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ORIGINAL ARTICLE

Detection of D-amino acids in purified proteins synthesized in *Escherichia coli*

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Abstract It has long been believed that amino acids comprising proteins of all living organisms are only of the L-configuration, except for Gly. However, peptidyl D-amino acids were observed in hydrolysates of soluble high molecular weight fractions extracted from cells or tissues of various organisms. This strongly suggests that significant amounts of D-amino acids are naturally present in usual proteins. Thus we analyzed the D-amino acid contents of His-tag-purified β-galactosidase and human urocortin, which were synthesized by *Escherichia coli* grown in controlled synthetic media. After acidic hydrolysis for various times at 110°C, samples were derivatized with 4-fluoro-7-nitro-2, 1, 3-benzoxadiazole (NBD-F) and separated on a reverse-phase column followed by a chiral column into D- and L-enantiomers. The contents of D-enantiomers of Ala, Leu, Phe, Val, Asp, and Glu were determined by plotting index D/(D + L) against the incubation time for hydrolysis and extrapolating the linear regression line to 0 h to eliminate the effect of racemization of amino acids during the incubation. Significant contents of D-amino acids were reproducibly detected, the D-amino acid profile being specific to an individual protein. This finding indicated the likelihood that D-amino acids are in fact present in the purified proteins. On the other hand, the D-amino acid contents of proteins were hardly influenced by the addition of D- or L-amino acids to the cultivation medium, whereas intracellular free D-amino acids sensitively varied according to the extracellular conditions. The origin of these D-amino acids detected in proteins was discussed.

Keywords D-amino acid · Chirality · *Escherichia coli* · β-galactosidase · Urocortin · Acidic hydrolysis

Abbreviations

NBD-F	4-fluoro-7-nitro-2,1,3-benzoxadiazole
HPLC	High performance liquid chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside

Introduction

Biomolecular homochirality has been solidly believed in, i.e., natural amino acids, other than Gly, comprising all living organisms are only of the L-configuration with a few exceptions in, e.g., eubacterial cell wall peptidoglycans and non-ribosomally synthesized peptidic antibiotics. However, recent studies have shown an unexpectedly wide distribution of free D-amino acids in a variety of organisms. In particular, free D-Asp and D-Ser are found in mammalian tissues, and their physiological roles are being investigated (Konno et al. 2007), that is, D-Asp regulates hormonal secretion in various endocrine organs (D'Aniello 2007; Homma 2007) and D-Ser is involved in neural transmission (Mustafa et al. 2004; Nishikawa 2005).

In addition, reports are increasing on peptidyl D-amino acids. D-Asp, as well as D/L-isoAsp, is found in lens α-crystallin from cataract patients and in amyloid β-peptide from Alzheimer's patients' brains (Fujii et al. 1994; Roher et al. 1993). The origin of such peptidyl D-amino acids has been

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explained by non-enzymatic isomerization associated with aging or diseases (Fujii et al. 1999). On the other hand, peptidyl D-amino acids were found in a funnel spider venom, ω-agatoxin (position 46, D-Ser), a cone shell venom, conotoxin (position 44, D-Phe), and a platypus venom, DLP-2 (position 2, D-Met), and so on. These D-amino acid residues were shown to be due to specific isomerases that act on each precursor translation product (Heck et al. 1994; Buczek et al. 2005; Whittington et al. 2009).

Nagata et al. (1998) detected D-amino acids in hydrolysates of soluble high molecular weight fractions extracted from cells or tissues of a wide range of organisms including eubacteria, archaea, fungi, plants, and animals. This report gave us the idea that significant amounts of D-amino acids are, counter to common sense, naturally present in usual proteins. If bacterial proteins generally contain D-amino acids, they cannot be explained by aging due to their fast turnover rates. To verify this, it is necessary to directly analyze individual purified proteins excluding the effect of possible racemization during hydrolysis.

For this purpose, in the present study, we arbitrarily chose two proteins to analyze; *Escherichia coli* β-galactosidase composed of 1023 amino acids per subunit of a tetramer and human urocortin, a neuropeptide belonging to the sauvagine/corticotropin-releasing factor/urotensin I family, composed of 40 amino acids, both of which were synthesized in *E. coli*. We suspected that an enzyme molecule containing D-amino acids, if any, may more or less have impaired molecular activity, and may tend to be lost during usual purification procedures in which fractions exhibiting lower specific activities are discarded. Thus we performed affinity chromatography involving histidine purification tags without monitoring the specific activities of the proteins of interest.

We here examined whether or not D-amino acid residues (Ala, Leu, Phe, Val, Asp, and Glu) are significantly detected in proteins purified from *E. coli* on orthodox amino acid analysis after hydrolysis in HCl. These six D-amino acids were easily distinguishable according to chirality with our system. We demonstrate that D-amino acids are in fact detected reproducibly after careful cancelling of the effect of racemization during acid hydrolysis. We further discussed the possibility that the intracellular conditions of amino acids influence the final contents of D-amino acids in purified proteins.

