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Attempt to simultaneously generate three chiral centers in 4-hydroxyisoleucine with microbial carbonyl reductases

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ABSTRACT

A panel of microorganisms was screened for selective reduction ability towards a racemic mixture of prochiral 2-amino-3-methyl-4-ketopentanoate (*rac*-AMKP). Several of the microorganisms tested produced greater than 0.5 mM 4-hydroxyisoleucine (HIL) from *rac*-AMKP, and the stereoselectivity of HIL formation was found to depend on the taxonomic category to which the microorganism belonged. The enzymes responsible for the AMKP-reducing activity, *Ap*AR and *Fs*AR, were identified from two of these microorganisms, *Aureobasidium pullulans* NBRC 4466 and *Fusarium solani* TG-2, respectively. Three AMKP reducing enzymes, *Ap*AR, *Fs*AR, and the previously reported *Bt*HILDH, were reacted with *rac*-AMKP, and each enzyme selectively produced a specific composition of HIL stereoisomers. The enzymes appeared to have different characteristics in recognition of the stereostructure of the substrate AMKP and in control of the 4-hydroxyl group configuration in the HIL product.

1. Introduction

The biocatalytic production of chiral compounds in the pharmaceutical, agrochemical, and fragrance industries has grown significantly in recent years. Two types of enzymatic methods are typically used for the generation of chiral compounds. The first method is the kinetic resolution of racemic compounds using enzymes able to recognize particular stereoisomers as their substrates.[1] The second method involves the asymmetric conversion of prochiral compounds using enzymes catalyzing stereospecific reactions.[2,3] Although each method has been utilized individually, less is known about the combination of the kinetic resolution and the asymmetric conversion in the production of chiral compounds from racemic prochiral substrates. If the combined methods were applied in chiral alcohol production, multiple chiral carbon centers could be simultaneously generated, and only the desired stereoisomer would be obtained from a racemic mixture of the prochiral ketone. In such a case, sophisticated carbonyl reductases with stereoselective features both of substrate recognition and carbonyl reduction would be required as the biocatalysts.

4-Hydroxyisoleucine (HIL) is a hydroxy amino acid originally found in the annual herbaceous plant, fenugreek (*Trigonella foenum-graecum* Leguminosae).[4] In HIL molecule, there are three chiral centers generating eight stereoisomers consisting of four pairs of enantiomers: (2*S*,3*S*,4*S*) and (2*R*,3*R*,4*R*)-HIL [*SSS*- and *RRR*-HIL]; (2*S*,3*S*,4*R*) and (2*R*,3*R*,4*S*)-HIL [*SSR*- and *RRS*-HIL]; (2*S*,3*R*,4*R*) and (2*R*,3*S*,4*S*)-HIL [*SRR*- and *RSS*-HIL]; and (2*S*,3*R*,4*S*) and (2*R*,3*S*,4*R*)-HIL [*SRS*- and *RSR*-HIL]. Of these, *SRS*-HIL is the major stereoisomer naturally present in fenugreek.[5] *SRS*-HIL was reported to have potential for the treatment of non-insulin-dependent type II diabetes mellitus, where it stimulated insulin secretion depending on plasma glucose level.[6] Besides *SRS*-HIL, non-natural stereoisomers were also reported to have antidiabetic activities.[7,8] Therefore, HIL stereoisomers are potential candidates for a novel class of antidiabetic agent acting on the essential dysfunctions of type II diabetes.

Recently, we reported that *Bt*HILDH, an NAD-dependent alcohol dehydrogenase derived from *Bacillus thuringiensis* 2e2, oxidized *SRS*-HIL into (2*S*,3*R*)-2-amino-3-methyl-4-ketopentanoate [*SR*-AMKP].[9] *Bt*HILDH also catalyzed the reverse carbonyl reducing reaction, or asymmetric formation of *SRS*-HIL from *SR*-AMKP; however the reactivity of *Bt*HILDH towards other AMKP stereoisomers,

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or its ability to produce other HIL stereoisomers, has not been examined.

