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Expression of recombinant human histidine decarboxylase with full length and C-terminal truncated forms in yeast and bacterial cells

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Histamine-synthetic enzyme (HDC) from human basophilic leukemia cell line KU-812-F was expressed by yeast, cell-free, and bacterial expression systems. A full length form of 74-kDa HDC was expressed in *S. cerevisiae* and purified by using ion exchange and gel filtration chromatographies. In the purification process, the 74-kDa HDC was found in an enzymatically inactive fraction, while degraded HDCs were detected in an enzymatically active fraction. The 74-kDa HDC expressed in the wheat germ cell-free system was also found in an inactive fraction. When a C-terminal truncated form of HDC expressed in *E. coli* was purified, the enzyme had high specific activity. Our results suggest that the enzyme activity exhibited by the degraded HDCs expressed in *S. cerevisiae* could be due to the C-terminal truncation.

Key words: histamine, HDC, recombinant

Histidine decarboxylase (HDC, EC: 4.1.1.22) catalyzes a decarboxylation reaction of histidine to produce histamine, a bioactive amine, that influences physiological reactions including allergy, gastric acid secretion,

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Abbreviations: AroDC, aromatic amino acid decarboxylase; DTT, dithiothreitol; *E. coli*, *Escherichia coli*; GAD, glutamate decarboxylase; HDC, histidine decarboxylase; OPA, *o*-phthalaldehyde; PEG, polyethylene glycol; PLP, Pyridoxal 5'-phosphate; *S. cerevisiae*, *Saccharomyces cerevisiae*

capillary dilatation, neurotransmission, and smooth muscle contraction. In human, several cell types capable of producing histamine have been identified. Since the HDC expression pattern in individual cells are not entirely understood, it is possible that the histamine production may be found in other cell types.

Human HDC cDNA was isolated and characterized from basophilic leukemia cells [1], and rat and mouse HDC cDNAs were isolated from fetal liver [2] and mastocytoma P-815 cells [3], respectively. Generally, the HDC cDNAs encode a protein with a molar mass of 74-kDa. Taguchi *et al.* and Ohmori *et al*. purified HDC from fetal rat liver [4] and from mouse mastocytoma P-815 cells [5], respectively. The molecular mass of the both HDCs was estimated to be 53-54-kDa, that is about 20-kDa smaller than the size estimated from the full length cDNAs. The difference in the molecular mass is explained by the post-translational processing that truncates C-terminal about 20-kDa region [6]. Several attempts made to understand the detailed mechanism of the post-translational processing did not clear the activity profile on 74-kDa HDC [6-11]; thus it is important to closely examine whether or not the full length form of HDC exhibits enzyme activity.

There were several attempts made to produce recombinant 74-kDa HDC; such as baculovirus system, reticulocyte cell lysate system and yeast system. HDC protein expressed by the baculovirus system was initially insoluble where, after the solubilization, the enzymatic activity was detected [12]. On the other hand, mouse recombinant 74-kDa HDC expressed by the baculovirus system was concluded to be inactive [13] and rat recombinant 74-kDa HDC expressed by the reticulocyte cell lysate system was also inactive [7]. In the both cases, the solubility of the enzyme was not documented. Since the properties of the recombinant 74-kDa HDC remain unclear, we need to obtain a soluble 74-kDa HDC protein in homogeneous preparation in order to carry out biochemical studies.

In our previous studies, we were able to successfully express human recombinant 74-kDa HDC as a soluble form in yeast, however the enzyme was not purified [14]. In the present study, the purification of the 74-kDa HDC produced by this yeast expression system was carried out. As a reference, we also examined the 74-kDa HDC by using wheat germ cell-free expression system, since this *in vitro* expression system supposed to be protease-free and the full-length product would be expected. Furthermore, an expression system of 54-kDa HDC, the C-terminal truncated form, was attempted with *E. coli*. We evaluated the enzyme activity of the purified 74-kDa HDC forms produced by the yeast and wheat germ systems as well as the 54-kDa HDC obtained by the bacterial system.

