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D-Amino acid production by *E. coli* co-expressed three genes encoding hydantoin racemase, D-hydantoinase and *N*-carbamoyl-D-amino acid amidohydrolase

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Abstract

Three genes respectively encoding D-specific hydantoinase (DHHase), *N*-carbamoyl-D-amino acid amidohydrolase (DCHase) and hydantoin racemase (HRase) were co-expressed in *E. coli* in a system designed for the efficient enzymatic production of D-amino acids via a combination of hydantoin hydrolysis and hydantoin racemization. With the use of whole cells, the D-forms of eight amino acids – D-phenylalanine, D-tyrosine, D-tryptophan, *O*-benzyl-D-serine, D-valine, D-norvaline, D-leucine and D-norleucine – were efficiently converted from the corresponding DL-5-monosubtituted hydantoin compounds.

Keywords: D-Amino acid; Hydantoin racemase; D-Hydantoinase; *N*-Carbamoyl-D-Amino acid amidohydrolase

1. Introduction

D-Amino acids have been extensively used for pharmaceutical intermediates. D-*p*-Hydroxyphenylglycine, an important intermediate for amoxicillin, is commercially produced from DL-5-*p*-hydroxyphenylhydantoin by the hydantoin hydrolysis method [1–10]. However, the methods designed to efficiently produce the other D-amino acids have all been thwarted by the rate-limiting effect of the spontaneous racemization step of 5-monosubstituted hydantoin compounds. The racemization rate depends on the 5-substituted moiety in hydantoin ring, and 5-*p*-hydroxyphenylhydantoin appears to be the hydantoin compound with a rate that is not slow [11]. One study has demonstrated an enhanced rate of racemization at a higher temperature and under an alkaline condition [12], and this has prompted the development of a system using thermostable enzymes [13–16]. As an alternative, one of the most useful methods to produce D-amino acid is to combine enantioselective hydrolases with HRase (Fig. 1). Enzymatic racemization of hydantoin compounds has been reported previously [17], and the HRase gene has been isolated from several kinds of microorganisms [11,17–22]. Other studies have reported successful D-amino acid conversion using *E. coli* separately expressed as biocatalysts [23]. Earlier reports demonstrated L-amino acids production from DL-5-monosubstituted hydantoin with HRase, hydantoinase, and *N*-carbamoyl-L-amino acid amidohydrolase [24–26].

In a previous study on the properties of HRase from *Microbacterium liquefaciens* AJ3912 [17,27] and DHHase and DCHase from *Flavobacterium* sp. AJ11199 [28], we found that their wide substrate specificities could be readily applied to the production of various D-amino acids. In this paper, we report the co-expression of three genes encoding HRase, DHHase and DCHase in *E. coli* and the efficient production of various D-amino acids by whole cells.

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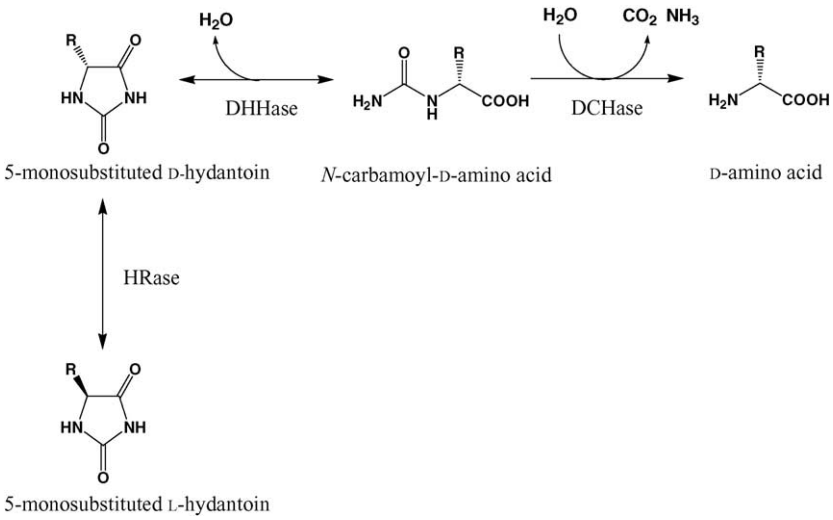

Fig. 1. Reaction scheme for the hydrolysis of 5-monosubstituted hydantoin to D-amino acid using D-hydantoinase (DHHase) and *N*-carbamoyl-D-amino acid amidohydrolase (DCHase) combined with hydantoin racemase (HRase).