In present study, we tested various microorganisms for their ability to react with a racemic mixture of prochiral AMKP (*rac*-AMKP), and observed a tendency to form HIL isomers depending on the microbial taxonomic category. Moreover, we successfully purified and identified two carbonyl reductases acting on AMKP from *Aureobasidium pullulans* NBRC 4466 and *Fusarium solani* TG-2. These NADPH-dependent AMKP reductases, *Ap*AR and *Fs*AR, were minutely estimated together with *Bt*HILDH as biocatalysts selectively producing specific HIL stereoisomers from *rac*-AMKP.

2. Materials and methods

2.1. Conditions for rapid amino acid analysis by achiral high-performance liquid chromatography (achiral HPLC)

Amino acids were derivatized using the AccQ-Tag Derivatization Kit (Waters, MA, USA) according to the manufacturer's instructions and analyzed with an Alliance 2695 HPLC system (Waters). An XBridge C_{18} column (5 μm, 2.1 × 150 mm; Waters) was used for separation at 40 °C. The mobile phases used were 10 mM ammonium acetate at pH 5.0 (eluent A) and methanol (eluent B), and the flow rate of the eluent was 0.3 ml/min. The eluent gradients were 0–1% (v/v) B for 0–0.5 min, 1–5% B for 0.5–18 min, 5–9% B for 18–19 min, 9–17% B for 19–29.5 min, 17–60% B for 29.5–40 min, and 60% B for 40–43 min. The AccQ-Tag derivatives were detected with a fluorescence detector (Ex. 250 nm, Em. 395 nm).

2.2. Microbial screening for reduction activity of AMKP

rac-AMKP was prepared according to the chemical synthesis previously reported.[10] Approximately 10 mg of air-dried cells of each strain preserved in our laboratory was added to 100 μl of the reaction mixture in a test tube (10 × 105 mm). The reaction mixture contained 35 mM *rac*-AMKP, 1 mM NADH, 1 mM NADPH, 250 mM d-glucose, and 15 U/ml glucose dehydrogenase, in 50 mM Tris-HCl buffer (pH 7.4). The reaction was carried out at 28 °C for 16 h with shaking (300 rpm). After 16 h, the reaction mixture was centrifuged at 1200×*g* for 15 min, and the supernatant was analyzed by achiral HPLC.

2.3. Construction of expression strains for AMKP reductases, ApAR and FsAR

Total RNA was prepared from *A. pullulans* NBRC 4466 and *F. solani* TG-2. Approximately 0.1 g of wet cells of each strain was added into a 2-ml stainless blender tube with a metal cone, pre-cooled in liquid nitrogen, and then disrupted at 1700 rpm for 10 s with a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). The total RNA fraction was extracted with ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. The cDNA was prepared from the total RNA using a PrimeScript RT-PCR Kit (Takara Bio, Shiga, Japan). The *Ap*AR gene was amplified from cDNA obtained from *A. pullulans* NBRC 4466 cells by PCR using the following primers: ACAGGATCCATGGACACCTCAAAAGCCATCAACCCTTCG and AGTAAGCTTTTAGTATGTCGCGGGCATCATGCTCAGGCG. The *Fs*AR gene was amplified from cDNA obtained from *F. solani* TG-2 cells by PCR using the following primers: ATAGGATCCATGGCTTCCGAAAACGCGAATAGCAGCGA and TATAAGCTTTCAATTGGCCGCAGGGAAATTGCCCAGACG. The PCR was carried out with Prime STAR polymerase (Takara Bio) under the following conditions: 30 cycles of 10 s at 98 °C, 10 s at 58 °C, and 1.5 min at 72 °C. The PCR product was digested with *Bam*HI and *Hin*dIII endonucleases, and cloned into the expression vector pQE80L (QIAGEN, CA, USA), which was digested with the same endonucleases. The resultant plasmids, pQE-*Ap*AR and pQE-*Fs*AR were transformed into *E. coli* JM109. A *Bt*HILDH-expressing strain, pET-*Bt*HILDH *E. coli* Rosetta2 (DE3), was constructed as previously reported.[9]

