An Enzymatic Method to Estimate the Content of L-Hydroxyproline

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Abbreviations:

L-Hyp, L-hydroxyproline; T4LHyp, *trans*-4-hydroxy-L-proline; T3LHyp,
trans-3-hydroxy-L-proline; HypE, hydroxyproline 2-epimerase; HypDH,
cis-4-hydroxy-D-proline dehydrogenase; T3LHypD, T3LHyp dehydratase; Pyr2CR,
 Δ^1 -pyrroline-2-carboxylate reductase; CC, calibration curve.

Abstract

Post-translational hydroxylation of the L-proline residue mainly occurs in collagen; therefore, the L-hydroxyprolines (L-Hyp) synthesized, including *trans*-4-hydroxy-L-proline (T4LHyp) and *trans*-3-hydroxy-L-proline (T3LHyp), are important markers for directly measuring the content of collagen in several biological samples. The most frequently used method to estimate the content of L-Hyp is high-performance liquid chromatography (HPLC), which is inconvenient. In the present study, we attempted to estimate the content of L-Hyp using coupling systems with metabolic enzymes of the T4LHyp (hydroxyproline 2-epimerase (HypE) and *cis*-4-hydroxy-D-proline dehydrogenase (HypDH)) and T3LHyp pathways (T3LHyp dehydratase (T3LHypD) and Δ^1 -pyrroline-2-carboxylate reductase (Pyr2CR)) from microorganisms. We constructed a functional expression system of recombinant HypDH with a heterooligomeric structure in *Escherichia coli* cells. Enzymological characterization revealed that the β -subunit acted as a catalytic subunit, and also that assembly with other subunit(s) improved the kinetics for *cis*-4-hydroxy-D-proline and thermostability. By using a spectrophotometric assay with different wavelengths, the contents of T4LHyp and T3LHyp were successfully estimated within the ranges of 0.004~1 mM and 0.05~1 mM, respectively, and were consistent with those determined by HPLC. This enzymatic method was used to measure the content of T4LHyp in the acid-hydrolysate of collagen, and blood plasma.

1. Introduction

Approximately 25% of amino acid residues in mammalian collagens are L-proline, and nearly 40% of these L-proline residues are post-translationally converted to L-hydroxyproline (L-Hyp), with *trans*-4-hydroxy-L-proline (T4LHyp) and *trans*-3-hydroxy-L-proline (T3LHyp) being synthesized by prolyl 4-hydroxylase (EC 1.14.11.2) and prolyl 3-hydroxylase (EC 1.14.11.7), respectively (Adams and Frank, 1980). Since this hydroxylation is almost entirely specific to the collagen protein, L-Hyp, especially T4LHyp, is an important marker for directly measuring the content of collagen in several biological samples, including foods and tissue fibrosis. Serumal and urinary L-Hyp have attracted attention as an important biomarker of bone resorption and many human diseases (Simsek et al., 2004).

Therefore, a simple, fast, inexpensive, and sensitive method needs to be established to determine the content of L-Hyp. After the common approach of acid-hydrolysis in 6 N HCl, the content of L-Hyp in the hydrolysate can be successfully measured using many different techniques. Although ion-exchange separation using amino acid analyzer (high-performance liquid chromatography (HPLC)) is one of the most common methods (Le Boucher et al., 1997), this method is time-consuming, expensive, and requires a large apparatus. Enzymatic detection (particularly using a spectrophotometer) represents a useful alternative to HPLC because of its high specificity for substrates and ability to perform measurements using aquatic solvents under mild conditions. On the other hand, although the oxidative deamination reaction by L-amino acid oxidase (EC 1.4.3.2) allows L-amino acids to be measured easily by detecting the H₂O₂ yielded, the application of this method has been limited due to broad substrate specificity, except for L-glutamate oxidase (Upadhyay et al., 2006). To the best of our knowledge, no specific L-amino acid oxidase for T4LHyp or T3LHyp has yet been reported (Satomura et al., 2011, 2012).