Materials and methods

E. coli strains and chemicals

E. coli strains MG1655 (provided by Dr. A. Imaizumi, Ajinomoto Co., Inc.) and BL21 (DE3) (purchased from Novagen, USA) were cultured in L-broth (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, and 0.5% NaCl) or M9 minimum medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.1% NH_4Cl, 0.05% NaCl, 1 mM $MgSO_4$, and 0.1 mM $CaCl_2$), containing either 0.4% glucose or 0.4% galactose at 37°C.

L-asparagine and glycine were purchased from Wako Pure Chemical Industries (Osaka, Japan), and D-glutamine was from Sigma (St. Louis, MO, USA); other D- and L-amino acids were from Peptide Institute Inc. (Osaka, Japan). β-galactosidase (II) was obtained from Toyobo Co., Ltd. (Osaka, Japan). Chemically synthesized urocortin (human) was purchased from Peptide Institute Inc. 4-Fluoro-7-nitro-2, 1, 3-benzoxadiazole (NBD-F) was obtained from Dojindo Laboratories (Kumamoto, Japan). Other chemicals were of the highest grade or HPLC grade available from commercial sources.

The mixture of 20 amino acids, with a total concentration of 1%, was prepared according to the amino acid occurrence frequency in total proteins encoded by the *E. coli* genome (Table 1), and sterilized with a 0.22 μm membrane filter (Millipore, Billerica, MA, USA). This mixture was added to M9 medium to a final concentration of 0.5%. "L20" denotes a mixture of 19 L-amino acids and Gly. "D(DNEQ) + L16" is a mixture of D-Asp, D-Asn, D-Glu, D-Gln, Gly, and 15 other L-amino acids. "D(ASVLF) + L15" is a mixture of D-Ala, D-Ser, D-Val, D-Leu, D-Phe, Gly, and 14 other L-amino acids. Mixtures

Table 1 D- and L-amino acids added to media

Amino acid	Final concentration (mM)
Ala	5.32
Arg	1.31
Cys	0.33
His	0.54
Lys	1.20
Met	0.93
Pro	1.92
Ser	2.76
Thr	2.26
Asp	1.92
Glu	1.94
Asn	1.31
Gln	1.51
Ile	2.28
Leu	4.05
Phe	1.18
Trp	0.37
Tyr	0.70
Val	3.02
Gly	4.88

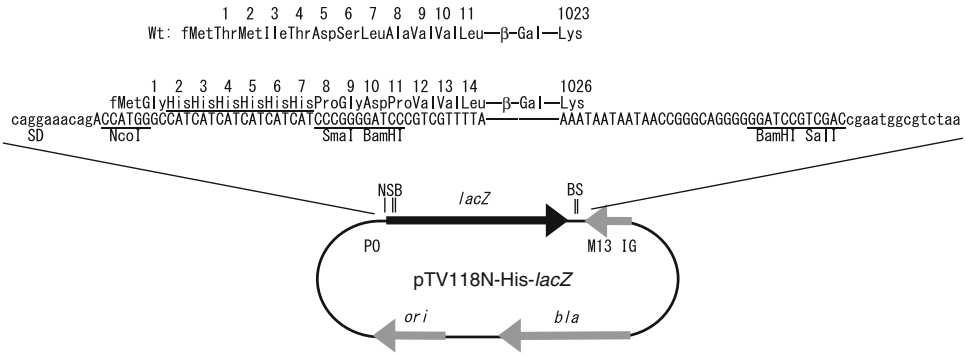

Fig. 1 Structure of pTV118N-His-*lacZ*. DNA sequences derived from pTV118N are depicted in lower case letters. The His-tagged *β*-galactosidase encoded by the plasmid is shown with amino acid numbers and aligned with the wild type sequence

"D(DNEQ)" and "D(ASVLF)", devoid of L-amino acids and Gly, contain the same amounts of the indicated four and five D-amino acids as "D(DNEQ) + L16" and "D(ASVLF) + L15", respectively.

Production and purification of *β*-galactosidase

The *Sma*I-*Sal*I fragment of pMC1871 (Casadaban et al. 1983), encoding almost the whole *lacZ* gene, was recloned into pTV118N between the *Sma*I and *Sal*I sites, newly generated in such a way that the α-peptide-coding segment of pTV118N was replaced by the whole *lacZ* gene. Then the *Nco*I-*Sma*I fragment at the 5′ end of the *lacZ* gene was exchanged with a synthetic DNA, CATGGGCCATCAT CATCATCATCATCCC, to create a $(His)_6$-tag at the *N*-terminus of *β*-galactosidase. Thus the *N*-terminal eight amino acids of the original *β*-galactosidase, Thr-Met-Ile-Thr-Asp-Ser-Leu-Ala, should be replaced by 11 amino acids, Gly-$(His)_6$-Pro-Gly-Asp-Pro, in the *β*-galactosidase produced by pTV118N-His-*lacZ* (Fig. 1).