2. Materials and methods

2.1. Chemicals

Hydantoin compounds and *N*-carbamoylamino acids were prepared from corresponding amino acids with potassium cyanate as described previously [29]. Restriction enzymes, *Taq* DNA polymerase, pUC19 and pSTV29 were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). pKK223-3 was purchased from Amersham Bioscience Corp. (NJ, USA). All other chemicals were of the highest grade commercially available.

2.2. Plasmids and bacterial strains

A pSTV29 derivative expression vector was constructed as follows. The *Eco*O109I-*Hin*dIII fragment of the trp promoter region obtained from pTrp4 [28] and the rrnB terminator obtained from *Hin*dIII/*Hin*cII-digested pKK223-3 were inserted into *Eco*O109I/*Pvu*I-digested pSTV29, resulting in pTrp8.

The cassette oligonucleotides 1 and 2 for the trp promoter were synthesized, annealed, and inserted into the *Eco*RI/*Kpn*I site of pHSG298 to give pHSG298EK. Next, pHSG299EX was obtained in the same manner by inserting cassette oligonucleotides 3 and 4 into the *Eco*RI/*Xba*I site of pHSG299.

The DCHase gene was amplified with oligonucleotides 5 and 6 from AJ11199 genomic DNA, digested with *Kpn*I/*Xba*I and inserted into *Kpn*I/*Xba*I site of pHSG298EK. The *Eco*RI/*Xba*I fragment containing the trp promoter and DCHase gene was subcloned into pHSG299, resulting in pTrp299DCHase1. DHHase gene was amplified with oligonucleotides 7 and 8 from AJ11199 genomic DNA as a template, digested with *Eco*RI/*Xba*I and subcloned into pHSG298EK. A PCR product harboring the trp promoter and DHHase gene was obtained from this plasmid with the oligonucleotide 9 and 10, digested with *Xba*I/*Pst*I and inserted into pTrp299DCHase1 to give pTrpHrCr. The DCHase gene and DHHase gene were amplified by the oligonucleotide 11 and 12 with pTrpHrCr as a template, digested with *Nde*I/*Eco*RI, and inserted into pTrp8, resulting in pTrp8CH.

The HRase gene was amplified with oligonucleotide 13 and 14 from AJ3912 genomic DNA, digested with *Eco*RI/*Xba*I and inserted into pTrp299EX. The *Eco*RI/*Pst*I fragment of the resulting plasmid was ligated to pSTV29 to give pTrp29R. Lastly, the HRase gene was amplified with oligonucleotide 15 and 16, digested with *Nde*I/*Eco*RI and inserted into pTrp4, resulting in pTrp4R.

The oligonucleotides used are listed in Table 1, and the sequence of the amplified fragments by PCR was confirmed by a DNA sequencer (CEQ2000, Beckmann Coulter, Inc., CA, USA). These plasmids were transformed into *E. coli* JM109 to express three genes, and the transformants obtained are summarized in Table 2.

2.3. Cultivation

One mililitre of preculture with LB medium was inoculated into 300 ml of medium I (25 g/l D-glucose, 1 g/l $MgSO_4{\cdot}7H_2O$, 5 g/l $(NH_4)_2SO_4$, 1.4 g/l KH_2PO_4, 2.28 g/l sodium citrate• $2H_2O$, 20 mg/l $FeSO_4{\cdot}7H_2O$, 20 mg/l $MnSO_4{\cdot}5H_2O$, 1 mg/l thiamine HCl) in a 1000 ml scale jar fermenter (Able-Biott, Tokyo, Japan). After cultivation for 24 h, 15 ml of seed culture was inoculated into 285 ml of medium I in 1000 ml scale jar fermenter. The antibiotics (ampicillin (100 μg/ml) and either kanamycin (50 μg/ml) or chloramphenicol (50 μg/ml)) were added when required. All of the cultures were cultivated at 35°C with aeration at 1vvm, a controlled agitation speed to keep the dissolved oxygen (DO) level above 1.5 ppm, and ammonia gas added to maintain a pH of 7.0. After the cells had grown and the glucose in