In contrast to mammals, a few bacteria, including *Pseudomonas aeruginosa* PAO1, *Pseudomonas putida* KT2440, and *Azospirillum brasilense* ATCC 29145, convert T4LHyp to α -ketoglutarate through four enzymatic steps (Watanabe et al., 2007, 2012, 2013). Hydroxyproline 2-epimerase (HypE; EC 5.1.1.8) first catalyzes the isomerization of T4LHyp to *cis*-4-hydroxy-D-proline (C4DHyp), which is then oxidized to Δ^1 -pyrroline-4-hydroxy-2-carboxylate by C4DHyp dehydrogenase (HypDH) (Fig. 1A). We recently reported that *A. brasilense* (but not the two other *Pseudomonas* species) grew using not only T4LHyp, but also T3LHyp as the sole carbon source, and also that the metabolic pathway consisted of two enzymes, T3LHyp dehydratase (T3LHypD; EC

4.2.1.77) and NAD(P)H-dependent Δ^1 -pyrroline-2-carboxylate (Pyr2C) reductase (Pyr2CR; EC 1.5.1.1), which converted T3LHyp to L-proline via Pyr2C (Watanabe et al., 2014a) (Fig. 1B). In addition, each homologous protein to T3LHypD and Pyr2CR was functionally characterized from the psychrophilic bacterium *Colwellia psychrerythraea* 34H and hyperthermophilic archaeon, *Thermococcus litoralis* DSM 5473 (Watanabe et al., 2014a, b). These Pyr2CR are members of the L-ornithine cyclodeaminase/ μ -crystallin superfamily, whereas another type of Pyr2CR is involved in the metabolism of D-lysine and D-proline from *Pseudomonas* species: the so-called DpkA (LhpD in this study) (Muramatsu, et al., 2005).

Since HypE and T3LHypD utilize only T4LHyp and T3LHyp as the substrate in the microbial metabolic pathways of T4LHyp and T3LHyp, respectively, and not any other natural L-amino acid such as L-proline, difficulties have been associated with directly assaying their activities. On the other hand, the activities of HypDH and Pyr2CR (which act on C4DHyp and Pyr2C, converted by HypE and T3LHyp) can be readily monitored using a spectrophotometer, in spite of difficulties in assaying the activities of HypE and T3LHyp, which directly act on T4LHyp and T3LHyp (Watanabe et al., 2012). The aim of this study was to apply coupling systems with HypE/HypDH and T3LHypD/Pyr2CR to the enzymatic estimation of the contents of T4LHyp and T3LHyp, respectively. Among the four enzymes used, the most serious drawback was found with HypDH. There are two different types of HypDH with heteromeric and homomeric structures, and only the former was useful in the present study due to its significant stability (Watanabe et al., 2012). However, this protein (from *P. aeruginosa*) is only functionally expressed in *P. putida* as a host cell, and its expression level is still insufficient for bioindustries. Therefore, we constructed an expression system for heteromeric HypDH from *A. brasilense* in *Escherichia coli* cells, and briefly characterized several of its properties including the catalytic function of each subunit. On the other hand, combinations between T3LHypD and Pyr2CR from several organisms were optimized to achieve more sensitive measurements of T3LHyp with a small amount of enzymes. By using both these coupling systems, we successfully measured the contents of T4LHyp and T3LHyp (and these coexistence) within the ranges of 0.004~1 mM and 0.05~1 mM, respectively, which was consistent with those determined by HPLC. We also attempted to estimate the content of T4LHyp in the acid-hydrolysate of collagen, and blood plasma, and showed that this enzymatic method has a potential to substitute HPLC analysis.

2. Materials and methods

2.1. Plasmid construction

The primer sequences used in this study are shown in Table S1. The prefixes Ab (*A. brasiliense*), Cp (*C. psychrerythraea*), Pa (*P. aeruginosa*), Tl (*T. litoralis*), and Hs (*Homo sapiens*) were added to the gene symbols or protein designations when required for clarity. The (His)₆-tag was attached to the N-terminus of all genes (proteins) using the pETDuet-1, pACYCDuet-1, or pCOLADuet-1 vector (Novagen, Madison, WI, USA). T3LHypD genes from *A. brasiliense* (BAO21621; AbLhpH), *C. psychrerythraea* (CPS_1453; CpLhpH), *T. litoralis* (OCC_00387; TlLhpH), and *H. sapiens* (C14orf149; HsLhpH), and Pyr2CR genes from *A. brasiliense* (BAO21622; AbLhpI), *C. psychrerythraea* (CPS_1455; CpLhpI), *T. litoralis* (OCC_00362(7); TlLhpI), and *P. aeruginosa* (PA1252; PaLhpD) were cloned into pETDuet-1 to obtain pET/AbLhpH, pET/CpLhpH, pET/TlLhpH, pET/HsLhpH, pET/AbLhpI, pET/CpLhpI, pET/TlLhpE, and pET/PaLhpD, respectively, as described previously (Watanabe et al., 2014a, b). The (Putative) HypE gene from *A. brasiliense* (BAN78527; AbLhpA) was introduced into the BamHI-HindIII sites of pETDuet-1 to obtain pET/AbLhpA. The (Putative) HypDH genes from *A. brasiliense*, consisting of *LhpB* (BAN78526; encoding β -subunit), *LhpE* (BAN78524; α -subunit), and *LhpF* (BAN78525; γ -subunit), were introduced into the BamHI-PstI sites of pACYCDuet-1, BamHI-HindIII sites of pETDuet-1 and BamHI-PstI sites of pCOLADuet-1 to obtain pACYC/AbLhpB, pET/AbLhpE, and pCOLA/AbLhpF, respectively.