The *lacI* gene with its own promoter was PCR amplified from the *E. coli* W3110 genome with the addition of *Cla*I and *Hind*III sites at the 5′ and 3′ ends, respectively, and introduced between the *Cla*I and *Hind*III sites of pACYC184, giving rise to pACYC184-*lacI*.

MG1655 cells carrying both pTV118 N-His-*lacZ* and pACYC184-*lacI* were grown at 37°C for 12 h in L-broth medium containing 100 μg/ml ampicillin and 30 μg/ml chloramphenicol. These two drugs were also present in the following media. Cells were collected by centrifugation, washed twice with M9 minimum medium containing 0.4% glucose and resuspended in 100 ml of the same medium. After cultivation at 37°C to $OD_{660} = 0.6–0.8$, cells were collected and resuspended in 400 ml of M9 medium containing 0.4% galactose for further cultivation. When OD_{660} reached 0.5, 1 mM IPTG was added to induce expression of the His-tagged *lacZ* and cells were grown at 37°C for an additional 3–12 h in M9 minimum medium containing 0.4% galactose and supplemented with an amino acid mixture.

The cells producing the *N*-terminal His-tagged *β*-galactosidase were ultrasonicated in binding buffer

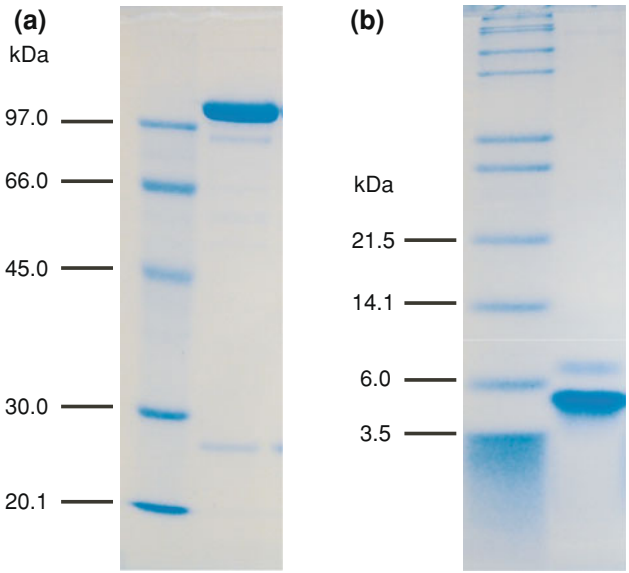

Fig. 2 SDS-polyacrylamide gel electrophoresis of His-tag purified *β*-galactosidase (**a**) and urocortin (**b**), which were prepared from *E. coli* and stained with Coomassie G250. Gel concentrations, 10% (**a**) and 15% (**b**)

comprising 20 mM potassium phosphate, pH 7.4, 500 mM NaCl. Imidazole, pH 7.0, was added to a final concentration of 100 mM, and then the mixture was applied to a His SpinTrap (GE Healthcare Bio-Science Corp., Piscataway, NJ, USA). After washing with 20 mM potassium phosphate, pH 7.4, 500 mM NaCl containing 100 mM imidazole, His-tagged *β*-galactosidase was eluted with 500 mM imidazole, desalted with 20 mM potassium phosphate, pH 7.4, and concentrated with a VIVASPIN 500 (Vivascience AG, Hannover, Germany) (Fig. 2a).

Production and purification of urocortin

Human urocortin has a sequence composed of 40 amino acids, DNPSLSIDLTFHLLRTLLELARTQSQRERAEQ NRIIFDSV. A pair of single-stranded DNAs, 5′-GACAAC CCGTCTCTGTCTATCGACCTGACCTTCCACCTGCT GCGTACCCTGCTGGAACTGGCGCGTAC-3′ and 5′-AA CAGAGTCGAAGATGATACGGTTCTGTTCCGCACGT

TCACGCTGAGACTGGGTACGCGCCAGTTCC-3′, were annealed each other with the underlined nucleotides and then incubated with Klenow fragment to generate a double-stranded DNA encoding human urocortin and consisting of codons optimized as to the expression in *E. coli*. Then the DNA fragment was PCR amplified with the forward primer 5′-GGGAATTCATCATATGGACAACCCGTCTCTGTC TATC-3′ to introduce an *Nde*I site (underlined) containing the initiation codon and the reverse primer 5′-AATCTC GAGTTAATGATGATGATGATGATGAACAGAGTCG AAGATGATACG-3′ to introduce a *C*-terminal $(His)_6$-tag and an *Xho*I site (underlined). The amplified fragment was cloned between the *Nde*I and *Xho*I sites of pET43.1a(+), giving rise to pET43a-urocortin-His. *E. coli* BL21 (DE3) carrying this plasmid was grown at 37°C for about 12 h in L-broth containing ampicillin (100 μg/ml), which was also present in the following media. After 12 h cultivation, cells were collected, washed twice, and grown in 100 ml of M9 medium containing 0.4% glucose to OD_{660} of 0.5. Cells were collected and resuspended in 400 ml of M9 medium containing 0.4% glucose for further cultivation. When OD_{660} reached 0.6–0.8, IPTG was added to 0.4 mM to induce the production of urocortin. The cells were grown at 37°C for 6 h in M9 minimum medium (0.4% glucose) supplemented with amino acids.