2.4. Expression and purification of recombinant AMKP-reducing enzymes

Each of the *E. coli* transformants carrying pQE-*Ap*AR, pQE-*Fs*AR, and pET-*Bt*HILDH were cultured at 28 °C in LB medium comprised of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (v/v) NaCl with the addition of appropriate antibiotics. At an OD_{600} = 1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and the cultures were incubated for 16 h at 28 °C with shaking at 300 rpm. The cell suspension (250 mL) was centrifuged at 3000 rpm for 10 min and the cell pellet was suspended in 10 ml of binding buffer containing 20 mM Tris-HCl buffer (pH 7.4), 0.5 M NaCl, 20 mM imidazole, and 1 mM DTT, and disrupted for 1 h by sonication with an Insonator 201 R (KUBOTA, Osaka, Japan). The lysate was centrifuged at 12,000*g* for 30 min, and the supernatant was filtered with a 0.45-μm Millex Syringe-driven Filter Unit (Millipore, MA, USA). The protein solution was applied to a Ni-Sepharose column (His Trap HP 5 ml; GE Healthcare Bioscience) equilibrated with binding buffer. The column was washed with binding buffer, and the proteins were eluted with a linear gradient of 0.02–0.50 M imidazole in binding buffer. The fractions containing the recombinant enzyme were pooled, concentrated by ultrafiltration and used as the purified enzyme. The specific activities of these enzymes towards *rac*-AMKP as a substrate were measured and expressed in μmol/min/mg (U/mg).

2.5. Simultaneous analysis of eight stereoisomers of HIL and four stereoisomers of AMKP by chiral HPLC

Amino acid solutions (0.5 mg/ml amino acids and 4 mg/ml triethylamine in 50% acetonitrile) were mixed in equal amounts with 2 mg/ml 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) in acetonitrile, and were reacted for 30 min at RT. The amino acid derivatives were analyzed using a LC-10A HPLC system (Shimadzu, Kyoto, Japan) equipped with a UV detector. The CAPCELL PAK C18 column (5 μm, 4.6 × 250 mm; Shiseido, Tokyo, Japan) was used for the separation at 45 °C. The mobile phases were 10 mM KH_2PO_4 (pH 2.8; eluent A) and acetonitrile (eluent B), and the flow rate of the eluent was 0.3 ml/min. The eluent gradients were 20–25% (v/v) B in 0–60 min and 25% (v/v) B in 60–70 min. HIL and AMKP diastereomers were kind gifts from Ajinomoto Co., Inc (Kawasaki, Japan).[11] The GITC-derivatives of stereoisomers were detected spectrophotometrically at 250 nm.

2.6. Reaction conditions for AMKP-reducing enzymes

The standard reaction conditions were as follows: 35 mM *rac*-AMKP, 1 mM NADH or 1 mM NADPH, and 0.1 U/ml *Ap*AR, *Fs*AR, or *Bt*HILDH in 50 mM Tris-HCl buffer (pH 7.4) at 28 °C. The enzyme activity was spectrophotometrically determined by measuring the decrease in the absorbance at 340 nm resulting from the oxidation of NAD(P)H. The amount of NAD(P)H was estimated using a molar absorption coefficient of $6.22 \times 10^3\ M^{-1}\cdot cm^{-1}$. The effect of pH on the activity was examined by varying the reaction pH between 3.0 and 10.0. The effect of temperature on the activity was examined by varying the reaction temperature between 10 °C and 70 °C. For substrate specificity analysis, various carbonyl compounds were used as the substrate in place of *rac*-AMKP. In order to produce HIL stereoisomers, 250 mM D-glucose and 15 U/ml glucose

dehydrogenase were added to the standard reaction mixture. Determination of HIL stereoisomers formed in the reaction mixture was performed by the chiral HPLC analysis.

3. Results

3.1. Survey of the AMKP-reducing enzyme activity of microorganisms

A total of 1093 microbial strains from our culture collection were analyzed for their ability to reduce AMKP into HIL. In 16 of the strains, including 2 strains of bacteria, 5 strains of ascomycetous yeasts, 7 strains of filamentous ascomycetes, and 2 strains of basidiomycetes, HIL production of greater than 0.5 mM from 35 mM *rac*-AMKP was observed in the reaction mixture (Table 1). These results indicated that the ability to reduce AMKP was widely distributed across various genera of microorganisms. Of these, four strains produced more than 5 mM of HIL (*Bacillus thuringiensis* NBRC 3951, *Sporobolomyces gracilis* NBRC 1033, *Aureobasidium pullulans* NBRC 4466, and *Crinipellis stipitaria* NBRC 30259), with *S. gracilis* NBRC 1033 in particular showing the highest production (11.6 mM).