2.2. Expression and purification of (His)₆-tagged recombinant proteins

E. coli strain BL21(DE3) (for pET/AbLhpA, pET/AbLhpH, pET/CpLhpH, pET/AbLhpI, pET/CpLhpI, pET/PaLhpD, pACYC/AbLhpB, pET/AbLhpE, and pCOLA/AbLhpF), BL21-CodonPlus (DE3)-RIL (for pET/TlLhpH and pET/TlLhpI) or Rosetta2(DE3) (for pET/HsLhpH) (Novagen) was used as a host cell for the expression of recombinant proteins. *E. coli* harboring each plasmid was grown at 37 °C to a turbidity of 0.6 at 600 nm in Super broth medium (pH 7.0, 12 g tryptone, 24 g yeast extract, 5 ml glycerol, 3.81 g KH₂PO₄, and 12.5 g K₂HPO₄ per liter) containing chloramphenicol (30 mg/liter for pACYCDuet-1), ampicillin (50 mg/liter for pETDuet-1), and/or kanamycin (50 mg/liter for pCOLADuet-1). After the addition of 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG), the culture was incubated for a further 6 h at 37 °C (for AbLhpA, AbLhpH, AbLhpI, HsLhpH, TlLhpH, and TlLhpI) or for 18 h at 18 °C (for CpLhpH, CpLhpI, PaLhpD, AbLhpB, AbLhpE, and AbLhpF) to induce the expression of the His₆-tagged protein. Cells were harvested and resuspended in

1 Buffer A (50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl and 10
2 mM imidazole). The cells were then disrupted by sonication, and the solution was
3 centrifuged. The supernatant was loaded onto a Ni-NTA Superflow column (Qiagen,
4 Valencia, CA, USA) equilibrated with Buffer A linked to the BioAssist eZ system
5 (TOSOH, Tokyo, Japan). The column was washed with Buffer B (50 mM sodium
6 phosphate buffer (pH 8.0) containing 300 mM NaCl, 10% (v/v) glycerol, and 50 mM
7 imidazole). The enzymes were then eluted with Buffer C (pH 8.0, Buffer B containing
8 250 mM imidazole instead of 50 mM imidazole). To purify AbLhpB, AbLhpE, and/or
9 AbLhpF, a buffer system containing 2 mM MgCl₂ and Tween-20 (1% (v/v) for Buffer
10 A; 0.1% (v/v) for Buffers B and C) was used. The purified proteins were concentrated
11 by ultrafiltration with Centriplus YM-30 (Millipore, Bedford, MA, USA), dialyzed
12 against 50 mM Tris-HCl buffer (pH 8.0) containing 50% (v/v) glycerol, and stored at
13 -35 °C until use. If necessary, the purified recombinant proteins were analyzed by
14 SDS-PAGE (Laemmli, 1970).

16 2.3. *HypDH* assay

17 HypDH was assayed using a Shimadzu UV-1800 spectrophotometer (Shimadzu
18 GLC Ltd., Tokyo, Japan) by a previously described method (Watanabe et al., 2012). To
19 estimate potential C4DHyp oxidase activity in AbHypDH, the production of H₂O₂ was
20 measured using the 4-aminoantipyrine peroxidase system (Fischer et al., 1996). Briefly,
21 an enzyme solution was incubated with a reaction mixture (1 ml) consisting of 50 mM
22 Tris-HCl (pH 9.0), 10 mM C4DHyp, 1.5 mM 4-aminoantipyrine, 0.04% (w/v)
23 *N,N*-diethyl-*m*-toluidine (NNDT), and 2.5 unit of horseradish peroxidase (Oriental
24 Yeast, Osaka, Japan) at 30 °C. The reaction was stopped by adding 1 ml of 2% (w/v)
25 SDS solution. The millimolar absorption coefficient (ϵ) for quinoneimine dye (reduced)
26 was 28.2 mM⁻¹·cm⁻¹ at 545 nm. D-Amino acid oxidase (from porcine kidney;
27 Sigma-Aldrich, Japan) was used as a control for this assay.

28 Protein concentrations were determined by the method of Lowry et al. (1951) with
29 bovine serum albumin as the standard. SDS-PAGE was performed as described by
30 Laemmli (1970). K_m and V_{max} values were calculated by a Lineweaver-Burk plot (also
31 those for T3LHypD and Pyr2CR).