Table 1
Oligonucleotides used in this study

No.	Sequence
1	5′-AAT TCT GTT GAC AAT AAT TCA TCG AAC TAG TTA ACT AGT ACG CAA GTT CAC GTA AAA AGG GTG GTA C-3′
2	5′-CAC CCT TTT TAC GTG AAC TTG CGT ACT AGT TAA CTA GTT CGA TGA ATT ATT GTC AAC AG-3′
3	5′-AAT TCT GTT GAC AAT AAT TCA TCG AAC TAG TTA ACT AGT ACG CAA GTT CAC GTA AAA AGG GTT-3′
4	5′-CTA GAA CCC TTT TTA CGT GAA CTT GCG TAC TAG TTA ACT AGT TCG ATG AAT TAT TGT CAA CAG-3′
5	5′-GGG GTA CCG GAG AGG AAT ATG CCA GG-3′
6	5′-GCT CTA GAG CCG CGC CGC TCA GAC GG-3′
7	5′-GGG GTA CCA TGA CCC ATT ACG ATC TC-3′
8	5′-GCT CTA GAC GTC CTG TCC TTT CCG CC-3′
9	5′-CGT CTA GAT GTT GAC AAT TAA TCA T-3′
10	5′-CGC TGC AGT CAG GCC GTT TCC ACT TCG CCC GT-3′
11	5′-GGA ATT CCA TAT GCC AGG AAA GAT CAT TCT CGC G-3′
12	5′-CGG AAT TCT CAG GCC GTT TCC ACT TCG CC-3′
13	5′-GCT CTA GAG CGA TGC GTA TCC ATG TC-3′
14	5′-GGC TGC AGC GCT CCT TCT CGT TAG AG-3′
15	5′-CGG GAA TTC CAT ATG CGT ATC CAT GTC ATC AA-3′
16	5′-CGC GGA TCC TTA GAG GTA CTG CTT CTC GG-3′

the medium was exhausted, 500 g/l D-glucose was added at a rate of 3.5 ml/h.

2.4. SDS-PAGE

The cells were harvested by centrifugation ($8000 \times g$, 10 min, 4 °C), washed twice with 50 mM potassium phosphate buffer (pH 8.0), and disrupted by sonication (Biorupter, Tosho Electronics Ltd., Japan). The insoluble fraction was obtained by centrifugation at $18{,}000 \times g$ for 20 min at 4 °C to separate the soluble fraction as the supernatant, and resuspended with the same buffer. SDS-PAGE was performed on a 10–20% polyacrylamide gel (Daiichi Pure Chemicals, Tokyo, Japan) to detect the insoluble aggregates.

Table 2
Plasmids and transformants for gene expression used in this study

Plasmids or transformants	Relevant characteristics or carrying plasmids
Plasmids	
pTrpCrHr	pHSG 298 derivative containing DCHase gene and DHHase gene with trp promoter
pTrp4CH	pUC19 derivative containing DCHase gene and DHHase gene with trp promoter and rrnB terminator
pTrp8CH	pSTV28 derivative containing DCHase gene and DHHase gene with trp promoter and rrnB terminator
pTrp29R	pSTV29 derivative containing HRase gene with trp promoter
pTrp4R	pUC19 derivative containing HRase gene with trp promoter and rrnB terminator
Transformants[a]	
D1	pTrpCrHr, pTrp29R
D2	pTrp4CH, pTrp29R
D9	pTrp8CH, pTrp4R

[a] *E. coli* JM109 was used as the host strain.

2.5. Reaction conditions

The reaction mixture contained 50 g/l of DL-hydantoin compound, 20 mM of potassium phosphate buffer, 1 mM of $MnSO_4$ and 5% (v/v) of the culture broth in 300 ml. The reaction was carried out at 37 °C with moderate stirring, and the pH was controlled by adding of NaOH and H_2SO_4. In the reactions to produce D-Tyr, *O*-benzyl-D-Ser, and D-Trp, the atmosphere in the reaction system was replaced with nitrogen gas to prevent the substrate and the product from oxidizing. The activity for D-amino acid formation was expressed as D-amino acid produced from DL-hydantoin compound after 3 h at pH 7.5. One unit was defined as the amount of enzyme catalyzing the formation of 1 μmol of D-amino acid per minute.