Among the 16 strains, their reduction reaction products from *rac*-AMKP differed in the composition of HIL enantioisomers (Fig. 1). In most cases, the composition was dependent on the taxonomic category to which these microorganisms belong. Bacterial strains such as NBRC 3951 and JCM 3205 produced only *SRS*/*RSR*- and *SSR*/*RSS*-HIL isomers as their HIL products. All yeast strains tested, NBRC 1033, NBRC 0385, NBRC 0715, NBRC 0879, and NBRC 4466, produced mainly *SRS*/*RSR*- and *SSS*/*RRR*-HIL isomers. Interestingly, basidiomycetous strains such as NBRC 30259 and NBRC 30604 also produced *SRS*/*RSR*- and *SSS*/*RRR*-HIL isomers. Six strains of filamentous ascomycetes, TG-2, NBRC 6605, NBRC 6813, NBRC 30538, 20-14, and NBRC 9435, showed low selectivity in the AMKP reduction reaction and produced all enantioisomers of HIL. One filamentous ascomycete, however, NBRC 30015, showed a similar tendency to bacteria to produce *SRS*/*RSR*- and *SSR*/*RSS*-HIL isomers.

Table 1
Microorganisms possessing AMKP reducing activity to produce HIL.

Taxonomic category	Strain	HIL production (mM)
Bacteria	*Bacillus thuringiensis* NBRC 3951	5.2
	Rhodococcus ruber JCM3205	1.7
Ascomycetous yeasts	*Sporobolomyces gracilis* NBRC 1033	11.6
	Cryptococcus albidus NBRC 0385	2.6
	Rhodotorula pallida NBRC 0715	2.3
	Rhodotorula marina NBRC 0879	2.0
	Aureobasidium pullulans NBRC 4466	5.1
Filamentous ascomycetes	*Fusarium solani* TG-2	3.5
	Gibberella fujikuroi NBRC 6605	1.0
	Acremonium fusidioides NBRC 6813	3.6
	Acremonium terricola NBRC 30538	3.9
	Acremonium strictum 20-14	3.5
	Verticillium albo-atrum NBRC 9435	0.6
	Pithomyces maydicum NBRC 30015	0.5
Basidiomycetes	*Crinipellis stipitaria* NBRC 30259	5.8
	Tricholoma matsutake NBRC 30604	2.0

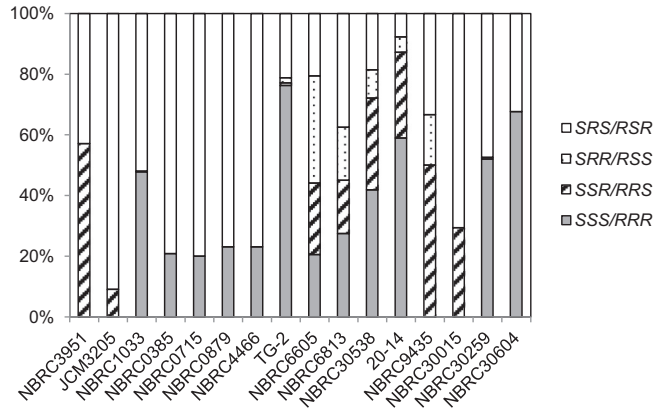

Fig. 1. Selective production of HIL enantioisomers with various microorganisms. HIL formed in the reaction mixture was analyzed with the achiral HPLC. Percentage composition of enantioisomers produced from *rac*-AMKP is indicated.

These results suggest that microorganisms may possess several AMKP-reducing enzymes with different characteristics.