The *C*-terminal His-tagged urocortin was purified as in the case of *β*-galactosidase, except that the washing buffer comprised 20 mM potassium phosphate, pH 7.4, 500 mM NaCl, 50 mM imidazole, and 6 M urea, and that the elution buffer contained 300 mM imidazole. Desalting and concentration of the protein were carried out with Sep-Pak C18 Cartridges (Waters, Milford, MA, USA). The solution was freeze-dried and dissolved in miliQ water (Fig. 2b).

Hydrolysis and determination of D- and L-amino acids

β-galactosidase (10 μg) and urocortin (5 μg) were hydrolyzed with a Pico Tag™ workstation (Waters) for 3, 6, 16, and 24 h at 110°C under 6 M HCl vapor. In this hydrolysis reaction, Asn and Gln are converted to Asp and Glu, respectively. Dried-up samples were dissolved in 50 mM borate buffer, pH 9.5. A 20 μl aliquot of each sample was mixed with 10 μl of 50 mM NBD-F in dry acetonitrile, followed by incubation for 5 min at 60°C, which was terminated by the addition of 1% trifluoroacetic acid. The resultant fluorescent NBD-derivatives were filtered through a 0.45 μm filter (Millex-LH; Millipore) and separated on a TSK-gel ODS-80Ts column (250 × 4.6 mm i.d.; Tosoh, Tokyo, Japan) maintained at 33°C by HPLC as previously described (Hamase et al. 1997). The isolated NBD-amino acids (including the D- and L-isomers of individual amino acids) were further separated into D- and L-enantiomers on a Sumichiral OA-3100 column (250 × 4.6 mm i.d.; Sumika Chemical Analysis Service, Osaka, Japan) maintained at 35°C by isocratic elution with 3 mM citric acid in methanol. In the case of the separation of NBD-derivatives of Asp and Glu, a column switching HPLC system was employed as described in a previous report (Long et al. 2001); in the first reverse-phase mode, Mightysil RP-8 GP (150 × 4.6 mm i.d.; Kanto Chemical Co., Inc., Tokyo, Japan) or TSK-gel Octyl-80Ts (150 × 4.6 mm i.d.; Tosoh) was used, and in the second normal phase mode for enantioseparation, Sumichiral OA-3200 (250 × 4.6 mm i.d.; Sumika Chemical Analysis Service) and Sumichiral OA-3100 (250 × 4.6 mm i.d.) were used for Asp- and Glu-derivatives, respectively.

The amounts of the D- and L-enantiomers of each amino acid were determined based on the peak heights in chromatograms. The D-amino acid ratios at each time point during the hydrolysis reaction were calculated as (amount of D-amino acid)/(amount of D-amino acid + amount of L-amino acid: (D/(D + L))). The content of the D-enantiomer of a certain amino acid in a protein concerned was determined by plotting each ratio against the reaction time for hydrolysis, and extrapolating the linear regression line to 0 h. Data were expressed as the mean values for independent experiments, $n = 2$, 4 or 6.

Determination of free D-amino acids in the *E. coli* cytosol

E. coli BL21 (DE3) cells grown at 37°C to $OD_{660} = 0.6$ in 50 ml of M9 medium supplemented with D- or L-amino acids were harvested, washed twice with PBS, resuspended in 750 μl PBS, and then disrupted by ultrasonication. A 200 μl aliquot of the supernatant obtained on centrifugation for 5 min at 15000 rpm at 4°C was mixed with 800 μl methanol and then incubated overnight at −20°C. The supernatant obtained on centrifugation for 10 min at 15000 rpm at 4°C was recovered as the intracellular free amino acid fraction.

A 200 μl aliquot of the free amino acid fraction was dried up and dissolved in 40 μl of 50 mM borate buffer, pH 9.5, mixed with 30 μl of 15 mM NBD-F in dry acetonitrile, and then incubated for 5 min at 60°C. The analytical procedures for D-amino acid contents were as described for protein hydrolysates.