2.6. Analysis

The substrates and products were analyzed by a high-performance liquid chromatograph packed with Inertsil Ph-3 (4.6 mm × 250 mm) (GL Science Inc., Tokyo, Japan). The detection was performed at 210 nm at 40 °C, and 50 mM sodium phosphate buffer (pH 2.8):acetonitrile (95:5) was used as the mobile phase. In alternative experiments, CHIRALPAK WH (4.6 mm × 250 mm) (Daicel Chemical Industries, Ltd., Tokyo, Japan) was used with 5 mM CuSO4:MeOH (100:5) as the mobile phase. The optical purities of amino acids were analyzed with a CROWNPAK CR(+) column (Daicel Chemical Industries, Ltd.) with diluted $HClO_4$ (pH 1.5).

3. Results and discussion

3.1. Co-expression of the HRase, DHHase, and DCHase genes in E. coli

We constructed three transformants – D1, D2, and D9 – to effectively co-express three genes encoding HRase, DHHase and DCHase (Table 2), and then estimated their ability to produce D-Phe from DL-5-benzylhydantoin using their broth as an enzyme source. After 24 h cultivation at 35 °C with or without antibiotics, all three of the transformants yielded more than 40 g of dry cells/l. The transformant D2 elicited the highest activity (Table 3), and the activity by D9 cells was higher than that by D1 cells even though the former harbored a plasmid (pTrp8CH) with lower copy numbers for the DCHase and DHHase genes.

Noting some instability in the activity obtained by the D1 and D2 cells without antibiotics, we analyzed plasmid stability. pTrpCrHr and pTrp4CH, the plasmid harboring the DCHase gene and DHHase gene in strain D1 and D2, respectively, were not maintained. pTrp8CH and pTrp4R in strain D9, on the other hand, remained stable throughout about 100

Table 3
Activities of the transformants

Transformants	Antibiotics addition	Dry cell weight (g/l)	Converting activity (U/mg dry cell)
D1	–	38.1	0.02
	+	40.3	0.09
D2	–	37.2	0.26
	+	41.5	0.31
D9	–	44.2	0.11
	+	43.2	0.12

After 24 h cultivation, the activity and the dry cell weight were measured with the culture broth. The activity was assayed by using DL-5-benzylhydantoin as the substrate. The formation of D-Phe was analyzed by HPLC (CHIRALPAK WH) after 3 h under the standard condition.

generations under the culture condition (data not shown), and their activities were almost the same with and without antibiotics (Table 3).

To confirm the level of gene expression, the soluble fractions and insoluble fractions prepared from the cells of these strains were separately analyzed on SDS-PAGE (Fig. 2). No bands corresponding to the three enzymes were detected in the insoluble fractions from the D1 and D9 cells. In contrast, two bands corresponding to DHHase and DCHase, respectively, were detected with high intensity in the insoluble fraction from the D2 cells. The overexpression of DHHases and DCHases gene from several microorganisms in *E. coli* has been reported to result in the formation of inclusion bodies [6,30]. One group attempted to prevent the formation of insoluble aggregates by altering culture temperature and introducing molecular chaperones (GroEL/GroES, DnaJ/DnaK) and thioredoxin [30]. In our study, the higher copy plasmid harboring the DHHase and DCHase genes was not maintained during cultivation, and the highest level expression without insoluble aggregates was obtained in D9 cells cultivated at 35 °C. Judging from these results, we selected the D9 strain as the best transformant and used it for the subsequent experiments.