3.2. Purification and identification of microbial AMKP reductases

In order to purify AMKP reductase, we first tested for AMKP-reducing activity in the cell-free extract of the above 16 strains. Reproducible HIL production was observed in one bacterium (NBRC 3951), four yeasts (NBRC 0385, NBRC 0715, NBRC 0879, and NBRC 4466), three filamentous actinomycetes (TG-2, NBRC 6813, and 20-14), and a basidiomycetes (NBRC 30259). These nine strains were then subjected to the enzymatic purification procedure, and at present two proteins from *A. pullulans* NBRC 4466 and *Fusarium solani* TG-2 have been successfully identified as enzymes responsible for AMKP-reducing activity.

3.3. ApAR, an AMKP reductase from A. pullulans NBRC 4466

The enzyme possessing AMKP-reducing activity was purified from the cell-free extract of *A. pullulans* NBRC 4466 through seven successive separation steps. Details of the purification procedure are described in the Supplemental materials (Table S1). Homogeneity of the purified enzyme, *Ap*AR, in the final preparation was confirmed by SDS-PAGE and the estimated molecular weight was approximately 36 kDa. Although the *N*-terminus of *Ap*AR was blocked to Edman degradation, its internal amino acid sequences were successfully determined, as shown in Table S3. The full-length genomic region containing the *apaR* gene was determined by degenerate PCR and inverse PCR, which are described in detail in the Supplemental materials. The *apaR* gene consisted of 1010 bp of nucleotides including two introns and three exons, and the protein-encoding region of the gene obtained from the cDNA was 909 bp. The deduced amino acid sequence of *Ap*AR showed 95% homology with a short-chain dehydrogenases/reductases (SDR) family enzyme of *Aureobasidium melanogenum* CBS 110374 (GenBank ID: KEQ58950.1) on a BLAST search.

3.4. FsAR, an AMKP reductase from F. solani TG-2

F. solani TG-2 was isolated as a fungal strain utilizing triacrylonitrile.[12] The enzyme possessing AMKP-reducing activity was purified from the cell-free extract of *F. solani* TG-2 through seven successive separation steps. Details of the purification procedure are described in the Supplemental materials (Table S2). Homogeneity of the purified enzyme, *Fs*AR, in the final preparation was confirmed by SDS-PAGE and the estimated molecular weight was

approximately 30 kDa. Although the *N*-terminus of *Fs*AR was blocked to Edman degradation, its internal amino acid sequences were successfully determined, as shown in Table S4. The full-length genomic region containing the *fsaR* gene was determined by degenerate PCR and inverse PCR, which are described in detail in the Supplemental materials. The *fsaR* gene consisted of 1047 bp of nucleotides including three introns and four exons, and the protein-encoding region of the gene obtained from the cDNA was 888 bp. The deduced amino acid sequence of *Fs*AR showed 98% homology with an SDR family enzyme of *Nectria haematococca* mpVI 77-13-4 (GenBank ID: XP_003043314.1) on a BLAST search.

3.5. Multiple alignment analysis of AMKP reducing enzymes

The amino acid sequences of *Ap*AR and *Fs*AR were used for the multiple alignment analysis together with that of *Bt*HILDH derived from *B. thuringiensis* 2e2, which we previously found as a NAD-dependent HIL dehydrogenase (Fig. 2). *Ap*AR and *Fs*AR possessed amino acid motifs for the NAD(P)-binding region (TGXXXGXG) and for the active site (YXXXK), which are conserved in classical short-chain alcohol dehydrogenase family enzymes.[13] *Bt*HILDH possessed the conserved active site motif but a slightly different NAD(P)-binding motif (SGXXXGXG). The sequence identities were 35% between *Ap*AR and *Fs*AR, 26% between *Ap*AR and *Bt*HILDH, and 28% between *Fs*AR and *Bt*HILDH.

3.6. Catalytic properties of microbial AMKP reductases

The three types of AMKP reducing enzymes, *Ap*AR, *Fs*AR, and *Bt*HILDH, were heterologously expressed in *E. coli* and purified to determine the various catalytic parameters for the reduction of AMKP. *Ap*AR and *Fs*AR strictly preferred NADPH as a coenzyme, in contrast to *Bt*HILDH, an NADH-dependent enzyme. The maximum activities of *Ap*AR and *Fs*AR were both observed at pH 8.5 and 37 °C, and *Bt*HILDH showed maximum activity at pH 6.5 and 45 °C. Specific activities of *Ap*AR, *Fs*AR, and *Bt*HILDH were determined to be 5.5, 6.9, and 353 U/mg for *rac*-AMKP, respectively.