Results

D-amino acid contents of authentic L-amino acid samples

The D-amino acid contents of commercially available L-Ala, L-Ser, L-Leu, L-Phe, L-Val, L-Asp, and L-Glu samples were determined as a mock experiment before the

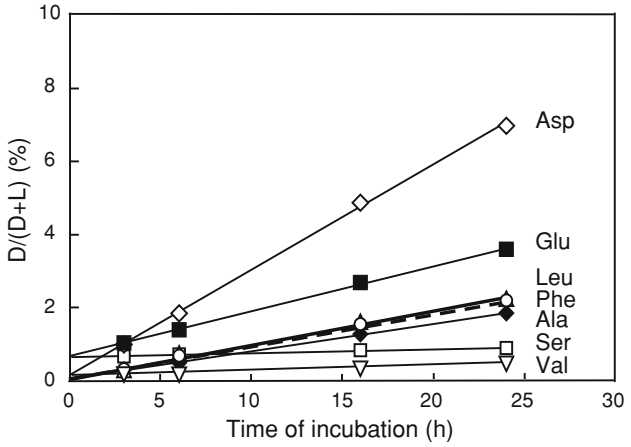

Fig. 3 Time-dependent racemization of free L-amino acids during the hydrolytic incubation. Samples were incubated for 3, 6, 16, and 24 h at 110°C under 6 M HCl vapor. The mean values for two independent experiments for Ala (*closed diamonds*), Ser (*open squares*), Leu (*triangles*), Phe (*open circles with a dotted line*), Val (*inverted triangles*), Asp (*open diamonds*), and Glu (*closed squares*) are plotted against incubation time with linear regression lines

following examination of protein hydrolysates. Incubation at 110°C under 6 M HCl vapor, which are the same conditions as those for protein hydrolysis, led to time-dependent racemization of these free L-amino acids (Fig. 3), and the original D-enantiomer content values obtained by extrapolating the incubation time to 0 h are shown in Table 2 as "Mock." The D-enantiomer contents of these commercially available samples were also determined without the incubation at 110°C under 6 M HCl vapor, and are shown in Table 2 as "Measurement." The D-enantiomer content values (D/(D + L)) determined as "Mock" and "Measurement" were close or similar for most "L-amino acid" samples. Although the L-Glu sample showed an exceptionally high value of 0.65%, measurement of the sample without hydrolysis gave the relatively close value of 0.63%. In contrast, the L-Asp sample showed an extrapolation value of 0.12% but an undetectable D-Asp content without hydrolysis. Thus, with this extrapolation method together with the acid hydrolytic reaction, free L-amino acids generate D-amino acid contents of no more than approximately 0.1% during the hydrolysis treatment performed.

Free D-amino acids within *E. coli* cells

To consider the possibility that naturally occurring D-amino acids are directly incorporated on protein synthesis in *E. coli* cells, we first determined the levels of intracellular free D-amino acids (Table 3). The ratios of both D-Ala and D-Glu, required for the peptidoglycan synthesis, remained roughly comparable to their L-enantiomers in the cells grown in either M9 minimum medium or that

Table 2 D-amino acid contents of commercially available free L-amino acids

	D/(D + L) (%)						
	Ala	Ser	Leu	Phe	Val	Asp	Glu
Mock[a]	0.03	0.61	0.01	0.04	0.11	0.12	0.65
Measurement[b]	0.01	0.56	0	0.02	0.02	0	0.63

[a] The D-amino acid contents were determined by plotting each D-amino acid ratio against the reaction time at 110°C under 6 M HCl vapor and extrapolating the linear regression line to 0 h, as shown in Fig. 3. The mean values for two independent experiments are presented

[b] The D-amino acid contents were determined without incubation at 110°C under 6 M HCl vapor. The mean values for four independent experiments are presented. The details are given under "Materials and methods" section

Table 3 D-amino acid contents of the intracellular free amino acid fractions of *E. coli* BL21 (DE3)

Culture medium	D/(D + L) (%)						
	Ala	Ser	Leu	Phe	Val	Asp	Glu
M9	44	0.6	0.3	0	0	0.2	25
L20	52	1.3	0	0	0.3	0.2	38
D(ASLFV)	50	1.1	3.3	10	6.4	0.6	31
D(ASLFV) + L15	51	0.9	5.8	12	15	0.2	26
D(DNEQ) + L16	–[a]	–[a]	–[a]	–[a]	–[a]	3.0	83

[a] Not determined

supplemented with D- or L-amino acids. In contrast, the contents of other D-amino acids examined are usually very low, but the addition of D-amino acids to the medium increased the intracellular ratios of the corresponding D-amino acids as much as, in some cases, more than 10%. Similar results were obtained for intracellular D-amino acids in cells grown to the stationary phase in the presence of 1 mM IPTG (data not shown). These observations suggest easily changeable intracellular contents of D-amino acids depending on the extracellular conditions.

D-amino acid content of β-galactosidase

The *N*-terminal His-tagged β-galactosidase produced by *E. coli* MG1655 harboring pTV118 N-His-*lacZ* and pACYC184-*lacI* was purified with a Ni column (Fig. 2a). The contents of D-Ala, D-Leu, D-Phe, D-Val, D-Asp, and D-Glu of the purified β-galactosidase were determined, as shown in Fig. 4 and Table 4. The NBD derivatives of D-enantiomers were significantly and reproducibly detected for all amino acids tested, except for that of D-Ser, whose peak was unclear and undetectable due to the lower racemization rate during the hydrolytic reaction. Detection of