33 2.4. *Enzymatic detection of T4LHyp*

34 The standard reaction mixture contained 0.25 mM *p*-iodonitrotetrazolium violet
35 (INT; artificial electron acceptor), 0.06 mM phenazine methosulfate (PMS;
36 electron-transfer intermediate), and purified AbLhpA (10 µg) in 50 mM Tris-HCl buffer

(pH 8.0). After the addition of 100 mM T4LHyp solution (100 μ l), the mixture was incubated for 5 min at 30 °C. The reaction was started by adding the purified HypDH protein (20 μ g) with a final reaction volume of 1 ml (ϵ for INT = 15.0 mM⁻¹·cm⁻¹ at 490 nm). In the assay procedure without a time interval, HypE and HypDH were first added to the standard reaction mixture, and the reaction was then started by the addition of T4LHyp.

2.5. Enzymatic detection of T3LHyp

The reaction mixture (1 ml) consisted of 50 mM Tris-HCl (pH 8.0), 10 mM T3LHyp, 0.15 mM NADH (for TILhpI) or NADPH (for LhpI proteins, except TILhpI), and purified T3LHypD (10 μ g). After an interval of 5 min, the reaction was started by the addition of a small amount of purified Pyr2CR. One unit of enzyme activity referred to 1 μ mol NAD(P)H oxidized/min.

2.6. Detection of L-Hyp by HPLC

L-Hyp was also assayed on an amino acid analyzer (L-8900; Hitachi, Tokyo, Japan), using ion exchange chromatography followed by post-column derivatization with ninhydrin (Le Boucher et al., 1997). The retention times of T4LHyp and T3LHyp were appropriately 14.2 and 9.5 min, respectively.

2.7. Preparation of acid-hydrolysate of collagen

Powder collagen (~10 mg) (Nitta Gelatin, AP-250) was hydrolyzed in 6 N HCl (5ml) in sealed tubes at 110 °C for 24 h and subsequently evaporated to dryness to remove acid. The residue was dissolved in water (10 ml) and filtered.

3. Results and discussion

3.1. Expression and purification of recombinant HypDH from *A. brasiliense*

The putative amino acid sequences of the AbLhpB, AbLhpE, and AbLhpF showed 68, 60, and 63% identities to those of *P. aeruginosa* (Watanabe et al., 2013), suggesting the potential function of HypDH. In contrast to PaHypDH, significant dehydrogenase activity with C4DHyp was detected in the cell-free extract prepared from *E. coli* cells co-expressing the *AbLhpB*, *AbLhpE*, and *AbLhpF* genes. When the protein(s) was induced at 18 °C by IPTG, specific activity was 5.2-fold higher than that at 37 °C (data not shown). The (His)₆-tagged protein(s) was purified homogeneously with a nickel-chelating affinity column (referred to as AbLhpBEF) (Fig. 1C): yield of ~5 mg

per liter of the culture. The enzyme showed 2.98 unit/mg protein of specific activity for C4DHyp, and couldn't utilize oxygen as an electron acceptor (no oxidase activity). Inactivation was not detected after 6 months at -35°C .

3.2. Characterization of AbHypDH

Cloning of the *AbLhpB*, *AbLhpE* and *AbLhpF* genes into different plasmid vectors enabled each subunit of heteromeric HypDH to be functionally characterized. HypDH activity was detected not only with AbLhpBEF (native structure), but also with AbLhpB and AbLhpBE (co-expression of AbLhpB+E): 2.69 and 2.90 unit/mg protein of specific activities for C4DHyp, respectively (Table 1). This result clearly indicated that the β -subunit encoded by the *AbLhpB* gene functioned as a catalytic subunit, as reported previously in heteromeric L-proline dehydrogenase (Kawakami et al., 2004, 2005) and sarcosine oxidase from archaea (Lee et al., 2012) with sequential similarities to AbHypDH.

The spectra of these active proteins (orange-brown; Fig. 2A, inset) commonly showed the characteristics of a typical flavoprotein (Fig. 2A). We previously demonstrated that PaHypDH ($\alpha_4\beta_4\gamma_4$) may contain 2 FAD (α - and β -subunits) and 1 FMN (between α - and β -subunits) within the structural unit of $\alpha\beta\gamma$ (Watanabe et al., 2012). Only FAD was extracted from AbLhpB (~ 0.8 mol per mol of protein), while FAD and FMN were extracted from AbLhpBEF and AbLhpBE (the molar ratio of FAD:FMN was 1.85:1) (Fig. 2B). Although FAD was detected in the extracts of AbLhpE and AbLhpEF, its content was very low (~ 0.05 mol FAD per mol protein). These results indicated that the γ -subunit (AbLhpF) had no effect on the binding of FMN, and the α -subunit by itself could not adequately bind FAD.