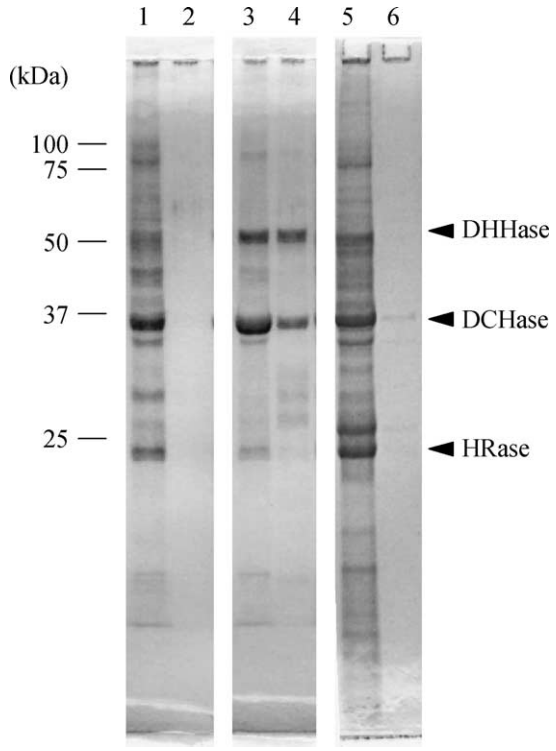

Fig. 2. Formation of insoluble aggregates by gene expression. After 24 h cultivation with antibiotics, the soluble and insoluble fractions were prepared from the cells of D1 (lane 1, soluble; lane 2, insoluble), D2 (lane 3, soluble; lane 4, insoluble), and D9 (lane 5, soluble; lane 6, insoluble) and then applied onto SDS-PAGE.

3.2. *Effect of pH on* D-*amino acid production*

To investigate the optimal pH of D-Phe production, we performed the reaction at various pH levels in the range of pH 6.9–8.3 (Fig. 3). After 20 h reaction, DL-5-benzylhydantoin remained at pH 7.2, and *N*-carbamoyl-D-Phe was accumulated in the reaction mixture under alkaline condition. These results consistent with the optimal pH ranges measured in DCHase and DHHase earlier, i.e., pH 6.5–7.0 and pH 8.0–9.0, respectively [28]. The HRase had an optimal pH of about 8.0 [17], but the enzymatic racemization did not seem to be rate-limiting under the tested conditions. In fact the ratio of D- and L-5-benzylhydantoin remained constant during the reaction, and the effective production of D-Phe was observed concurrently with the racemization of L-5-benzylhydantoin after a 48 h reaction at pH 7.5 (98% molar yield) (Fig. 4). On the basis of these results, we kept the pH at 7.5 in our subsequent reaction experiments.

3.3. D-*Amino acid production*

D-Amino acid production was investigated using the racemic hydantoin compounds (50 g/l) corresponding to Phe, Tyr, Trp, *O*-benzyl-Ser, Val, Leu, norvaline and norleucine (Fig. 5A and B). Among these reactions, those to produce

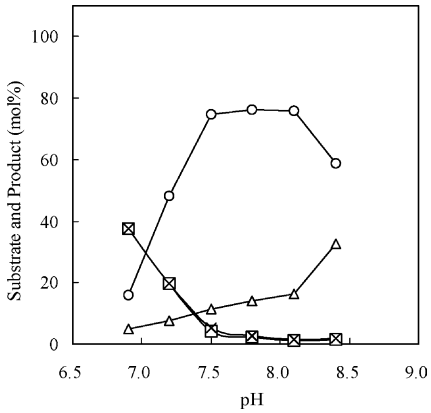

Fig. 3. The effect of pH on D-Phe production. The reaction with D9 cells was performed at pH 6.9–8.3 under a standard condition. D-Phe (◯), *N*-carbamoyl-D-Phe (△), D-5-benzylhydantoin (□) and L-5-benzylhydantoin (×) were quantified by HPLC (CHIRALPAK WH) after 20 h.

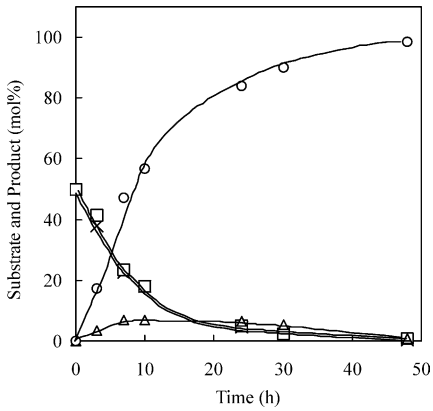

Fig. 4. Profile of D-Phe production. The reaction with D9 cells was performed at pH 7.5, and D-Phe (◯), *N*-carbamoyl-D-Phe (△), D-5-benzylhydantoin (□) and L-5-benzylhydantoin (×) were quantified by HPLC (CHIRALPAK WH).