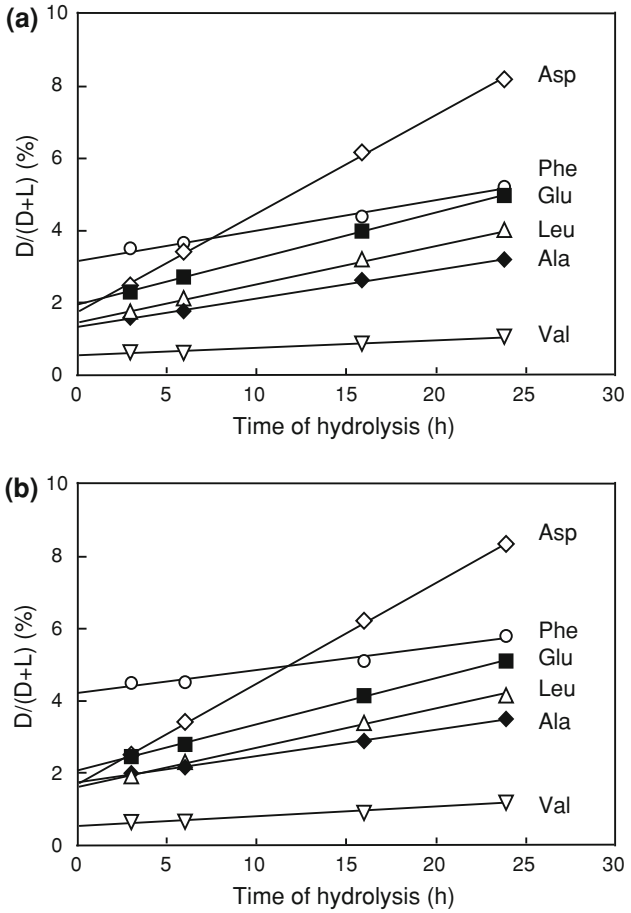

Fig. 4 D-amino acid ratios (D/(D + L)) during hydrolysis of the commercial purified β-galactosidase (**a**) and the His-tag purified β-galactosidase synthesized by *E. coli* (**b**). The mean values for four (**a**) and two (**b**) independent experiments for Ala (*closed diamonds*), Leu (*triangles*), Phe (*open circles*), Val (*inverted triangles*), Asp (*open diamonds*), and Glu (*closed squares*) are plotted against incubation time with linear regression lines. The details are as in Fig. 3

Table 4 D-amino acid contents of ß-galactosidase

Culture medium	D/(D + L) (%)					
	Ala	Leu	Phe	Val	Asp	Glu
Commercial[a]	1.34	1.47	3.18	0.57	1.75	1.96
M9	1.77	1.63	4.19	0.56	1.74	2.08
L20	1.80	1.64	3.82	0.55	1.41	2.00
D(ASLFV)	1.57	1.58	4.05	0.58	–[b]	–[b]
D(ASLFV) + L15	1.85	1.75	4.17	0.70	–[b]	–[b]
D(DNEQ)	–[b]	–[b]	–[b]	–[b]	1.88	2.21
D(DNEQ) + L16	–[b]	–[b]	–[b]	–[b]	1.53	2.03

The D-amino acid contents were determined as in Table 2 and Fig. 3

[a] A commercially obtained purified sample without a purification tag

[b] Not determined

The mean values for two independent experiments are shown. For the commercially obtained sample, the mean values for four independent experiments are shown

D-amino acids other than D-Ala and D-Glu suggests that they were not derived from peptidoglycan.

Furthermore, we examined the influence of changing the intracellular environment of amino acids on the contents of D-amino acids in synthesized proteins. β-galactosidase samples were purified from cells grown in M9 media without or supplemented with D- or L-amino acids, expecting that the addition of D-amino acids to the medium would increase their intracellular contents (Table 3), and finally increase the D-amino acids detected in the purified protein (Table 4). Contrary to our expectation, the levels of D-amino acids in β-galactosidase samples did not vary so much even between the samples with forced addition of D- and L-amino acids (Table 4).

A commercially available sample of purified β-galactosidase was also examined by the same method (Table 4). Similar values were obtained to those for the β-galactosidase samples purified by means of the His tag.

D-amino acid content of urocortin

The results of the analysis of β-galactosidase synthesized by *E. coli* suggest that proteins synthesized by *E. coli* generally contain D-amino acids. To confirm this, the D-amino acid contents were examined for another protein synthesized by *E. coli* and also for a chemically synthesized one as a biosynthesis control; we expected that different ways of synthesis lead to different patterns of the D-amino acid contents. Thus, *C*-terminal His-tagged human urocortin was synthesized using *E. coli* BL21 (DE3) cells grown in M9 minimum medium (0.4% glucose). Since this urocortin was insoluble, 6 M urea was added for solubilization in the purification step (Fig. 2b). D-Ala, D-Leu, D-Phe, D-Val, D-Asp, and D-Glu were also significantly detected in purified urocortin (Fig. 5 and Table 5). However, it was noted that the D-amino acid content profile was not similar to that of β-galactosidase. The addition of D-amino acids to the media did not influence the D-amino acid contents of urocortin, which was also the case for β-galactosidase (data not shown).