MATERIALS AND METHODS

Construction of the expression vectors - The plasmid pWIH31, previously constructed for the expression of human HDC [14], was used for the expression of 74-kDa HDC in *Saccharomyces cerevisiae.* pWIH31 was also used as a template to obtain 74-kDa HDC cDNA for wheat germ expression system and 54-kDa HDC cDNA for bacterial expression system. The plasmid pEU-E01 was used for the expression of 74-kDa HDC in the wheat germ cell-free expression system (CellFree Sciences, Ehime, Japan). The plasmid pET21a (Novagen, Madison, WI), was used for the expression of

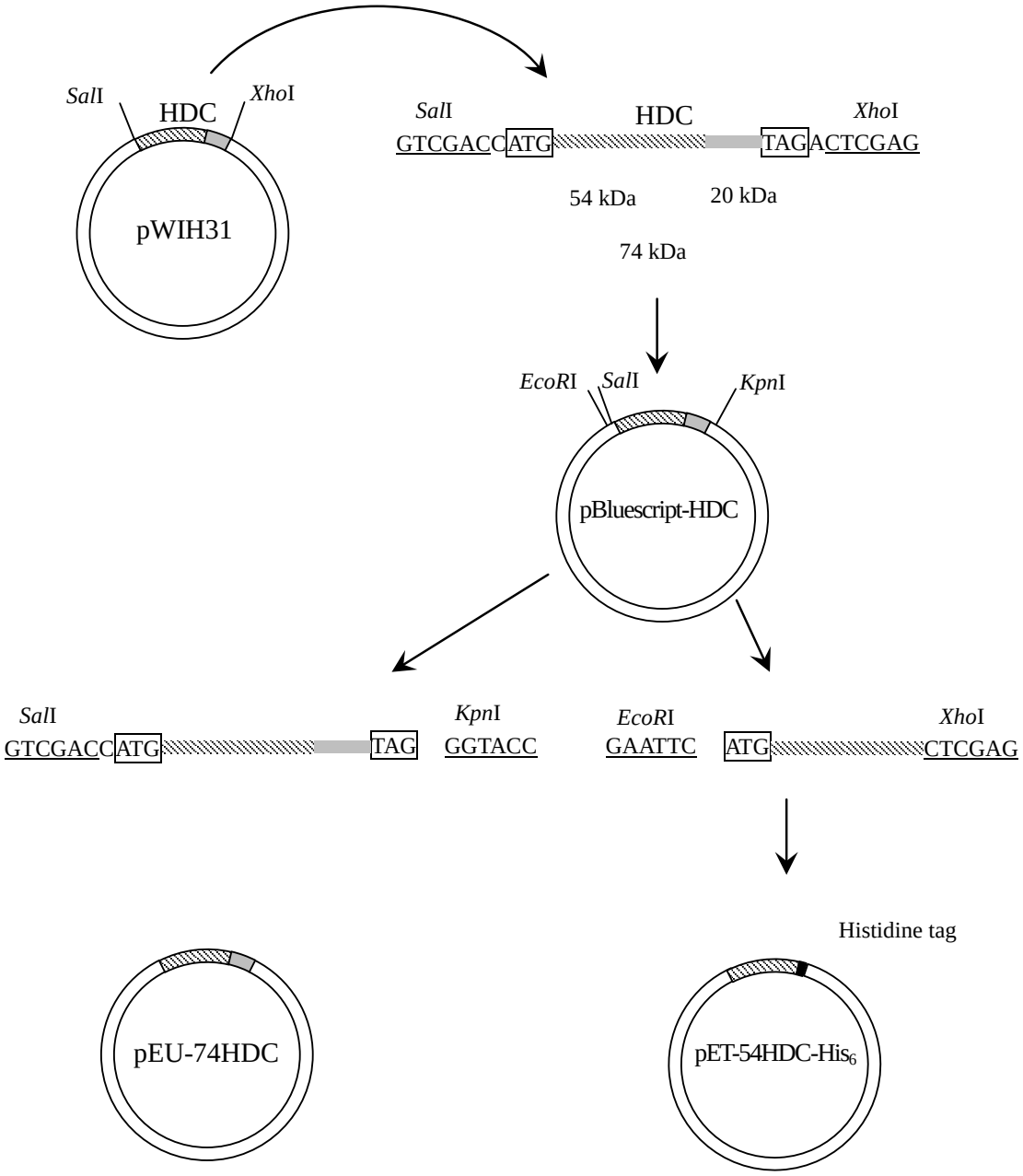

Fig 1. Structure and construction of the recombinant expression vector pEU-74HDC and pET-54HDC-His_6.

polyhistidine-tagged 54-kDa HDC in *Escherichia coli* strain BL21(DE3).

A DNA fragment containing HDC coding region was obtained from plasmid pWIH31 after digestion with *Sal*I and *Xho*I. It was inserted into pBluescript at the *Sal*I and *Xho*I sites to give pBluescript-HDC. The plasmid vector, pBluescript-HDC, was then digested with *Sal*I and *Kpn*I to give a fragment containing HDC coding region, and then the fragment was inserted into pEU-E01 by using the *Sal*I and *Kpn*I sites to construct pEU-74HDC.

For C-terminal truncated HDC, 54-kDa HDC cDNA was amplified by PCR with two primers, 5'- GAG AAT TCG ATA TCA AGC TTA TCG ATA CCG -3' and 5'- TCC TCG AGT GAC TCA GGA TGA GAG -3', and pBluescript-HDC was used as a template. Those primers were designed to introduce *Xho*I site at downstream of Ser476 codon. The PCR product was digested with *EcoR*I and *Xho*I and inserted into the same sites of the pET21a to construct pET-54HDC-His_6, so that the product, 54-kDa HDC-His_6, was designed to have a poly-His tag at its C-terminus end. The plasmid construction was summarized in Fig. 1. The constructed plasmid was confirmed by the restriction enzyme digestion analysis and DNA sequencing.

