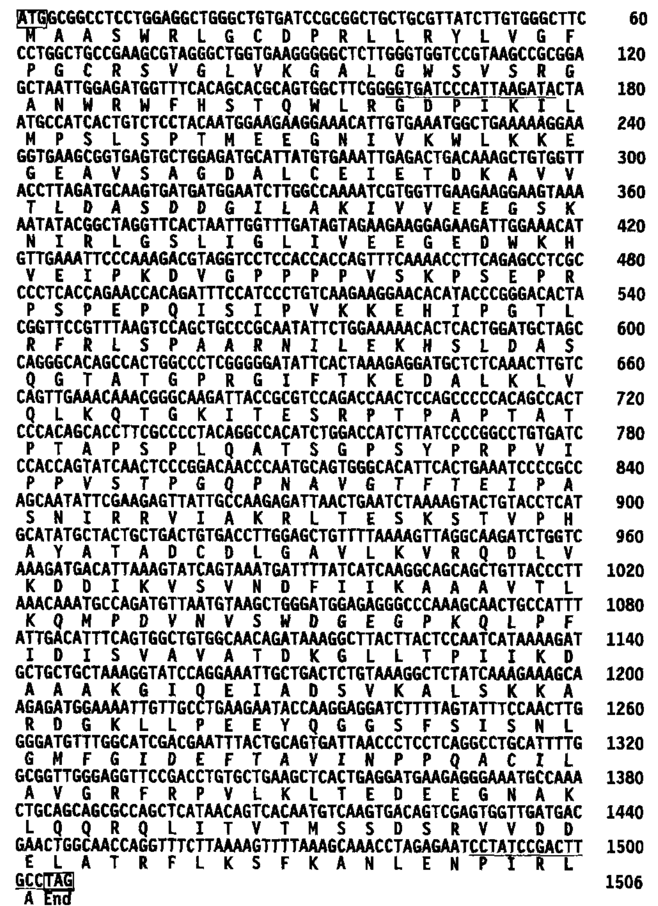

Fig. 1. Nucleotide and deduced amino acid sequence of human E3BP.

Numbering starts at the 5' start codon. The position of the sequences encoding the putative mitochondrial target sequence (leader peptide sequence) is 1 to 159. The oligonucleotide sequences used for generating PCR primers (E3BPF and E3BPR) are underlined. Start and termination codons are shown in boxes.

at 43-44 residues from the amino terminus of mammalian E2 proteins. A lysine residue was located at a position of 44 in the deduced sequence of mature E3BP, and this lysine occurred in a typical consensus sequence for the

Table 1. Comparison of amino-terminal amino acid sequences of mammalian E3BPs.

The amino acid sequences of bovine heart and kidney E3BP are compared with the deduced amino acid sequence of the human E3BP [11, 15]. Conserved amino acids of all three kinds of E3BPs are present in boxes, and conserved amino acids of human E3BP and bovine heart E3BP or human E3BP and bovine kidney E3BP are shown by underlining.

Source	Amino acid sequence
Bovine heart E3BP	ADPIKILMPSLSPTMEEGNIVK
Human E3BP	GDPIKILMPSLSPTMEEGNIVKWLKKEG
Bovine kidney E3BP	AGPIKILMPSLSPTMEEGNIVKWLIKEG

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A. Lipoyl-bearing domain

Human E2     135  VLLPALSPTMTMGTVQRWEKKVGEKLSEGDLLAEIETDKA 174
Human E3BP     6  ILMPSLSPTMEEGNIVKWLKKEGEAVSAGDALCEIETDKA  45
Yeast E3BP     5  FSMPAMSPTMEKGGIVSWKYKVGEPFSAGDVILEVETDKS  44
                                                         *

Human E2     175  TIGFEVQEEGYLAKILVPEGTRDVPLGTPLCIIVEKEAD 213
Human E3BP    46  VVTLDASDDGILAKIVVEEGSKNIRLGSLIGLIVEEGED  84
Yeast E3BP    45  QIDVEALDDGKLAKILKDEGSKDVDVGEPIAYIADVDDD  83

B. Hinge region

Human E2     214  ISAFADYRPTEVTDLKPQVPPPTPPPVAAVPPTPQPLAPT 253
Human E3BP    85  WKHVEIPKDVGP-----------PPPVSKPSEPRPSPEPQ 113
Yeast E3BP    84  LATIKLPQEANTANAKSIEIKKPSADSTEATQQHLKKATV 123

Human E2     254  PSAPCPATPAGPKGR 268
Human E3BP   114  ISIPVKKEHIPGTLR 128
Yeast E3BP   124  TPIKTVDGSQANLEQ 138

C. Inner core

Human E2     525  MMSVTLSCDHRVVDGAV 541
Human E3BP   413  LITVTMSSDSRVVDDEL 429
Yeast E3BP        -----------------
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Fig. 2. Comparison of the deduced amino acid sequences of human E2, human E3BP, and *S. cerevisiae* E3BP in the lipoyl-bearing domain and the hinge region.