At a concentration of 10 mM, AbLhpBEF utilized C4DHyp (100%), *trans*-4-hydroxy-D-proline (T4DHyp) (107%), and D-proline (34%), while its activities with other D-amino acids were only 1% less than those with C4DHyp (Table S2). The specific activity values of AbLhpB for T4DHyp and D-proline were only 3 and 1%, respectively, relative to C4DHyp (100%). Lower activities towards these substrates were also found in AbLhpBE. These proteins were subjected to further kinetic analyses with the active substrates and the determined parameters are listed in Table 1. The $V_{\max}/K_m$ value of AbLhpBEF for C4DHyp was ~ 124 - and ~ 736 -fold higher those for T4DHyp and D-proline, respectively, and this was attributed to the markedly lower K_m values. On the other hand, the high specificity of AbLhpB for C4DHyp was achieved not only by lower K_m values, but also higher $V_{\max}$ values. The kinetic constants of AbLhpBE were more similar to those of AbLhpBEF than AbLhpB. These results

clearly suggested that both AbLhpBEF and AbLhpB were strictly specific to C4DHyp, while the heteromeric subunit assembly of AbLhpBEF significantly increased catalysis (39-fold higher $V_{\max}/K_m$ value than that of AbLhpB), and this may have been caused by the modification of an active site in the β -subunit.

The AbLhpBEF protein was the most thermostable, and inactivation was not detected by a treatment at 50 °C for 10 min (Fig. 2C). The enzyme activity of the AbLhpB protein decreased to 70% after an incubation at 40 °C, and no activity was observed in a treatment at 50 °C. The AbLhpBE protein was the most thermolabile, and the thermal unfolding transition temperature ($T_{1/2}$) was 25 °C lower than that with AbLhpBEF. Indeed, liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis using tryptic peptides of each protein band of the AbLhpBE revealed that the α -subunit is partially digested between Pro⁶⁹-Asn⁷⁰ and Val⁷¹-Thr⁷², probably due to proteolysis in *E. coli* cells (data not shown). These results indicated that the γ -subunit significantly contributed to stabilizing the $\alpha_4\beta_4\gamma_4$ structure (and the α -subunit), but not to enzyme catalysis. Therefore, we only used AbLhpBEF and AbLhyB as HypDH in subsequent experiments.

3.3. Enzymatic detection of T4LHyp and optimization of conditions

HypE belongs to the proline racemase family, containing the archetypical proline racemase (EC 5.1.1.4), T3LHypD, and diaminopimelate epimerase (EC 5.1.1.7) (Goytia et al., 2007; Visser et al., 2012). Of these, three enzymes, except for T3LHypD, catalyze the deprotonation/reprotonation of the chiral carbon ($C\alpha$) of both amino acid enantiomers, resulting in the stereoinversion of chiral centers, and require no co-factor in catalysis, in contrast to other pyridoxal 5'-phosphate-dependent enzymes. Furthermore, HypE can only epimerize T4LHyp (and the *cis*-enantiomer), and not any other natural L-amino acids, including L-proline and T3LHyp (Watanabe et al., 2015). Therefore, (low) activity with D-proline in HypDH (Table 1) had no effect on the detection of T4LHyp, even if L-proline was contained in the samples such as the acid-hydrolysate of collagen (see below; Fig. 4A, inset).

The putative amino acid sequence of the AbLhpA shows 74% identity to that of PaHypE, which has been characterized functionally (Goytia et al., 2007). Although the epimerization of HypE has typically been assayed by measuring changes in optical rotations using a polarimeter, this method is inconvenient. Alternatively, when T4LHyp (10 mM) was incubated in the presence of purified AbLhpA (10 μ g) and AbLhpBEF (20 μ g), a significant increase was observed in absorbance at 490 nm, indicating the successful detection of T4LHyp spectrophotometrically. The optimum temperature and

pH of this system were 30 °C and 8.0~9.0, respectively.

3.4. Enzymatic detection of T3LHyp and optimization of conditions

Although T3LHypD can be assayed using a colorimetric method based on the reaction of 2-aminobenzaldehyde with Pyr2C, which yields a yellow reaction product (Visser et al., 2012), this procedure is inconvenient and has low sensitivity, as well as assay method of HypE. Alternatively, when T3LHyp (10 mM) was incubated in the presence of purified HsLhpH and PaLhpD (10 µg each; referred to as HsLhpH(10)/PaLhpD(10)) as T3LHypD and Pyr2CR, respectively, a decrease in absorbance at 340 nm, caused by the oxidization of NADPH, was observed, indicating that T3LHyp was continuously converted to L-proline via Pyr2C.