D-amino acids were also detected in chemically synthesized urocortin (Fig. 5 and Table 5). The D-Ala and D-Val contents of the urocortin synthesized by *E. coli* were significantly greater than those of the chemically synthesized urocortin ($P < 0.001$), whereas those of D-Leu and D-Glu of the chemically synthesized one were significantly greater ($P < 0.05$). The D-Phe and D-Asp contents showed no difference between the two urocortin samples.

Discussion

In this study, we detected significant amounts of D-amino acids in acid hydrolysates of two proteins, *E. coli*

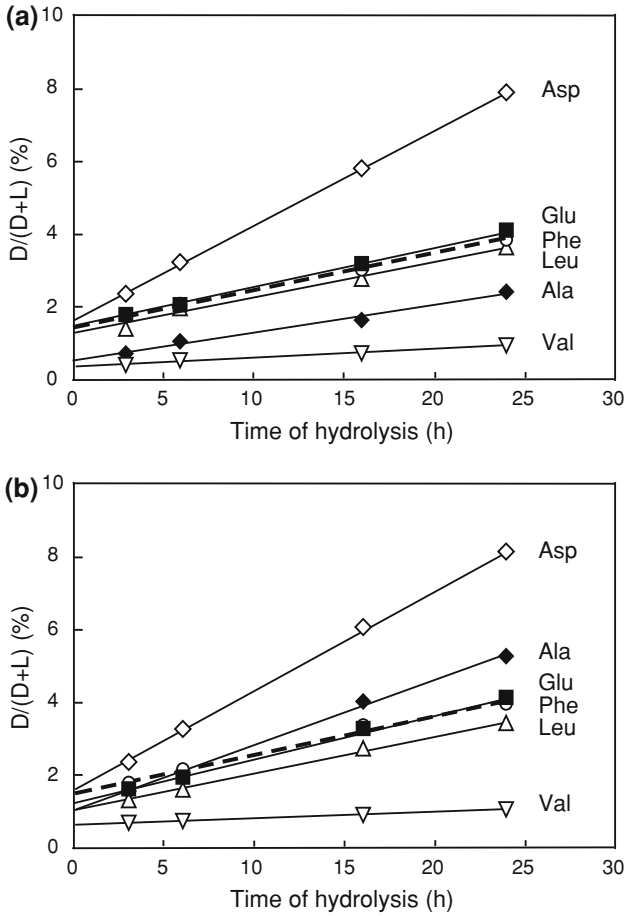

Fig. 5 D-amino acid ratios (D/(D + L)) during hydrolysis of the urocortin synthesized chemically (**a**) and the urocortin synthesized by *E. coli* BL21 (DE3) (**b**). The mean values for six independent experiments are plotted. The symbols are the same as in Fig. 4

β-galactosidase and human urocortin, which were produced by and purified from *E. coli*. The mock experiment with free L-amino acids, as a control, showed that extrapolation of the linear regression line of index D/(D + L) to 0 h rationally cancelled the effect of racemization of free amino acids during the incubation at 110°C under 6 M HCl vapor, mimicking hydrolysis, making the application of this method to the estimation of innate D-amino acids in protein hydrolyzates very credible (Table 2). The slope of regression lines corresponds to the rate of racemization of each amino acid during incubation. The slope for each free L-amino acid (Fig. 3) was almost identical to that found on protein hydrolysis (Figs. 4 and 5), and disagreement of D/(D + L) values of an amino acid among different samples can be attributed to their different *Y*-intercepts. This indicates that the effect of racemization of amino acids released on protein hydrolysis is in fact canceled by extrapolating the linear regression line to 0 h. In other words, the *Y*-intercept does not include the effect of racemization of the amino acid but mostly represents the D-amino acid contained in the original protein. Exceptionally, the slope of the regression line for Ala in the urocortin synthesized by *E. coli* was sharper than that for free L-Ala (Figs. 3 and 5), although this was not fully explained. One of our final interests is whether the "0 h" incubation samples exhibit the same D-amino acid values as the *Y*-intercepts. In the present experiments, however, shorter incubation times than 3 h for hydrolyses release smaller amounts of free amino acids, making it difficult to obtain reliable data for D-amino acid contents.

Two *β*-galactosidase samples, commercial purified and His-tag-purified, showed almost identical racemization profiles along with hydrolysis (Fig. 4) and gave very close intercepts for possible innate D-amino acid contents, except that D-Ala and D-Phe in the commercial sample seemed slightly lower (Table 4). At present, it is possible, but not yet convincing, that these decreased values for the commercial sample reflect the exclusion of enzyme molecules with higher D-amino acid contents by a usual purification procedure, as suspected at first. Moreover, it should be noted that the D-amino acid contents of His-tag-purified *β*-galactosidase and urocortin were quite different. In particular, the content of D-Phe was 4.19% for *β*-galactosidase and 1.42% for urocortin, both obtained from *E. coli* cells grown in M9 medium. It is thus concluded that the D-amino acid content profiles are specific to individual proteins. As a natural protein other than of the *E. coli* origin, a commercial sample of purified human serum albumin was examined. Again, we found significant amounts of D-amino acids, though the content pattern was different from those of *β*-galactosidase or urocortin (data not shown).