Conserved amino acids of all three proteins are shown in boxes, and conserved amino acids of human E2 and human E3BP are shown by underlining.

lipoyl-attachment site, and therefore, was highly likely to be the site of lipoylation [2].

The hinge region separating the segments of human E3BP and E2 showed the least sequence identity (15%) of any region of these proteins. The hinge of E2 that connects the second lipoyl-bearing domain with the peripheral subunit-binding domain was rich in both Pro and Ala residues. The corresponding region of E3BP was rich in Pro but contained no Ala residues (Fig. 2). Human E3BP had no sequence identity with human E2 in the hinge region along with yeast E3BP.

E3BP is an essential component of PDC because it positions E3 to accept electrons from the reduced lipoyl group of the E2 component [1, 8, 14]. Recently, it has been suggested that the only function of E3BP may be to anchor E3 to the complex. The intrinsic catalytic activity of E3BP is still unknown at present. Sanderson and colleagues reported that collagenase-treated mammalian PDC (removal of E2-derived lipoyl domains) showed some residual activity (15%), indicating that protein X (E3BP)-linked lipoamide groups can substitute for the lipoyl domains of E2 in an overall complex catalysis [20]. They also showed that normal protein X diacetylation occurred in the absence of the E2 lipoyl domain, and proposed that mammalian E3BP might be considered as a minor isoenzyme of E2, having some specialized role in E3 binding, and retained a limited ability to function in the acetylation reactions of the complex. Human E3BP showed a high level of similarity with E2 in its amino-terminal and lipoyl-bearing domains, however, the mammalian E3BP and the yeast E3BP are very different in their carboxyl-terminal regions, based on the deduced amino acid sequences of human E3BP (Fig. 2). The histidine residue for transacetylase activities of all α-keto acid dehydrogenase complexes is not conserved in the human E3BP, as concluded from the deduced amino acid sequences. This histidine residue is invariably present in the highly conserved sequence of DHRXXDG in proteins with acyltransferase activity [17]. The corresponding sequence in human E3BP is DSRXXDD (residues 221 to 226) (Fig. 2). The active site histidine is substituted by a serine residue, and the carboxyl-terminal glycine of the motif is substituted by an aspartate residue. According to these results, it is safe to mention that human E3BP does not have any acetyltransferase activity.

Cloning and Expression of Human E3BP cDNA

According to the DNA sequence from human E3BP cDNA, PCR primers were designed for amplification of the fragment that encoded the mature and processed E3BP. Since the first 53 amino acid sequences are thought to be a consensus sequence for mitochondrial targeting (leader peptide), the first 159 base pairs of the E3BP cDNA were excluded for PCR amplification. The resulting PCR product of approximately 1.35 kb (corresponding to bases 160 to 1,506 of E3BP cDNA) was digested with *NcoI/BamHI*. This fragment was ligated between *NcoI/BamHI* sites of bacterial expression vector pET32a (Novagen, U.S.A.) to generate the recombinant plasmid, pE3BP32a (Fig. 3). pET32a vector is designed for cloning and high-level expression of peptide sequences, and cloning sites are available for producing fusion proteins containing cleavable His-Tag sequences for detection and purification. The recombinant gene in the vector is under the control of the T7 promoter, and expression is induced by providing a source of T7 RNA polymerase under *lacUV5* control in the host cell. Therefore, expression of the recombinant gene can be induced by adding IPTG [22]. Human E3BP was expressed and purified using these characteristics of the vector and host. There was no critical difference in the induction of gene expression by the concentration of IPTG between 0.4 mM and 1 mM (data not shown). Therefore, the gene expression was induced by 0.7 mM IPTG to reach OD_{600} 0.7. The concentration of recombinant polypeptide was examined by SDS-PAGE. The concentration of the fusion protein content was about 20% of total proteins

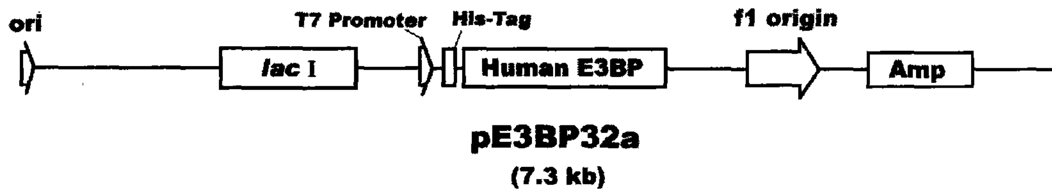

Fig. 3. Physical map of the expression vector, pE3BP32a.
pE3BP32a was constructed for expression of the human E3BP cDNA in *E. coli* BL21 (DE3). The PCR product for human E3BP cDNA is inserted into the vector and its expression is controlled by the T7 promoter.