To further optimize the reaction condition(s), we first screened the best combination of T3LHypD and Pyr2CR enzymes from several organisms. When AbLhpH, CpLhpH, and HsLhpH (10 µg) were each used together with PaLhpD (10 µg) (at 30 °C), a significantly sharp slope was detected with the two bacterial LhpH proteins, which may have been caused by their high specific activities (Table 2). As described in the Introduction section, both LhpD and LhpI function as Pyr2CR, and appeared by convergent evolution (Watanabe et al., 2014a; Muramatsu, et al., 2005). The most prominent difference in their properties is co-enzyme specificity: strict NADPH-dependent PaLhpD; strict NADH-dependent TlLhpI; NADPH-preferring CpLhpI; dual specific AbLhpI. Therefore, NADPH was used as a co-enzyme, except in the coupling system using TlLhpI. When AbLhpI, CpLhpI, and PaLhpD were each used together with AbLhpH, an approximately 10-fold increase in activity was observed in AbLhpI: the system of AbLhpH(10)/AbLhpI(1) achieved similar activity to that of AbLhpH(10)/CpLhpI(10) (or AbLhpH(10)/PaLhpD(10)). These results have been attributed to differences in the activities of LhpI proteins: the $V_{\max}/K_m$ value of AbLhpI was more than 40-fold higher than those of CpLhpI and PaLhpD (Table 2). The optimum temperature and pH for the system of AbLhpH(10)/AbLhpI(1) were 30~40 °C and pH 8.0, respectively. The optimum pH of the (extremely thermophilic) TlLhpH/TlLhpI system was pH 7.0~8.5, whereas its optimum temperature was clearly higher than that of (mesophilic) AbLhpH/AbLhpI; the assay could not be performed above 70 °C due to the thermolability of NADH (data not shown). The activity achieved using TlLhpH(1)/TlLhpI(1) at 70 °C was similar to that using AbLhpH(10)/AbLhpI(1) (at 30 °C), while no significant activity was found at 30 °C. Therefore, we optimized the combination of T3LHypD and Pyr2CR to AbLhpH(10)/AbLhpI(1), and an assay was carried out at 30 °C.

3.5. Comparison of enzymatic and HPLC methods

A calibration curve (Lineweaver-Burk plot) for T4LHyp was successfully established using AbLhpA(10)/AbLhpBEF(10) or AbLhpA(10)/AbLhpB(10) as a coupling system (referred to as CC4_{BEF} and CC4_B, respectively) with ranges between 0.004~0.2 mM ($R^2 = 0.998$) and 0.04~1 mM ($R^2 = 0.999$), respectively (Fig. 3A, B) (these were values in the reaction mixture, whereas the initial contents in the sample were 10-fold higher because of dilution (see the Materials and methods section)). Differences in detectable ranges between CC4_{BEF} and CC4_B were due to the high affinity of AbLhpBEF for C4DHyp (Table 1), allowing the quantification of T4LHyp at a broader range of concentration than that using either CC4_{BEF} or CC4_B. A calibration curve for T3LHyp was developed using AbLhpH(10)/AbLhpI(1) as the coupling system (referred to as CC3_{Ab}): range between 0.05~1 mM ($R^2 = 0.998$) (Fig. 3C).

The concentrations of several diluted samples of T4LHyp (samples 1~5 in Table 3) or T3LHyp (samples 6~9) were estimated using CC4_{BEF}, CC4_B, or CC3_{Ab}, whereas actual concentrations were determined by HPLC. The values of the two highest T4LHyp samples, 1 and 2 were not determined using CC4_{BEF}, which may have been due to the saturated reaction. A good linear relationship was obtained between the enzymatic and HPLC methods (Fig. S1), although T3LHyp values by the enzymatic method were slightly lower than those by HPLC. We then measured several diluted solutions (samples 10~13), in which T4LHyp and T3LHyp co-existed, using CC4_{BEF} and CC3_{Ab}. The results obtained showed that neither L-Hyp had any significant impact on estimating the contents of other L-Hyp.