3.7. Substrate specificity of microbial AMKP reductases

The recombinant *Ap*AR, *Fs*AR, and *Bt*HILDH were reacted with various carbonyl compounds, and the activities were determined by consumption of NAD(P)H measured spectrophotometrically at 340 nm (Table 2). Similar substrate specificities were observed for both *Ap*AR and *Fs*AR. Besides *rac*-AMKP, both enzymes showed reducing activities towards 1,3-dichloroacetone, 2,3-indolinedione, and levulinic acid. Relative activities for these substrates compared to *rac*-AMKP were 16%, 10%, and 9.3% for *Ap*AR, and 12%, 20%, and 30% for *Fs*AR, respectively. On the other hand, *Bt*HILDH had a different substrate spectrum, and reacted with ketopantolactone, 1,4-naphthoquinone, and various phenolic aldehydes much more than *rac*-AMKP.

3.8. Selective production of HIL stereoisomers with ApAR, FsAR, and BtHILDH

The recombinant *Ap*AR, *Fs*AR, and *Bt*HILDH enzymes were evaluated as biocatalysts for the selective production of HIL stereoisomers (Fig. 3). In the reaction mixture of *Fs*AR with *rac*-AMKP as the substrate, *SSS*-, *SRS*-, *RSS*-, and *RRS*-HIL were equally produced with the conversion of >99%. This result indicated that *Fs*AR catalyzed the stereoselective carbonyl reducing reaction to form the (*S*)-4-hydroxy group of HIL but did not recognize the stereo-configuration of 2-amino and 3-methyl groups. Although the same result was observed in the (*S*)-selectivity of AMKP reduction with *Ap*AR, its steric recognition of the substrate was somewhat different. Interestingly, *Ap*AR preferred *SR*-AMKP and also reacted well with *SS*- and *RR*-AMKP, but poorly utilized *RS*-AMKP, resulting in the conversion of 57%. This steric recognition ability of *Ap*AR corresponded to that of *Bt*HILDH, but *Bt*HILDH showed diverse stereoselectivity in AMKP reduction. *Bt*HILDH converted 30% of substrate and produced *SRS*-, *RRS*- and a small amount of *RSS*-HIL like *Ap*AR, however *SSR*-HIL was produced instead of *SSS*-HIL. Thus, there was a relationship between the stereo-configuration of the substrate and stereoselectivity of carbonyl reduction in the HIL-producing reaction by *Bt*HILDH.