Preparation of cell lysate - Cell growth and cell lysate preparation of *S. cerevisiae* were carried out as described previously [14]. Yeast cells were pre-cultured in a medium containing 1% yeast extract, 1% peptone, and 2% glucose for overnight and was cultured in a medium containing 0.67% yeast nitrogen base without amino acid, 2% histidine, 2% leucine, 2% adenine sulfate, 2% uracil, and 2% sodium acetate at 28 °C. Cells in the late-exponential phase were harvested by centrifugation. The precipitated cell pellets were suspended into 60 mM potassium phosphate buffer, pH 7.1, containing 0.1 mM DTT, 0.5 mM PLP and 1 mM EDTA, then, applied for the cell disruption. The suspended cells were placed in Bead-Beater (Biospec Products, Bartlesville, OK, U.S.A.) that was kept cold with ice and one-minute disruption was repeated for seven times. The suspension was centrifuged at 10,000 × g for 30 min and the supernatant was collected as a cell lysate.

In vitro protein expression was performed with wheat germ cell-free expression system by using wheat germ expression premium kit (CellFree Sciences, Ehime, Japan). Transcription and translation reactions were carried out according to the manufacture's protocol. The reaction products obtained were subjected for biochemical analysis.

E. coli strain transfected with pET21a-54HDC-His_6 was grown at 37 °C for overnight in LB medium containing 50 μg/ml ampicillin. This pre-culture was transferred into the fresh LB medium containing 50 μg/ml ampicillin to make an initial OD_{600} of 0.05, which was then inoculated at 37 °C until OD_{600} reached above 0.6. The HDC

protein production was carried out without any induction substances such as IPTG. The culture was continued to inoculate at 30 °C for another 15 h. After the centrifugation, the cell pellets were collected and re-suspended with an equal amount of PBS which was constituted with 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 8 g/l NaCl and 1.15 g/l Na_2HPO_4. The cell lysate was obtained by treating the suspension with ultrasonication and centrifugation at 10000 × g for 20 min at 4 °C.