When the urocortin synthesized by *E. coli* was compared with the chemically synthesized form, statistically significant differences were observed in their D-amino acid contents (Table 5). Since chemical peptide synthesis cannot exclude the possibility of a low level of isomerization, the higher D-Leu and D-Glu values ($P < 0.05$) for the chemically synthesized sample may reflect some isomerization during chemical synthesis. On the other hand, the urocortin synthesized by *E. coli* exhibited higher D-Ala and D-Val values ($P < 0.001$). This observation constituted another line of evidence that at least the Ala and Val contents of the biologically synthesized urocortin do include D-enantiomer fractions specific to biosynthesis. However, as mentioned above, only the biosynthesized urocortin exhibited a sharper slope of the Ala regression line than other samples (Fig. 5b), which possibly added an unknown bias to the *Y*-intercept of Ala. Furthermore, urocortin has a single Val at the *C*-terminus, where the His-tag was connected in the biosynthesis construct. Thus the possibility remains that the value for the *C*-terminal Val is affected by this situation.

Table 5 Comparison of the D-amino acid contents of urocortin chemically synthesized and that synthesized by *E. coli*

	D/(D + L) (%)					
	Ala	Leu	Phe	Val	Asp	Glu
Chemically synthesized	0.49 ± 0.21	1.36 ± 0.32	1.45 ± 0.25	0.36 ± 0.06	1.59 ± 0.31	1.46 ± 0.31
Synthesized by *E. coli*	0.98 ± 0.18**	0.99 ± 0.11*	1.42 ± 0.14	0.58 ± 0.09**	1.53 ± 0.16	1.17 ± 0.13*

The D-amino acid contents were determined as in Table 2 and Fig. 3, and the mean values ± standard deviation for six independent experiments are presented

* $P < 0.05$, ** $P < 0.001$ (Student's *t*-test), as compared to the values for the chemically synthesized sample

If the D/(D + L) values extrapolated to 0 h reflect innate D-amino acids directly incorporated in the course of protein synthesis, the D-amino acid contents of proteins should reflect the intracellular amino acid status, i.e., the amounts of free D-amino acids, which will, in turn, vary according to their contents in the culture medium. Some tRNAs have been reported to be aminoacylated in vitro with cognate D-amino acids, although the reactivities were lower than for the corresponding L-amino acids (Calendar and Berg 1967; Soutourina et al. 2000; Takayama et al. 2005). The incorporation of D-amino acids into proteins was, in fact, suggested in a special situation (Dedkova et al. 2006). Thus we first examined the intracellular contents of free D-amino acids in *E. coli* and found that D-amino acids sensitively vary in response to the extracellular conditions (Table 3). At least, it cannot be said that D-amino acids are especially rare substances for bacteria since natural media contain significant amounts of D-amino acids (data not shown).

Contrary to our expectation, β-galactosidase synthesized by *E. coli* cultured in various media, including ones with supplementation of D- or L-amino acids, showed almost no significant differences in D-amino acid contents (Table 4). A similar result was observed for urocortin synthesized by *E. coli* (data not shown). Therefore, variable intracellular concentrations of D-amino acids are not reflected by the D-amino acid contents of the final protein. This finding suggests that the D-amino acids detected in proteins are not derived through direct incorporation of intracellular D-amino acids during translation.

Based on the present data, three explanations are possible for the origin of the D-amino acids detected in proteins purified from *E. coli* cells. (1) D-amino acids were incorporated into the nascent proteins in translation, (2) posttranslational epimerization of proteins led to D-amino acid residues, and (3) some unidentified artifact during the analytical step generated D-amino acids. We expected the first possibility, but we have also discussed contradictory observations above. Non-enzymatic epimerization of peptides yielding D-Asp and D-Asn has been demonstrated (Geiger and Clarke 1987). Similar isomerization may occur for Glu and Gln residues, but epimerization is not known for other amino acids in polypeptides. In addition, non-enzymatic epimerization of proteins usually requires several decades, which is not applicable to bacterial proteins. Specific enzymes are known to function in posttranslational epimerization to yield a biologically active peptide containing a D-amino acid (Heck et al. 1994; Buczek et al. 2005; Whittington et al. 2009). But to explain our present data, unrealistic numbers of epimerases have to be presumed. We consider that the racemization of amino acids released on the hydrolysis of proteins is cancelled out, as discussed above. Nonetheless, very fast epimerization cannot be excluded, in particular, in the beginning stage of hydrolysis when most samples remain as peptides. Considering these possibilities, we are now trying another approach to reveal the origin of D-amino acids detected in purified proteins.

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