D-Phe, D-Tyr, *O*-benzyl-D-Ser, D-Leu, D-norvaline and D-norleucine proceeded efficiently, achieving a 98% molar yield after 48 h and an optical purity of more than 99% e.e. The initial reaction rate of D-norleucine production was shown to be the highest (0.43 U/mg dry cell), and the aliphatic D-amino acids were more efficiently produced than aromatic D-amino acids among the substrates tested. The rate of D-Val production and yield of D-Val after 48 h (88% yield) were lower than the rates and yields of the others, and an accumulation of *N*-carbamoyl-D-Val was observed during this relatively low-yield reaction (approx. 60 mM). Given that the activity of the DCHase for *N*-carbamoyl-D-Val was lower, as reported previously [28], these results suggest that the production rate may chiefly depend on the substrate specificity of DCHase from AJ11199. When 25 g/l of DL-*iso*-propylhydantoin was used as the substrate, 98% yield (99% e.e.) was achieved in 48 h (data not shown).

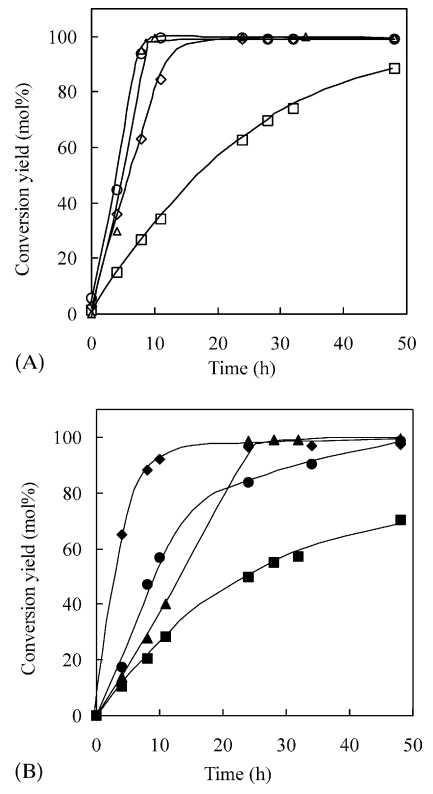

Fig. 5. D-Amino acid production by D9 cells. The reaction with D9 cells was performed at pH 7.5 with 50 g/l of the corresponding DL-5-monosubstituted hydantoin as substrate. (A) D-aliphatic amino acids. The production profile of D-Leu (◯), D-norleucine (△), D-Val (□) and D-norvaline (◊). (B) D-aromatic amino acids. The production profile of D-Phe (●), D-Tyr (▲), D-Trp (■) and *O*-benzyl-D-Ser (♦).

Among the reactions to produce the aromatic D-amino acids, the D-Trp production had the lowest rate and yield (70%). In this case, however, the reaction was accompanied by far less *N*-carbamoyl-D-Trp accumulation (less than 8 mM in total) than the reaction to produce D-Val. The isomers of 5-indorylmethylhydantoin were almost constant during the reaction. The activity of the DHHase for D-5-indorylmethylhydantoin was at almost the same level as that of DHHase for the corresponding compound to D-Phe, but the activity of DCHase for *N*-carbamoyl-D-Trp was lower. This may be one of the reasons why low yield of D-Trp was obtained, although 96% yield of D-Trp with optical purity of 99% e.e. was achieved in 48 h from 25 g/l of the substrate (data not shown). In addition, we speculated that DHHase and DCHase may be inhibited by D-Trp. The decrease of the production rate in D-Phe production after 16 h may be also caused by similar inhibition (Fig. 5B). In the case of D-Tyr and *O*-benzyl-D-Ser, the low solubility of the product seemed to scarcely affect on these enzymes. On the other hands, the efficient production of more than 350 mM of D-norvaline observed in our reactions suggested that ammonium ion scarcely inhibited the three enzymes.

4. Conclusion

We succeeded in constructing a useful system of whole-cell catalysis for D-amino acid production via co-expression of the HRase, DHHase and DCHase gene in *E. coli*. The use of this constructed system enabled the efficient production of various types of D-amino acid from the corresponding DL-5-monosubstituted hydantoin compound.

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