Enzyme activity assay - HDC activity was determined by quantitating histamine on an HPLC equipped with *o*-phthalaldehyde (OPA) derivatization system as described previously [15]. An assay mixture contained 0.1 mM DTT, 1% (v/v) PEG#300, 10 μM PLP, 3.2 mM L-histidine and the enzyme solution in 50 mM potassium phosphate buffer, pH 6.8,was prepared as described by Ohmori *et al*. [5]. The assay mixture was incubated at 37 °C for 30 or 60 min and the reaction was terminated by adding 60% perchloric acid at the amount of 5% of the assay volume. The protein concentration was estimated by using BioRad protein assay with bovine serum albumin as standard.

Enzyme purification procedure - Enzyme purification was carried out according to the procedure described by Ohmori et al. [5]. Ammonium sulfate precipitation was carried out by adding solid ammonium sulfate slowly into the solution to reach 25% saturation. After ammonium sulfate was completely dissolved and an additional 20-min stirring was completed, the suspension was centrifuged at 10,000 × g for 30 min to obtain a supernatant. Ammonium sulfate was then added to the supernatant to be 55% saturation. The suspension was centrifuged at 10,000 × g for 30 min and the pellets were dissolved with about 5 volumes of 20 mM potassium phosphate buffer, pH 6.8, and then the solution was dialyzed against the same buffer for overnight in the cold room. The dialyzed solution was centrifuged at 10,000 × g for 30 min and the supernatant containing the enzyme was collected. The obtained enzyme solution was applied on a Fractgel EMD DEAE 650(S) HPLC column (1.5 × 13 cm) that was pre-equilibrated with 20 mM potassium phosphate buffer, pH 6.8, containing 0.2 mM DTT, 1% (v/v) PEG#300, 10 μM PLP and 0.5 mM EDTA at a flow rate of 1.0 ml/min. The enzyme fraction was eluted within a 30 min by application of a linear gradient of potassium phosphate buffer, pH 6.8, containing 0.2 mM DTT, 1 v/v % PEG#300, 10 μM PLP and 0.5 mM EDTA from 20 to 500 mM. The fractions containing HDC activity were pooled and applied on a Sephacryl S-400 gel filtration HPLC column (2.5 × 45 cm) that was pre-equilibrated with 200 mM potassium phosphate buffer, pH 6.8, containing 0.2 mM DTT, 1% (v/v) PEG#300, 10 μM PLP and 0.5 mM EDTA at a flow rate of 0.7 ml/min. The column was eluted with the same buffer and aliquots of each of the fractions were examined for HDC activity,

SDS-PAGE and Western blot analysis.

The cell lysate of *E. coli* cells was applied on a column packed with cobalt based immobilized metal affinity chromatography resin (Clontech, Mountain View, CA, U.S.A.). The column was equilibrated with 50mM sodium phosphate buffer, pH 6.8, containing 0.3 N NaCl, 1% PEG#300, and 10 μM PLP, and washed with 30 mM imidazole containing buffer. The HDC containing fraction was eluted with 150 mM imidazole in 50mM sodium phosphate buffer, pH 6.8, containing 0.3 N NaCl, 1% PEG#300, and 10μM PLP. The eluted protein was concentrated by an Amicon ultrafiltration with YM 30 membrane.

Western blot analysis – Proteins on SDS-PAGE gel was electrically blotted to nitrocellulose membrane on a BioRad semi-dry blotter for 1 hr with the use of blotting buffer constituted with 20% methanol, 0.3% Tris and 1.44% glycine. After the blotting, the membrane was soaked in skim milk solution for 1 hr, washed with TTBS buffer, constituted with 20 mM Tris, 154 mM NaCl, 0.05% Tween-20, pH 7.4, and incubated with rabbit polyclonal antibody against HDC (Progen, CA, U.S.A.). The membrane was washed with TTBS and then incubated with secondary antibody, donkey anti-rabbit IgG antibody with HRP (GE Healthcare, CA, U.S.A.). The immuno-positive bands were visualized with ECL Western blotting detection reagent (GE Healthcare, CA, U.S.A.) according to the manufacturer's instruction. After the treatment with ECL reagent, the membrane was exposed to Polaroid film (Type 667) in a cassette (Invitrogen, CA, U.S.A.).