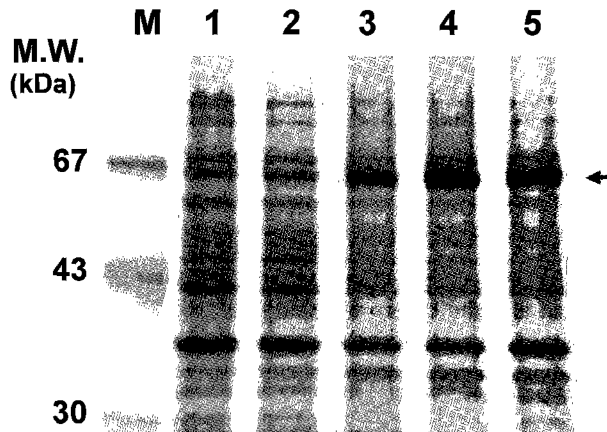

Fig. 4. SDS-PAGE analysis of the human E3BP expressed in *E. coli* BL21 (DE3).
The samples were loaded on 10% polyacrylamide gel. M, protein molecular weight standard; lane 1, total proteins of host with pET32a; lane 2, total proteins of host with pE3BP32a in no induction; lane 3, total proteins of host with pE3BP32a in 6 h induction; lane 4, total proteins of host with pE3BP32a in 12 h induction; lane 5, total proteins of host with pE3BP32a in 24 h induction. The overexpressed recombinant human E3BP is indicated by an arrow.

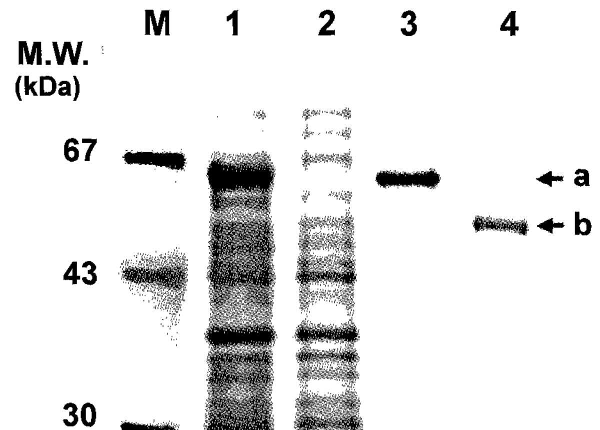

Fig. 5. SDS-PAGE analysis of the purified human E3BP from *E. coli.*
The human E3BP was expressed by IPTG induction and purified by Ni-NTA affinity column chromatography. The eluted protein was treated with enterokinase for cleavage between amino-terminal residues for affinity and recombinant E3BP polypeptide. M, protein molecular weight standard; lane 1, total proteins of *E. coli* BL21 (DE3) with pE3BP32a (12 h induction); lane 2, unbound fraction of Ni-NTA affinity column; lane 3, the eluted fraction containing the fused E3BP protein; lane 4, enterokinase-treated recombinant E3BP separated from amino-terminal residues for affinity. Label "a" indicates the overexpressed recombinant human E3BP and "b" indicates enterokinase-treated (cleaved) recombinant human E3BP (ca. 50 kDa).

produced in *E. coli*, and there was no significant difference in the expressed protein depending on the induction time (Fig. 4). Because the protein was fused with six consecutive histidine residues that have a nickel-binding property, the expressed protein was purified by nickel-bound affinity column chromatography. Nonspecific binding could be reduced by including a low concentration of imidazole (10 mM) in wash buffers. The molecular mass of the protein was calculated to be ca. 64 kDa. Considering that its amino-terminal residues (15 kDa) served as a vector itself for the recombinant E3BP polypeptide (ca. 50 kDa from nucleotide sequences), this molecular weight seems to be reasonable. The recombinant E3BP eluted by the buffer (50 mM of sodium phosphate, pH 8.0, 300 mM of NaCl, 100 mM of imidazole) was treated with enterokinase to remove the additional amino-terminal. The cleaved His-tag was separated by SDS-PAGE and the matured form of the recombinant human E3BP was purified (Fig. 5).

The measurement of the activity for transacetylase of this recombinant human E3BP purified from *E. coli* will be useful in providing direct proof or evidence to show the presence of the catalytic activity in the inner core domain of human E3BP and the functional difference(s) between human E3BP and E2.

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