3.6. Enzymatic detection of T4LHyp in the acid-hydrolysate of collagen

T4LHyp is more common in collagen than T3LHyp. Although T3LHyp is particularly abundant in collagen IV (up to 10% of the total L-Hyp content), collagen that contained a significant amount of T3LHyp was not available. Therefore, we used normal collagen (containing T4LHyp only) as a biological samples. In the typical acid-hydrolysate by 6 N HCl, the molar ratio of glycine, L-proline, L-alanine, and T4LHyp (the four main amino acid components) was 3.4:1.4:1.3:1.1, while the molar rate of T4LHyp to total amino acids was 0.11 (Fig. 4A, inset). Since it currently remains unclear whether the T4LHyp content(s) in the sample were within the range of CC4_{BEF}, several diluted solutions of the acid-hydrolysate were prepared, whereas the actual concentrations were determined by HPLC using no diluted samples. A good linear relationship was found between the enzymatic and HPLC methods as well as standard

solutions of T4LHyp (described above) (Fig. 4A). Furthermore, the dilution of samples had no significant effect: for example, 1.05, 1.09, and 1.17 mM for samples diluted 5-, 10-, and 20-fold, respectively, which was similar to 1.01 mM with the HPLC method (sample No. 4). These results suggest that other L-amino acids in the acid-hydrolysate of collagen did not significantly impact on the estimation of T4LHyp contents, thereby confirming the significantly high specificities of HypE and HypDH for T4LHyp and C4DHyp, respectively.

As described in the Introduction section, T4LHyp is important as a biomarker in blood plasma and urine. Blood plasma (one sample) was analyzed in the present study because of the successful detection of T4LHyp by HPLC. A rapid increase in absorbance was initially detected even when no enzyme was added, suggesting the presence of a compound(s) that reacts with PMS-INT in blood plasma (Fig. 4B). Therefore, we started to measure the slope at 5-min time intervals from the addition of blood plasma to the reaction mixture. The (almost linear) activity slope with the HypE/HypDH continuous reaction was significantly higher than that without the enzyme, and the concentration of T4LHyp could be estimated as 23.9 μ M from their differences using CC4_{BEF}, which was similar to the value estimated by HPLC (23.3 μ M). These results suggested that the enzymatic method was also applicable to clinical laboratory tests.

Ito et al. (1985) attempted to estimate free T4LHyp enzymatically in tissue hydrolysates using a similar strategy to this study, in which unpurified HypE and commercially available D-amino acid oxidase were used. Although the continuous reaction was spectrophotometrically monitored by a colorimetric assay according to the H₂O₂ subsequently produced, the procedure may need ~40 min for a complete analysis and only be suitable for a high content of T4LHyp in acid-hydrolysates (detectable range between 4 and 50 mM). Furthermore, T3LHyp must be undetectable by this method because HypE cannot utilize T3LHyp as a substrate. In the present study, we developed an enzymatic method capable of separately detecting T4LHyp and T3LHyp not only in standard solution, but also in tissue hydrolysates, with markedly higher sensitivity using the metabolic enzymes of bacteria: in particular, no oxidase activity in HypDH should be significantly important, compared with (commercially available) D-amino acid oxidase. This rapid and convenient method may have further advantages for clinical diagnoses using a microplate reader, as described above. These lines of study are in progress in our laboratory.

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Figure legends

Figure 1. Metabolic pathways of T4LHyp (A) and T3LHyp (B) by organisms. The former was only operative in bacteria, whereas the latter was operative in mammals, bacteria, and archaea. Among the metabolic enzymes involved in each pathway, the coupling systems of HypE/HypDH and T3LHypD/Pyr2CR could be utilized to estimate T4LHyp and T3LHyp, respectively. (C) SDS-PAGE of AbHypDH with 10% (w/v) gel. Lanes 1 and 3, cell-free extracts of *E. coli* expressing AbLhpB and AbLhpBEF, respectively (50 μ g); lanes 2 and 4, purified AbLhpB (10 μ g) and AbLhpBEF (30 μ g), respectively. (D) SDS-PAGE of purified AbLhpA (lane 1), HsLhpH (lane 2), AbLhpH (lane 3), CpLhpH (lane 4), TlLhpH (lane 5), PaLhpD (lane 6), AbLhpI (lane 7), CpLhpI (lane 8), and TlLhpI (lane 9), respectively. Five micrograms of purified protein was applied to a 12% (w/v) SDS-PAGE gel.

Figure 2. Characterization of AbLhypDH. (A) Absorption spectra. Blue, AbLhpB; red, AbLhpE; violet, AbLhpF; orange, AbLhpBE; green, AbLhpEF; light green, AbLhpBEF. A 5 mg/ml protein solution (photograph is inset) was used. (B) HPLC analysis of prosthetic groups. Prosthetic groups from HypDH proteins were extracted as described previously (Watanabe et al., 2012). (C) Thermal inactivation of the AbLhpB (triangle), AbLhpBE (square), and AbLhpBEF proteins (circle). Each purified protein was dialyzed against 50 mM Tris-HCl (pH 8) for 2 h at 4 °C to remove glycerol. Enzyme activity was determined after an incubation for 10 min at each temperature, and is shown as a relative value (means $\pm$ SD, n = 3) expressed as a percentage of the controls without the heat treatment.