RESULTS AND DISCUSSION

Expression of recombinant full-length HDC in S. cerevisiae and purification of HDC – In our previous study, human HDC was expressed in *S. cerevisiae* and exhibited enzymatic activity of 0.2 nmol/min/mg [14]. The crude extract gave a major band at around 74-kDa in Western blot analysis; however, we did not perform any extensive purification on the 74-kDa HDC. In this study, we applied the crude extract on a DEAE HPLC column to purify the 74-kDa HDC, after confirming the 74-kDa band on the electrophoretic analysis (Fig. 2). For each fractions containing the enzymatic activity in the DEAE column purification were collected, where the specific activity was at around 0.7 nmol/min/mg. The SDS-PAGE and

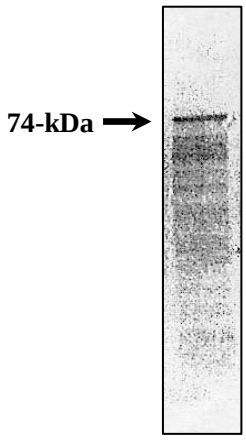

Fig. 2. Western blot of crude extract of *S. cerevisiae* transfected with pWIH31. Rabbit polyclonal antibody was used against HDC.

Western blot analysis on each pooled fractions gave several bands smaller than 74-kDa (data not shown), where those fractions exhibiting 74-kDa band did not show any significant enzymatic activity. The crude extract was also applied on gel filtration chromatography and the fractions containing the 74-kDa HDC were found to be enzymatically inactive (data not shown). Although the sequential purification with DEAE and gel filtration chromatography gave a fraction containing 74-kDa HDC protein, our further purification attempts were unsuccessful because of the low yield of the 74-kDa HDC in the fraction. Before attempting the further purification, we need more proteins to treat with. For this reason, development of expression system with higher protein expression levels is needed. Also desired would be the expression system that is capable to prevent the unnecessary proteolytic degradation in the host cells.

Expression of recombinant full-length HDC by using wheat germ cell-free expression system - Proteolysis-free wheat germ cell-free expression system was employed for the expression of 74-kDa HDC. SDS-PAGE and Western blot analysis of the reaction products indicated that the wheat germ cell-free expression system gave higher protein expression level than the *S. cerevisiae* system (Fig. 3). Aliquots of the reaction products were measured for HDC activity and protein concentration, where the specific activity of 0.3 nmol/min/mg was obtained, the value being nearly the same as that of *S. cerevisiae* system. Although the wheat germ cell-free expression system is supposed to be proteolysis-free, several bands appeared on Western blot analysis (Fig. 3), and they are apparently degraded HDC proteins. The supplier noted that the cell-free protein synthesis system is not completely devoid of wheat endosperm which could be the source of proteases. Our results implicate a possibility that human 74-kDa HDC is enzymatically inactive as was the recombinant mouse 75-kDa HDC [13]; however, the results were not totally conclusive.

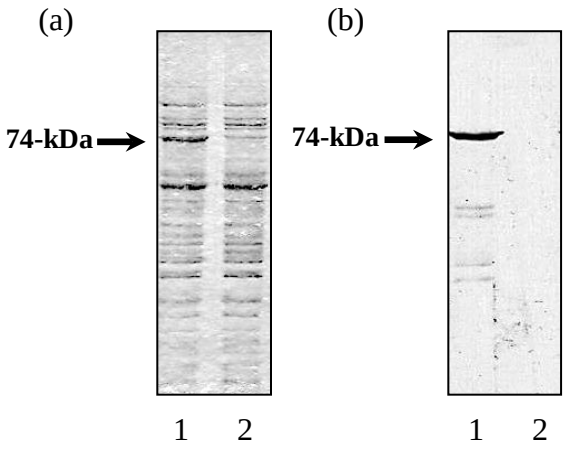

Fig. 3. (a) CBB-stained SDS-PAGE gel and (b) Western blot of reacted mixture of wheat germ cell-free expression system. Lane 1; pEU-74HDC, lane 2; pEU-E01 empty. Antibody was rabbit polyclonal antibody against HDC.