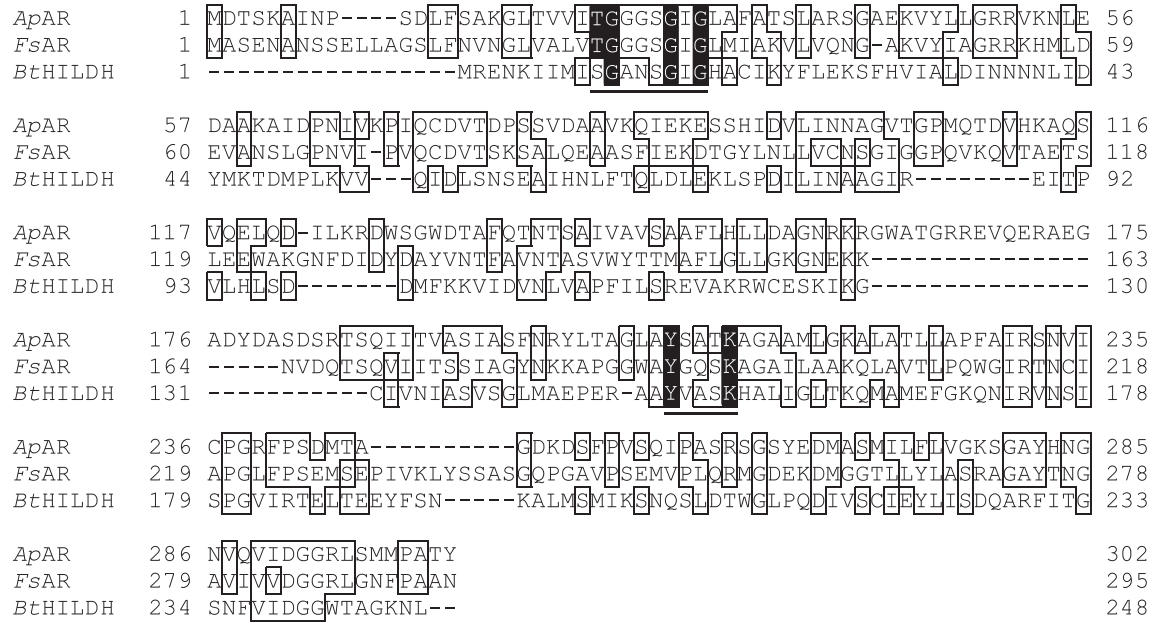

Fig. 2. Multiple alignment analysis of amino acid sequences of *Ap*AR, *Fs*AR, and *Bt*HILDH. *Ap*AR of *A. pullulans* NBRC 4466, *Fs*AR of *F. solani* TG-2, and *Bt*HILDH of *B. thuringiensis* 2e2 were used for the analysis. The common amino acid residues of these enzymes are in boxes. The NAD(P)-binding motif (TGXXXGXG) and active site motif (YXXXK) are underlined, in which the conserved amino acid residues are highlighted in black.

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Table 2
Substrate specificities of microbial AMKP reductases, *Ap*AR, *Fs*AR, and *Bt*HILDH towards carbonyl compounds.

Substrate	Relative activity (%)		
	*Ap*AR	*Fs*AR	*Bt*HILDH
Ketopantolactone	*n.d.*	*n.d.*	822
o-Chlorobenzaldehyde	*n.d.*	*n.d.*	478
1,4-Naphthoquinone	*n.d.*	*n.d.*	424
o-Nitrobenzaldehyde	*n.d.*	*n.d.*	375
p-Nitrobenzaldehyde	*n.d.*	*n.d.*	323
Terephthalaldehyde	*n.d.*	*n.d.*	297
m-Chrolobenzaldehyde	*n.d.*	*n.d.*	245
m-Nitrobenzaldehyde	*n.d.*	*n.d.*	126
Cuminaldehyde	*n.d.*	*n.d.*	122
rac-AMKP	100	100	100
p-Chlorobenzaldehyde	*n.d.*	*n.d.*	83
2,3-Butanedione	*n.d.*	*n.d.*	28
2-Decanone	*n.d.*	*n.d.*	27
Salicylaldehyde	*n.d.*	*n.d.*	12
2-Nonanone	*n.d.*	*n.d.*	6
1,3-Dichloroacetone	16	12	*n.d.*
2,3-Indolinedione	10	20	*n.d.*
Levulinic acid	9.3	30	*n.d.*

n.d., not detected.

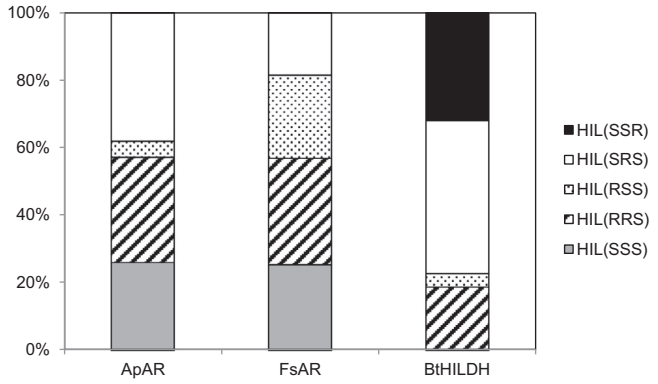

Fig. 3. Selective production of HIL stereoisomers using *Ap*AR, *Fs*AR, and *Bt*HILDH . HIL formed in the reaction mixture was analyzed with the chiral HPLC. Percentage composition of stereoisomers produced from *rac*-AMKP is indicated.