Figure 3. Calibration curves for T4LHyp (A) and T3LHyp (B) by an enzymatic method using coupling systems of AbLhpA/AbLhpBEF (left panel in A), AbLhypA/AbLhpB (light panel in A), and AbLhpH/AbLhpI. The Y-axis is the inverse number of the activity slope (initial change in the absorbance).

Figure 4. Enzymatic detection of T4LHyp in biological samples. (A) Collagen. The acid-hydrolysate (10 ml) was diluted 5- (yellow bar), 10- (light green bar), and 20-fold (blue bar) with water, and the content of T4LHyp was estimated enzymatically. The red bar indicates the actual concentrations determined by HPLC using non-diluted solution. (B) Blood plasma. A non-deproteinized sample (100 μ l) was directly added to the reaction mixture in the presence (red line) or absence of enzyme(s) (black line). Since a rapid increase in absorbance was initially detected even when no enzyme was added, the content of T4LHyp was calculated from the differences in these slopes (arrow).

1 **Table 1. Kinetic parameters of AbLhpB, AbLhpBE, and AbLhpBEF.**

Protein	Substrate	Specific activity ^a (unit·mg ⁻¹)	K_m (mM)	V_{max} (unit·mg ⁻¹)	V_{max}/K_m (unit·mg ⁻¹ ·mM ⁻¹)
AbLhpB	C4DHyp	2.69	1.31	3.65	2.78
	T4DHyp	0.0808	34.0	0.382	0.0112
	D-Proline	0.0258	6.87	0.0390	0.00571
AbLhpBE	C4DHyp	2.90	0.219	4.64	21.2
	T4DHyp	0.606	17.2	1.93	0.113
	D-Proline	0.220	32.1	1.01	0.0315
AbLhpBEF	C4DHyp	2.98	0.0628	6.74	107.5
	T4DHyp	3.20	8.14	7.06	0.869
	D-Proline	1.02	22.4	3.25	0.146

2 ^a Under standard assay conditions in the Materials and methods.

3

4

Table 2. Kinetic parameters of several T3LHypD and Pyr2CR enzymes.

Enzymes		Temperature ^b (°C)	Specific activity ^c (unit·mg ⁻¹)	K_m (mM)	V_{max} (unit·mg ⁻¹)	V_{max}/K_m (unit·mg ⁻¹ ·mM ⁻¹)
T3LHypD ^a	AbLhpH	30	26.1	14.3	59.2	4.15
	CpLhpH	30	25.4	2.79	32.9	11.8
	HsLhpH	30	7.37	9.34	13.9	1.49
	TiLhpH	50	44.6	0.298	42.6	145
Pyr2CR	AbLhpI	30	584	0.628	936	1507
	CpLhpI	30	30.2	5.90	206	35.0
	PaLhpD	30	42.3	0.447	66.4	149
	TiLhpI	50	23.9	0.944	26.9	28.6

^a Coupling assay using the PaLhpD (for AbLhpH, CpLhpH and HsLhpH) or TiLhpI (for TiLhpH) protein.

^b Assay temperature.

^c Under standard assay conditions in the Materials and methods.

Table 3. Comparison of enzymatic and HPLC methods for the estimation of L-Hyp.

Sample No.	T4LHyp (mM) ^a			T3LHyp (mM) ^a	
Methods	Enzyme		HPLC	CC3 _{Ab}	HPLC
	CC4 _{BEF}	CC4 _B			
1	— ^b	6.74	7.00	0	0
2	— ^b	3.50	3.80	0	0
3	1.64	1.31	1.50	0	0
4	0.49	0.50	0.50	0	0
5	0.28	0.32	0.30	0	0
6	0	0	0	3.14	3.42
7	0	0	0	1.92	2.15
8	0	0	0	0.98	1.19
9	0	0	0	0.66	0.71
10	0.14	— ^c	0.13	0.83	0.85
11	0.50	— ^c	0.50	1.20	1.06
12	0.18	— ^c	0.18	0.86	0.96
13	0.33	— ^c	0.33	1.79	1.92

^a Initial content of the sample, but not the reaction mixture (10-fold dilution).

^b Not determined due to the saturated reaction.

^c Only CC4_{BEF} was used as a calibration curve.

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Watanabe et al. Figure 2

Watanabe et al. Figure 3

Watanabe et al. Figure 4