Expression and purification of recombinant C-terminal truncated HDC – HDC belongs to the same family with glutamate decarboxylase (GAD) and L-aromatic amino acid decarboxylase (AroDC). Their amino acid sequence

homology indicates that they are PLP-dependent enzymes. When primary structure alignment was examined among these three decarboxylases, HDC showed an extended C-terminal of about 150 amino acids. We have been puzzling over why HDC has this extended C-terminal region of HDC. While working on rat HDC, Ichikawa's group raised a possibility that 74-kDa HDC was a precursor form and removal of the C-terminal 150 amino acids could activate the enzyme. Hence, we have re-designed our expression system so that C-terminal truncated HDC would be expressed in *E. coli* system. An *Xho*I site was introduced to the HDC cDNA, where C-terminal 20-kDa truncated protein was produced and the deletion size was corresponding to the residues from 477 to 622 (Fig. 1). The *Eco*RI-*Xho*I fragment was inserted into pET vector to make pET-54HDC-His_6 plasmid vector. After the transformation of *E. coli* host cells, the transformant cells were inoculated without IPTG since no induction substances were necessary to induce HDC synthesis. The grown cells were harvested, and lysed to give a soluble fraction, in which the specific activity of 2.9 nmol/min/mg was found. The lysate was loaded on a metal affinity chromatography that specifically binds to His-tag protein. Homogeneous protein fraction having an apparent size of 50-kDa and specific activity of 1,200 nmol/min/mg was obtained (Fig. 4). SDS-PAGE analysis indicated that there is a single band at the size near 54-kDa. Overall yield was about 5 mg HDC per 10 L culture (about 50 g wet cells). In order to evaluate the C-terminal and N-terminal parts of the eluted protein, Western blot and protein sequence analyses were performed. In the Western blot analysis, the band was visible with polyclonal antibody against HDC but it was not visible with a monoclonal antibody against polyHis-tag that supposed to be locating at the C-terminal end (data not shown). The sample was blotted on PVDF membrane which was then used for protein sequence analysis. The analysis gave a sequence of MEPEEY, which is the exact match with the N-terminal six amino acids as expected for human HDC based upon cDNA sequence. The *E. coli* system expressed a soluble form of HDC with its size of 50-kDa and enzymatic activity. The expressed protein has its C-terminal end truncated. However, a question remained why the monoclonal

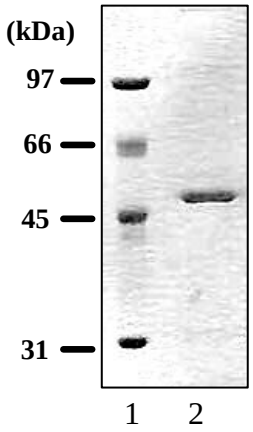

Fig. 4. CBB-stained SDS-PAGE gel of protein purified from *E. coli* transfected with pET-54HDC-His_6 (lane 2). Lane 1; marker.

antibody failed to detect polyHis-tag and needs to be clarified in the future research.

In conclusion, the full length recombinant human HDCs expressed by *S. cerevisiae* and wheat germ cell-free systems were enzymatically inactive. The residual enzyme activity exhibited in the *S. cerevisiae* and wheat germ cell-free systems probably arose from the degraded HDCs. The role of C-terminal 150 amino acids in 74-kDa HDC is still unclear; however, HDC mutant protein that lacks the C-terminal 150 amino acids clearly gave high enzyme activity. The present results support an assumption that post translational processing could be involved for the activation of human HDC. However, since a possibility of misfolding of the protein cannot be excluded, a further study is warranted to clarify if the full length recombinant human HDC has the enzymatic activity.

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