4. Discussion

According to BLAST search results of *Ap*AR and *Fs*AR, each of the AMKP reductases showed homology with function unknown SDR family enzymes of various species belonging to filamentous fungi. *Bt*HILDH is also an SDR family enzyme, but it has similarity with many bacterial SDR family enzymes. Thus, it is not surprising that the homologies are very low between *Ap*AR and *Bt*HILDH, or *Fs*AR and *Bt*HILDH. However, only 35% homology was observed between *Ap*AR and *Fs*AR, both fungal AMKP reductases. While *Bt*HILDH has been shown to convert *SRS*-HIL into *SR*-AMKP in bacterial secondary metabolism[9], the physiological roles of *Ap*AR and *Fs*AR are still unknown.

The catalytic characteristics of these three AMKP-reducing enzymes are summarized in Table 3. In spite of the low sequence similarity, *Ap*AR and *Fs*AR were found to both be NADPH-dependent enzymes catalyzing the stereoselective reduction of AMKP to produce (*S*)-4-hydroxy-HIL, and both had similar catalytic characteristics including optimum reaction temperature and pH. However, there was a slight difference in their steric preference for substrate AMKP, resulting in different compositions of the HIL stereoisomers produced, in particular *RSS*-HIL. *Fs*AR did not recognize the stereoconfiguration of 2-amino and 3-methyl groups of

Table 3
Summary of catalytic characteristics of AMKP-reducing enzymes.

Enzyme	Cofactor dependency	Stereoselectivity	
		Substrate recognition	Carbonyl reduction
*Ap*AR	NADPH	non-selective	4*S*
*Fs*AR	NADPH	2*S*/3*R* > 2*S*/3*S* = 2*R*/3*R* > 2*R*/3*S*	4*S*
*Bt*HILDH	NADH	2*S*/3*R* > 2*S*/3*S* = 2*R*/3*R* > 2*R*/3*S*	4*S* (2*S*/3*R*,2*R*/3*R*,2*R*/3*S*) 4*R* (2*S*/3*S*)

AMKP. On the other hand, *Ap*AR preferred *SR*-AMKP to *SS*- and *RR*-AMKP, and accepted *RR*-AMKP only slightly as its substrate. This may be due to some steric hindrance in the substrate binding pocket affecting access to *RR*-AMKP. The case of *Bt*HILDH was more complicated; its substrate preference was the same as *Fs*AR and it behaved like a (*S*)-selective carbonyl reductase, but an (*R*)-form alcohol, *SSR*-HIL, was unexpectedly produced from *SS*-AMKP. Structure analysis will be required to elucidate the structural features around the catalytic sites and substrate binding pockets of *Ap*AR, *Fs*AR, and *Bt*HILDH, that result in the generation of the various HIL stereoisomers.

The composition of HIL isomers produced was different between *Ap*AR and *Fs*AR (Fig. 3) and the corresponding original microorganisms, *A. pullulans* NBRC 4466 and *F. solani* TG-2 (Fig. 1). The cause might be that other predominant AMKP reductases with different selectivity or metabolic pathways for specific HIL-isomers are exist in the original microorganisms. The novel reductases and metabolic enzymes could be discovered from these microorganisms by reexamination of enzymatic purification method.

We have already developed two types of enzymatic production processes for the naturally occurring form of the stereoisomer *SRS*-HIL. In the first case, acetaldehyde, α-ketobutyrate, and L-glutamate were used for substrates of the cascade reaction with two chirality-generating enzymes, 4-hydroxy-3-methyl-2-keto-pentanoate aldolase and branched-chain amino acid transaminase.[14] In the second case, a chiral substrate, L-isoleucine, was directly hydroxylated by Fe(II)/α-ketoglutarate-dependent dioxygenase.[11,15] Compared to these processes, the carbonyl reductase-based method reported in this study has the advantage of generating three chiral carbon centers simultaneously from an inexpensive racemic substrate. However, selectivity of these AMKP-reducing enzymes is not enough to produce only a single stereoisomer. To realize this concept, advanced biocatalysts with selectivities both in substrate recognition and carbonyl reduction need to be obtained by further microbial screening or enzyme engineering approaches. For example, mutation introduction to amino acid residues around the substrate binding pocket is a promising approach to increase selectivity of substrate recognition, especially in the case of *Ap*AR and *Fs*AR that possess high selectivity for carbonyl reduction.

A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2017.06.044.

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